

Stimulatory effect of autologous adipose tissue-derived stromal cells in an atelocollagen matrix on wound healing in diabetic *db/db* mice

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Abstract

We aimed to evaluate the effectiveness of the application of an atelocollagen matrix containing autologous adipose tissue-derived stromal cells (ASCs) on wound healing in diabetic (*db/db*) mice. Cultured ASCs from *db/db* mice and from *db/+* mice secreted identical amounts of growth factors, cytokines, and type I collagen. ASCs from *db/db* mice proliferated at the same rate as those from *db/+* mice. When DiI-labeled ASCs were applied to full-thickness round skin wounds on the backs of diabetic *db/db* mice, histological observation at 2 weeks showed that red fluorescent-labeled tissues were formed in the epidermis, dermis, and capillaries. Twelve *db/db* mice were treated with either matrix alone or matrix containing ASCs and then sacrificed at 1 or 2 weeks. A histological examination demonstrated significantly advanced granulation tissue formation, capillary formation, and epithelialization in those wounds treated with atelocollagen matrix containing ASCs, compared with wounds treated with matrix alone.

1. Introduction

Although many tissues are known to contain lineage-committed progenitor cells for tissue maintenance and repair, several studies have also demonstrated the presence of uncommitted progenitor cells within the matrix of connective tissues [1]. For instance, human adipose tissue-derived multilineage (stromal) cells have the potential to differentiate into bone [2], cartilage [3], fat [4], myocardium [5], skin [6], skeletal muscle [7], and neurons [8, 9]. No significant differences have been observed between adipose tissue-derived stromal cells (ASCs) and human bone marrow-derived mesenchymal stem cells from the same patient with regard to yield of adherent cells, growth kinetics, cell senescence, differentiation capacity, or gene transduction efficiency [10]. Furthermore, transplantation of constructs cultured with human ASCs significantly stimulates skin repair, angiogenesis, and re-epithelialization in athymic mice when compared with constructs cultured with human fibroblasts [11]. The multipotent characteristics of ASCs, as well as their abundance in the human body, make ASCs a potential resource for wound repair and tissue engineering applications.

Wound healing proceeds in 3 overlapping phases: inflammation, proliferation (including formation of granulation tissue), and matrix formation and remodeling [12]. This sequential process requires the interaction of cells in the dermis and epidermis, as well as the activity of chemical mediators released from inflammatory cells, fibroblasts,

and keratinocytes [12]. An absence of the cellular and molecular signals required for normal wound repair processes—such as inflammation, angiogenesis, contraction, deposition of extracellular matrix, granulation tissue formation, epithelialization, and remodeling—may contribute to poor healing of some wounds, such as diabetic ulcers [12-14]. Numerous strategies have been investigated for coverage of such skin defects, including temporary skin substitutes (porcine xenografts, synthetic membranes, atelocollagen sponge, and allogenic substitutes) and permanent skin substitutes (cultured epidermis and dermal substitutes) [15, 16]. Artificial dermal substitutes, such as atelocollagen matrix (ACM; PELNAC; Johnson & Johnson Japan, Tokyo, Japan), are structurally optimized to incorporate into surrounding tissue and to allow cell invasion by fibroblasts and capillaries for subsequent dermal remodeling [16, 17]. Nevertheless, effective coverage is still not established when the area to be covered is large or the local conditions are poor—as in cases of severe contamination, very poor blood flow and vascularity, or congenital skin disorders like epidermolysis bullosa [16, 18].

A previous wound healing study using a mitomycin C-treated, healing-impaired rat model showed that the application of an ACM containing inbred ASCs onto an open wound significantly induced the formation of granulation tissue and capillaries and accelerated wound healing [19]. The effectiveness of ACM containing autologous ASCs has been evaluated using full-thickness skin incisions on the backs of healing-impaired

diabetic (*db/db*) mice [20]. The present study demonstrated that cultured ASCs from *db/db* mice proliferated well, secreted substantial amounts of growth factors and cytokines, and generated type I collagen, all of which are suitable for wound repair. Furthermore, the majority of the seeded labeled ASCs were incorporated into the regenerated epidermis, dermis, and capillaries.

2. Materials and Methods

2.1. Isolation of Adipose Tissue-Derived Stromal Cells from *db/db* and *db/+* Mice

Male mutant diabetic mice (C57BL/ksJ *db/db*) and their normal littermates (*db/+*) (CREA Japan Inc., Tokyo, Japan) were used. All mice were maintained on a standard laboratory diet and water *ad libitum* and were used experimentally after reaching 10 weeks of age. Prior to the experiments, urinary glucose and protein were analyzed using reagent strips (Uro-Labstix; Bayer Medical Ltd., Tokyo, Japan), and all of the *db/db* and *db/+* mice were diagnosed to be severely diabetic and normal, respectively.

Adipose tissue was obtained from the abdomens of the *db/db* and *db/+* mice. ASCs were prepared as described previously [2, 3, 19]. Briefly, approximately 1 g of adipose tissue from 1 *db/db* mouse was washed extensively with 10 ml of phosphate-buffered saline (PBS). Similarly, 1 g of adipose tissue was prepared from the inguinal adipose tissue of 3 10-week-old *db/+* mice. The extracellular matrices were digested with 0.075% collagenase at 37°C for 1 h. After the addition of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics, the samples were centrifuged at 1,200 *g* for 10 min. The cell pellet was resuspended in medium and incubated at room temperature for 10 min. After the cellular remains were filtered with a 100- μ m nylon mesh, the cells were resuspended with control medium, and about 5×10^5 cells were plated into each round ACM disc (1.5 cm in diameter;

PELNAC; Gunze Co., Kyoto, Japan). The ACM containing the ASCs was incubated in control medium at 37°C for 2 h before transplantation. The yield of adhered ASCs was estimated to be about 4×10^5 to 6×10^5 cells per gram of adipose tissue obtained from both *db/db* and *db/+* mice.

2.2. Monolayer Culture of ASCs and Immunohistochemistry

After removal of cellular remains through a 100- μ m nylon mesh, ASCs were incubated in DMEM containing 10% FBS and antibiotics in a dish (100 mm in diameter). When the adhered cells became subconfluent after 4 days of culture, they were subcultured. For the *in vitro* experiments, the cells were released from the culture dish by treatment with a trypsin-EDTA solution (1 \times ; 0.2 g EDTA and 0.5 g porcine trypsin per liter of HBSS; Sigma Aldrich Japan, Tokyo) at 37°C for 5 min, and the suspension was centrifuged to pellet the cells. The cells were then resuspended in DMEM containing 10% FBS and antibiotics and used as ASCs.

ASCs from *db/db* and *db/+* mice were cultured for 4 days in DMEM containing 10% FBS and antibiotics to an almost confluent monolayer. The cultures were washed with PBS and fixed for 30 min in a solution of 2% paraformaldehyde and 0.5% glutaraldehyde, followed by a wash with PBS. The culture plates were then incubated with primary polyclonal antibody (anti-mouse/rabbit Type I collagen; Cosmo Bio Co.

Ltd., Tokyo, Japan) at room temperature for 1 h and with secondary antibody (goat anti-rabbit biotinylated anti-IgG; Cosmo Bio Co. Ltd.) for 30 min, followed by a wash with PBS. The primary antibody was then visualized with ABC-peroxidase reagent and Novared substrate, according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK), and the culture plates were examined under a light microscope (Leica DM-IRB (SLR); Leica Microsystems, Tokyo, Japan).

2.3. Quantification of Growth Factor and Cytokines

ASCs from *db/db* and *db/+* mice were plated on 10-cm-diameter plastic dishes (1×10^6 cells per dish). ASC-conditioned medium was collected at 4 days after plating. At the end of the culture period, supernatants were collected and stored at -80°C until analysis. The presence of cytokines was analyzed using the BioPlex system (Bio-Rad Laboratories Co. Ltd., Tokyo, Japan). The following cytokines were analyzed: interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), basic fibroblast growth factor (b-FGF), platelet-derived growth factor bb (PDGF-bb), and vascular endothelial growth factor (VEGF). Hepatocyte growth factor (HGF) levels in the cell culture supernatants were measured using an ELISA kit (Institute of Immunology Co. Ltd., Tokyo, Japan).

2.4. Fluorescent Labeling of ASCs

Isolated ASCs from *db/db* mice (1×10^6 cells) were suspended in serum-free DMEM and fluorescently labeled by adding 5 μ l of Vybrant™ DiI cell-labeling solution (Cambrex BioScience Inc., Charles City, IA, USA) to the cell suspension for 20 min at 37 °C, according to the manufacturer's instructions. The labeled cells were washed, centrifuged, and resuspended in DMEM containing antibiotics. ACMs containing labeled ASCs were prepared and then applied to wounds, as described below.

2.5. Transplantation of ACM containing ASCs

Under general anesthesia with pentobarbital sodium (Dainippon Sumitomo Pharma Co. Ltd. Osaka, Japan), the dorsal area was totally depilated, and 2 full-thickness round wounds (approximately 1.5 cm in diameter) were created on the back of each *db/db* mouse with a pair of sharp scissors and a scalpel. An ACM alone (control) or an ACM containing freshly isolated ASCs was applied to the wound using 6-0 nylon sutures (Kono Seisakusho Co. Ltd., Chiba, Japan). The skin area surrounding the wound was removed from each *db/db* mouse for histological examination at 1 and 2 weeks after treatment ($n = 6$ for each time point). These animal experiments were approved and carried out following the guidelines for animal experimentation of the National Defense Medical College, Tokorozawa, Saitama, Japan.

2.6. Histological Examination

Removed skin samples including wound tissue were fixed in a 10% formaldehyde solution, embedded in paraffin, and sectioned in 4- μ m increments (Yamato Kohki Inc., Asaka, Saitama, Japan). Sections were made perpendicular to the anterior-posterior axis and perpendicular to the surface of the wound and were stained with hematoxylin-eosin reagent. For each section, a randomized area showing granulation and capillary formation was photographed ($\times 100$ magnification), and the thickness of granulation formation and number of capillary lumens per microphotograph were evaluated. Only mature vessels containing erythrocytes were counted. For immunohistochemistry, the frozen sections were also immunostained with rabbit anti-mouse CD31 antibody (Takara Bio Inc.).

2.7. Wound Closure Analysis

Digital photographs were taken 1 and 2 weeks after the silicon membrane was removed. The silicone membranes of the ACMs were removed after 1 week. Wound closure was quantified by the epithelialization rate (%), which was calculated as $(1 - \text{open wound area/original wound area}) \times 100$ (Table 1).

2.8. Statistical Analysis

Results are expressed as mean \pm SD. A paired Student's *t*-test was used to determine the probability of significant differences. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expansions of ASCs from *db/db* and *db/+* Mice and Immunohistochemistry

After cell isolation with enzymes as described above, more than 5×10^5 nucleated cells adhering to the culture plates were obtained from about 1 g of adipose tissue from *db/db* and *db/+* mice. After 4 days, cells from both types of mice were subconfluent and fibroblast-like. When detached and subcultured for 3 days, the 2 groups of cells showed identical growth rates (Figure 1). After a total of 8 days in culture (2 passages), the cells were collected and used as ASCs; at this point, the number of ASCs was at least 25 times higher than the number of originally plated cells. There was no significant difference in ASC yield between the *db/db* and *db/+* mice.

ASCs from *db/db* and *db/+* mice were cultured to an almost confluent monolayer and then immunostained with primary antibody (anti-Type I collagen). There was no significant difference between the cell cultures in the specific binding of the primary antibody (Figure 2).

3.2. Quantification of Growth Factors and Cytokines Secreted by ASCs

Cultured ASCs from *db/db* and *db/+* mice secreted significant amounts of growth factors and cytokines, including IL-6, b-FGF, PDGF-bb, VEGF, and HGF over a 4-day period (Figure 3). No statistically significant differences were observed between ASCs

from *db/db* and *db/+* mice in the secretion of those angiogenic factors. The generation of growth factors might be suitable for regenerative ASC therapy for healing-impaired wounds.

3.3. Fluorescent Labeling of ASCs from *db/db* Mice and Autologous Transplantation

About 25–30% of the ASCs isolated from *db/db* mice were labeled with DiI (Figure 4A). ACMs containing labeled ASCs were prepared and applied to wounds ($n = 6$) (Figure 4B). Figures 5A and 5B show CD31-immunostaining and DiI-labeled tissue, respectively, on the transplant area of *db/db* mice at 2 weeks. The transplanted DiI-labeled ASCs were mainly incorporated into regenerated granulation and epithelial tissues (Figure 5B). Some microvessels were double-labeled with fluorescence (DiI) and CD 31 (Figure 5C), suggesting that some of the seeded ASCs may incorporate into blood vessels as endothelial-progenitor cells.

3.4. Observations on Transplantation of ACM Containing Autologous ASCs

We observed no signs of undesirable inflammation, infection, neovascularization, or adipose tissue formation in wounds treated with ACM alone or with ACM containing ASCs. Wound areas were recorded with the aid of a digital camera at 1 and 2 weeks

(Figure 6). Although wound closures (epithelialization rates) did not appear to be stimulated in the wounds treated with ACM containing ASCs at 1 week, there was significantly enhanced wound closure at 2 weeks, compared with the control (ACM alone) wounds (Table 1).

3.5. Granulation Tissue Thickness and Capillary Number

Significant stimulation of granulation tissue formation was observed in the ACM containing ASCs at 1 and 2 weeks (Table 1). Significant stimulatory effects on capillary formation were also seen in the wounds treated with ACM containing ASCs at 1 and 2 weeks (Table 1). When the effects of treatment with ACM alone or ACM containing ASCs were assessed in the wounds of *db/+* mice, no statistically significant differences were observed regarding granulation, epithelial, and capillary formation; both wounds were almost repaired after 1 week (Table 1).

4. Discussion

Other researchers and we have previously reported that stromal cells derived from adipose tissue possess the ability to produce cartilage and bone matrix [1-3]. Large quantities of ASCs can be obtained from human adipose tissue by liposuction under local anesthesia; thus, cell multiplication under expensive and laborious cell-culture conditions is not required. Approximately 5×10^5 cells per gram of adipose tissue can be obtained from human adipose tissue by liposuction (data not shown). In contrast to a cultured dermal substitute, our ACM containing ASCs can be prepared within a few hours in a clinical setting. Furthermore, the use of autologous ASCs reduces the risks of infectious disease transmission and cellular rejection. Thus, ASCs represent an abundant, safe, practical, and appealing source for autologous cell replacement.

In this study, ASCs from *db/db* and *db/+* mice proliferated for 3 days with identical growth rates. There was no significant difference in the production of Type I collagen between the cell cultures from *db/db* and *db/+* mice. Furthermore, ASCs from *db/db* and *db/+* mice both showed high levels of CD34 and Sca1 but not of c-kit (data not shown), suggesting that the 2 sets of ASCs include similar populations of mesenchymal stem cells from bone marrow [21, 22].

Cultured ASCs from *db/db* and *db/+* mice secreted a number of angiogenic cytokines—including b-FGF, PDGF-bb, VEGF, and HGF—at similar, bioactive levels.

Introduction of ASCs *in vivo* might also up-regulate expression of these growth factors by autocrine and/or paracrine actions in the wound [23].

By using fluorescent (DiI) labeling of ASCs from *db/db* mice and autologous transplantation, we investigated the fates of autologous ASCs seeded into healing-impaired wounds, the differentiation of autologous ASCs, and the interactions between seeded autologous ASCs and their environment. The results suggested that the majority of seeded autologous ASCs were incorporated into the regenerated granulation and epithelial tissues. In addition, some of the seeded ASCs may incorporate into blood vessels as endothelial-progenitor cells [24]. In fact, application of ASCs has been suggested as having potential for angiogenic therapy for ischemic disease [21].

The application of ACM alone to an open wound in *db/db* mice resulted in minor effects on granulation tissue formation, epithelialization, and capillary number. However, parameters such as granulation tissue thickness, epithelium, and vessel formation were significantly increased in the wounds treated with ACM containing autologous ASCs at 1 or 2 weeks in the *db/db* mice. Although the mechanism responsible for impaired wound healing in *db/db* mice is not completely understood [12], it is likely that the presence of macrophages has a significant effect on the formation of wound granulation tissue and that macrophage accumulation is impaired in *db/db* mice [12]. Furthermore, a defect in the expression of vascular endothelial growth factor may be associated with

any wound-healing disorder [13].

It is interesting to note that the addition of autologous ASCs into the ACM had only minor effects on the degree of healing in normal (*db/+*) mice. As explained above, macrophages have a significant effect on the formation of wound granulation tissue [12]; it is likely that sufficient macrophages accumulate in *db/+* mice but not in *db/db* mice. Thus, it is possible that *db/+* mice have a sufficient amount of growth factors in the wound and wound fluid to permit a maximal rate of healing, and therefore, only a minor increase in wound repair can result from the application of autologous ASCs. In addition, the wound closure assay and histological examinations used in this study might not be sensitive enough to detect small effects.

5. Conclusion

ASCs from both *db/db* and *db/+* mice proliferated with identical growth rates, produced Type I collagen, and secreted a number of angiogenic cytokines at similar levels. Wound healing experiments using the *db/db* mouse model showed that the application of ACM containing autologous ASCs onto an open wound induced the formation of significant granulation tissue, epithelium, and capillaries, thereby accelerating wound healing.

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FIGURE CAPTIONS

FIG. 1. After 4 days of culture, ASCs from *db/db* (○) and *db/+* (▲) mice were detached with trypsin-EDTA solution and cultured for 3 days with identical growth rates.

FIG. 2. Photographs are representative of 6 wounds treated with ACM alone or with ACM containing ASCs immunostained with anti-Type I collagen.

FIG. 3. Growth factors and cytokines present in cell culture supernatants were analyzed on day 4 in ASCs from *db/+* (white bars) and *db/db* (black bars) mice. Data represent the mean \pm SD of duplicate samples from 3 culture supernatants.

FIG. 4. Labeling efficiency of Vybrant™ DiI-labeled ASCs isolated from *db/db* mice was about 25–30% (A). ACMs containing DiI-labeled ASCs were then prepared and applied to the wounds of *db/db* mice (B).

FIG. 5. CD31-immunostained (A) and DiI-labeled (B) tissue on the transplant areas of *db/db* mice at 2 weeks. The transplanted DiI-labeled ASCs were mainly incorporated into regenerated granulation and epithelial tissues. Some microvessels were double-labeled with DiI and CD 31 (C). Each photograph is representative of 6

experiments.

FIG. 6. ACM containing ASCs or ACM alone was applied to each wound by suture. The silicon membranes were removed at 1 week; the skin surrounding the wounds was removed at 2 weeks and sectioned for histological examination. Sections were made perpendicular to the wound surface. Histological examination of wound repair was performed at 2 weeks after the initial wounding. Photographs are representative of 6 wounds treated with ACM alone and 6 wounds treated with ACM containing ASCs. Tissue was stained with hematoxylin-eosin. Short arrows show blood vessels containing erythrocytes. Long arrows show the thickness of the granulation tissue.

FIG. 1

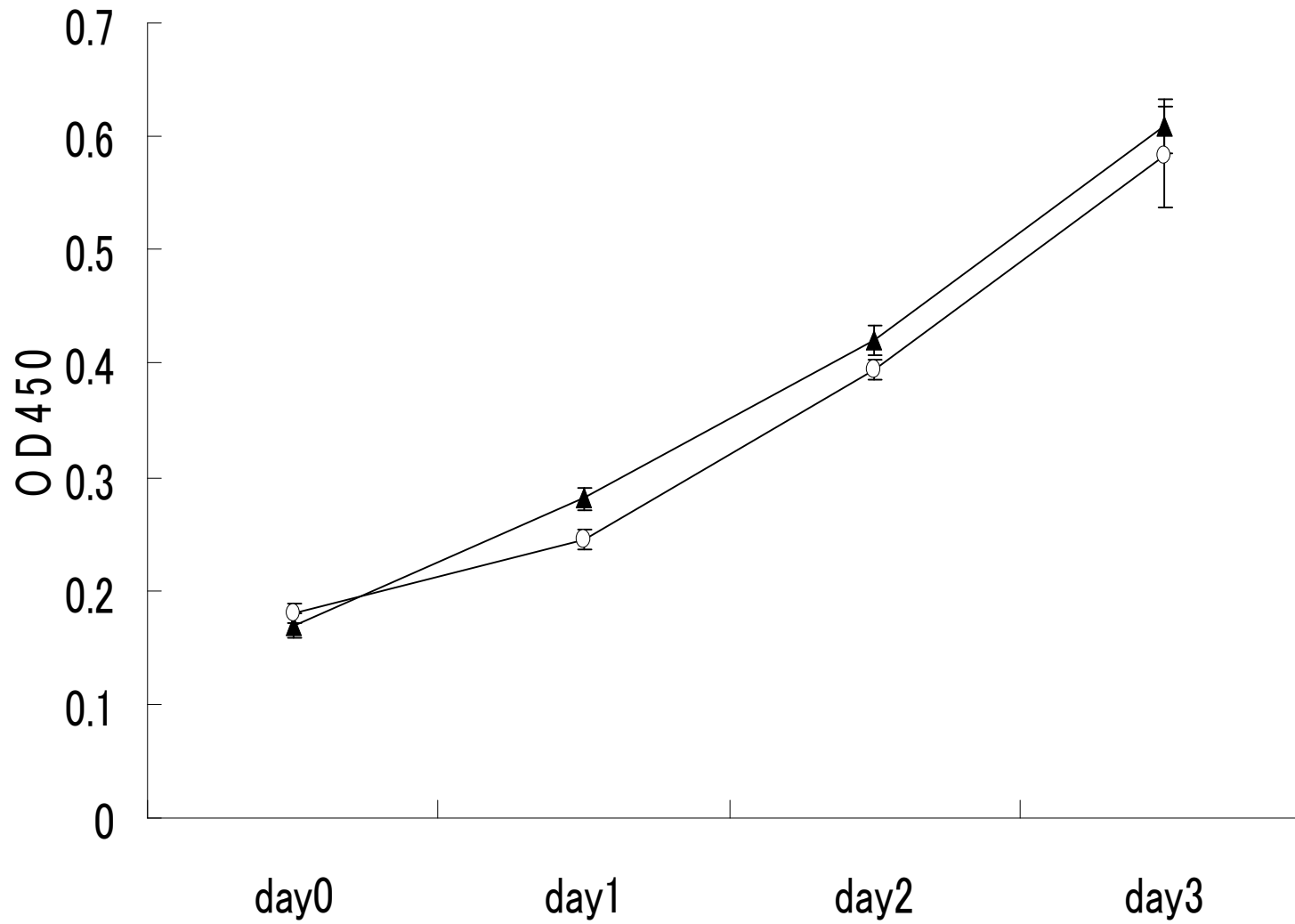
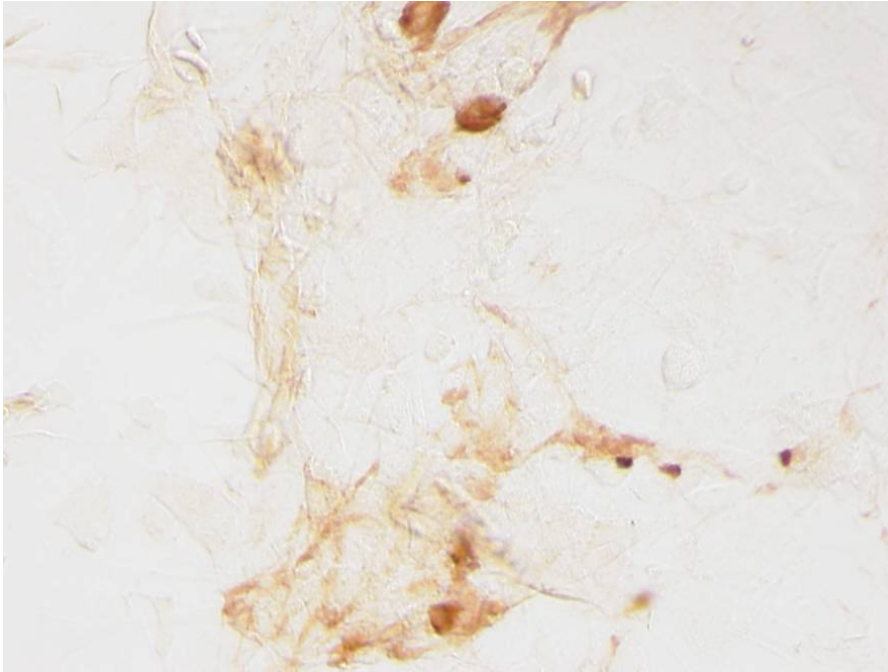
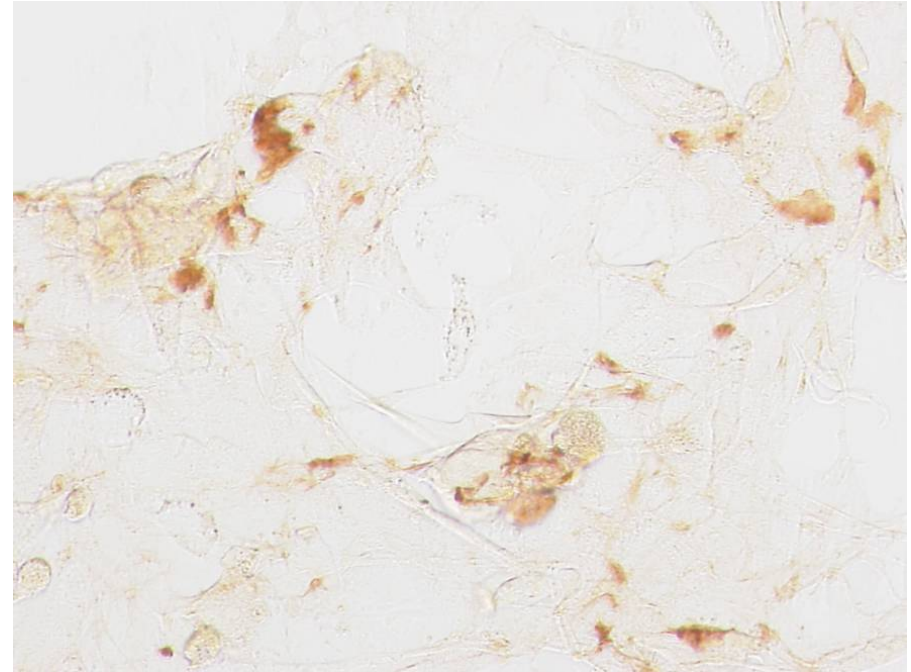


FIG. 2



db/+ mouse-derived ASCs



db/db mouse-derived ASCs

FIG. 3

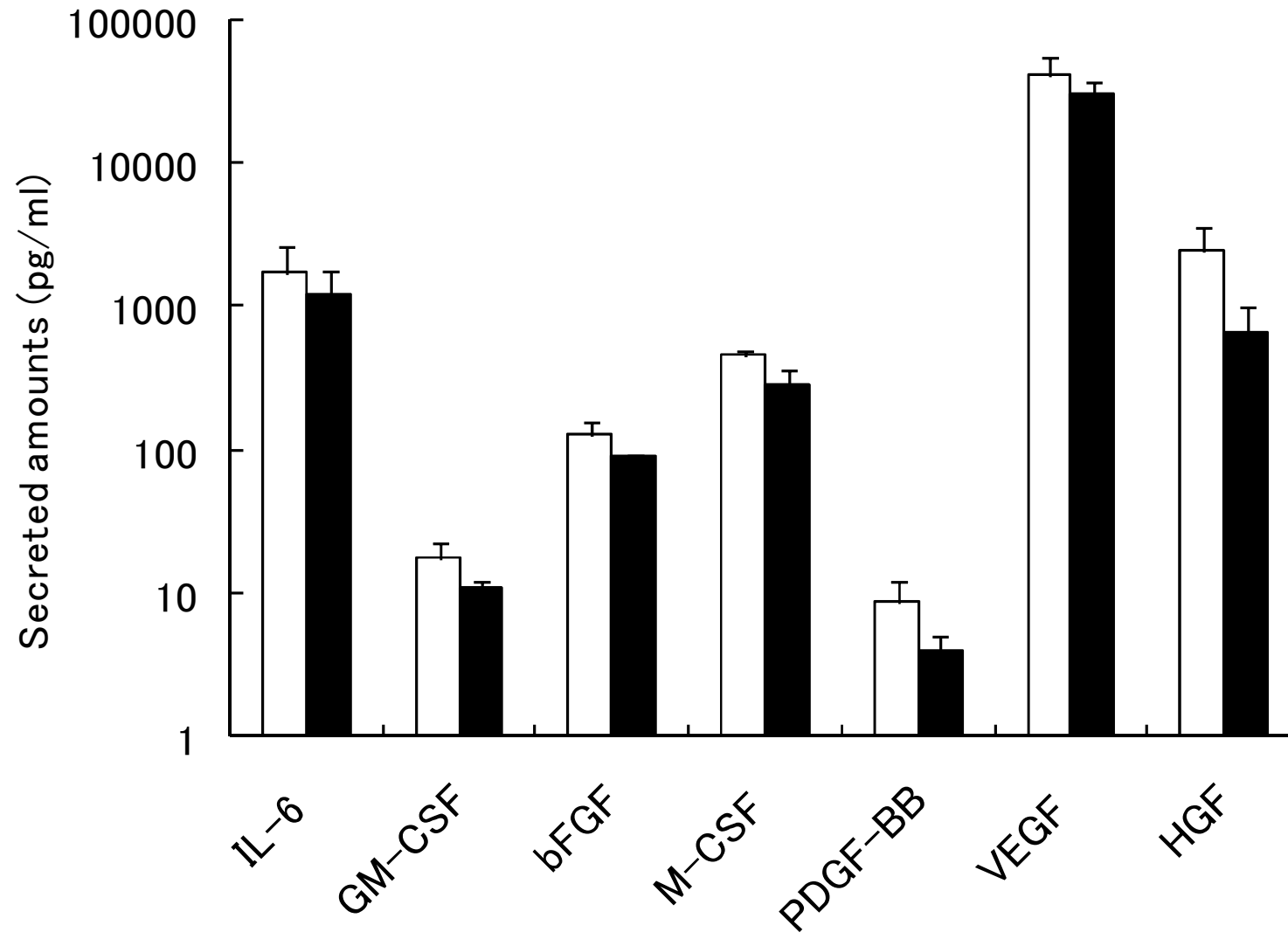
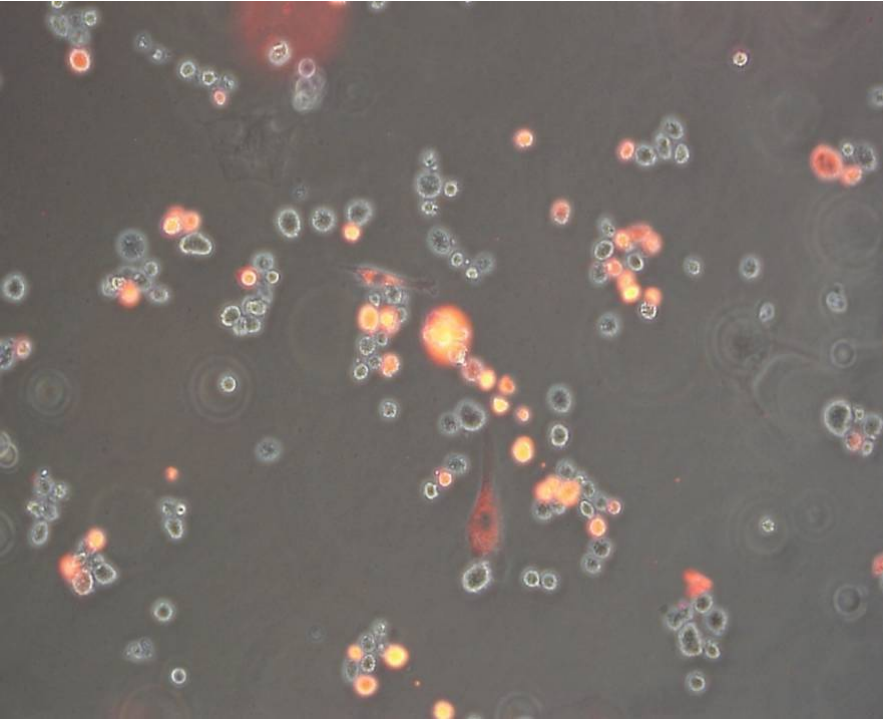


FIG. 4

A



B



FIG. 5

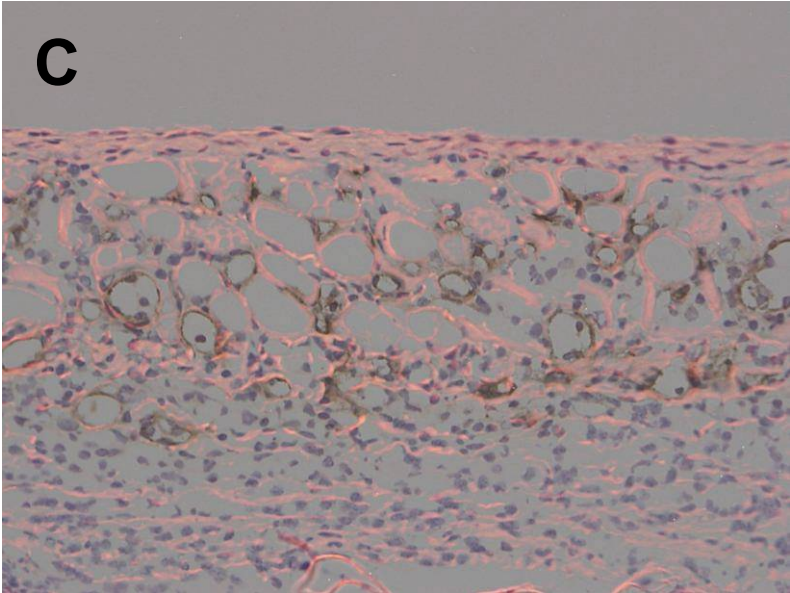
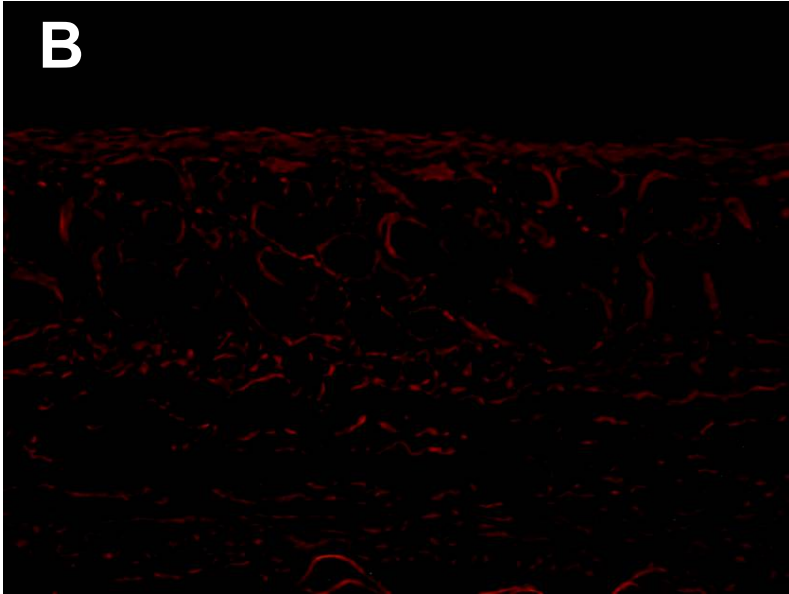
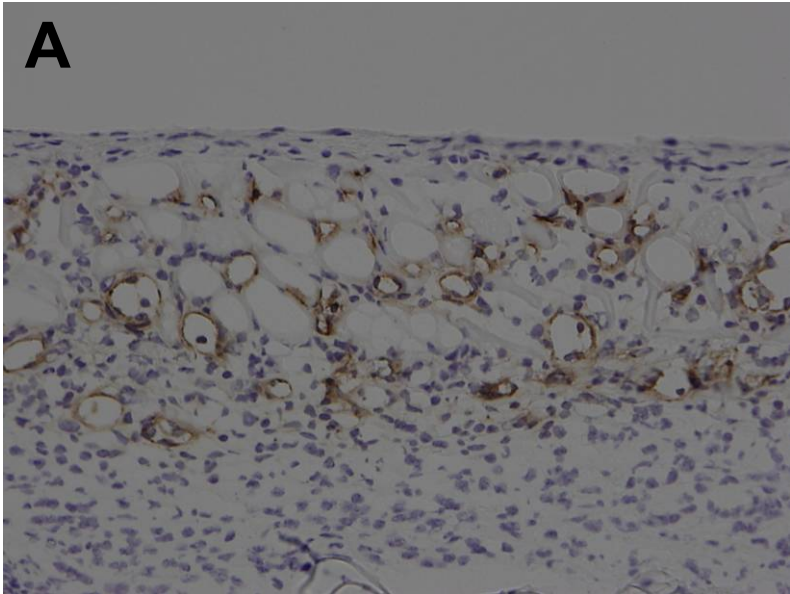


FIG. 6

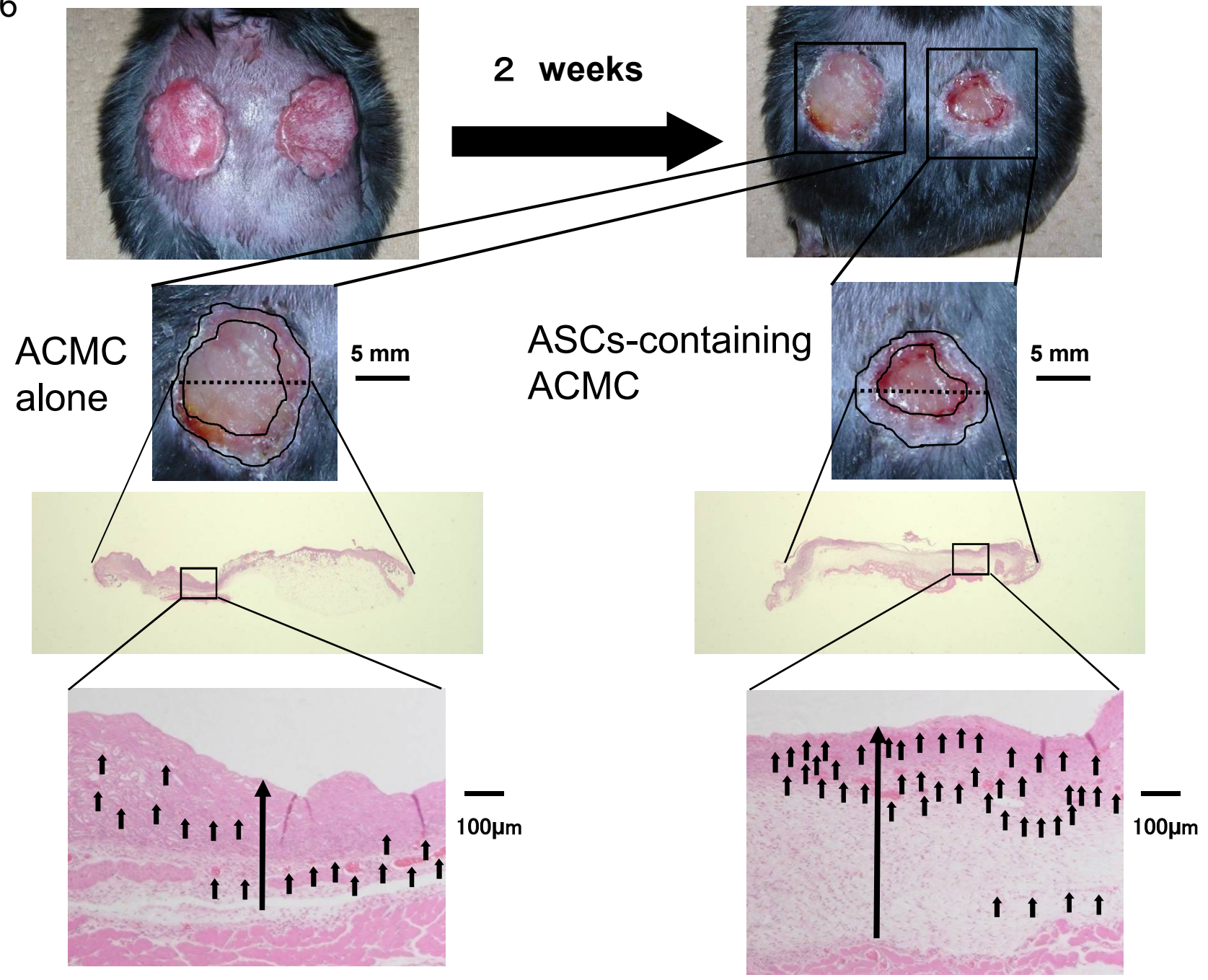


TABLE 1
Effects of ASCs-containing ACM on wound healing of db/db mice

	Control (ACM alone)		ASCs-containing ACM	
	<i>db/+ mice</i>	<i>db/db mice</i>	<i>db/+ mice</i>	<i>db/db mice</i>
Granulation tissue thickness (µm)				
1 week	745.0 ± 105.0	160.2 ± 26.0	762.0 ± 121.0	236.9 ± 35.8 *
2 weeks	not determined	290.5 ± 42.0	not determined	806.7 ± 124.0 **
Vessels (per sight)				
1 week	44 ± 17	17 ± 4	47 ± 18	32 ± 14 *
2 weeks	not determined	23 ± 5	not determined	55 ± 16 **
Epithelialization rates (Wound closure) (%)				
1 week	90.5 ± 5.0	14.8 ± 8.4	93.6 ± 4.8	16.8 ± 9.8
2 weeks	not determined	52.6 ± 11.8	not determined	87.3 ± 5.1 **

Epithelialization rates (%) were calculated by the equation “(1 – open wound area / original wound area) × 100”. Data represent mean ± SD. Student’s *t* test, **p*<0.05, ***p*<0.001.