Research Article

Stimulatory Effect of Autologous Adipose Tissue-Derived Stromal Cells in an Atelocollagen Matrix on Wound Healing in Diabetic db/db Mice

Masaki Nambu,1, 2 Masayuki Ishihara,3 Satoko Kishimoto,3 Satoshi Yanagibayashi,1 Naoto Yamamoto,1 Ryuichi Azuma,1 Yasuhiro Kanatani,4 Tomoharu Kiyosawa,1 and Hiroshi Mizuno 2

1 Department of Plastic and Reconstructive Surgery, National Defense Medical College, Saitama 359-8513, Japan
2 Department of Plastic and Reconstructive Surgery, Juntendo University School of Medicine, Tokyo 113-8421, Japan
3 Research Institute, National Defense Medical College, Saitama 359-8513, Japan
4 Policy Sciences, National Institute of Public Health, Saitama 351-0197, Japan

Correspondence should be addressed to Masaki Nambu, tmbs88@yahoo.co.jp

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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 experiments, urinary glucose and protein were analyzed using reagent strips (Uro-Labstix; Bayer Medical Ltd., Tokyo, Japan). 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].

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2.2. Monolayer Culture of ASCs and Immunohistochemistry. After the removal of cellular remains remains through a 100-µm nylon mesh, ASCs were incubated in DMEM containing 10% FBS and antibiotics in a dish (100 mm in diameter). 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.

2.3. Quantification of Growth Factor and Cytokines. 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 (antimouse/rabbit Type I collagen; Cosmo Bio Co. Ltd., Tokyo, Japan) at room temperature for 1 h and with secondary antibody (goat antirabbit 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).

2.4. Fluorescent Labeling of ASCs. Isolated ASCs from db/db mice (1 × 10^6 cells) were suspended in serum-free DMEM and fluorescent labeled by adding 5 µL of Vybrant DiI cell-labeling solution (Cambrex BioScience Inc., Charles City, Iowa, 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-µm 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 immunohistochemistry, the frozen sections were also immunostained with rabbit antimouse 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 – open wound area/original wound area) × 100 (Table 1).

2.8. Statistical Analysis. Results are expressed as mean ± SD. A paired Student’s t-test was used to determine the probability of significant differences. A value of P < .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 1 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 4(a)). ACMs containing labeled ASCs were prepared and applied to wounds (n = 6) (Figure 4(b)). Figures 5(a) and 5(b) 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 5(b)). Some microvessels were double-labeled with fluorescence (DiI) and CD 31 (Figure 5(c)), 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
Table 1: Effects of ASCs-containing ACM on wound healing of db/db mice.

|                      | Control (ACM alone) | ASCs-containing ACM |
|----------------------|---------------------|---------------------|
|                      | db/+ mice           | db/db mice           | db/+ mice           | db/db mice           |
| 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 < .05, **P < .001.

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

Figure 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 ± SD of duplicate samples from 3 culture supernatants.

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 $5 \times 10^5$ 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 $db/+\text{ }$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 $db/+\text{ }$mice. Furthermore, ASCs from $db/db$ and $db/+\text{ }$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

Figure 4: Labeling efficiency of Vybrant Dil-labeled ASCs isolated from $db/db$ mice was about 25–30% (a). ACMs containing Dil-labeled ASCs were then prepared and applied to the wounds of $db/db$ mice (b).

Figure 5: CD31-immunostained (a) and Dil-labeled (b) tissue on the transplant areas of $db/db$ mice at 2 weeks. The transplanted Dil-labeled ASCs were mainly incorporated into regenerated granulation and epithelial tissues. Some microvessels were double-labeled with Dil and CD31 (c). Each photograph is representative of 6 experiments.
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 upregulate 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 regenerating 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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