Effects of Adipose-derived Stromal Cells and of their Extract on Wound Healing in a Mouse Model

In this study, the authors investigated the effects of adipose-derived stromal cells (ADSCs) and of their extract on wound healing. After creating wound healing splint model on the backs of mice, ADSCs and their extract were applied. Wound healing rates were calculated at 3, 5, 7, 10, and 14 days after the wounding, and tissues were harvested at 7 and 14 days for histological analysis. Wound healing rates were significantly higher at 7, 10, and 14 days in the cell group than in the control, but in the cell extract group wound healing rates were significantly decreased ($P<0.05$). Histological scores and capillary densities in the cell group were significantly higher at 2 weeks ($P<0.05$). In the cell group, thick inflammatory cell infiltration and many capillaries were observed at 1 week, and thick epithelium and numerous large capillaries were observed at 2 weeks. The present study suggests that ADSCs accelerate wound healing as known, and the effects of ADSCs on wound healing may be due to replacing insufficient cells by differentiation of ADSCs in the wound and secreting growth factors by differentiated cells, and not due to the effect of factors within ADSCs.

Key Words: Adipose-derived Stromal Cell; Cell Extract; Wound Healing

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penicillin and 100 units/mL streptomycin (Gibco BRL, Grand Island, NY, USA). After mincing the fat pads, the minced fat tissue was digested at 37°C for 1 hr with intermittent shaking in PBS with 0.03% collagenase (Sigma) and neutralized with same amount of basic media (Dulbecco's Modified Eagle's Media high glucose [DMEM-hg, Gibco]) containing 10% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin and 100 units/mL streptomycin (Gibco). After filtration through a 25-μm filter and centrifugation (250 g, 10 min), the floating fat and adipocytes were removed. The cells in precipitated layer were cultured in basic media in humidified air with 5% (v/v) CO₂ at 37°C. The non-adherent cells were washed with PBS after 24 hr and the media was changed every 3 days. Before the cells were confluent, the cells were subcultured after isolation with 0.25% trypsin-EDTA (Gibco). The cells thus obtained were regarded as ADSCs, which were used for the experiments at passages 1-5.

**Wound healing model and ADSCs transplantation**

The experimental mice were randomly divided into three groups and the excisional splinting model was generated as described previously (8). In brief, after hair removal from the dorsal surface and anesthesia, two 6-mm full thickness skin defects on the back of mouse with silicone rings. (Fig. 1). The animals were housed individually. We tested the adhesive on the skin in mice prior to this experiment and did not find any skin irritation or allergic reaction.

**Wound analysis**

Digital photographs of wounds were taken at days 3, 5, 7, 10, and 14. Time to wound closure was defined as the time at which the wound bed was completely re-epithelialized and filled with new tissue. Wound area was measured by tracing the wound margin and calculated using an image analysis program (Image J; National Institute of Health, Bethesda, MD, USA). The investigators who measured the wound were blinded. The wound healing rate was calculated as follows: (Area of original wound-Area of remaining wound)/Area of original wound × 100.

**Histological examination**

Mice were sacrificed at 7 and 14 days, when skin samples including the wound and 5 mm surrounding skin, were harvested. The specimens were fixed in 10% neutral buffered formalin for at least 25 hr at room temperature. After fixation, perpendicular sections to the anterior-posterior axis of the wound were embedded in paraffin. Five μm-thick sections were stained with hematoxylin and eosin. As part of the histological evaluation, all slides were examined in blind fashion under ×40 to ×100 magnifications. Each slide was given a histological score ranging from 1 to 12: 1-3, none to minimal cell accumulation, no granulation tissue or epithelial travel; 4-6, thin, immature granulation, dominated by inflammatory cells with a few fibroblasts, capillaries, or collagen deposition, and minimal epithelial migration; 7-9, moderately thick granulation tissue, dominant inflammatory cells, more fibroblasts and collagen deposition, extensive neovascularization, and minimal to moderate migrating epithelium; 10-12, thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition, and epithelium partially to completely covering the wound (9).

**Immunohistochemical examination**

Five μm-thick sections were placed on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). The sections were defaraffinized, rehydrated through graded alcohols, pretreated with 3% hydrogen peroxide in PBS for 15 min to block endogenous peroxidase activity, and then blocked for non-specific protein binding with 3% rabbit serum (Sigma) in PBS. Primary rat anti-mouse CD31 antibodies (platelet endothelial cell adhesion molecule-1 [PECAM-1], BD Phar-
mingen™, San Diego, CA, USA) are diluted 1:50 in PBS and applied on the sections. The slides were incubated with the antibody overnight at 4°C in a humid chamber. After three washes with PBS for 3 min each, sections were flooded and incubated for 40 min at 36°C with anti-rat IgG-peroxidase (Sigma) diluted 1 in 500 in PBS. The slides were washed in PBS. Then, the detected antigens were visualized by incubation with 3,3′-diaminobenzidine tetra-hydrochloride solution (DAB peroxidase substrate, Sigma). Finally, sections were counterstained with Mayers hematoxylin and mounted.

Capillary density was assessed morphometrically by examining three fields per section of the wound between the edges in six successive sections after immunofluorescence staining for endothelial cells with anti-CD31 antibody. Capillaries, identified by positive staining for CD31 and appropriate morphology, were counted by a single observer in blind fashion under ×400 magnification. Capillary density was calculated as mean number of capillaries per high power field (HPF) (10).

Statistical analysis

All results were expressed as mean ± SEM. Statistical analysis was performed with ANOVA followed by Duncan’s new Multiple Range Test using SPSS for Windows (Version 15.0). P values <0.05 were considered to denote statistical significance.

RESULTS

Wound healing rates

Wound healing rates were measured at 3, 5, 7, 10, and 14 days after the wounding. The rates were 29.7 ± 9.5%, 32.2 ± 9.3%, 45.6 ± 16.4%, 61.0 ± 19.1%, and 89.6 ± 6.6%, respectively, in the control group; 13.8 ± 10.3%, 28.6 ± 15.3%, 57.7 ± 18.6%, 80.1 ± 5.8%, and 98.3 ± 2.5% in the cell group; and 10.0 ± 4.0%, 16.2 ± 10.2%, 22.4 ± 20.6%, 53.8 ± 28.5%, and 92.0 ± 4.2% in the cell extract group, respectively. In all groups, wound healing rates increased with time. Wound healing rates in the two experimental groups were significantly lower than in the control group at 3 days after wounding, but were significantly higher at 7, 10, and 14 days after wounding (P < 0.05).

In the cell extract group, wound healing rates were significantly lower at 3, 5, and 7 days after the wounding and were not significantly different from those of the control at 10 days. Furthermore, wound healing rates were lower in the cell extract group than in the cell group from 5 days after wounding (P < 0.05) (Fig. 2).

Histology and immunohistochemistry

The histological scores of wounds were 3.3 ± 0.6 (control group), 4.5 ± 0.7 (cell group), and 3.1 ± 0.6 (cell extract group) at 1 week after the wounding and 5.2 ± 0.8 (control group), 8.0 ± 1.9 (cell group), and 6.2 ± 0.9 (cell extract group) at 2 weeks. The cell group had significantly higher scores than the other two groups at 1 and 2 weeks after wounding (P < 0.05), and no significant difference was observed between the control and cell extract groups (Fig. 3).

Histological evaluation of week 1 and 2 wounds disclosed that the cell group had enhanced cellularity (Fig. 4) and increased vasculature (also shown in Fig. 5). Granulation tissue in the cell group appeared to be thicker and larger than other groups (Fig. 4). In addition, the cell group wounds appeared to have increased reepithelialization. However, in the cell extract group, cellularity and granulation tissue were similar to those of the control at 1 and 2 weeks after wounding (Fig. 4), and decreased vascularity (also shown in Fig. 5).

Mean capillary densities were 9.0 ± 3.6/HPF (control group), 9.8 ± 2.4/HPF (cell group), and 9.3 ± 6.2/HPF (cell extract group) at 1 week, and there was no significant difference between the groups. At 2 weeks, mean capillary densities were...
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17.3 ± 3.0/HPF (control group), 24.5 ± 6.7/HPF (cell group), and 19.1 ± 4.9/HPF (cell extract group) and all groups showed higher capillary densities than those at 1 week after the wounding. In particular, capillary density was significantly

![Fig. 4. Micrographs of wound bed and wound margin in each group at 1 and 2 weeks after wounding (H&E stain, 40). Wound edges are indicated by arrows. At 1 week after wounding, the control group shows thin granulation tissue containing many neutrophils and lymphocytes (A), the cell group shows thick inflammatory cell infiltrations with many capillaries (B), and the cell extract group has more granulation tissues than in the control group (C). At 2 weeks after wounding, the control group shows thick granulation tissues with little re-epithelialization (D), the cell group shows slightly thicker epithelium and greater granulation tissue (E), and the cell extract group shows less re-epithelization and granulation tissue than in the cell group at 2 weeks (F).](image1)

![Fig. 5. Micrographs of wound bed and wound margin in each group at 1 and 2 weeks after wounding (Immunohistochemical staining for CD31, × 100). Capillaries with endothelial cells are stained brown (thin arrows). Wound edges are indicated by thick arrows. At 1 week, some small numbers of capillaries are observed in granulation tissues in the control group (A) and the cell extract group (C), but large numbers of capillaries are observed in the cell group (B). At 2 weeks after wounding, increased capillary development is noted in the control group (D), the cell group (E), and the cell extract groups (F). Particularly, in the cell group numerous larger capillaries are observed in comparison with other groups.](image2)
higher in the cell group than those in the other groups at 2 weeks ($P<0.05$) (Fig. 6).

**DISCUSSION**

Cutaneous wound healing process requires interactions between cells in the dermis and epidermis and the release of chemical mediators from inflammatory cells, fibroblasts, and keratinocytes. The proliferation of mesenchymal cells and capillaries, as well as the influx of macrophages into granulation tissue, serves to replace the dermal defect and to provide substrates and inducers for re-epithelialization. Because various cell types participate in wound healing process, various cell-based therapies offer promising therapeutic strategies to improve wound healing in physiological and pathological conditions. In recent studies, the transplantation of BMSCs has been reported to activate the healing process due to their capacity to differentiate in the skin epidermis and appendages, thus to mediate dermal regeneration (6). And also several studies have recently demonstrated accelerated rates of wound closure after transplantsations of BMSCs (11), mesenchymal stem cells (12), or ADSCs (7). In our experiments, the ADSC-treated group showed significantly more rapid wound healing rates and had higher histological scores than those of the control group. Authors used the wound healing splint model, which is invented to prevent skin contraction around the wound, allow the wounds to heal via granulation and re-epithelialization (8). Acceleration of wound healing rates by ADSCs treatment in this model is thought to be caused by the substitution of deficient cells after differentiation of ADSCs as stated in previous reports (6).

Neovascularization is a crucial step in wound healing process (2), which is necessary to sustain newly formed granulation tissue and ensure the survival of keratinocytes. Wu et al. (6) demonstrated that BMSC-treated wounds have higher capillary densities, and suggested that BMSCs promote angiogenesis. In our study, ADSC-treated wounds had significantly higher capillary densities than that of the control group, which suggested that ADSCs, like BMSCs, promote neovascularization. The promoting neovascularization by ADSCs is considered one of factors which accelerate the wound healing by ADSCs.

Many types of cytokines and growth factors are responsible for inflammation, re-epithelialization, the formation of granulation tissue, and neovascularization during the healing process. Thus the application or induction of these cytokines and factors is known to accelerate wound healing (13). Kim et al. (7) reported that paracrine factors secreted by ADSCs induce collagen synthesis and promote wound healing through fibroblast activation, migration, and proliferation. Gaustad et al. also reported that cell extracts, which contain growth and differentiation factors, can be used as an alternative to stimulate surface molecules to promote the differentiation of ADSCs to target cells (14). Cell extract was thought to differentiate mesenchymal stem cells, which are located in tissues around wound, into cells required for the wound healing process. In order to confirm the note of cell extract, we performed this comparative study on the effect of ADSCs and ADSC cell extract on wound healing to investigate whether cell extract from ADSCs could have beneficiary effect on wound healing. Our results showed that the cell extract group showed rather lower wound healing rates than those of the control group throughout experimental periods, and no significant difference versus the control until 2 weeks. Because ADSCs are not differentiated cells, the promotion of the specific differentiation of mesenchymal stem cells by cell extract of ADSCs would not occur. This result was in contrast to a previous report (14). Accordingly, we consider that ADSC cell extract has no effect on wound healing, and that many factors in ADSC cell extract are less helpful in wound healing.

In conclusion, our experiments showed that ADSCs treatment enhanced wound healing as previously reported. Furthermore, our results suggest that this beneficiary effects of ADSCs on wound healing may be not caused by factors in ADSCs, but rather by the substitution of deficient cells after differentiation of ADSCs.

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