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Original Research Article

Mesenchymal stem cells promote incision wound repair in a mouse model

Dong-Jie Li, Chuan-An Shen, Tian-Jun Sun, Lin Zhang, Hu-Ping Deng and Jia-Ke Chai*

Department of Burns and Plastic Surgery, The First Affiliated Hospital of PLA General Hospital, Beijing, 100048, China

For correspondence:* ***Email:* *chaijiake403@gmail.com;* ***Tel:*** *0086-10-66867072;* ***Fax:*** *0086-10-68989181*

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Abstract

Purpose: *To investigate the wound healing process via the application of mesenchymal stem cells (MSCs) in a mouse model.*
Methods: *MSCs were collected from the bone marrow of the femur and tibia of 6 – 12-week-old C57BL/6 mice. Full-thickness cutaneous wounds (4 × 2 cm) were made by incision on the dorsal side of the mice. The wound was then subjected to one of four random treatments: phosphate-buffered saline (PBS) solution, 3T3 fibroblasts, naive MSCs, or interferon gamma-activated MSCs. Chalkley method was used to determine vascular density. A score was given, for each field examined, for CD31-positive areas, and the results of blind analysis were confirmed by independent analysis of a second evaluator.*
Results: *The tensile strength of the wound area was significantly lower in older versus younger mice ($p \leq 0.0007$). Only one quarter of the mean force was required to disrupt wound integrity in older mice compared to young mice. Treatment with MSCs showed positive effects on wound healing. Activated MSCs showed the greatest efficacy at a dosage of 5×10^4 activated MSCs/8 cm^2 of wound area or 6, 250 cells/cm^2.*
Conclusion: *The results suggest that MSC therapies enhance the tissue regeneration capacity in mice, especially in older populations, through effective transdifferentiation into the epithelium.*

Keywords: *Mesenchymal stem cell, wound healing, mouse*

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INTRODUCTION

Tissue damage, especially skin defects, remains a major health issue all over the world. This is mainly due to microbial infections, congenital diseases, poor conditioning, or damage sustained over a very large area. Numerous strategies have been employed to treat skin defects, such as the use of synthetic membranes, porcine xenografts, allografts, and permanent skin substitutes. However, these approaches have met with limited success. Typically, wound healing has three phases: inflammation, proliferation, and remodeling [1]. These stages require fibrin clot formation, cell migration, extracellular matrix deposition, dermal reconstitution, and re-epithelialization [2–5]. The main growth factors involved in the process of wound healing include the fibroblast growth factor (FGF) family, platelet-derived growth factor (PDGF), tumor necrosis factor (TNF)-α, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF) [6]. Studies on the positive effects of exogenous growth factors on wound healing have failed to confirm their efficacy in clinical trials.

Mesenchymal stem cells (MSCs) are self-renewing and can differentiate into various cell and tissue types, such as skin cells. Apart from being exogenous growth factors, MSCs have been shown to play important roles in the wound healing process, making them a promising candidate for cell-based therapy. The multipotency of MSCs has been well established *in vitro* [7–10], although there have been few *in vivo* studies.

Autologous MSC-based therapy for tissue regeneration in older populations eliminates the need for extensive research on inflammatory responses upon usage of this therapy for tissue regeneration. Administration of MSCs in the wound region enhances regeneration by recruiting endothelial cells and activated macrophages, and also increases vascularity [11,12].

The functions of macrophages are greatly restricted in older individuals due to various factors, such as altered cytokine production, diminished expression of Toll-like receptors, and signaling via the ERK and MAPK pathways [13]. As macrophages play important roles in the secretion of angiogenic growth factors and recruitment of other molecules responsible for wound healing, alterations in these cells may hamper wound healing responses. This phenomenon is especially prominent in older populations [14]. The present study was performed to investigate the effects of MSCs on the healing of incisional wounds in both young and aged C57BL/6 mice.

EXPERIMENTAL

Animals

Both young (6 – 12 weeks old) and aged (18 months old) male C57BL/6 mice were obtained from the breeding stock of PLA General Hospital, Haidian District, Beijing, China. All mice were housed in the Department of Burn and Plastic Surgery vivarium (Beijing, China) under specific pathogen-free conditions with controlled light (12-hour light/dark schedule; lights on 06:00 – 18:00) and humidity. Sterile water and food were provided *ad libitum*. Bedding consisted of wood shavings (aspen shavings; NEPCO, Warrensburg, NY). Cages were cleaned and sterilized every 2 weeks. The protocol of the animal experiment was approved by the Animal Care Committee of the Department of Burn and Plastic Surgery, Affiliated Hospital of PLA General Hospital (approval no. 4203441). All experimental procedures were conducted in accordance with institutional guidelines and conformed to the National Research Council Guide for Care and Use of Laboratory Animals [15].

Cell culture

Preparation and culture of MSCs were conducted according to established protocols [16–18]. MSCs were collected from the bone marrow (BM) of femurs and tibias of young C57BL/6 mice (6 – 12 weeks old). BM was flushed through a 40-mm filter into MSC medium, which consisted of 10 % fetal bovine serum (Sigma Aldrich, St. Louis, MO), 40 % F-12 nutrients (Invitrogen, Carlsbad, CA), 40 % alpha-modified Eagle's medium (Invitrogen), and 1 % antimycotic solution (Invitrogen). The cells were plated in MSC medium at a density of 2×10^7 per 9.6 cm^2 plate and maintained at 37 °C in a 5 % CO_2 incubator. After 72 hours of proliferation, the non-adherent cells (non-MSCs) were removed and the adherent cells (MSCs) were cultured for an additional 7 days. Depletion of contaminating macrophages was carried out by immunomagnetic selection with biotinylated antibodies to CD11b (eBioscience, San Diego, CA), CD45 (eBioscience) and MACS anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The negative control cell culture was performed at a density of 10^6 cells per flask. Flow cytometry of the isolated immune-depleted cells indicated < 3 % contamination with $CD45^+$ cells. Thereafter, MSCs (passage 4 cells) were cultured with recombinant murine interferon gamma at 500 U/mL, administered every 3 days. These MSCs were stained with carboxy-fluorescein diacetate and administered at specified doses.

Wound healing model

A total of nine mice were used in the experiments in each group. Using a #15 surgical blade, a 4 × 2 cm (8 cm^2) incisional wounds was created on the dorsal side of the C57BL/6 mice under anesthesia (ketamine and xylazine @ 40 mg kg^{-1}). The wound was than subjected to four treatments: phosphate-buffered saline (PBS) solution, 3T3 fibroblasts, naive MSCs, or interferon gamma-activated MSCs. The dosage of activated MSCs was 50,000 cells per wound. Following wound closure (three staples in each wound), the mice were treated with buprenorphine for postoperative analgesia. Macrophage depletion was performed using clodronate (dichloro methylene diphosphonate) [19]. Administration was performed intraperitoneally (0.2 mL) at 3-day intervals for 7 days before opening of the wound, and continued until the sixth day after the operation.

Characterization of cell lines

The experimental animals were treated with either enhanced green fluorescent protein (eGFP)-positive BM cells or MSCs ($3^{rd} – 4^{th}$ passage) at a concentration of 10^6 cells/mL or 10^5 cells/ml, respectively. The MSCs were prepared by washing and suspension in 0.8 % methylcellulose (Sigma Aldrich). Methylcellulose was used to create a semisolid environment that could successfully prevent cell loss from the wound region. The viability of cells was also assessed after maintenance in culture in methylcellulose for 7 days. Following incubation for 30 minutes at 4 °C, immunophenotyping of cells was performed using fluorescein isothiocyanate-conjugated antibodies against murine CD11b, CD29, CD31, CD44, and CD45 (BD Pharmingen, Heidelberg, Germany). Fluorescence-activated cell sorting (FACS) with a 488 nm argon laser was used for the analyses, which were performed using CellQuest software (Becton Dickinson, San Jose, CA) for a total of at least 10,000 events.

Analysis of vascular density

Anti-CD31 antibody (BD Pharmingen) was used for immunohistochemical detection of blood vessels. Cryosections 4-mm thick, blocked with 1 % bovine serum albumin (Sigma Aldrich) and preincubated with hydrogen peroxide in methanol, were incubated with primary antibody at 4 °C – 8 °C overnight. Sections were then incubated for 1 hour at room temperature with peroxidase-conjugated secondary antibody. Then, the samples were treated with diaminobenzidine (DAB)–hydrogen peroxide (Sigma) and counterstained with hematoxylin before analysis by optical microscopy. Chalkley method was used for the determination of vascular density [20]. The slides were examined at 250 × magnification, at a randomly distributed point in a specific area (0.196 mm^2). The scores were given per field examined for CD31-positive areas. The results of the blinded analysis were confirmed by independent analysis by a second evaluator.

Statistical analysis

Each experiment was conducted on three different animals concurrently and the whole experiment was repeated three times. Comparisons were performed after converting the tensile strength to a percentage of that in PBS solution-treated control animals. Comparison of paired samples was performed by Student's paired *t* test, multiple group comparisons were performed by analysis of variance (ANOVA), and differences between groups were determined using Bonferroni's method. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

RESULTS

The effects of aging on tensile strength were determined by examining the wound tensile strength in both young and aged mice. The results showed that the wound tensile strength in older mice was considerably lower than that in young mice ($p \leq 0.0007$). Compared to young mice (0.2 ± 0.003 N), about one quarter of the mean force (0.05 ± 0.002 N) was required to disrupt wound integrity in older mice (Figure 1).

Treatment with a single dose of MSCs increased wound healing by about 10 % relative to the control group treated with PBS. However, activated MSCs showed the greatest efficacy (25 % ± 2.8 % higher than controls) at a dosage of 5×10^4 activated MSCs/8 cm^2 of wound area or 6, 250 cells/cm^2. This effect was extended even up to day 14. This dose was lower than those reported in other clinical studies showing the effects of activated MSCs on wound healing.

Upon determination of the optimal dosage, i.e., 6, 250 cells/cm^2, three separate experiments ($n = 6$) were performed for direct comparison of tensile strength between young and aged mice. Treatment of young and aged mice was performed simultaneously to eliminate differences in various parameters. Comparison of the results was carried out based on histological observation after excision of wounds on day 7.

Immunohistochemistry with anti-CD31 antibody (Fig. 2) was performed to analyze the vascular density of the dermal substitute. Only a slight increment was detected in the MSC-treated mice compared to the controls.

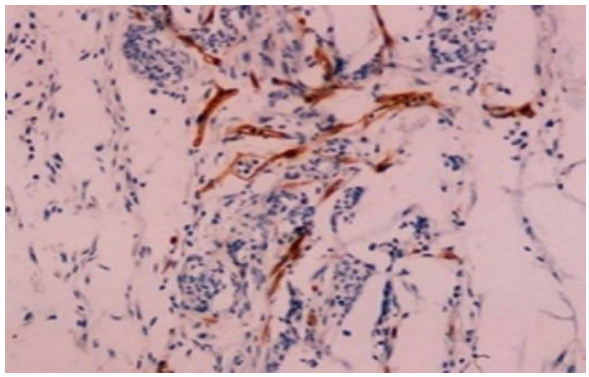

Figure 2: Immunohistochemical analysis of the dermal substitute with anti-CD31 antibody to assess vascular density

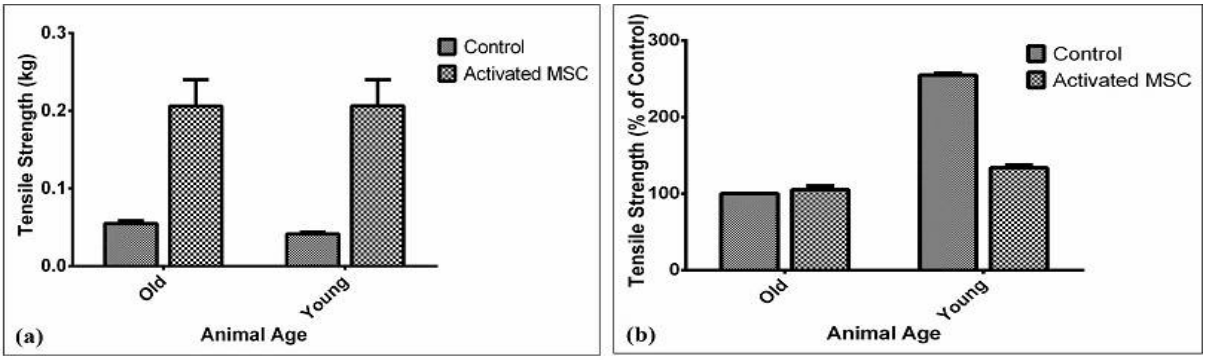

Figure 1: Effect of activated mesenchymal stem cell (MSC) administration on tensile strength in incisional wounds in young and aged animals; (a) data are expressed as the means of three replicates; (b) aggregate data of the replicates normalized to control animals and expressed as the percentage change from the controls

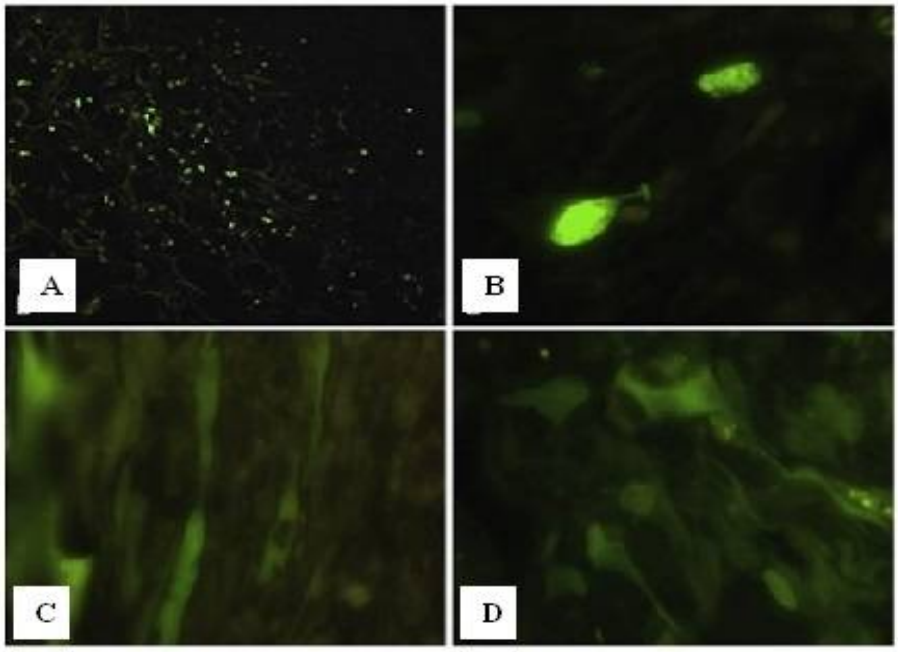

Figure 3: Representative fluorescence microscopy images of MSCs in incisional wounds on days 4 and 8. (A) Four days after the procedure, eGFP cells were seen only among the fibers of the dermal substitute. (B) On day 8, some eGFP cells were visible in the adjacent tissue. (C) On day 4 after the procedure, eGFP-positive elongated MSCs were only present in the wound bed. (D) On day 8, MSCs were still predominantly seen in the wound bed, but had a different shape. Original magnification 100× (A) and 400× (B – D)

Increase in cell number was evident for both bone marrow (BM) cells and MSCs in day-8 versus day-4 samples. Four days after the procedure, enhanced green fluorescent protein (eGFP)-positive cells were seen only among the fibers of the dermal substitute (Figure 3A). On day 8, a few oval eGFP-positive cells were also seen in the adjacent tissue (Figure 3B). Samples from MSC-treated animals on day 4 after the procedure showed eGFP-positive elongated MSCs among the cells present in the wound bed (Figure 3C). On day 8, MSCs were still predominantly seen in the wound bed, but began to show alterations in their morphology (Figure 3D) due to the regeneration of cells in the wound area.

DISCUSSION

Wound healing is influenced by a variety of factors, including wound location, personal history of disease, allergy, and other types of skin defects. There has been a great deal of research regarding application of various scaffolds, cells, and growth factors to aid and accelerate the process of skin regeneration [21]. The marked regenerative ability of the skin epidermis is due to the presence of epidermal stem cells that continuously produce keratinocytes. This principle has been utilized to develop various types of tissue-engineered skin grafts in clinical applications. However, the inability to regenerate skin appendages, such as

sweat glands and hair follicles, are major limitations. Thus, there is a need for research on skin grafts that complement its structural and functional properties [22]. MSCs have the ability for site-specific differentiation in response to cues provided by different organs. MSCs can differentiate not only into cells of mesenchymal lineage, but also into endothelium and endoderm cells *in vitro* [23]. This phenomenon suggests the suitability of MSCs for cutaneous wound healing. Therefore, we assessed the possible therapeutic value of MSCs in terms of promoting cutaneous wound healing.

Under normal conditions, small amounts of MSCs are present in the peripheral blood system, which increases markedly under stressful conditions, such as large cuts or acute burns [24]. Technically, MSCs can be harvested with greater efficiency from the BM, where they are present in higher concentrations. The observed exponential growth of MSCs in the peripheral blood can be due to their release from the BM. The suitability of BM, rather than the blood system, for MSC isolation has also been demonstrated [25]. MSC concentration depends on age as well as the type and size of injury. In older individuals, the concentrations of MSC precursors are considerably reduced in both peripheral blood vessels and BM. Thus, we hypothesized that the regeneration mechanism could be improved in older individuals by external infusion of MSCs. The increased regeneration capability observed in older mice upon treatment with activated MSCs validated this hypothesis. We speculate that macrophage function is restored by MSCs, thereby improving the regenerative process.

The tensile strength, defined as the breaking strength per unit thickness of tissue, is used to describe the healing rate of wounds and is a useful comparative measurement for wounds [26]. We investigated the impact of transplanted MSCs on tensile strength as a measure of the degree of wound healing. An optimal scar is one that most resembles the collagen structure, architecture, and tensile strength of normal skin. Thus, the biological status of the repaired wound depends on proper chemical reorganization of collagen fibers during the remodeling stage, which affects the tensile strength. The plot of tensile strength as a function of time has a characteristic exponential curve [27]. During the first days of the wound reparative process, the tensile strength increases moderately. Then, acceleration of the healing process occurs with a rapid increase in the tensile strength, which reaches a steady state at about 8 weeks with a maximum peak more than 1 year after the injury [27]. Any interference with the collagen synthesis process will be reflected in the biomechanical properties of the tissue, and in the wound recuperation period [27]. In this study, tensile strength was significantly greater after surgery in the group treated with MSCs compared with the control group. Although the strength of repaired skin incision did not reach that of uninjured skin, it was demonstrated previously that incisional skin wounds regain only 40 % of the tensile strength of unwounded tissue 120 days after wounding [27].

In the present study, incisional wounds treated with MSCs regained almost double the strength compared with untreated wounds, thus validating our hypothesis. Experiments on healing excisional wounds in a human mesenchymal stem cell (hMSC)-populated porcine skin substitute showed that the wound size in nude rats was significantly smaller in the stem cell-treated groups compared with controls [28]. Moreover, a positive role of hMSCs in limiting radiation-induced skin lesions (delay in lesion development, reduced radiation dermatitis, and faster healing rate) has been demonstrated [29]. These previous studies support our observations and highlight the promise of MSCs for wound healing.

CONCLUSION

We have demonstrated the ability of activated MSCs to promote wound healing in acute incisional wounds, as reflected in regained tensile strength. The process is slow and further studies are required before human trials can be carried out. The significant improvement in the amount of tensile strength regained in the mouse model provides a potential approach to overcoming age-related barriers in healing capacity, and also shows the possibility of overcoming various surgical complexities in aged patients.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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