Original Article
Platelet-derived growth factor and stromal cell-derived factor-1 promote the skin wound repairing effect of bone mesenchymal stem cells: a key role of matrix metalloproteinase 1 and collagens

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Abstract: Bone marrow mesenchymal stem cells (BMSCs) have been applied to treatment of skin wounds. In the current study, the effect of BMSCs on secretion of several cytokines during skin wound healing was investigated. In addition, the mechanism underlying the promoting effect of platelet-derived growth factor (PDGF) and stromal cell-derived factor-1 (SDF-1) on BMSCs was also preliminarily explored. BMSCs were isolated from femur and tibia of rats and characterized by surface antibodies. Skin wounded rats was employed as the in vivo model for evaluating the effect of BMSCs on skin healing and secretion of basic fibroblast growth factor (bFGF), interleukin 1β (IL-1β), PDGF, SDF-1, and tumor necrosis factor α (TNF-α). The promoting effect of SDF-1 and PDGF on the function of BMSCs was also assessed by focusing on the activity of matrix metalloproteinase 1 (MMP-1) signaling and deposition of collagens. Administration of BMSCs promoted skin wound healing and induced the production of bFGF, IL-1β, PDGF, SDF-1, and TNF-α. Moreover, PDGF treatment recruited BMSCs to injured sites and strengthened the effect of BMSCs on skin wound by suppressing activity of MMP-1. Similarly, SDF-1 treatment also increased the treating effect of BMSCs on skin injury, which was through the deposition of collagen I and collagen III. Both indicators exerted their effect on BMSCs in a dose-dependent manner. Findings outlined in the current study indicated that administration of BMSCs accelerated wound healing and enhanced the production of PDGF and SDF-1, which would contribute to recruitment of BMSCs in injured area and deposition of collagens.

Keywords: Bone mesenchymal stem cells, platelet-derived growth factor, skin wound, stromal cell-derived factor-1, matrix metalloproteinase 1, collagen

Introduction

Chronic skin wound is defined as a process during which injured skin fails to achieve anatomic and functional integrity orderly and timely [1]. There are complicated factors driving the onset of chronic skin wounds, including repeated trauma, continued pressure, ischemia or localized and systemic illness, which all result in deficient body ability to handle the wounds [2]. According to the investigation conducted in 2010, the incidence of chronic skin wounds in United States is five to seven million cases each year [3]. Worse still, more than 50% of the chronic wounds poorly response to current clinical treatments [4] due to the fact that wound repair is a highly complex biological process which requires the involvement of several cell types, the extracellular matrix, and other regulatory factors [5]. Therefore, emerging theories infer that the wound-repair treatments should depend on the development of products with similar composition to that of skin [6].

In recent years, mesenchymal stem cells (MSCs) have captured substantial interests for their therapeutic potential for tissue engineering [7-9]. Since the first clinical trial performed with stem cells offered curing hope for patients, numerous researchers have explored the function of the cells in types of disorders in animal models or preclinical trials [7-9]. MSCs is characterized by high differentiation and expansive potential, ease of isolation and culture,
PDGF and SDF-1 strengthen skin injury repairing function of BMSCs

and lack of promotion of immune-rejection [5], especially those derived from bone marrow (BM).

The BM stroma contains two types of precursor cells, which can differentiate into hematopoietic cell (HC) and MC lineages. Among which, bone marrow mesenchymal stem cells (BMSCs) are defined as lineage expresses CD29, CD73 and CD90 while lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules [10]. Application of BMSCs for treatment of different diseases has been performed extensively in recent years. For example, Amodo et al. have used ex vivo-expanded allogeneic BMSCs for treatment of swine ischemic myocardium and found that BMSCs administration led to alleviated contractile dysfunction and suppressed pathologic scarring of the infarcted left ventricular wall [11]. In another study conducted by Li et al., the authors indicated that intravenous injection of BMSCs reduced brain infarction area in the brains of stroke rats [12]. Regarding the improving potential of BMSCs on skin wounds, lots of studies have provided solid lab evidence for BMSCs’ effect to accelerate wound healing [5, 13]. It is well recognized that it’s the cytokines necessary for wound healing secreted by BMSCs that contribute to the wound closure, which include epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), stromal cell-derived growth factor-1 (SDF-1), etc [14, 15]. Among all these cytokines, SDF-1 is proved to play an important role in the recruiting of BMSCs to wound area [16]. Furthermore, the expression of SDF-1 in endothelial cells is induced by platelet-derived growth factor (PDGF) which strengthens the differentiation potential of BMSCs [17]. Given that SDF-1 and PDGF themselves are also considered as promoters for wound healing [18], it is reasonable to assess the roles of SDF-1 and PDGF in the wound healing effect of BMSCs.

Thus, in the current study, it was hypothesized that the wound healing accelerating function of BMSCs should be further promoted by SDF-1 and PDGF. A series of assays were performed with skin wounded rats to assess the involvement of SDF-1 and PDGF in the skin wound treating effect of BMSCs. Moreover, the promoting effect of the two factors on the function of BMSCs was also assessed. Findings outlined in our study indicated the administration of BMSCs would increase the levels of SDF-1 and PDGF in rat models and co-administration of BMSCs with either factor would strengthen the wound healing effect of the cells.

Materials and methods

Chemical and antibodies

Antibody against matrix metalloproteinase-1 (MMP-1) (bs-4597R) was purchased from Bioss (Beijing, China). Antibodies against tissue inhibitors of matrix metalloproteinase (TIMP) (WL0259), collagen I (WL0088) and collagen III (WL0136) were purchased from Wanleibio (Shenyang, China). Antibody against β-actin (sc-47778) was purchased from Santa Cruz (USA). Secondary goat anti-rabbit (A0208) and goat anti-mouse (A0216) IgG-HRP antibodies were purchased from Beyotime Biotechnology (Shanghai, China). Total Protein Extraction Kit (WLA019) was purchased from Wanleibio (Shenyang, China). PDGF and SDF-1 were purchased from Peprotech (Rocky Hill, NJ). PKH-26 (PKH-26) was purchased from Sigma (USA). Enzyme-linked immuno sorbent assay (ELISA) kits for basic fibroblast growth factor 2 (FGF2) (CEA551Ra), interleukin 1β (SEA563Ra), PDGF (SEC921Ra), SDF-1 (SEA-122Ra), and tumor necrosis factor α (TNF-α) (SEA133Ra) were purchased from Uscn Life Science Inc. (Wuhan, China).

Animals

Health male Sprague-Dawley (SD) rats were provided by Changsheng Biotechnology (Liaoning, China) and housed in cages at room temperature (20-25°C) with a constant humidity (55 ± 5%) with food and water available ad libitum. All the assays with animals were performed following the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of The People’s Hospital of Liaoning Province.

Isolation and characterization of BMSCs

Femur and tibia of isoflurane anesthetized rats was carefully dissected and excised. Bone marrow were collected with Dulbecco’s modified eagle medium (DMEM) and the collected suspension was subjected to 157 g for 10 min.

8254 Int J Clin Exp Pathol 2017;10(8):8253-8262
Afterwards, stock collagenase solution was added to the suspension to make a final concentration of 0.5 Units/ml. Then the plates with contents were placed on a Shaker and incubated with constant agitation for 60 ± 15 min at 37°C. After incubation, the content was triturated with a 25 ml pipette for 2-3 min and filtered with 70 μm nylon net and centrifuged at 157 g for 10 min. After being resuspended with PBS and centrifuged at 157 g for 10 min for three times, the collected cells were cultured in DMEM at 37°C in an atmosphere of 5% CO₂ for 24 h. Then the suspension cells were discarded and the adherent cells were used as BMSCs for subsequent assays. The osteogenic differentiation and adipogenic differentiation potential of BMSCs were detected using Alizarin Red S and Oil Red O methods, respectively. 30 minutes before transplantation, the expression of specific indicators on the surface of ADMSCs, including CD29, CD45, and CD90 were detected using a FACS flow cytometer (Accuri C6, BD, USA). Then the BMSCs were labeled with PKH-26 for in vivo illustration.

**Induction of skin injured rat model and BMSC administration**

Before induction of skin injuries, rats were subjected to tail vein infusion of 1 × 10⁷/ml PKH26 labeled BMSCs. One week after BMSC injection, back skin of isoflurane anesthetized rats were scratched with a scalpel for induction of skin injuries. At the 3rd, 6th, 9th, and 12th day after model induction, blood samples of rats were collected from tail vein for ELISA. 14 days after model induction, rats were sacrificed and skin tissues around the injuries area were sampled and preserved for subsequent assays.

For rats in PDGF and SDF-1 treated groups, different concentrations of the two agents (5 ng/ml, 50 ng/ml, 100 ng/ml, and 500 ng/ml for PDGF; 1 ng/ml, 2 ng/ml, 10 ng/ml, and 50 ng/ml for SDF-1) were injected into the injured area at multiple points. Then all the rats were raised under same condition for 14 days before being sacrificed for further investigations. All the assays were represented by at least six replicates.

**ELISA**

The production of basic fibroblast growth factor (bFGF), interleukin 1β, PDGF, SDF-1, and TNF-α were determined using ELISA kits according to the manufacturers’ instruction.

**H&E staining**

The histological changes of skin tissues were observed using H&E staining: tissues under different treatments were placed into Bouin solution (4% formaldehyde) for perfusion fixation. Then they were dehydrated using different concentration of alcohol and vitrified in dimethylbenzene. Samples were embedded in paraffin, sectioned and stained with H&E and the results were detected under a microscope at 100 × magnification.

**Masson staining**

To assess the accumulation of collagens, Masson trichrome was utilized to demonstrate the change in tissue samples: briefly, sections were deparaffinized with toluene and rehydrated with graded alcohol. Three different dyes were used in order to differentiate between cells and extracellular matrix: (1) celestine blue solution was used for cell staining (dark blue-black); (2) acid fuchsin solution was used for elastin (pink); and 3) methyl blue solution for collagen (blue). The results were detected under a microscope at 100 × magnification.

**Immunofluorescent assay**

Skin tissues were permeabilized with 0.5% Triton X-100 for 30 min and then blocked with 10% goat serum for 15 min. Afterwards, tissues were washed and stained with 4,6-diamino-2-phenyl indole (DAPI) for 5 min at room temperature. The distribution and expression of PKH-26 in cells were imaged with a fluorescent microscope at 100 × magnification.

**Western blotting**

Total protein was extracted using the Total Protein Extraction Kit according to the manufacturer’s instructions and the concentration was determined using the BCA method according to the manufacturer’s instructions. Then 40 μg protein (20 μl) from each sample was subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, the membranes were rinsed with TTBS and were incubated with the primary antibodies against
Figure 1. Isolation and characterization of BMSCs. A. Representative images of morphology of BMSCs; the cells were shuttle shaped. B. BMSCs were characterized by positive expression of CD29 and CD90 and negative expression of CD45.
PDGF and SDF-1 strengthen skin injury repairing function of BMSCs

MMP1 (1:5000), TIMP (1:5000), collagen 1 (1:1000), collagen 3 (1:1000), β-actin (1:1000) (internal reference protein for total protein) for 1 h at room temperature. After three washes with TTBS, the membranes were incubated with secondary HRP-conjugated IgG antibodies (1:5000) for 40 min at 37°C. Following six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent and the images were recorded in the Gel Imaging System.

**Statistical analysis**

The data were expressed as mean ± SD. Difference between two groups was analyzed using Student’s t test with a significant level of 0.05 (two-tailed P value). All the statistical analyses were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA).

**Results**

**Isolation and characterization of BMSCs**

BMSCs were isolated from femurs and tibias of SD rats and identified by flow cytometry. As shown in **Figure 1**, the cells were shuttle shaped and positively expressed CD29 (72.8% of BMSCs vs. 3.9% of control) and CD90 (66.2% of BMSCs vs. 4.1% of control) while negatively expressed CD45 (4.4% of BMSCs vs. 6.6 of control), which was typical characteristics of MSCs. Moreover, as shown in **Figure S1A**, deposition of Alizarin Red could be detected for BMSCs while bone marrow cells (BMCs) had no reaction with Alizarin Red, representing the osteogenic differentiation potential of BMSCs. Similarly, in Oil Red O assays, deposition of Oil Red O could only be detected in BMSCs (**Figure S1B**). The above validation indicated the successful generation of BMSCs in the current study.

**BMSCs accelerated skin wound healing and increased expression of pro-healing factors**

In the current study, the BMSCs were labeled with PKH-26 for an in vivo illustration of the existence and distribution of the cells. Health rats and skin injured rats were all subjected to BMSCs administration and the existence of BMSCs was detected with a immunofluorescent microscope: BMSCs were stained red (**Figure 2**). As illustrated by H&E staining, induction of skin injuries destroyed the integrity of skin tissues with cell walls being broken and cells being irregular shaped. After administration of BMSCs, the shapes of cells were restored and infiltration of cells were alleviated (**Figure 3**).

Moreover, it was found that in health rats treated with BMSCs, the expressions of SDF-1α, TNF-α, IL-1β, bFGF, and PDGF stayed unchanged during the experiments (**Figure 4A-E**). In rats with skin injuries, the expression of all the indicators were initiated by BMSCs, representing a promoting effect of BMSCs on the secretion of...
the factors accelerating skin wound healing. However, the effect of BMSCs on the production of pro-healing factors increased during the first nine days while declined at 12\textsuperscript{th} day, possibly indicating that the effect of single administration of BMSCs would recede with time.

\textit{Administration of PDGF strengthened the wound healing effect of BMSCs by decreasing the expression of MMP-1}

To evaluate the effect of PDGF on the function of BMSCs, skin injured rats were co-administration with BMSCs and PDGF at different concentrations. As shown in Figure 5A, administration of PDGF increased the accumulation of BMSCs. The fibrosis process of injured skin was also ameliorated by co-administration of BMSCs and PDGF: fibrosis was stained blue in injured sites (Figure 5B). At molecular level, administration of PDGF decreased the expression of MMP-1 while increased the production of TIMP-1, which would accelerated the formation of scar tissues (Figure 5C) [19].

\textit{Administration of SDF-1 strengthened the wound healing effect of BMSCs by increasing the expression of collagens}

The effect of SDF-1 on the wound healing function of BMSCs was also detected in the current study. Administration of SDF-1 increased the
PDGF and SDF-1 strengthen skin injury repairing function of BMSCs

Figure 5. Co-administration of PDGF increased the skin injured healing effect of BMSCs via inhibition of MMP-1 signaling. A. Representative images of immunofluorescent detection of PKH-26. PDGF induced accumulation of BMSCs to injured area in a dose dependent manner. B. As illustrated by Masson staining, the accumulation of collagens (stained blue) was increased by PDGF co-administration. C. Representative images of western blotting validation of expression of MMP-1 and TIMP-1. The level of former indicator decreased with PDGF concentration while that of latter increased with PDGF concentration.

accumulation of BMSCs to injured sites (Figure 6A). However, it was found that the median concentration of SDF-1 (2 ng/ml) had the strongest inducing effect on BMSCs accumulation while higher concentration of SDF-1 restricted the distribution of BMSCs in injured area (Figure 6A). But in contrary to the changing pattern with BMSCs accumulation, SDF-1 also exhibited a promoting effect on BMSCs treatment in a dose-dependent manner similar to the effect of PDGF: as shown in Figure 6B, higher concentration of SDF-1 induced a stronger healing process as illustrated by H&E staining (Figure 6B). Moreover, administration of SDF-1 induced the production of collagen I and collagen III at injured sites (Figure 6C), which was corresponding to the scar tissue formation inducing effect of PDGF by suppressing MMP-1.

Discussion

Skin is the largest organ of body, which plays determining roles in protecting bodies, sweating, and feeling temperature [20]. To achieve those functions, composition and structure of skin are complicated, a “full-thickness” damage of which will result in impairments on multiple structures and cell types [21]. In ideal conditions, a regulated mode of wound healing process will take place with coagulation, formation of a fibrin clot, and progresses to an inflammatory phase following skin damages to maintain tissue homeostasis of skin [1]. However, skin wound healing process can be seriously dys-regulated due to complicated factors, which contributes to the pathological changes in many circumstances [20]. Therefore, skin wounds, especially those being chronic, still remain a challenge in clinical practice. Based on the above information, emerging studies have been performed in recent years by focusing on acceleration of the healing process of skin wounds. Among different potential therapeutic strategies, application of BMSCs has brought hopes to solve the matters following skin injuries. In the current study, our results...
PDGF and SDF-1 strengthen skin injury repairing function of BMSCs

further confirmed that administration of BMSCs could promote the healing process of skin wounds. Moreover, co-administration of BMSCs with PDGF and SDF-1 strengthened the skin repairing effect BMSCs, respectively.

Many factors, including growth factors, cytokines, interleukins, and tumor necrosis factors (TNFs) influence the wound healing process of skin injuries. Based on the results of ELISA, it was found that administration of BMSCs increased the secretion of bFGF, TNF-α, IL-1β, PDGF, and SDF-1α during the first nine days after skin injury induction. The time period was corresponding to the skin wound healing process as being illustrated by H&E staining. However, BMSCs administration had no effect on the expression of the above factors in heal skin tissues, which indicated that effect of BMSCs was to accelerate skin repair instead initiating pathways involved in the process. In addition, the levels of all the indicators were significantly down-regulated at 12th day after model induction. Such results might indicate that the administration of single dose of BMSCs would recede with time. In addition, among the indicators detected in the current study, SDF-1 and PDGF have been proved to be involved in the recruitment and differentiation of BMSCs [16, 17]. Therefore, the effect of the two factors on the function of BMSCs was further assessed.

Following BMSCs treatment, models rats were furthered injected with different concentration of PDGF and SDF-1. Based on the histological staining, it was found that co-administration of PDGF and BMSCs alleviated fibrosis process after skin wound by inhibiting the expression of MMP-1. As being previous reported, PDGF alone poorly induced the expression of MMP-1 [22]. When concatenated administration with other cytokines, such as IL-1α, PDGF can induce the expression of MMP-1 and induce degradation collagens [23]. In the current study, co-administration with BMSCs and PDGF dramatically down-regulated the expression of...
MMP-1 and up-regulated the expression of TIMP-1. MMPs are a family of zinc endopeptidases which degrade all the components of the extracellular matrix [24-26]. MMP-1 belongs to the collagenase subfamily, function of which will be inhibited by TIMP-1 [27]. The ratio of MMP-1 to TIMP-1 has been reported to be an indicator for wound healing in clinical samples: there is a significant correlation between a high ratio of MMP-1/TIMP-1 and good healing [27]. However, based on the western blotting assay in the current study, lower MMP-1/TIMP-1 ratio was associated with the promoted skin wound healing process. The differences between results of ours and Muller's study might indicate multiple functions of MMP-1 in the skin wound healing process. Although the decreased MMP-1/TIMP-1 ratio in the current study was contrary to previous report based on clinical investigation, it was associated with the increased production of collagen I and collagen III after co-administration of SDF-1 and BMSCs. Upon skin being injured, onset of collagen secretion can be detected [28], contributing to the formation of scar tissues. Additionally, as being previously validated, the expression of SDF-1 is induced by PDGF in endothelial cells [16]. Combined with our results, administration of PDGF potentially influenced the function of BMSCs by suppressing the function of MMP-1 and inducing the secretion SDF-1, which further facilitated accumulation of BMSCs and deposition of collagen I and collagen III in injured area. Nevertheless, some shortcomings exist with our studies: although we indicated a promoting effect of PDGF on the secretion of SDF-1, the validation was not performed in the current study. The pathways underlying the interaction between PDGF, SDF-1, and BMSCs were only preliminarily investigated. Additionally, the effect of single administration of BMSCs on skin injury would recede with time, which demanded a solution for its practical application.

In summary, our study confirmed the promoting effect of BMSCs on skin wound healing by inducing the production of several factors, including PDGF and SDF-1. Moreover, the two indicators could strengthen the function of BMSCs in a positive loop. It was hypothesized based on our results that administration of BMSCs enhanced the production of PDGF and SDF-1 and the former one further induced the production of SDF-1, which would contributed to recruitment of BMSCs in injured area and deposition of collagens. However, due to the restriction of experimental design and device, the hypothesis was only preliminarily explored in the current study. Additional studies are needed to be performed to reveal the pathways underlying the interaction between BMSCs, PDGF, and SDF-1.

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Disclosure of conflict of interest
None.

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PDGF and SDF-1 strengthen skin injury repairing function of BMSCs


Figure S1. Identification of BMSCs. A. Representative images of osteogenic differentiation of bMSCs as detected by Alizarin Red S method and osteogenic bMSCs were stained red. B. Representative images of adipogenic differentiation of BMSCs as detected by Oil Red O method and adipogenic BMSCs were stained red. Magnification: 400 ×.