Hematopoietic stem cells can differentiate into pericytes and myofibroblast in wound healing, not bone Mesenchymal stem cells

Yanan Kong

Anhui Medical University <https://orcid.org/0000-0001-8021-8007>

Liuhanhang Cheng

General Hospital of People's Liberation Army: Chinese PLA General Hospital

Min Xuan

People's Liberation Army General Hospital of Southern Theatre Command

Hao Ding (✉ dingh2327@sina.com)

Anhui Medical University

Biao Cheng

PLA General Hospital of Southern Theatre Command: People's Liberation Army General Hospital of Southern Theatre Command

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Abstract

Background

Hematopoietic stem cells(HSCs) and mesenchymal stem cells(MSCs) can participate in wound healing. However, very few studies had shown HSCs and MSCs could arrive to the wound and differentiate into tissues. In this study, we intend to investigate the role of bone marrow HSCs and MSCs in wound healing.

Methods

We first removed the bone marrow of mice by irradiation. Furthermore, we injected different colours of fluorescent HSCs and MSCs into the tail vein of irradiated mice to reconstruct bone marrow function. We prepared wound models on the back of these mice. In vivo imaging and immunohistochemical staining were used to track the expression of fluorescent protein.

Results

HSCs and MSCs have been isolated and cultured. HSCs expressed Sca1, not lineage, CD34 or CD48. MSCs expressed CD29 and CD44, not CD34 or CD45. HSCs labeled with green fluorescent protein reached the wound and co-expressed with desmin and α -SMA. MSCs didn't stay on the wound.

Conclusions

The results show HSCs in the bone marrow of mice can directly participate in wound healing and differentiate into pericytes and myofibroblasts.

Introduction

Skin is the largest organ in the body, defending the foreign populations and protecting the body[1]. So it's often subjected to various injuries and forms wound. The problem affects an estimated twenty million people around the world. This number may significantly increase due to ageing population and escalating incidence of civilization diseases such as obesity and diabetes. Globally, by 2020 the wound care market is projected to surpass 22 billion USD per year. Treatment of these wounds can be long-lasting and huge-cost[2]. Some wounds lack effective methods to cure.

Researchers had tried many types treatments, such as stem cells, cell factors, tissue engineering and so on[3]. Stem cells are proven to promote wound healing by several direct and indirect mechanisms, including residing cells stimulation, growth factors release, angiogenesis and inflammation[4]. Mesenchymal stem cells and hematopoietic stem cells are the most commonly used cells. Mesenchymal

stem cells and hematopoietic stem cells are often injected in blood or around the wound. However, these methods have difficulties in tissue targeting and high cell attrition rate[5]. Very few studies had shown bone mesenchymal stem cells(BMSC) and hematopoietic stem cells(HSC) could arrive to the wound and differentiate into tissues[6]. In this study we want to investigate BMSC and HSC whether reaching the wound and participating in wound repair in mice.

Wound healing has four overlapping phases: hemostasis, inflammation, proliferative phase and tissue remodeling[7]. The process of angiogenesis is the major link in wound healing. Many types of cells participate in angiogenesis, such as endothelial cells, stromal cells, pericytes, and endothelial progenitor cells[8]. In the past, people mainly focused on the role of endothelial cells in angiogenesis, pericytes are often overlooked. Pericytes, also known as adventitial cells or Rouget cell, were discovered by Eberth and Rouget in the 1870s. In recent years, with the discovery and application of more markers of pericytes, the effects of pericytes on angiogenesis caught the attention of scientists. Even more, some studies suggested that pericytes play an important leading role in formation of neovascular buds[9]. Therefore, the role and mechanisms of pericyte in angiogenesis process are very important.

The clinical significance of pericytes research lies in controlling angiogenesis and stabilizing status of blood vessel. However, its exact origin and differentiation mechanisms are still unclear. Some researchers found BMSC differentiate into pericytes, another researchers thought HSC differentiate into pericytes. In short, the origin and differentiation of pericytes still have a lot of controversy. Although bone marrow is the exact source of pericytes in the angiogenesis process of wound healing[10–12]. The pluripotent stem cells originated in the bone marrow have hematopoietic stem cells and marrow mesenchymal stem cells[13]. It's unclear what type of stem cells is the source of pericytes. It will be the basis of this study research topics.

Materials And Methods

Chemicals And Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Lineage negative selection cocktail kit, Sca-1 positive selection cell isolation kit and StemSpan serum-free expansion medium were purchased from StemCell Technologies (Vancouver, BC, Canada). Stem-cell factor (SCF), thrombopoietin (TPO) and Flt3 ligand (Flt-3) were purchased from R&D Systems (Minneapolis, MN, USA). Anti-mouse lineage-Percp-cy5.5, Sca1-PE, CD34-FITC, CD48-APC-cy7, CD45-FITC, CD29-PE, CD44-PE, CD34-PE antibodies were obtained from BD Biosciences (San Jose, CA, USA). Primary antibodies against RFP, EGFP, α -SMA, K19 and desmin were purchased from Abcam(Cambridge, United Kingdom).

Animals

Eight-week-old enhanced green fluorescent protein(EGFP) C57BL/6 mice were purchased from Model Animal Research Center of Nanjing University. Eight-week-old red fluorescent protein(RFP) C57BL/6 mice

were purchased from Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences. All C57BL/6 mice were purchased from Guangdong Medical laboratory Animal Center. Experimental procedures were approved by the Bioethics Committee of General Hospital of Guangzhou Military Command. ALL protocols were approved by the Bioethics Committee of General Hospital of Guangzhou Military Command.

Isolation of bone-marrow-derived HSCs

To get bone-marrow-derived HSCs, six weeks old to eight weeks old C57BL/6 mice(n=10) were isolated as before[14]. Briefly, bone marrow-derived cells were collected by flushing the femurs and tibias. Then the red blood cells of bone marrow-derived cells were removed by red blood cell lysis buffer. Lineage-negative(Lin-) bone marrow cells were obtained through lineage positive cells depletion using Lineage negative selection cocktail kit by Dynabeads. Sca-1 positive(Sca-1+) lin- bone marrow cells were obtained through Sca-1 negative cells depletion using Sca-1 positive selection cell isolation kit by magnetic cell sorting again. After that we got lin-Sca+ HSCs. To obtain HSCs marked fluorescent protein, the EGFP or RFP mice were used.

In vitro culture of HSCs

Lin-Sca+ HSCs were cultivated in 6-well plates or 25 cm² flasks (Costar, Cambridge, MA) at a concentration of 10⁶/mL nucleated cells in StemSpan serum-free expansion medium supplemented with 50 ng/ ml SCF, 50 ng/ ml TPO and 50ng/ml Flt-3. Cultures were incubated at 37°C in a 5% CO₂ atmosphere[15, 16]. The cells grew in suspension. When the cells grew to 3-4 layers, suspending cells were harvested and expanded in more flasks. Three to four passages of Lin-Sca+ HSCs were used for follow-up studies.

Isolate and culture bone-marrow-derived MSCs

MSCs were isolated as before[17]. Briefly, bone marrow cells were collected by flushing the femurs and tibias of 4 weeks old mice(n=10). These cells were cultivated in 6-well plates or 25 cm² flasks (Costar, Cambridge, MA) at a concentration of 10⁶/mL nucleated cells in DMEM, with low glucose (4.5 mM), GLUTAMAX I (Gibco), 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). No cytokines were added at any stage. Cultures were incubated at 37°C in a 5% CO₂ atmosphere. After 72 hours, nonadherent cells were removed. When 70% to 80% confluent, adherent cells were trypsinized, harvested, and expanded in larger flasks. The adherent spindle-shaped cells were further propagated for three passages. To obtain MSCs marked fluorescent protein, the EGFP or RFP mice were used.

HSCs and MSCs were identified byFlow Cytometry

Bone marrow-derived HSCs of the 3rd-4th passage were harvested directly, and rinsed with PBS containing 2%FBS 3 times. Then they were incubated with anti- lineage-Percp-cy5.5, Sca1-PE, CD34-FITC,

CD48-APC-cy7 antibodies for 30 min according to the manufacturers' instructions. After that, the cells were rinsed with PBS containing 2%FBS 2 times and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

The bone marrow derived MSCs at the 2rd–3rd passage were trypsinized, centrifuged, and rinsed with PBS containing 2%FBS 3 times. Then they were incubated with anti- CD34-PE, CD45-PE, CD29-FITC, CD44-FITC antibodies for 30 min according to the manufacturers' instructions. After that, the cells were rinsed with PBS containing 2%FBS 2 times and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Normal mice with bone marrow derived HSCs marked EGFP and MSCs marked RFP models were prepared.

Forty C57BL/6 mice, weighting 20-25g, 6-8 weeks old, were randomly divided into irradiated group(n=20) and control group(n=20). Mice in irradiated group(IR) received a split dose of 8 Gy of g irradiation[18]. Then bone marrow derived HSCs labeled EGFP and MSCs labeled RFP were injected into blood through tail vein. Mice in irradiated group randomly received tail vein injections of 10^6 HSCs and 10^6 MSCs (IR+TP group) or equal amounts of solvent (sterile PBS), each group have 10 mice. Mice in control group randomly received tail vein injections of 10^6 HSCs and 10^6 MSCs (TP group) or equal amounts of solvent (sterile PBS, Ctr group), each group have 10 mice.

The mice bone marrow function was evaluated by clone culture

Bone marrow cells of mice in irradiated group were used for clonal culture. The mice were divided into transplantation group (IR+TR group) and PBS group according to receiving stem cells injection or PBS injection. The clonal culture was executed according to the instruction manual. Briefly, bone marrow-derived cells were collected by flushing the femurs and tibias. Then the red blood cells of bone marrow-derived cells were removed by red blood cell lysis buffer. Resuspend cells to 10^5 cells/ml using IMEM containing 2% FBS. 100 μ l of cells were added to 1 ml of clonal medium and implanted in a 35mm culture dish for culture. Cultures were incubated for 12 days at 37°C, 5% CO₂ and 95% humidity atmosphere. We observed clone formation under an inverted microscope.

Preparation of wound model

To investigate the effect of bone marrow derived HSCs and MSCs on wound healing, three group mice were prepared as wound healing models on the second day. The wound healing models were prepared as before[19]. Briefly, three groups mice were euthanized by 10% chloral hydrate (0.002 ml/g body mass). Full-thickness skin wounds of 6mm in diameter were created on each mouse on the dorsal-right or -left shaved skin with sterile puncher.

Traces of HSCs and MSCs on wound healing were tracked by live imaging system

The fluorescent protein of mice were detected by live imaging system (Roper Scientific, Martinsried, Germany), anesthetized with chloral hydrate. The pictures were taken on day 1, 3, 5, 7 after the wound formation.

Traces and differentiation of HSCs and MSCs on wound healing were detected by immunohistochemistry

The skin were harvested on day 5 after the wound formation. Two mice were randomly equally selected and killed each time in one group. The dorsal skins around the wound (area 1.5×1.5 cm) were carefully dissected and fixed in 4% paraformaldehyde and residual samples were immediately frozen in liquid nitrogen, and further processed for the morphometric analyses as described below [20].

The paraffin specimens were serial sectioned. The serial sections were immunostained with EGFP, RFP, desmin, α -SMA and K19. Briefly, sections were deparaffinized with immersion in dimethylbenzene and rehydrated, then heated in citrate buffer (0.01 M, pH 6.0) for 5 min at 100 and were then treated with endogenous peroxidase (3% hydrogen peroxide solution) for 5 min at room temperature. After blocking in 10% goat serum for another 30 min at room temperature, sections were immunostained with primary antibodies for EGFP (1:500; Abcam, Cambridge, UK) or RFP, desmin, α -SMA, K19 containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) overnight at 4°C. After washing three times with PBST (PBS supplemented with 0.1% Tween-20), sections were incubated with secondary antibodies, avidin-biotin-peroxidase complex and DAB reagent [19, 21]. Subsequently, all sections were double stained with hematoxylin and visualized under the microscope (BX51, Olympus, Japan).

Results

Characterization Of Isolated HSCs

Bone marrow derived HSCs were isolated by magnetic bead sorting using lineage negative and Sca1 positive. The HSCs grew in suspension and colonies formed (Fig 1A). Cell surface markers were assessed using flow cytometry to characterize isolated HSCs. The cells before sorting mainly expressed Lin+Sca1- (Fig 1B1). After sorting, about 70% cells expressed Sca1, not lineage, CD34 or CD48 (Fig 1B2, B3), which is consistent with markers of HSCs.

Characterization Of Isolated MSCs

Cell surface markers were assessed using flow cytometry to characterize isolated MSCs. About 90% MSCs expressed CD29 and CD44, not CD34 or CD45 but (Fig. 2B), which is consistent with previous reports.

Evaluation of HSCs and MSCs transplantation effect after mice bone marrow suppression

Mice in irradiated group received a split dose of 8 Gy irradiation. The mice would die totally within 7 days, if the mice wouldn't receive the transplantation of HSCs and MSCs (dates not shown). Mice were irradiated (8Gy dose) to induced bone marrow suppression. After that, EGFP labeled HSCs and RFP

labeled MSCs (IR+TP group) or PBS (PBS group) were transplanted to mice by tail intravenous injection. All mice with HSCs and MSCs transplantation were normally alive. To evaluate HSCs and MSCs transplantation effect, the bone marrow cells of mice with HSCs and MSCs transplantation were colony cultured. There are many cells in the bone marrow of the mice in the IR + TP group and few cells in the bone marrow of mice in the PBS group (Fig3 B, C). The colony culture showed the cells in IR+TP group differentiated into erythroid (BFU-E), granulocyte macrophage cell line (CFU-GM) and granulocyte macrophage red line (CFU-GEMM) colonies. However, the bone marrow cells of mice with PBS transplantation can't form colonies (Fig3 C, D).

The location of HSCs and MSCs in wound of mice using live whole-body fluorescence imaging technique

After the mice received HSCs labeled EGFP and MSCs labeled RFP or PBS transplantation, the remaining three groups of mice were carried out wound model. The live whole-body fluorescence imaging technique and immunohistochemical staining were used to monitor the location and differentiation of stem cells on day 1, 3, 5, 7 after the wound formation. The whole-body fluorescence imaging showed there were much red fluorescence and green fluorescence in the wound area of irradiated mice and normal mice on day 1. However, the red fluorescence disappeared, only green fluorescence appeared around the wound on 3th day. There were no any fluorescence in the wound area of normal mice. The same is true on the 5th and 7th days. On 7th day, the healed wound showed green fluorescence, no red fluorescence (Fig4). In order to eliminate the effects of fluorescent proteins, we repeated the experiments using MSCs labeled EGFP and HSCs labeled RFP and achieved the same results(dates not shown).

The differentiation of HSCs and MSCs in wound of mice using immunohistochemical staining

On 5th day, we used serial sectioning to analyze the wound and its peripheral skin. The models injected by MSCs labeled RFP and HSCs labeled EGFP were analyzed. The immunohistochemical staining showed lots of EGFP expressed, no RFP expressed. Desmin and α -SMA expressed in the areas where green fluorescent protein is expressed. A small amount of K19 expressed in the place where EGFP expressed. Desmin appeared around the blood vessels and muscle tissues, but EGFP and desmin co-colored around the blood vessels. α -SMA and EGFP co-expressed in the granulation tissue area of the wound (Fig5).

Discussion

Skin wound has become one among the 3 major diseases of the aged inpatients[2]. Many studies focused on skin wound healing. Stem cells were used to cure wound healing[5]. In our study, BMSCs and HSCs were used to cure wound healing in mice. All mice would die in 7 days if the mice received 8 Gy of irradiation and no other treatment. The BMSCs and HSCs were injected to tail vein of mice. All mice receiving stem cells tail vein injection survived. To confirm the role of transfused hematopoietic stem cells and mesenchymal stem cells, We used the bone marrow cells of the mice for clonal culture on 7 days after the infusion of stem cells. Bone marrow cells from mice receiving stem cell injection can form

BFU-E, CFU-GM and CFU-GEMM. So the given BMSCs and HSCs reestablish the function of bone marrow[22].

To study the role of bone marrow cells in skin wounds, wound model of mouse back skin was made. To track the role of stem cells, different colors of fluorescence labeled stem cells were used. We identified the origin of stem cells through the color of fluorescence. Red fluorescence and green fluorescence appeared around the wound on day 1. It means both MSCs and HSCs took part in the wound healing on day 1. However, only green fluorescence appeared around the wound on 3th day, not the red fluorescence. It means that only HSCs are involved in wound repair from the third day. To determine the repair role of stem cells, we detected markers for stem cell differentiation. The immunohistochemical staining showed Desmin and α -SMA expressed in the areas where green fluorescent protein is expressed. Desmin appeared around the blood vessels and muscle tissues, but EGFP and desmin co-colored around the blood vessels. It suggested that HSCs differentiate into pericytes, not MSCs. Angiogenesis is an important step in wound healing[8]. Pericytes play an important role in maintaining the stability of blood vessels. There has been controversy about the origin of pericytes in wound healing. We confirmed that during wound healing, HSCs can directly differentiate into pericytes. Interestingly, we found that EGFP and α -SMA were co-expressed. α -SMA is a marker of myofibroblasts[23]. It suggested that HSCs can differentiate into myofibroblasts. Studies have shown that myofibroblasts are cells with multiple differentiation potentials. During wound healing, myofibroblasts can convert to a completely different lineage, such as adipocyte[24]. It suggested that HSCs have an important positive role in wound repair. In the study, we also found that K19 and EGFP were co-expressed and located on a similar newly formed hair follicle structure. This indicated that HSCs differentiate into epithelial cells. It confirmed that HSCs play an important role in wound healing again. Previous studies have found that the number of HSCs in the peripheral blood of diabetic patients and mice is reduced and the differentiation potential is impaired[25]. Diabetic patients and diabetic animals often have difficulties in wound healing[26]. Is this related to impaired HSC function? More research is needed to confirm.

Previous studies have suggested that MSCs participate in wound repair in several ways, directly differentiate into wound repair cells, secrete growth factors, and provide microenvironmental support[27, 28]. When MSCs directly participate in the differentiation into wound repair cells, only a very small number of MSCs can differentiate[29]. In our study, MSCs did not directly participate in the differentiation and formation of wound repair cells. It may be related to the different mice models used to construct the wound models. The recipient cells could migrate to the damaged site[30, 31]. In our study, mice bone marrow is damage. The recipient MSCs may migrate to bone marrow. This may be the reason that our experiment did not find MSCs appear in the wound. However, in our study, whether MSCs affect wound healing through growth factors, it remains to be studied.

In a conclusion, the HSCs and MSCs were injected through tail vein after the complete loss of bone marrow cells function in mice. In the mice skin wound healing, HSCs differentiated into pericytes and myofibroblasts, not MSCs. Under such conditions, the role of MSCs in wound healing needs further study.

Abbreviations

HSCs: Hematopoietic stem cells; MSCs: Mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; SCF: Stem-cell factor; TPO: Thrombopoietin; Flt-3: Flt3 ligand; EGFP: Enhanced green fluorescent protein; RFP: Red fluorescent protein; Lin⁻: Lineage-negative; Sca-1⁺: Sca-1 positive; IR + TR group: Irradiated group + transplantation group.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were approved by the Bioethics Committee of General Hospital of Guangzhou Military Command. ALL protocols were approved by the Bioethics Committee of General Hospital of Guangzhou Military Command.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Yanan Kong and Liuhanhang Cheng carried out the research, collected and analyzed the data, wrote the manuscript. Hao Ding and Biao Cheng performed conception and designed the research. Min Xuan collected the data.

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Figures

Figure 1

Culture photo and flow cytometry identification results of Hematopoietic stem cells. A shows the photo of HSCs growing in suspension. B1 shows the results of flow cytometric identification of cells in bone marrow before magnetic bead sorting. B2 and B3 show the results of flow cytometry identification after cells sorting in bone marrow, mainly expressing lineage-, Sca1+, CD34-, CD48- .

Figure 2

Culture photo and flow cytometry identification results of Mesenchymal stem cells. A shows the photo of MSCs growing adherently. B shows the results of flow cytometry identification after MSCs sorting in bone marrow, more than 90% expressing CD34⁻, CD45⁻, CD29⁺, CD44⁺.

Figure 3

Results of bone marrow function evaluation in mice after MSC and HSC transplantation. A shows the experimental design pattern diagram. B shows that there are many cells in the bone marrow of the mice in the IR + TP group and few cells in the bone marrow of mice in the PBS group. C shows that a large number of cells and colonies can be seen in the culture in TP group, and no cells or colonies have been cultured in PBS group. D shows that there are various colonies in TP group. (IR + TP means irradiation + transplantation).

A

B

Figure 4

Whole-body fluorescence imaging results of mouse wounds. MSCs labeled red fluorescent protein. HSCs labeled green fluorescent protein. In the IR+TP group, green fluorescence was visible 7 days after the wound formation, red fluorescence was only visible 1 day after the wound formation. The mice in the TP group showed green and red fluorescence only 1 day after the wound formation. No fluorescence in the control group. The red box marks the location of the wound. (IR+TP means irradiate and transplantation. TP means transplantation. CTR means control.)

Figure 5

Results of immunohistochemical staining of wound specimens. No red fluorescent protein expressed in wound specimen. Green fluorescent protein, α -SMA, Desmin and K19 had coexpression. The scale in the pictures is 100 μ m.

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