Transplantation of Dermal Multipotent Cells Promotes the Hematopoietic Recovery in Sublethally Irradiated Rats

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Radiation/Dermal multipotent cells/Hematopoietic recovery.

Our previous study indicated that dermal multipotent cells with the differentiation capacity to form cells with the phenotypic properties of osteocytes, adipocytes, chondrocytes, and neurons in specific inducing media could be isolated from the enzymatically dissociated dermal cells of newborn rats by their adherence to culture dish plastic. We have also observed that the systemic transplantation of dermal multipotent cells could not repopulate the hematopoietic system in lethally irradiated rats. In this paper, we found that a transplantation of plastic-adherent dermal multipotent cells into sublethally irradiated rats led to a significant increase of white blood cells in peripheral blood, nucleated cells, CFU-GM, and CFU-F colonies in bone marrow. FISH analysis, using a Y-chromosome specific probe, showed that dermal multipotent cells could engraft into bone marrow in recipients. Flow cytometry (FACS) analysis also showed that the proportion of CD2 and CD25 positive lymphocytes in peripheral blood did not change significantly in two weeks after transplantation. By these results, we infer that dermal multipotent cells may represent an alternative origin of mesenchymal stem cells to restore marrow microenvironment and promote the survival, engraftment, and proliferation of hematopoietic cells.

INTRODUCTION

In the past few years, the existence and biological properties of multipotent stem cells from various adult tissues have been widely studied.1,2) Adult stem cells show promising prospects in regenerative medicine. In this regard, bone marrow mesenchymal stem cells have been the model of choice for many researchers. Simultaneously, several research groups have investigated alternative sources of adult stem cells.2,3) The cells of dermis are highly accessible and there is increasing evidence that dermis contains stem cells. Stem cells obtained from skin dermis have been shown to have the capacity to produce neural as well as mesodermal derivatives.4,5) Unlike some cell types whose stem cell potential is being investigated, the functional activity of multipotent cells in dermis is not well known. In a previous paper, we reported that dermal multipotent cells (DMCs) could be isolated from the enzymatically dissociated dermal cells of newborn rats by their adherence to culture dish plastic.5) Our previous work also showed that dermal multipotent cells could support the growth of CFU-GM and CFU-E hematopoietic colonies in vitro, but the systemic transplantation of these cells could not repopulate the hematopoietic system in lethally irradiated rats (unpublished data). In this paper, we report that single sorted plastic-adherent multipotent cells from newborn rat dermis can contribute to the acceleration of the hematopoietic recovery without an obvious activation of allogenic lymphocytes in sublethally irradiated rats. This may have some therapeutic implications for using the alternate sources of bone marrow mesenchymal stem cells in hematopoiesis.

MATERIALS AND METHODS

Materials

Wistar rats were purchased from the Center of Laboratory Animals of the Third Military Medical University. All materials were purchased from Sigma Aldrich (St. Louis, Missouri, USA), unless otherwise stated. Iscove’s Modified Dulbecco’s Medium (IMDM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Culture plastic flasks and dishes were purchased from Corning Incorporated (Acton, MA, USA). Mouse antirat CD31, CD34, and CD45 monoclonal antibodies were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, California, USA). Mouse antirat nestin monoclonal antibody was purchased from Pharmingen (San Diego, CA, USA). FITC-labeled monoclonal antibodies for rat CD44, CD59, CD90, and mouse...
IgG1 and IgG2b were purchased from Serotec (Oxford, U.K.). Other antibodies, including antirat pan-cytokeratin, cytokeratin19, vimentin, ICAM-1, VCAM-1, Factor VIII, α-smooth muscle actin, desmin, NF-200, collagen II, and HRP-labeled secondary antibodies were purchased from Boster (Wuhan, China).

Cell isolation and culture

The procedures were described previously.20 Full-thickness skin tissue was obtained from the dorsum of one-day-old rats after they were killed by ether. It was transferred to phosphate buffered saline (PBS) solution containing 0.25% trypsin and enzymatically dissociated at 4°C, overnight. The dermis tissue layer (proved by histological examination) was dissociated by flushing with D-Hank’s solution, and the suspension at a density of 5 × 10⁶ cells/ml was filtered through a nylon mesh to remove cellular debris and centrifuged. The cell pellet was resuspended and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% FBS, 100 U/ml penicillin, and 100 microgram/ml streptomycin at a density of 10⁶ cells/mm². Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Six hours later, the non-adherent cells were removed and the adherent cells were harvested with 0.25% trypsin and 1 mM ethylenediaminetetraacetate (EDTA). The cells were then serially diluted into culture flasks. These colonies were studied as candidate plastic-adherent dermal multipotent cells. Once the cells were more than 80% confluence, they were recovered with trypsin/EDTA and replated at a ratio of 1:3. The expanded cells were used for the characterization of their differentiation capacity.

To induce differentiation, the cells were nontreated in osteogenic medium (osteogenic: IMDM supplemented with 10% FBS, 0.1 µM dexamethasone, and 0.1 µg/ml BMP-2; chondrogenic medium: IMDM supplemented with 10% FBS, 0.1 µM dexamethasone, and 10 ng/ml TGF-beta 1; adipogenic medium: IMDM supplemented with 10% FBS, 0.5 mM isobutyl-methylxanthine (IMBX), and 1 µM dexamethasone; neural medium: IMDM supplemented with 10% FBS, and 5 × 10⁻⁵ M retionionic acid) for 21 days, and differentiation was confirmed by using histological staining and immunohistochemistry study (osteogenic: alkaline phosphatase activity and alizarin red stain; chondrogenic: collagen II synthesis and alcian blue stain; neural: nestin and NF-200 production). The added agents such as dexamethasone, growth factors and other supplements were changed every 3 days by half the volume of the culture media. Cells maintained in control medium were examined as negative controls. The cells with the multilineage differentiation capacity that could be expanded effectively in vitro were used in the following experiments.

Irradiation and transplantation

Female Wistar rats (six-week-old and weighing about 120 g) were divided into three groups: normal nonirradiation control (Group N), irradiation control (Group R), and DMC transplantation after irradiation (Group T). Before cell transplantation, the recipient rats of group R and group T were irradiated over the whole body with a sublethal dose of 5 Gy of gamma rays from a 60Co source. The absorption rate was 31.02–31.98 cGy/min. In six hours after irradiation, 2 × 10⁶ cultured plastic-adherent DMCs in 1 ml physiological saline were injected into a tail vein of each recipient of group T. The animals of group R were injected with the same volume of normal saline. All the animals were fed commercial laboratory food and purified tap water and were housed under standard conditions. Nucleated cells, CFU-F, CFU-GM, and CFU-E hematopoietic colonies in bone marrow; white blood cells, and hemoglobin (Hb) in peripheral blood were assessed after transplantation.

Preparation of bone marrow cells and hematopoietic progenitor colonies assay

Bone marrow cells were obtained from anesthetized rats by aseptic isolation of the femurs followed by a flushing of the marrow with IMDM medium, using a 25-gauge needle. The cells were suspended in the medium, and single cell suspensions were made.

To determine the CFU-GM (colony forming unit of granulocyte/macrophage progenitors) derived colonies, 2 × 10⁵ bone marrow nucleated cells were cultured in triplicate in 24-well dishes in the presence of methyl-cellulose (3.0%) in RPMI 1640, t-glutamine (2.0 mM), fetal bovine serum (15%), 2-mercaptoethanol (0.1 mM), penicillin, and streptomycin (100 U/ml and 100 µg/ml, respectively), and GM-CSF (10 µg/ml). The cells were incubated in a humidified incubator containing 5% CO₂ and at 37°C. After incubation for 7 days, the colonies containing 50 or more cells were counted under an inverted microscope.

To determine the CFU-F (fibroblastic-colony forming unit cultures) derived colonies, 10⁵ bone marrow nucleated cells were plated in triplicate in 24-well dishes, in DMEM containing 10% fetal bovine serum, 10⁻⁶ M hydrocortisone, and 50 µg/ml ascorbic acid. The cells were incubated in a humidified incubator containing 5% CO₂ and at 37°C. After incubation for 28 days, the colonies containing 50 or more cells were counted under an inverted microscope.

Flow cytometry (FACS) analysis

Whole blood was directly labeled with fluorescein isothiocyanate (FITC) conjugated CD2 and CD25 monoclonal antibodies, as previously described7; 10,000 cells from each
sample were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). To discriminate cell autofluorescence, negative control samples were prepared by using cells alone. Moreover, peripheral blood lymphocytes (PBL) were acquired inside an electronic gate, which discriminated lymphocytes from the other leucocytes.

**Fluorescence in situ hybridization (FISH) studies**

The engraftment of DMCs was assessed by in situ hybridization with FITC-labeled Y-chromosome probe on 3 µm frozen tissue sections. The Y-chromosome probe was generated by PCR and labeled with FITC. The slides were placed in methanol:acetic acid (3:1) for 30 s, dried, and treated with proteinase K, 10 µg/ml, for 45 min at 37°C. After washing, the slides were incubated with probe overnight at 37°C. With extensive washings, coarse debris, which might have been mistaken for a positive signal, was minimized. The slides were observed under a fluorescence microscope, and marrow tissue from female rat served as negative control tissue.

**Statistical analysis**

All experiment data were expressed as mean ± standard deviation and evaluated for statistical significance by Student’s *t*-test. Statistical significance was assumed at the *p* value less than the 0.05 level.

**RESULTS**

**Biological characterization of cultured DMCs**

When cultured in vitro, only a very small percentage (lower than 0.01%) of the dissociated primary dermal cells adhered to the plastic. For clonal analysis, adherent cells were seeded by low density for the presence of one single cell in one culture well. Some single cells formed colonies with one layer, and a few (lower than 5%) single cells formed colonies with more than two layers. These colonies, which formed multiple layers, were isolated and tested as candidate dermal multipotent cells. After expansion, most cultured DMCs were fibroblast-like cells (Fig. 1) and were negative for lineage-specific

![Fig. 1. Morphology of cultured DMCs under light microscope. \(\times 100\).](image1)

![Fig. 2. Multilineage differentiation of DMCs.](image2)
surface markers (includes pan-cytokeratin, cytokeratin19, Factor VIII, CD31, CD34, α-smooth muscle actin (α-SMA), desmin, collagen II, and nestin), but were positive for CD90, CD59, CD44, VCAM-1, ICAM-1, and vimentin. The doubling time of DMCs was about 40 h when cultured in vitro. Following induction, DMCs differentiated into cells with phenotypic properties of osteocytes, adipocytes, chondrocytes, and neurons in specific inducing media in vitro. DMCs formed alizarin red-positive nodules in osteogenic medium on day 21, but the controls did not (Fig. 2A). When cultured in chondrogenic medium for 21 days, positive cells for collagen II staining and alcian blue staining were evident in histological sections (Fig. 2, B and C). When cultured in adipogenic medium for 21 days, DMCs formed oil red positive adipocytes (Fig. 2D). DMCs also produced NF-200 and nestin and became typical neuron-like cells in morphology in neural medium on day 21 (Fig. 2, E and F).

Effects of DMCs transplantation on the hematopoietic recovery in irradiated rats

To observe the effects of DMCs on hematopoiesis in vivo, experimental rats were exposed to 5 Gy sublethally γ-ray-irradiation. Then 2 × 10⁶ DMCs were infused into the tail veins of each animal after irradiation. For animals that received 5 Gy irradiation, the white blood cell (WBC) counts of the group R and group T at 7 days reduced more significantly than those of group N because of the hematopoietic damage caused by irradiation (Fig. 3). The WBC count of group T with DMCs transplantation at 15 and 21 days was significantly higher than that of group R (Fig. 3). Hemoglobin (Hb) was not significantly different among the three groups (data not shown). Furthermore, the number of nucleated cells, CFU-F, and CFU-GM colonies in bone marrow of group T were also significantly higher than of group R on 15 days after transplantation (Fig. 4). Donor-origin cells were detected in recipient marrow tissues 15 days after the DMC transplantation by FISH analysis, using Y-chromosome specific probe (Fig. 5). These results suggest that in vivo use of DMCs promotes the hematopoietic recovery from sublethal irradiation-induced damage.

Analysis of the CD2 and CD25 positive peripheral lymphocytes in peripheral blood after DMC transplantation

To assess the immune status of recipient rats, we analyzed the proportions of CD2 (expressed on the whole lymphocytes) and CD25 (activated lymphocytes) POSITIVE lymphocytes in peripheral blood. There is no significant difference of the proportions of CD2 and CD25 positive lymphocytes at 15 days between the DMC-treated group and the control group (Fig. 6, A and B). These results indicated that the transplanted DMCs did not induce an obvious activation of recipient peripheral lymphocytes.

Fig. 3. Kinetics of peripheral white blood cells of group N (gray), group T (black), and group R (blank). The results were collected from six parallel experimental animals. The bars represent standard deviation. *p < 0.05, compared with group T; #p < 0.05, compared with group N.

Fig. 4. Numbers of nucleated cells, CFU-GM and CFU-F colonies in bone marrow on days 15 of group N (gray), group T (black) and group R (blank). The results were collected from six parallel experimental animals. The bars represent standard deviation. ###p < 0.01, compared with group T; **p < 0.01, compared with group N.

Fig. 5. FISH results with Y-chromosome probe. The marrow tissues were collected from the animals on day 15 after DMC transplantation. ×200.
DISCUSSION

Marrow stromal cells provide the structural and physiological support for the engraftment, survival, proliferation, and differentiation of hematopoietic stem cells. The transplantation of stromal cells may promote the reconstitution of hematopoiesis in adults after HSC transplantation. Mesenchymal stem cells (MSCs) from marrow have the potential to develop into distinct mesenchymal tissues, including marrow stroma. The cotransplantation of MSCs and HSCs has obtained a fast recovery of hematopoiesis. However, the autotransplantation of bone marrow mesenchymal stem cells is limited, especially when the bone marrow has been injured in some kinds of hematopoietic diseases characterized by stromal failure, such as multiple myeloma, spontaneous cytopenias, and aplastic anemia. Host-derived stromal cells are also functionally altered after aggressive radio/chemotherapy or from nuclear accidents. Under these conditions, there required other sources of multipotent stem cells for replacement therapy instead of bone marrow mesenchymal stem cells. The utilization of adult stem cells is a certain solution in the current state of replacement therapy. In this study, we report that dermis-derived multipotent cells isolated by their interaction and regulate the hematopoietic functions. Our other study also indicated that DMCs could not repopulate hematopoiesis in lethally irradiated rats (unpublished data). Bartholomew et al have reported that baboon MSCs (bMSCs) could suppress the proliferative activity of allogeneic peripheral blood lymphocytes in vitro and prolong skin graft survival in vivo. In our studies, we also observed that the proportions of CD2 and CD25 positive lymphocytes did not increase within 2 weeks following systemic transplantation of DMCs. These data from in vivo experiments at least showed that the systemic transplantation of DMCs did not lead to an obvious activation of recipient lymphocytes, and this will benefit for the engraftment and survival of implanted cells. By these data, we infer that dermal multipotent cells may at least represent an alternative origin of mesenchymal stem cells to restore marrow microenvironment and promote the survival, engraftment, and proliferation of HSCs. The relevant mechanism of DMCs on hematopoiesis and whether the cotransplantation of DMCs and HSCs results in clinical benefit are to be further studied.

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