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Transplantation of bone marrow mesenchymal stem cells on collagen scaffolds for the functional regeneration of injured rat uterus

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ABSTRACT

Serious injuries of endometrium in women of reproductive age are often followed by uterine scar formation and a lack of functional endometrium predisposing to infertility or miscarriage. Bone marrow-derived mesenchymal stem cells (BM-MSCs) have shown great promise in clinical applications. In the present study, BM-MSCs loaded onto degradable collagen membranes were constructed. Collagen membranes provided 3-dimmensional architecture for the attachment, growth and migration of rat BM-MSCs and did not impair the expression of the stemness genes. We then investigated the effect of collagen/BM-MSCs constructs in the healing of severe uterine injury in rats (partial full thickness uterine excision). At four weeks after the transplantation of collagen/BM-MSCs constructs, BM-MSCs were mainly located to the basal membrane of regenerative endometrium. The wounded tissue adjacent to collagen/BM-MSCs constructs expressed higher level of bFGF, IGF-1, TGFβ1 and VEGF than the corresponding tissue in rats receiving collagen construct alone or in spontaneous regeneration group. Moreover, the collagen/BM-MSCs system increased proliferative abilities of uterine endometrial and muscular cells, facilitated microvasculature regeneration, and restored the ability of endometrium to receive the embryo and support its development to a viable stage. Our findings indicate that BM-MSCs may support uterine tissue regeneration.

1. Introduction

Uterine endometrium is a unique tissue that undergoes dynamic, cyclical processes of growth, differentiation, sloughing, and renewal in each menstrual cycle. Regeneration of the endometrium is essential for embryo implantation and the maintenance of pregnancy. Severe endometrial damage caused by myomectomy, curettage, endometritis or endometrial tuberculosis lead to endometrium scar formation which may result in abnormal uterine bleeding, miscarriage, pregnancy complications, or infertility [1–3]. Several strategies have been adopted for the treatment of endometrial fibrosis. However, few methods have adequately addressed problem of endometrial severe scar formation following surgery [4–7]. A few studies in animal model have been made for the *in vivo* reconstruction of uterus with new trauma, but still fail to restore the reproductive function of the operation area [8–10].

Bone marrow-derived mesenchymal stem cells (BM-MSCs), localized in the stromal compartment of bone marrow, are multipotent stem cells capable of differentiation into osteoblasts,

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adipocytes, chondrocytes as well as many other cell types [11–12]. Their easy acquisition, self-renewal, multipotent differentiation potential, and weak immunogenicity make them a promising cell source for regenerative medicine [13–14]. A number of studies have demonstrated that BM-MSCs accelerated the repair of bone, cartilage, skin and peripheral nerve [15–22] and have improved the function of organs following ischemia including heart, brain and kidney [23–29]. Previous reports have also shown that BM-MSCs also contributed to the endometrial repair, representing potential progenitor cells for the endometrial stromal fibroblast [30–35]. It was not clear whether BM-MSCs can promote the regeneration of fertile uterus endometrium and directly differentiate into specific cell types within uterus.

Collagen, one of the basic structural elements of extracellular matrix, has been widely used in wound repair and tissue regeneration due to its abundance, biodegradability, and biocompatibility [36–39]. Collagen acts not only as a framework for structural support of tissue, but also as a substance for regulating cell behavior including cell adhesion, migration and differentiation. In previous studies, porous collagen sponges with pore diameters in the range of 120–200 μm were used for 3D culture of many cell types, such as fibroblasts, marrow cells and oral keratinocytes [40–41]. In this study, we sought to characterize of BM-MSCs after loading onto collagen membranes and the effect of this collagen/BM-MSCs constructs in the regeneration of rat uterus following full-thickness injury.

2. Materials and methods

2.1. BM-mscs isolation and monolayer culture

All animals were treated in accordance with the guidelines of the Experimental Animals Management Committee (Jiangsu Province, China). Rat BM-MSCs were isolated and cultured *in vitro* as previously described [42]. In brief, two-month-old female rats were sacrificed with an overdose of ketamine. Both femora and tibias were isolated and rinsed with phosphate-buffered saline (PBS; Gibco, Grand Island). After muscle and extraossial tissues were trimmed, the marrow cells were flushed from bone cavities with low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Gibco). After filtering with 100 μm cell strainers (BD Bioscience), the flushed mixture was centrifuged, washed and re-suspended in LG-DMEM containing 12.5% fetal bovine serum (FBS; Hyclone, Logan), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 1× Insulin-Transferrin-Selenium (ITS; Gibco) and 10 ng/mL basic fibroblast growth factor (bFGF; Gibco). The re-suspended cells were seeded in 100-mm cell culture dish and incubated at 37 °C in a humid atmosphere consisting of 5% CO_2. The non-adherent cells were removed after 72 h of culture and fresh medium was added. The medium was changed every other day and passage was conducted when cells reached confluence (80–100%). Rat BM-MSCs of passages 3–5 were used for the following experiments.

2.2. Flow cytometric analysis

FACS analysis of rat BM-MSCs was performed at the fifth passage. Cells were detached from the culture dish using 0.05% trypsin-EDTA (Gibco), centrifuged, rinsed and re-suspended in PBS at a concentration of 10^5 cells/mL. Re-suspended cells were incubated with 5 μL of fluorescein isothiocyanate-labeled anti-rat CD90 (BD Pharmingen, San Diego), CD45 (Invitrogen, Carlsbad), CD44 (AbD Serotec, Kidlington), CD29 (AbD Serotec) and phycoerythrin (PE)-conjugated anti-rat CD34 (Santa Cruz Biotechnology, Santa Cruz) in the dark at 4 °C for 30 min. After being washed twice with PBS, cytometric analysis was performed using a flow cytometer (BD FACSCalibur).

2.3. BM-mscs culture on the collagen scaffolds

Collagen scaffolds were obtained from Zhenghai Biotechnology (Yantai City, Shandong Province, China). Scaffolds were rinsed with culture medium and placed onto a 24-well culture plate. After withdrawal of excess fluid from the scaffolds using absorbent paper, 50 μL of the BM-MSCs suspension (5×10^5 cells/cm^2 scaffold) was dripped equally onto each scaffold. The cell-seeded scaffolds were incubated in humid air consisting of 5% CO_2 at 37 °C for 1 h. The scaffolds were then maintained in complete culture medium for the following experiments.

2.4. Morphology of BM-MSCs cultured on the collagen scaffolds

The histological appearances of BM-MSCs within the scaffolds after 1 h, 3 h, 12 h, 24 h, 48 h and 72 h culture were examined under light microscopy while their cellular morphology was studied with scanning electron microscopy (SEM) at 1 h, 3 h, 12 h, 24 h and 72 h in culture. For light microscopic studies, the samples were fixed in 4% paraformaldehyde overnight, dehydrated in graded alcohols and embedded in paraffin. Five μm thick sections were stained using a standard hematoxylin and eosin (HE) staining protocol. For SEM studies, the samples were fixed in 4% glutaraldehyde, then post-fixed with 1% OsO4, dehydrated in a graded ethanol, and dried in a critical point drier (Hitachi, Tokyo, Japan). After the samples were coated with gold in a JFC-1100 unit (Jeol Inc., Japan), they were observed under a scanning electron microscope (Hitachi Model S-3000N, Tokyo, Japan).

2.5. Quantitative real time PCR (qRT-PCR)

Total RNA was isolated using TRIzoL® reagent (Invitrogen, Carlsbad). RNA samples were then treated with DNase I(Promega, San Luis Obispo) to remove any contaminating genomic DNA. The quality of the RNA was evaluated using spectrophotometry and denaturing agarose gel electrophoresis. cDNA was synthesized from 1 μg of purified total RNA using a PrimeScript RT reagent kit (Bio-Rad Laboratories, Hercules), according to the manufacturer's instructions. The specific primers used for quantitative PCR analysis are listed in Table S1. Each real-time PCR reaction had the following components: 1 μL of RT product, 10 μL of SYBR Green PCR Master Mix (Bio-Rad Laboratories), and 500 nM each of the forward and reverse primers. QRT-PCR for stemness genes was performed by the blow procedure (95 °C 3 min; 94 °C 10 s, 60 °C 30 s, 72 °C 30 s, 40 cycles). Melt curve analyses (55–95 °C, 0.5 °C increments, 10 s) showing single melting peaks were conducted to ensure the specificity of the amplification of the expected DNA fragments. Each sample was analyzed in triplicate. The experiment was repeated three times. The relative transcript abundance was determined according to the $2^{-\triangle\triangle C(T)}$ method, and the fold change in expression of each gene was normalized to an endogenous control (*GAPDH* gene).

2.6. Rat uterine horn damage model and BM-MSCs transplantation

Vaginal smears were obtained daily between 08:00–10:00 AM. In total, 92 female Sprague–Dawley rats (250–300 g) with four consecutive 4-day estrus cycles were randomly assigned to four groups, including a sham operated group ($n = 32$ uterine horns), a spontaneous repair group ($n = 32$ uterine horns), a collagen/PBS group ($n = 38$ uterine horns), and a collagen/BM-MSCs group ($n = 82$ uterine horns). After the rats were anesthetized, the uterine horns were exposed through a low abdominal midline incision. A segment of 1.5 cm in length and 0.5 cm in width was resected from the horn of the uterus, and the side of mesometrium was left intact. The two different kinds of collagen scaffolds (collagen/PBS and collagen/BM-MSCs) (1.5 cm × 0.5 cm) were sutured in place to replace the excised segment. For the spontaneous regeneration group, after excision, the uterine horns with complete hemostasis and marked defects were left open for spontaneous healing without any scaffolds. For the sham operated group, after exposure by an abdominal midline incision, the uterine horns were left intact in the abdominal cavity without excision. The rectus fascia and skin were then repaired with sutures. All rats received intramuscular injections of penicillin twice a day for three days after the surgery.

2.7. immunofluorescence analysis

Rat BM-MSCs were labeled by CM-DiI (C7000, Molecular probe, Invitrogen, Eugene) before transplantation for the trace of the stem cells according to the instructions. Rat uterine tissues containing collagen/BM-MSCs constructs were removed at day 30 and embedded into OCT (Leica, Nussloch, Germany) and frozen at −30 to −35 °C for 5–10 min. Finally, the frozen tissues were continuously sectioned to 6 μm thickness using a cryostat (Leica). The sections were adhered to slides treated by 0.1% polylysine and expression of fluorescence was observed immediately under the fluorescence microscope (Leica). Part of slides was stained with antibodies of Desmin (1:200, ab15200, Abcam, Cambridge) and Vimentin (1:1000, ab137321, Abcam, Cambridge) for differentiation of BM-MSCs *in vivo*.

2.8. enzyme-Linked immunosorbent assay

The bFGF, IGF-1, VEGF and TGF-β1 concentrations in the regenerated uterus were measured using an enzyme-linked immunosorbent assay. For Enzyme-linked immune sorbent assay (ELISA), uteruses were collected 3 days, 7 days and 14 days postoperative, washed in PBS and stored at −80 °C for further analysis. Finally, these samples were homogenized in pre-chilled lysis buffer (20 mM HEPES, 0.5 mM EGTA, 1 mM DTT and 0.32 M sucrose; PH 7.4) containing protease inhibitors cocktail (Sigma, St. Louis), then incubated at 4 °C for 30 min. The samples were centrifuged at 15,000 rpm in a pre-chilled centrifuge at 4 °C for 10 min. The supernatant from uterus lysate was collected and assayed for protein concentration using the BCA Protein Assay reagent kit. Samples containing equal amounts of total proteins were analyzed by IGF-1 ELISA kit (Westang Bio, Shanghai, China), bFGF ELISA kit (Cusabio Biotech, Wuhan, China), TGF-β1 and VEGF ELISA kit (eBioscience, San Diego) according to the manufacturer's protocols.

Sensitivity of the IGF-1 assay was 1 ng/mL, the intra-assay coefficient of variation was 10%, and the inter-assay coefficient of variation was 10%. For the bFGF assay, the sensitivity was 0.22 pg/mL, the intra- and inter-assay coefficient were 8% and 10%, respectively. Values for the TGF-β1 and VEGF assay were 7.8 pg/mL and 27 pg/mL