DIFFERENTIATION OF LYMPHATIC ENDOTHELIAL CELLS FROM BONE MARROW MESENCHYMAL STEM CELLS WITH VEGFS

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ABSTRACT

Although there have been many experimental studies demonstrating that bone marrow-derived mesenchymal stem cells (MSCs) have the potential to differentiate into mesenchymal tissues such as osteocytes, chondrocytes, and adipocytes in vivo and in vitro, little information is available regarding their potential to differentiate into lymphatic endothelial cells. Therefore, we chose to investigate differentiation of MSCs into lymphatic endothelial cells using stimulation with members of the vascular endothelial growth factor (VEGFs) family. Rat MSCs were isolated from bone marrow aspirate of Sprague-Dawley rats as previously described and characterized with flow cytometry for surface markers CD14, CD34, CD29, and CD90. Purified MSCs were plated and cultured in the presence of VEGF-A, VEGF-C, or the combination of both for 10 days. We examined the cells for Prox-1 and LYVE-1 by immunocytochemistry, RT-PCR, and Western blot analysis. Results demonstrated that expressed Prox-1 and LYVE-1. Our results indicate that MSCs induced by VEGFs are capable of differentiating into lymphatic endothelial-like cells in vitro, and this response has the potential to make them attractive candidates for the development of autologous tissue grafts for future therapy.

Keywords: differentiation, lymphatic endothelial cell, mesenchymal stem cell (MSC), VEGF-A, VEGF-C

Lymphedema is a problem of tissue fluid imbalance due to defects in lymphatic uptake and/or transport (1-3). Primary lymphedema often results from congenital dysplasia of the lymphatic system, whereas the more common secondary lymphedema arises as a consequence of surgical, malignant, inflammatory, infectious, or traumatic disruption of the lymphatics. Although secondary lymphedema is a common clinical condition, treatment for this disabling condition remains limited and largely incomplete (4,5). Regeneration or reconstruction of the lymphatic pathways is a potential option for future treatment, but these methods are still under study.
Recent molecular studies have elucidated lymphangiogenesis, which has been shown to be stimulated by various cytokines, including those in the vascular endothelial growth factor (VEGF) family (6-11): VEGF-A, VEGF-B, VEGF-C, VEGF-D, Orfivirus-encoded VEGF-E, and the placenta growth factor. These ligands bind to VEGF receptors VEGFR-1, VEGFR-2, and VEGFR-3 with partially overlapping receptor specificities. Traditionally, VEGF binds VEGF receptors 1 and 2 and is angiogenic. VEGF-C binds to receptors 2 and 3 and is either hemangiogenic or lymphangiogenic in different assays. In addition, the affinity of VEGFs toward their receptors is regulated by proteolytic processing. The affinity of mature, proteolytically processed form of VEGF-C is 40 times higher for VEGFR-3 than for VEGFR-2. Furthermore, in adults VEGFR-3 is largely absent from blood vessel endothelium and remains predominantly expressed in the lymphatic endothelium. Targeted gene inactivation and transgene approaches in mice (11,12) have also revealed the critical roles of VEGF-C in lymphatic vessel function with adenoviral expression of VEGF-C inducing lymphangiogenesis in the skin of normal mice.

In order to resolve questions about lymphangiogenic specificity of VEGF family in lymphedema, previous investigators found that Embryonic Stem Cells treated with VEGF-A and VEGF-C individually promote lymphatic endothelial cell formation in vitro and enhance formation of lymphatic vessel structures in specific culture conditions or in 3-dimensional collagen matrix. These results provide a powerful new in vitro tool to dissect molecular mechanisms in lymphatic vessel formation.

Bone marrow stromal cells (BMSCs) (13-16) are also known as mesenchymal stem cells or colony-forming units (CFU), and they have become popular for use in tissue and cell repair. Although previous studies have demonstrated that bone marrow-derived mesenchymal stem cells (MSCs) have the potential to differentiate into mesenchymal tissues like osteocytes, chondrocytes, and adipocytes in vivo and in vitro, little information is available regarding their potential to differentiate into lymphatic endothelial cells (17-21). Therefore we undertook an investigation of the differentiation of rat MSCs into lymphatic endothelial cells (LEC).

MATERIALS AND METHODS

Animals

Four week-old male Sprague-Dawley rats were purchased from animal center of Shandong University, and all animal experiments were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 and the National Institutes of Health guidelines for the care and use of laboratory animals for experimental procedures. In addition, all procedures were approved by the Committee for Animal Experiments of the Shandong University.

Isolation and Culture of MSCs

Rat MSCs were isolated from bone marrow aspirate of Sprague-Dawley rats as previously described (13,14). Briefly, under sterile conditions, the femur and tibia of the rats (approximately 100 g) were excised, with special attention given to remove all connective tissue attached to bones. Bone marrow plugs were extracted from the bones by flushing the bone marrow cavity with 10 ml of MSC medium Dulbecco’s modified Eagle’s medium (DMEM)-low glucose supplemented with 10% fetal bovine serum and 100U/ml antibiotic (penicillin and streptomycin) solution. Marrow plug were dispersed by passing through subsequent pipettes of decreasing sizes. After a homogenous cell suspension was achieved, cells were centrifuged (1400 rpm, 8 minutes), resuspended in complete culture medium, plated (5x10⁶ cells per 75-cm² culture flask), and incubated at 37°C humidified atmosphere...
with 5% CO₂ for 3 days before the first medium change. The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate (14,22). Fresh complete medium was added and replaced every 2 or 3 days for approximately 10 days. At 90% confluence, the cells were trypsinized (0.125% trypsin, Sigma) and were passed to 75-cm² flasks at 1:3 ratio. First-passage MSCs were used in experiments for induction of osteocytes and adipocytes. Cells were washed with D-Hank’s and trypsinized for release conventional culture medium containing 10% fetal bovine serum (FBS) to stop trypsinization. MSC cells were plated into 6-well at 2x10⁴/ml. After culture using conventional culture medium containing 10% FBS until ~90% confluent, culture medium was changed into either osteogenic induction culture medium (10 mM/L β-sodium glycero-phosphate; 0.05 mM/L vitamin C; 100 mM/L dexamethasone; 85% HG-DMEM; 15% super new bovine serum) or adipogenic induction culture medium (L-DMEM; 10% FBS; 1 mM/L dexamethasone; 10 mg/L insulin, 0.5 mM/L IBMX; 0.2 Mm/L indomethacin; 100 U/ml Penicillin-Streptomycin). After 2 weeks of induction, cells were stained by Alizarin red or Oil red O respectively. Cells had a typical spindle-shaped appearance, and the MSC phenotype was confirmed by differentiation into osteocytes and adipocytes with specific differentiation media (23).

Induction of LEC Differentiation

At 90% confluence, MSC cells were trypsinized and passed to 24-well plates in complete medium. When cells reached 70-80% confluence, medium was changed to endothelial cell culture medium with 2% FBS, 10 ng/ml FGF, 5µg/ml heparin, 50 mg/ml endothelial cells growth supplement and either 20 ng/ml VEGF-A, 50 ng/ml VEGF-C and 20 ng/ml VEGF-A + VEGF-C (all ProSpec) for 10 days with medium change every 2 days.

Immunofluorescence Staining

Staining of cultured cells on plates was performed according to the following protocol. Cells were fixed in 4% paraformaldehyde (PFA/PBS) for 10 minutes on ice, washed with PBS, incubated in 0.1% Triton, and blocked with 1% BSA/PBS for 30 minutes at room temperature. The fixed cells were stained with mouse anti-Prox1 (1:100, Chemicon) or goat anti-LYVE-1 (1:50, Santa Cruz Biotechnology) followed by FITC-conjugated anti-goat antibodies or TRITC-conjugated anti-mouse antibodies (Zhongshan, Peking). Nuclei were counterstained with hochest33324 and viewed with fluorescent microscope (IX-FLA, Olympus). Images were captured with a Sanyo color CCD camera (VCC-2972, Sanyo Electric, Osaka, Japan).

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

The mRNA expression of lymphatic endothelial markers by cells was evaluated by semi-quantitative reverse transcription/polymerase chain reaction (RT-PCR). In brief, total RNA was extracted by using an RNeasy Mini Kit (QIAGEN) according to the directions of manufacturer. First-strand complementary DNA (cDNA) was prepared from the RNA template (5 g) by using an oligo(dT)12-18 primer and SuperScript II reverse transcriptase (Invitrogen). The
RT-reaction profile was 42°C for 60 min, followed by 70°C for 10 min. PCR amplification was performed by denaturation at 94°C for 30s, annealing at 58°C or 53°C for 1 min, and extension at 72°C for 1 min and 45s, by using template cDNA and TaKaRa Ex Taq (Takara, Otsu, Japan, http://www.takara.co.jp). The sequences of the primers were as follows:

Prox-1 Forward, 5'-GAGGAGCCTGTGTTCTGTCC-3',
Prox-1 Reverse, 5'-AGAGGCCCTAGCCAGTGTTGA-3',
LYVE-1 Forward, 5'-CACAGGGGAAACACACTCCT-3,
LYVE-1 Reverse, 5'-CATCGGCAAACATGAAGAGA-3',
VWF Forward, 5'-TGGGTCAGAAGGATTCCTATGT-3',
VWF Reverse, 5'-CAGCCTGGATAGCAACGTACA-3'

The PCR products were electrophoresed on 3% agarose gels and detected with ethidium-bromide staining. Lymphatic endothelial cells were used as the positive control and undifferentiated MSC cells as the negative control.

Western Blot Analysis

Cells from culture were washed three times in PBS and incubated in the lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.02% sodium azide, 100 µg/ml PMSF, 1 µg/ml peptin, and 1 µg/ml aprotinin) for 30 min on ice. The homogenate was centrifuged at 16,000 xg for 45 min at 4°C. Equal amount of protein from each lysate was subjected to 12% SDS PAGE and transferred onto a nitrocellulose membranes. After blocking for 1.5 h in 5% nonfat dried milk containing 0.1% Tween 20, the membrane was incubated at 4°C overnight in the presence of antibodies to mouse anti-rat Prox-1, goat anti-rat LYVE-1, and β-actin (Santa Cruz Biotechnology). The membrane was then washed and further incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. Following washing, immunoreactive bands were detected using the Western Lightning Chemiluminescence Reagent (Amersham Biosciences). Lymphatic endothelial cells were used as the positive control and undifferentiated MSC cells as the negative control.

RESULTS

Cell Culture of MSCs

MSCs were isolated according to standard techniques for the isolation of mononuclear cells from bone marrow. Phase contrast microscopy from cells in passage P0 demonstrated a fibroblast-like, spindle-shaped morphology (Fig. 1). In later passages (>P5), the spindle-shaped cells began to display a broadened, flat morphology. Therefore, endothelial experiments were performed only on cells of P3. Using flow cytometry (Fig. 2), MSCs typically express CD90 and CD29 and did not express CD14 and CD34 which are specific surface markers of vascular endothelial cells. The ability of the MSCs to differentiate into osteocytes and adipocytes was tested in osteogenic medium and in adipogenic medium. When cultured in osteogenic medium for 15 days: on day 12-15, cells began to mineralize their matrix and were positive for Alizarin red staining (Fig. 3). In the adipogenic medium, cells were also able to differentiate into adipocytes and demonstrated lipid vacuoles (Fig. 4).

Differentiation of MSCs into Lymphatic Endothelial-Like Cells

We introduced differentiation into endothelial-like cells by cultivating confluent MSCs in the presence of 20 ng/ml VEGF-A,
50 ng/ml VEGF-C or 20 ng/ml VEGF-A/VEGF-C with endothelial cell culture medium for 10 days. Cell morphology showed no difference compared with undifferentiated MSCs, Phase contrast microscopy from cells demonstrated a fibroblast-like, spindle-shaped morphology. We did not identify any differences among the presence of VEGF-A, VEGF-C, or VEGF-A/VEGF-C with endothelial cell culture mediums (Fig. 5).

**Immunohistochemical Staining Analysis**

Positive immunohistochemical staining for Prox-1 (Fig. 6) and LYVE-1 (Fig. 7) was demonstrated as characterization of lymphatic endothelial-like cells. Undifferentiated MSCs showed almost no specific staining for Prox-1 and LYVE-1, but after 10 days of culture the overall fluorescence intensity of the differentiated MSCs was markedly enhanced in VEGF-A, VEGF-C or VEGF-A/VEGF-C endothelial cell culture mediums.

**Fig. 1.** Phase contrast microscopy from cells in passage P0 demonstrate a fibroblast-like, spindle-shaped morphology (A) while cells in later passages (P5) demonstrate spindle-shaped cells beginning to display a broadened, flat morphology (B).

**Fig. 2.** Flow cytometry of cells at passage 3 demonstrates that MSCs express CD90 and CD29 as markers of stem cells and lack expression for CD14 and CD34 which are the specific surface markers for vascular endothelial cells.
Fig. 3. The ability of MSCs to differentiate into osteocytes was examined. Cells cultured in osteogenic medium for 15 days and tested on days 12-15 began to mineralize their matrix and stained positive for Alizarin red (A). Control cells (B) stained negative.

Fig. 4. The ability of the MSCs to differentiate into adipocytes was examined. Cells cultured with adipogenic medium differentiated into adipocytes as demonstrated by positive oil red O staining. Control cells stained negative (not shown).

Fig. 6. Immunohistochemical staining for Prox-1, a marker characterizing lymphatic endothelial-like cells. Undifferentiated MSCs showed almost no specific staining for Prox-1, but after 10 days of culture, overall fluorescence intensity of differentiated MSCs was markedly enhanced in the induction medium containing VEGF-A, VEGF-C, or VEGF-A/VEGF-C.

Fig. 7. Immunohistochemical staining for LYVE-1, a marker characterizing lymphatic endothelial-like cells. Undifferentiated MSCs showed almost no specific staining for LYVE-1, but after 10 days of culture, overall fluorescence intensity of differentiated MSCs was markedly enhanced in the induction medium containing VEGF-A, VEGF-C, or VEGF-A/VEGF-C.
RT-PCR and Western Blot Analysis

Matching the immunohistochemical staining, RT-PCR (Fig. 8) and Western blot analysis (Fig. 9) produced similar findings after 10 days of culture with both Prox-1 and LYVE-1 expression documented in differentiated MSCs using VEGF-C, VEGF-A, and VEGF-A/VEGF-C culture medias. Control analysis using undifferentiated MSCs showed no specific expression for Prox-1 and LYVE-1.

DISCUSSION

The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for autologous or allogeneic cell therapy. Clinical trials for the treatment of osteogenesis imperfecta (24), metachromatic leukodystrophy, and Hurler syndrome (25,26) support the therapeutic relevance of transplanted MSCs. The use of autologous vascular endothelial progenitor cells seems attractive for the development of engineered vessels as well as for the vascularization of engineered tissues and may also be useful to augment vessel growth in ischemic tissue (13,18,19). However, there has been little information available regarding the potential of MSCs to differentiate into lymphatic endothelial cells.

Our study shows for the first time that rat bone marrow-derived CD90+, CD29+ MSCs are capable of differentiating into lymphatic endothelial-like cells in vitro, which make them attractive candidates for the development of autologous tissue grafts. Serial analysis of gene expression (SAGE) revealed that single cell-derived colonies of MSCs expressed mRNAs of multiple cell lineages, including characteristic endothelial cells for vasculogenesis and angiogenesis, which are currently not fully understood. The pivotal role of VEGF-A and VEGF-C for both processes was demonstrated, and they both should be included in “cocktails” for the in vitro differentiation of either endothelial progenitor cells or hematopoietic stem cells into endothelial cells in vitro (27-31).

Several populations of bone marrow-derived cells also have the potential to differentiate into endothelial-like cells. CD133+ HSCs cultivated at high cellular density and in the presence of endothelial growth factors like VEGF-A and VEGF-C were shown to acquire vascular endothelial features and lymphatic endothelial features (11,32), and CD34+ HSCs isolated from peripheral blood can differentiate into endothelial cells in vitro and contribute to vascularization in animal models (21,26). A distinct population of Embryonic Stem Cells has been described by the Verfaillie group (33) which is capable of differentiating...
into lymphatic endothelial cells in vivo and in vitro (34).

The major advantages of MSCs are the vast number of cells that can be isolated from one bone marrow aspirate and the genetic stability over many passages (23,35). One major criticism of studies describing plasticity of bone marrow stem cells is the heterogeneity of the cell population. Whereas MSCs in our system were both CD31 and CD133 negative; this feature was not seen in the MAPCs used by the Verfaillie group and also in EPCs (23,35). A major criticism of studies describing plasticity of bone marrow stem cells is the heterogeneity of the cell population. and endothelial molecules like Epican and Keratins 8 and 10 (21). These data suggest that the in vitro differentiation potential of MSCs is not restricted to mesodermal lineages and MSCs can also transdifferentiate...
into other lineages like lymphatic endothelial cells in vitro and in vivo.

Earlier work describes the molecular mechanisms responsible from VEGF-C stimulation in upregulating expression of VEGFR-3 and VEGFR-2. These play a major role in lymphangiogenesis in vivo and contribute together with matrix metalloproteases to the formation of capillary-like structures (36). In our study, differentiation of MSCs with VEGF-A and VEGF-C also upregulates the expression of the VEGFR-3 and VEGFR-2.

Differentiated MSCs could also be beneficial in the engineering of complex tissues, where vascularization of the tissue is an essential feature for the successful engraftment. Future clinical studies are needed to examine whether systemic application of pre-differentiated lymphatic endothelial MSCs may have positive effects in patients with lymphedema and lymphatic-related diseases. Although we cannot rule out the possible existence of subpopulations of committed cells, it appears unlikely since cells do not proliferate during the differentiation in presence of 2% fetal calf serum and VEGFs. Differentiation experiments with single cell-derived MSCs will ultimately test the plasticity of MSCs.

In our differentiation system, MSCs acquire major characteristics of mature lymphatic endothelial-like expression of LYVE-1 and Prox-1 after a 10-day differentiation, which indicates that these markers are expressed later in lymphatic endothelial maturation. Prolongation of differentiation time may also lead to an upregulation of these markers.

After differentiation, the immunohistochemical staining, RT-PCR, and Western blot analysis shows that rat stroma cells can also be differentiated into lymphatic-like cells with VEGF-A and VEGF-C. We also found that MSCs form tube-like structures when cultivated in semisolid medium; the presence of VEGFs markedly enhanced this behavior. Interestingly, the numbers of capillary-like cells in this assay were strongly enhanced in pre-differentiated MSCs. In addition, hypoxia upregulates several genes involved in angiogenesis including basic fibroblast growth factor, VEGF-A, VEGF-3 and VEGFR-2, and components of the plasminogen system (37) and these interactions need to be examined.

Our findings may support the development of tissue engineered lymphatic vascular grafts based on autologous MSCs. Differentiated MSCs could also be beneficial in the engineering of complex tissues, where lymphatic vascularization of the tissue is an essential feature for the successful engraftment. Clinical studies are needed to evaluate whether the systemic application of pre-differentiated MSCs may offer an option for patients with lymphedema and lymphatic-related diseases.

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