ISOLATION, CULTURE AND DETECTION OF MESENCHYMAL STEM CELLS IN TISSUES FROM CRITICAL LIMB ISCHEMIA MODEL IN RAT

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Abstract

Introduction. *Rat mesenchymal stem cells (MSCs) represent a small portion of the cells in the stromal compartment of bone marrow and have the potential to differentiate into new blood vessel and other tissues.*

MSCs transplantation in tissues from critical limb ischemia model in rat may represent a therapeutic applications of vascular regeneration.

Aim. *The aim of this study was to isolate with a simple method the rat bone marrow stromal cells. Then the adherent cells were labeled with 5-bromo-2-deoxyuridine (BrdU, Sigma) and injected in the gastrocnemius and adductor muscle of ischemic hind limbs in order to demonstrate their presence in the critical limb ischemia model in rat.*

Material and methods. *MSCs were isolated from Wistar rats, 8 weeks of age. The MSCs were labeled in vitro for later identification by adding 10 μg/mL 5-bromo-2 –deoxyuridine (BrdU, Sigma).*

Results. *Small colonies of fibroblast-like cells were seen after several days of primary culture. These colonies increased in size and were subcultured after 15-18 days.*

Conclusion. *The MSCs obtained in this study presented a stable undifferentiated phenotype under normal culture conditions.*

MSCs are easy to isolate, culture, and detect in in vivo culture. These cells are characterized by high plasticity and could have an important role in angiogenesis.

Keywords: mesenchymal stem cells, critical limb ischemia model in rat, angiogenesis.

Introduction

Mesenchymal stem cells (MSC), a distinct type of adult stem cell, are easy to isolate, culture, and manipulate in ex vivo culture [1].

Rat mesenchymal stem cells (MSCs) represent a small portion of the cells in the stromal compartment of the bone marrow. MSCs are defined as a self-renewing population and have the potential to differentiate into several mesenchymal cell lineages (bone, cartilage, fat, new blood vessel and other tissues) and represent a promising source for therapeutic applications [2]. MSCs can be isolated from the bone marrow and can be easily separated from the hematopoietic stem cells (HSCs) due to their plastic adherence [3].

Critical limb ischemia is recognized as the most severe stage of peripheral arterial disease, and is associated with a high risk of amputation, along with significant morbidity and mortality.

MSCs therapy generates new blood vessels and promotes neovascularization and tissue repair. MSCs transplantation in tissues from critical limb ischemia model in rat can represent therapeutic applications of vascular regeneration.

Thus, designing, describing and validating these

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new therapies in an experimental model of critical limb ischemia is essential.

Aim

The aim of this study was to isolate with a simple method the rat bone marrow stromal cells. Subsequently the adherent cells were labeled with 5-bromo-2-deoxyuridine (BrdU, Sigma) and injected in the gastrocnemius and adductor muscle of ischemic hind limbs in order to demonstrate their presence in critical limb ischemia model in rat.

Material and methods

Experimental model of critical limb ischemia in rat

Critical limb ischemia was induced in Wistar 8 weeks rats weighing 200-240 g by progressive ligation and excision: first left femoral artery and, after two weeks, left iliac artery.

Mesenchymal stem cells isolation and culture

Bone marrow was collected from Wistar, 8 weeks of age rats. The animals were sacrificed by cervical dislocation according to Institutional Animal Care and Use Committee guidelines. The tibiae and femora were aseptically removed and adherent soft tissue was debrided. The bone marrow was collected by flushing femurs and tibias with complete medium DMEM-LG (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma), glutamine 2 mM (Sigma), 100 U/ml penicillin, 50 mg/ml streptomycin (both from Sigma) and heparin at a final concentration of 5 U/ml. Collected marrow samples were mechanically disrupted by passing them successively through 18 gauge and 20 gauge needles.

After 72 hours, nonadherent cells were removed and adherent cells further cultured. The cells were subcultured (50% to 60% confluence) by treatment with 0.25% trypsin in 1 mM EDTA (Sigma) for 10 min at 37ºC, and the resulting cell suspension was centrifuged at 1,500 rpm for 5 min, resuspended in complete culture medium and counted with a hemacytometer.

After 3 weeks with weekly medium changes, fibroblastic-like cells became the predominant cells in culture.

Flow cytometry

For cell surface antigen phenotyping, cells ($1x10^6$) were detached and stained with FITC conjugated antibodies (CD44, Becton Dickinson) and analyzed with FACSCanto II (Becton Dickinson).

Colonogenic assays

After 4 and 5 passages, cells were counted and plated in a concentration of 200 cells in each of triplicate 100 mm plates in normal culture medium with medium changes every 3–4 days for 2 weeks. The colonies were fixed and stained with 1% crystal violet (Sigma) for visualizing colonies produced at cultures. We also measured the size of colonies.

Calculation of population doubling time (PDT)

For the determination of cell capacity to undergo proliferation at given culture conditions, the population doubling time was calculated. Population doubling number (PDN) was calculated according to the equation PDT=culture time (CT)/PDN. To determine PDN, the formulae PDN=log N/N0×3.31 was used [4,5].

Cell proliferation assay

For later identification and evaluation of the level of proliferation we used the thymidine analogue bromodeoxyuridine (BrdU) (Sigma) incorporation (10 µg/mL). The BrdU was added to the culture medium 48 h prior to transplantation in rats with critical limb ischemia. MSCs were injected in the gastrocnemius and adductor muscle of ischemic hind limbs 30 days after surgery (progressive ligation and excision of femoral iliac artery).

Results

In order to examine the morphology and the degree of adherence of the cells daily microscopic examinations of cell cultures were performed. In the first days of culture, the total cell plate, adherent cells were 25% (figure 1, 2).

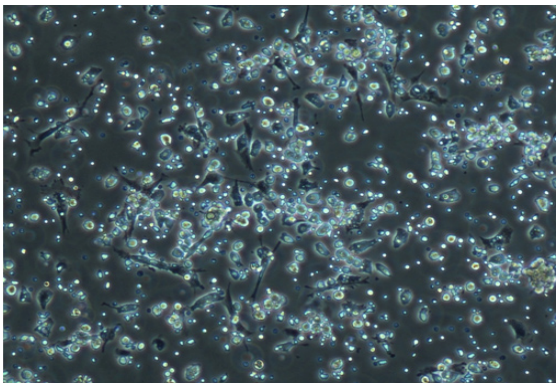

Figure 1.

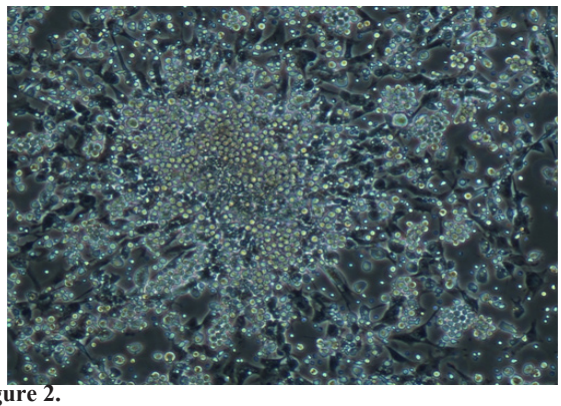

Figure 2.

Figure 1, 2. Light microscopic observation of cells after 1st day of culture.

After 72 h of culture, the culture medium was changed and the non-adherent hematopoietic cells in the culture were removed.

Starting with the 3rd day of culture, the adherent cells adopted some fibroblastic character, becoming elongated, spindle, round (figure 3).

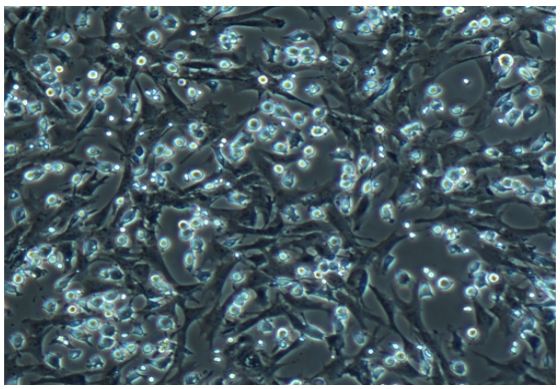

Figure 3. Cell morphology after 3rd day of culture.

The colonies of fibroblastic-like cells began to appear in the culture flasks after 7 days. These colonies increased in size and were passed after 15-18 days. The number of round cell count decreased progressively with the number of passages and after passages 3 were observed only fibroblasts like cells with bipolar character (figure 4).

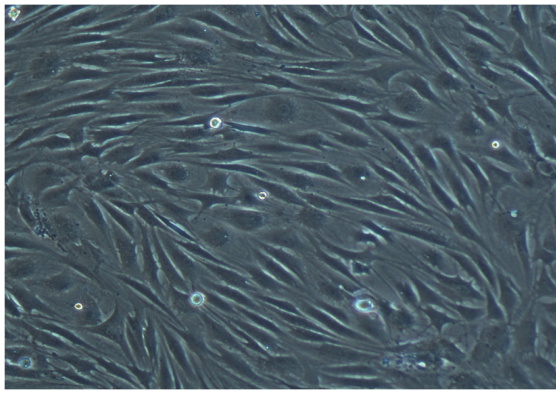

Figure 4. Morphology of rat bone marrow derived mesenchymal stem cells after passages 3.

After several passages, the cultures were composed of a homogenously fibroblastic cell and presented a stable undifferentiated phenotype.

Cells characterization by flow cytometry revealed that the cells isolated from rat were positive (95.1%) for CD44 (figure 5).

The colonogenic assay results show an average of 22±2 colonies at passage 4 and an average of 36.33±3.21 after passage 5. The differences were not statistically significant. PDT value was 35±16 hr for passage 4 and 34±16 for passage 5, and the cells in both culture started proliferating immediately after seeding.

5-bromo-2 –deoxyuridine (BrdU) is a thymidine analogue that is included in the DNA of dividing cells during S-phase and can be used as a methodological tool for in vivo investigations following in vitro prelabeling of isolated stem cells for subsequent cell tracking within the recipient host [6].

Once injected in the ischemic tissue of critical limb ischemia model, MSCs generate new collateral vessels (figure 6).

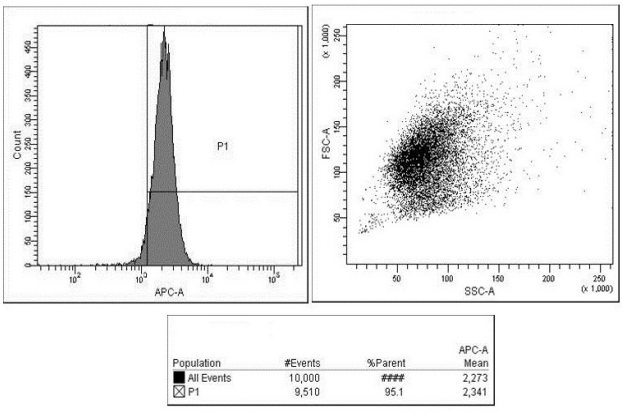

Figure 5. Results of flow cytometric analysis of cells harvested from rat bone marrow.

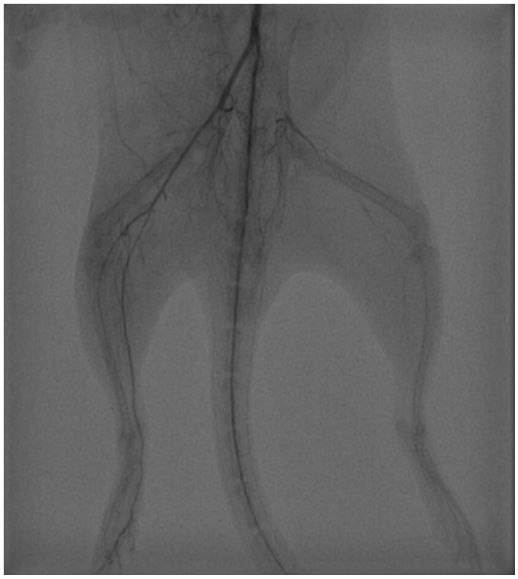

Figure 6. Angiography of critical limb ischemia model (ligation and excision of left femoral and iliac artery). New collateral vessels were generated 30 days after injecting MSCs.

Discussion

MSCs are multipotent stem cells characterized by their ability to self-renew and differentiate into mesoderm- and nonmesoderm-derived tissues [7,16,17]. MSCs isolated from bone marrow or from other tissue have similar surface markers and differentiation potential, but bone marrow is the major source of MSCs [16,22,23,26]. The transplantation of autologous stem cells from adult bone marrow might

therefore be a promising strategy for the treatment of critical limb ischemia [7,18,19,25]. These cells have the advantage that they can be easily isolated from healthy donor tissue. Our study does no report tumor formation, however it was reported by other studies [8,20,24].

Mesenchymal stem cells provide a useful tool for regenerative medicine due to their differentiation capacity [9,10,11].

The pluripotent potential of MSCs was demonstrated by identifying CD44 expression on their surface. Expression of CD 44 is involved in embryonic stem cell renewal [12,13].

New collateral vessels generated by MSCs were identified by angiography [14,15,25,26].

Conclusion

In this study we found a simple and safe method to isolate, culture, and detect MSCs *in vivo* culture. These cells, characterized by a high expression of CD44 on their surface can be easily isolated from the bone marrow by standard isolation techniques.

As a result of their high proliferating potential, MSCs generate new collateral vessels, therefore they could have an important role in therapeutic angiogenesis in critical limb ischemia.

We created an experimental model of critical limb ischemia in rat that could be the basis for a future clinical trial.

Based on these important characteristics, our study shows that MSCs represent an important source for regenerative medicine.

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