ISOLATION OF BONE MARROW MESENCHYMAL STEM CELLS

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SUMMARY

Mesenchymal Stem Cells (MSCs) have a high ability to renew and differentiate themselves into various lineages of conjunctive tissues. This study aimed to isolate the MSCs from murine bone marrow by using two different growth media and to characterize them with immunostaining with antivimentin antibody. We used six 2-week old BALB/c mice. Bone marrow was collected from mice's tibial and femoral channels and re-suspended in a final strength of 6x105 in Knockout-DMEM and high-glucose-DMEM media, supplemented by 10% FBS, and kept in a humidified 5% CO2 incubator at 37^0 C for 72 h, when non-adherent cells were removed during the change of medium. The number and density of adherent fibroblast-like colonies was greater with the Knockout-DMEM medium (within 5 days of culture) versus 10-20 days in DMEM-high glucose to get the same cellular concentration. The cells in both groups were highly positive for antivimentin antibody, characterizing them as MSCs. Obtaining MSCs as quickly as possible is essential for cell therapy field, especially when those cells are intended to be used for the repair of tissues from mesenchymal sources.

Keywords: Stem Cells; Cell Culture; Mice; Bone Marrow; Vimentin.

INTRODUCTION

Bone marrow stromal cells, also known as mesenchymal stem cells or fibroblastic colony-forming units, are multipotent non-hematopoietic stem cells adhering to culture plates[1,2].

Mesenchymal Stem Cells (MSC) of the bone marrow have the ability to renew and differentiate themselves into multiple lineages of conjunctive tissues, including bone, cartilage, adipose tissue, tendon, muscle, and bone marrow stroma[3,4]. Those cells have been first described by Friedenstein et al.[5], who found that MSCs adhere to culture plates, look like in vitro fibroblasts, and build up colonies[1,6]. Recently, MSCs have been brought to the attention of many researchers, because these cells are of great interest for treating various human diseases.

Many studies have isolated MSCs and controlled, in vitro, its differentiation into cartilaginous tissue and bone using specific growth factors, with the objective of using this new technology for repairing injured tissues of mesenchymal origin[7-9].

The proposition of this study was to standardize the best culture medium that should be employed in order to quickly isolate MSCs and characterize them with immunostaining by the antivimentin antibody, due to our interest in differentiating them in vitro in a cartilaginous tissue, aiming to, in the future, use it for repairing injured joint cartilage.

OBJECTIVES

The objective of this study was to standardize culture medium that should be employed for a faster isolation of the MSCs and their characterization with immunostaining by antivimentin antibody.

MATERIALS AND METHODS

MSCs were collected from the bone marrow of six two-week old BALB/c mice, supplied by the Experimental Laboratory of Infectious Diseases, Botucatu Medical College – UNESP. The animals were sacrificed with a lethal dose of sodium pentobarbital. This research was approved by the Committee of Ethics in Animal Experiments (CEEA) of this university according to the protocol nr. 345.

Culture medium tested was the Knockout DMEM (Dulbecco's modified Eagle's medium) (DMEM; Catalog nº 10829-018, Lot: 1209477) and high-glucose- DMEM (DMEM; Catalog nº 12100-046, Lot: 1181947), supplemented by 10% fetal bovine serum ([FBS] Catalog nº 10270-106, Lot: 40Q3534K) and 10U/ml penicillin G, 10ug/ml streptomycin and 25mg/ml anphotericin B (Catalog nº 15240-096, Lot: 1185890) all supplied by GIBCO® Invitrogen Corporation.

MSCs were collected from femoral and tibial bone marrow of three mice by inserting a 26-gauge syringe at bone cavity, washing it with 10 ml of Knock-out DMEM and high-glucose DMEM in the remaining three mice.

After centrifuging at 1200 rpm for 10 minutes, bone marrow cells were re-suspended in 1 ml at the corresponding culture media for cell counting and cell feasibility verification by using a Neubauer camera. The number of vital cells was determined by a technique that excludes non-vital cells stained by a trypan blue solution (GIBCO® Invitrogen Corporation), following the recommendations by Freshney[11]. For counting, 0.1 ml of the cell suspension was used, which, in turn, had used 0.1 ml of trypan blue and 0.8 ml of culture

Study conducted at the Botucatu Medical College, Cell Culture Laboratory, Hemocenter – UNESP - São Paulo - Brazil.

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medium. This cell suspension was transferred to the Neubauer camera with the aid of a Pasteur pipette and then cells were counted, excluding those showing blue staining (non-feasible cells). For calculating the number of cells, the following equation was used: NC x D x 10^4/#Q, where NC= number of counted vital cells; D= sample dilution (10) and #Q= number of squares at the Neubauer camera used for counting cells. Feasibility was always higher than 95%, and the number of plated cells was 6x10^5/plate.

A coverslip was placed inside culture plate to allow cells to grow over it for subsequent analysis with antivimentin antibody. Cultures were maintained in a heater at 37° C with 5% CO^2 for 72h. After 72h of culture, medium was refreshed in an interval of three to four days. When cells reached 80% confluence, presenting many fibroblastic colonies on the plate, coverslips were removed from culture and cells were fixed with 70% ethanol and washed twice with 2 ml phosphate buffer saline (PBS), pH 7.4. Cultivated coverslips were treated with 3% hydrogen peroxide in methanol in order to block endogenous peroxidases. Then, they were sequentially incubated with 3% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis MO, USA). In PBS, and the primary mouse antivimentin antibody (Santa Cruz Biotech, Santa Cruz CA, USA). Subsequently, coverslips were incubated with goat IgG antimouse antibody conjugated with peroxidase (Sigma Chemical Co., St. Louis MO, USA). Immunoperoxidase response was revealed with diaminobenzidine.

The mean area occupied by mesenchymal cells on plates was determined by the measurement of 10 fields randomly with a 5X magnification, from a slide of each donor mouse. Measurements were taken by using digital images, with an image analyzer Leica Q-win software Version 3 for Windows™.

Values achieved were expressed as average ± standard deviation. The non-paired Student's t-test was used, of which results with a significance level ($p \leq 0.05$) were considered as significant. Statistical test was performed in Instat (Version 3.0, GraphPad, Inc., San Diego, CA).

RESULTS

Colonies of cells with a fibroblastoid morphology started to appear on culture plates in 72 hours with the Knockout DMEM medium and in 5 days with the high-glucose DMEM. Non-adherent cells were removed from culture during medium refreshment. After 5 days of culture, the number and density of colonies of fibroblastoid-like cells with the Knockout DMEM medium (Figure 1a) were greater than with the high-glucose DMEM, which took nearly 10 days to reach the same cell concentration of the first medium (Figure 1b). Cells maintained in culture in both protocols were positive to antivimentin, characterizing them as MSCs. After 10 days, culture plate containing the Knockout DMEM medium was thoroughly covered by MSCs, as confirmed by antivimentin antibody response (Figure 1c and 1d). However, the plate where the high-glucose DMEM medium was used did not present the same cell strength (Figure 1e and 1f).

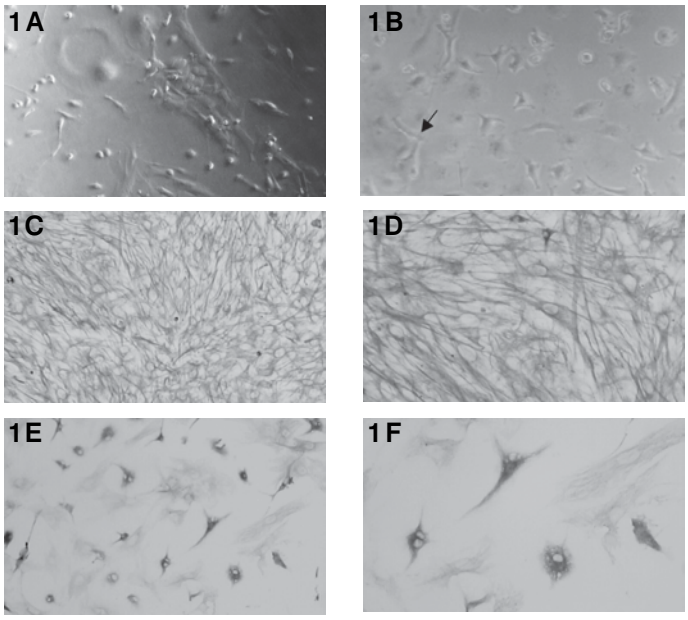

Figure 1- *Phase contrast photomicrography of mesenchymal cells in culture (A-B). Presence of cell colonies with fibroblastoid morphology after 5 days in culture with the Knockout DMEM medium (A) and in high-glucose DMEM medium, cells formed colonies within 10 days (B). Magnification: x400 respectively. Photomicrography of mesenchymal stem cells stained by antivimentin antibody (C-F). Notice the great cellular density with elongated morphology cultured in Knockout DMEM medium (C, D). Cell concentration was lower in the high-glucose DMEM medium (E, F). Magnification: (C, E) x200 (D, F) x400.*

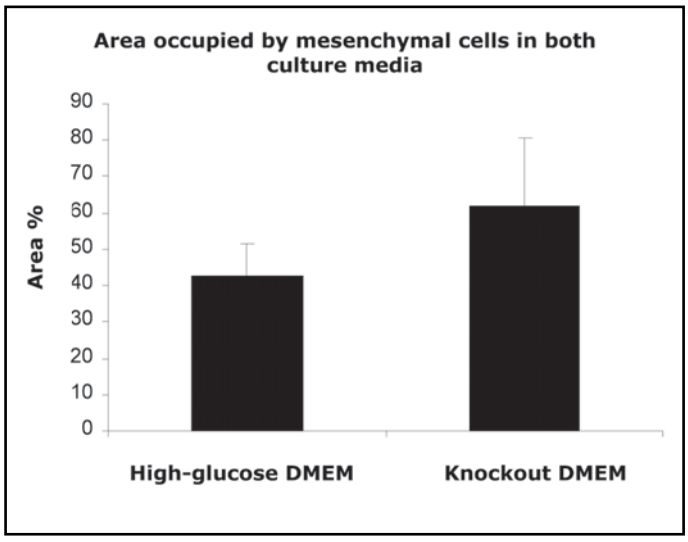

Figure 2 - *Comparison of the effects between both culture media on the area occupied by mesenchymal cells. Values represent the average ± standard deviation. Cells cultured in Knockout DMEM medium occupied a plate area approximately 50% larger than those cultured in high-glucose DMEM medium, showing a statistically significant difference, p<0.01.*

The mean area occupied by mesenchymal cells as analyzed by morphometry (Figure 2) was approximately 61.89%, with standard deviation of 18.64 for Knockout DMEM versus 42.88%, with standard deviation of 8.81 for high-glucose DMEM, with $p<0.01$, showing a statistically significant result.

DISCUSSION

Results show that it is possible to obtain MSCs in a short time by using the Knockout DMEM culture medium. This was possible due to its ability to be used specifically in embryonic stem cells. In other studies, the culture of MSCs with low-glucose DMEM was performed, and the authors achieved the first adherent fibroblastoid-like cells colonies only after 5 days[4,10].

In this study, we observed a significant difference between

both culture media, and those differences were more evident when cells were stained with the antivimentin antibody.

CONCLUSIONS

1. The use of both media enabled to show the superiority of Knockout DMEM, which produced a large number of cells when compared to the amounts achieved when the high-glucose DMEM was used.

2. The use of the Knockout DMEM medium could be an alternative for a quicker isolation of the MSCs in culture, especially when it is desired to differentiate them in other mesenchymal lineage tissues, such as cartilage.

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