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Isolationand Identification of mouse bone marrow derived mesenchymal stem cells

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Abstract:

Bone marrow derived mesenchymal stem cells (BM-MSCs) were successfully used in regenerative medicine. The purpose of this study was to isolate and characterize mesenchymal stem cells from mouse bone marrow for their subsequent use in researches. Previous data suggest that BM-MSCs are typically enriched by plastic adherent cultures, fibroplastoid cell fraction. However, Identification of MSCs achieved through their morphology, phenotypic characteristics and their biological behavior. The cellular morphology played a major part in identifying MSCs in vitro. In general, immature MSCs appeared as small, spindle-shaped cells, whereas mature MSCs was displayed as larger cells with a flat, polygonal morphology. Cells also tended to be locally confluent, growing in distinct colonies. Immunophenotypic analysis demonstrated that mouse BM-MSCs at passage three uniformly positive for CD44, CD90, CD105 and CD106. However, MSCs were always found to be negative for heamatopoietic specific markers CD34, CD45, and endothelial marker CD31. By concluding, mesenchymal stem cells can be successfully derived from mouse bone marrow by direct plating method. These cells represent a valuable source of stem cells for restoring the damaged organs.

Key words: *mesenchymal stem cells, mouse bone marrow, bone marrow stem cells, identification, isolation*

Introduction:

Stem cells are generally characterized as clonogenic and undifferentiated cells derived from different sources such as embryos, umbilical cord, amniotic tissue and adult stem cells (ASCs) (1). Stem cells can be induced to become different types of differentiated cells with specialized functions such as liver cells, cardiac myocytes, and neurons, etc. (2). However, for many years, therapeutically useful ASCs have been harvested from bone marrow (BM) (3). Adult stem cells localized in different tissues were thought to be restricted to replenishing only one or two lineages specific to that tissue (4). Adult bone marrow shelters different types of stem cells, including haematopoietic (HSCs) and Mesenchymal stem cells (MSCs) (5). One of the most extensively studied populations of multipotent ASCs was MSCs from the BM (6). The advantages of MSCs are their minimal invasive isolation, ability to self-renew, multi-lineage differentiation potential; express specific surface markers, and modulation of immune responses (7).Identification of MSCs achieved through their morphology, phenotypic characteristics.The cellular morphology played a major part in identifying MSCs in vitro. (8).

Phenotypically, MSCs express a number of markers, none of which, unfortunately, is specific to MSCs. the standard nomenclature for cell surface molecules (9). It was generally agreed that BM- MSCs do not express the haematopoietic markers CD45 (pan-leukocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14, or CD11 (monocyte and macrophage marker). They also do not express the stimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule), CD18 (leukocyte function-associated antigen-1), or CD56 (neuronal cell adhesion molecule-1), but they can express CD105 also known endoglin, CD73, CD44 (matrix molecules synthesize, lineage differentiation regulator), CD90, CD71, and Stro-1(highly specific for BM CFU-F) as well as the adhesion molecules CD106 (vascular cell adhesion molecule, CD166 (activated leukocyte cell adhesion

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molecule, intercellular adhesion molecule, and CD29 (10). There are several reports that describe the isolation of both human and rodent MSCs using antibody selection based on the phenotype of MSCs. Some have used a method of negative selection to enrich MSCs, whereby cells from the hematopoietic lineage are removed (11). Others have used antibodies to positively select MSCs (12). These principles have been utilized in BM-MSCs isolation by flow cytometry, magnetic beads, FACS (fluorescence-activated cell sorting) (13) Phenotypic characterization of MSCs is usually carried out using immunocytochemical detection of cell surface molecule expression (14).In order to identify MSCs, the International Society for Cell Therapy proposed for the minimal identification of MSCs (15) the following criteria:

1- Adherence to plastic in standard culture conditions

2- 2- Phenotype Positive (/ 95 % +) Negative (/+2 %/)

Positive	Negative
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
CD44	CD79a or CD19
	HLA-DR

3- In vitro differentiation: osteoblasts, adipocytes, chondroblasts

Accordingly, the present study was designed to investigate if the BM-MSCs isolated by direct plating are similar to those describedby the International Society for Cell Therapyfor use in tissue engineering and regenerative medicine.

Materials and methods:

The care of animals was in accordance with ethics committees in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Al-Mustansiriyah University. Briefly, young (4-6 weeks old) male albino mice were used to derive BM-MSCs cultures using a described technique by (16)with modification:

-Prepare Crude Bone Marrow Cell suspension

Mouse MSCs were harvested from the BM of the femurs and tibiae, Utilize pools of 4-6 miceweresacrificed by cervical dislocation. Femurs and tibiae were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the BM was collected by flushing with syringe through a 25 gauge needle containing 1 ml of prewarmed complete growth medium RPMI-1640 (US-Biological-USA) containing 100U/ml penicillin, 100µg/ml streptomycin and 10%FBS. This operation was continued until all bones were demarrowed for three mice for each culture. Single cell suspensions were prepared by gently mixing the cells with a pipette.

- Isolation of bone marrow –mesenchymal stem cellsUsing Primary explant culture method (direct plating)

According to the original working of Friedenstein (17) utilizing adherent cultures of untreated whole bone marrow, after preparation of mouse bone marrow uniform cell suspension. The cell suspension was centrifuged at 1000 rpm for 5 minutes at 18ºC, Supernatant containing thrombocytes and erythrocytes was discarded and the cell pellet was resuspended with complete growth medium RPMI-1640 with 10%FBS.

- Culturing and Expansion of BM-MSCs

The cell suspension were seeded in plastic tissue culture flasks 25cm2 with 5ml culture medium RPMI-1640 supplemented with 10% FBS and antibiotics at a plating density of 16 × 106 cells/ml using direct plating method. The culture was incubated at 37Cº in control medium for growth until complete confluent monolayer cell culture was reached. The MSCs were isolated based on their ability to adhere to the culture plates. After 24 hours, red blood cells and other non-adherent cells were removed and washed 2-3 with PBS then fresh medium was added to allow further growth. This feeding process then lasted twice in a week until we made the first passage of MSCs. The adherent MSCs grown to 80% confluency in 4-5 days were defined as passage zero (P0) cells. And was expanded by passaging. The P0 MSCs were washed with PBS and detached by incubating with 1ml of 0.25% trypsin-EDTA solution (US-Biological-USA) for 2 min at 37ºC. In order to dislodge the cells, the flask gently rock, then 5ml of culture media which converted to MEM plus 10% FBS was added to inactivate the trypsin, the single cell suspension were centrifuged at 2000 rpm for 10 min, the cells pellet were resuspended in 1ml complete medium plus MEM 10% FBS and counted manually using a Hemocytometer. The cell suspension was plated as P1 in two plastic tissue culture flasks 25cm2 at densities of 1 X 106 cell/ ml, complete medium was changed to remove non adherent cells at 48 h after seeding and every 3 days thereafter, when the first passaged become nearly confluent the cells were recultured in similar conditions. This study used bone marrow mesenchymal stem cells at passage 3, in a good growth state.

Immunocytochemistry analysis of BM-MSCs

Immunocytochemical analysis were performed on cells grown on coverslips. A coverslip was placed inside tissue culture dish (9cm2) to allow cells to grow over it for subsequent analysis at density 1×106 cell/ml. After the cells had grown to near 80% confluency (P3), the culture attached cells on coverslip were washed three times with PBS and fixed with 4% formalin prepared in PBS and left for 10 minutes and then dried. The following cluster of differentiation markers were used for phenotypic identification of MSCs. The cells were incubated with mouse anti-human CD45(US-Biological-USA), mouse anti-human CD105(US-Biological-USA), mouse anti-human CD90(US-Biological-USA), mouse anti-human CD31(Dako-America), Rat anti-mouse CD44(US-Biological-USA), mouse anti-rat CD106(US-Biological-USA) and Goat anti-mouse CD34(US-Biological-USA).The procedure of immunocytochemistry of MSCs was applied in room temperature according to the manufacture instruction of Santa Cruz Company as follows:

Solution of (0.1-1%) hydrogen peroxide H2O2was added and incubated in a humidified chamber for 10 minutes, then washed twice with PBS. The cells were incubated for one hour in 1.5% blocking serum. The cells were washed twice with PBS for 5 minutes, then the PBS was removed and 100µl of the primary antibody of the following CD markers (CD44,

CD34, CD105, CD90, CD45, CD31 and CD106) were added, and then incubated for 30 minute except the incubation period with CD90was remained overnight at 4Cº. The cells were washed three time with PBS for 5 minute. Biotinylated secondary antibody approximately 1-3 drops was added to the coverslip for 30 minutes. (An appropriate Mouse, Rat, and Goat secondary antibodies added to specific primary antibody). Then the cells were washed three time with PBS for 2 minute each.The mixture of streptavidin conjugated to horseradish peroxidase was incubated at room temperature for (30) minutes before use, then added to cover the cells, and then the cells were washed with PBS three times. Each plate was received 1-2 drops of DAB solution and this step was made in darkness and then left for 10-20 minutes. The cells were washed one time with distilled water. Two drops of Harris Haematoxylin stain were added and left for 5-10 seconds, and then washed with distilled water. Then left for five minutes to dry.Drops of DPX were added to the slide, coverslip were adhered carefully, and finally the slide were inspected by light microscope for detection of MSCs.

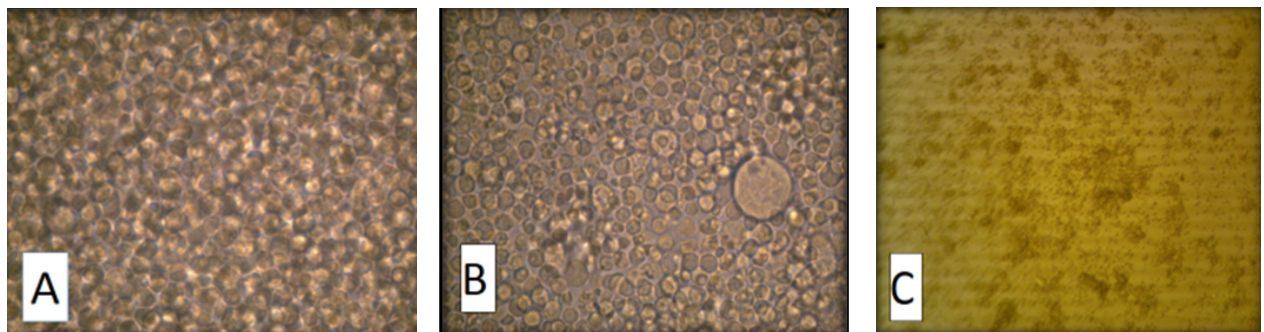
Results:

Morphology of bone marrow cultures

Bone marrow cells were isolated from the femurs and tibias of albino mice and plated into culture flasks. Non adherent cells were carefully removed after 6 and 24 hours by washing the adherent cells with complete media RPMI-1640 for three times and 5ml of fresh complete isolation media (CIM) RPMI-1640/10%FBS were replaced. Then adherent cells (passage 0) were received fresh complete medium every 72 hours and the culture were observed via phase contrast microscopy for their morphology. The initial culture (time 0) of the bone marrow suspension, cells were found to be round and glistening contained a heterogeneous cell populations, including Erythrocyte, B-lymphocytes, granulocytes, monocytes, adipocyte, very small embryonic-like cells (VSEL) mesenchymal, hematopoietic, and endothelial stem cells etc. (Fig. 1A). Mesenchymal stem cells were isolated based on the ability to form adherent monolayer in culture and the lack of adherence of other cells in the bone marrow stroma such as HSCs, erythrocyte, adipocytes and macrophages. Hence, change media performed after 6 hours, were many of MSCs had attached to the base of the tissue culture flask (Fig. 1 B). These adherent cells were remained attached to the bottom of the flask, formed high numbers of scattered plastic adherent colonies (at 24 hours) (Fig. 1 C). Some of adherent cells assumed spindle morphology, a characteristic oval body with the flattened ends. When they slowly elongate, others appeared as individual small size spindle-shaped cells (Fig. 1 D). While the floating round cells remained suspended in the medium and were mostly eliminated from the culture with subsequent media changes. The adherent cells tend to be locally confluent, growing in distinct colonies at about 48 hours (Fig.1E). Selective removal of cells in the suspension allowed the growth of adherent uniform spindle morphology cells formed colony-forming units fibroblast (CFU-F) were a large number of cells grew in a radial manner in neat rows (Fig.1F), and the density increased significantly which fused to form the confluent monolayer (Fig.1G).

Culturing and sub culturing

Upon reaching 80-90 % confluence before day 5 of primary isolation, the MSCs were subjected to passaging, Because CFU-F adherence was sensitive to trypsinization, 80-90% were detached after treatment of adherent cells with trypsin, and re-seeded at the density of 106 cells/ml at the split ratio of 1:2 using complete expansion media (CEM) α-MEM/ 10% FBS. However, as the culture progressed with trypsinization, the round non-adhesive cells gradually decreased and eventually disappeared by week 1. Fibroblast-like cells remained homogeneous morphologically (Fig.2A). These cells showed prolonged proliferative capacity without any morphological changes for more than 3 passages (Fig. 2B). The total number of adherent MSCs in the end of first passage (70-80% confluency) were reached 9.8 × 106 cells/ ml. However, morphologically three distinct-cell subpopulation of MSCs can be distinguished in direct plating culture: major fibroblast-like cells, minor round cells, very small number of large, flat cells (Fig. 2 A, B, C).



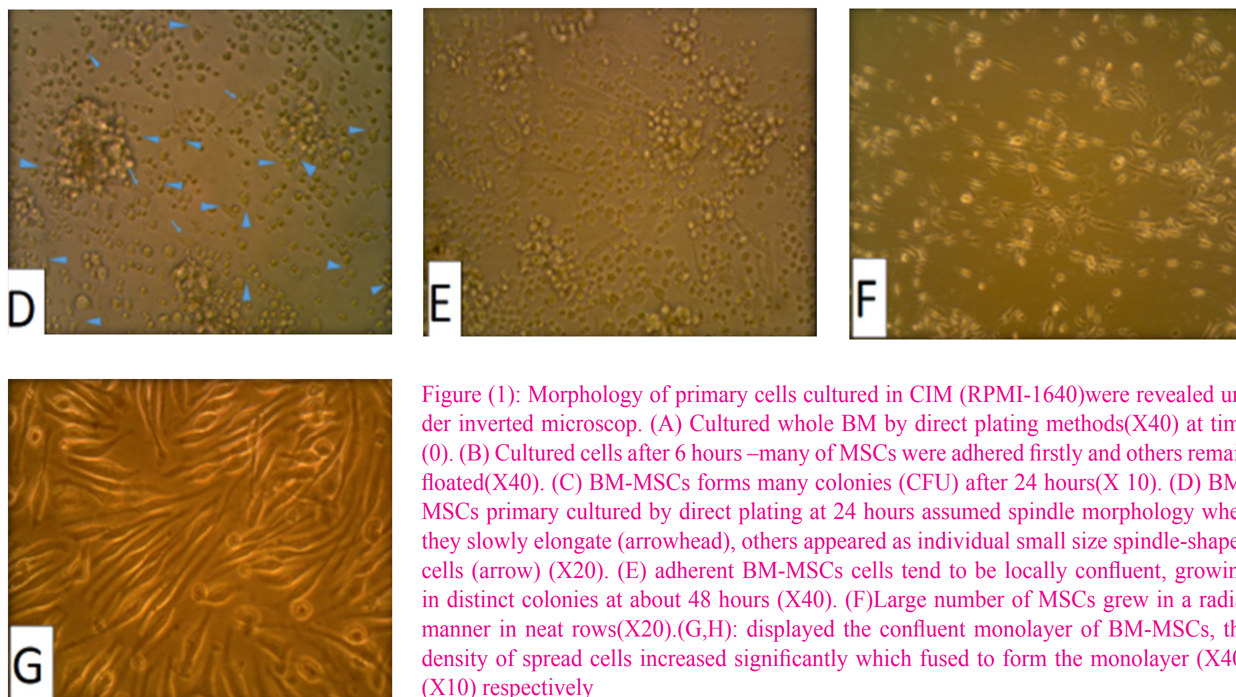


Figure (1): Morphology of primary cells cultured in CIM (RPMI-1640)were revealed under inverted microscop. (A) Cultured whole BM by direct plating methods(X40) at time (0). (B) Cultured cells after 6 hours –many of MSCs were adhered firstly and others remain floated(X40). (C) BM-MSCs forms many colonies (CFU) after 24 hours(X 10). (D) BM-MSCs primary cultured by direct plating at 24 hours assumed spindle morphology when they slowly elongate (arrowhead), others appeared as individual small size spindle-shaped cells (arrow) (X20). (E) adherent BM-MSCs cells tend to be locally confluent, growing in distinct colonies at about 48 hours (X40). (F)Large number of MSCs grew in a radial manner in neat rows(X20).(G,H): displayed the confluent monolayer of BM-MSCs, the density of spread cells increased significantly which fused to form the monolayer (X40) (X10) respectively

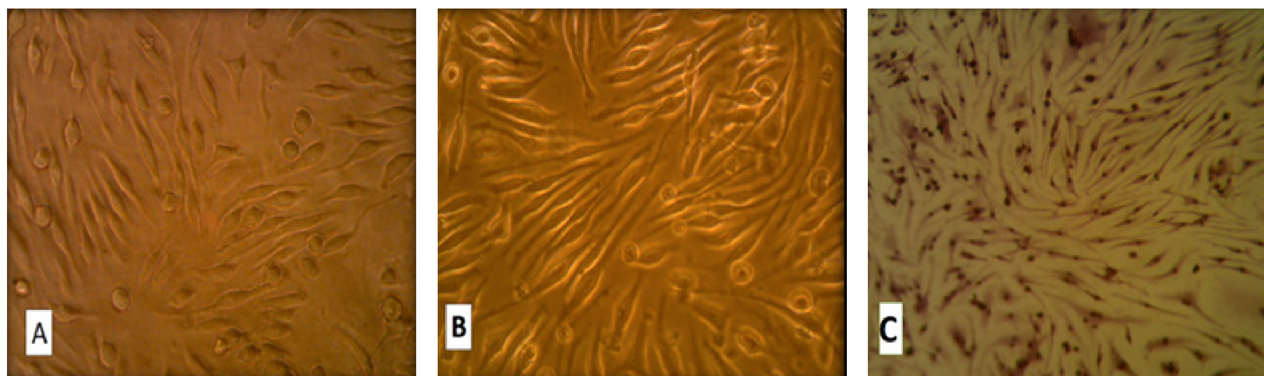


Figure (2): Expansion of BM-MSCs which cultured in CEM (MEM/ 10% FBS) revealed under inverted microscope (X40). (A): BM-MSCs confluent monolayer at 3 dayes of the first passage were progressed with trypsinization. (B): third passages of BM-MSCs after 3 dayes. (C): H&E staining of BM-MSCs culture at the fist passage Examined under light microscope (X40).

- Immunophenotypic characterization of BM-MSCs

The morphological homogeneity of the monolayer cultures at the third passage on a cover slip was also apparent by immunocytochemistry staining analysis, for the BM-MSCs isolated by direct plating of whole BM. The antigenic phenotype was carefully checked by using seven groups of cell surface molecules (CD44, CD34, CD105, CD90, CD45, CD106 and CD31). The immunocytochemical staining results were shown in (Figs. 3 and 4). Take to the interest all immunoreactivity staining was performed on the same sample. Immunophenotypic evaluation demonstrated that mouse BM-MSCs uniformly positive for CD44, CD90, CD105, and CD106 (Fig. 3) and negative for hematopoietic specific markers CD34, CD45, and endothelial marker CD31 (Fig.4). Cells immunostained with positive marker stained strongly brown. Negative control and cells immunostained with negative one showed no staining.

The results showed that MSCs are present in the mouse BM, by using direct plating culture techniques, can be successfully isolated and expanded in vitro. Although the initial cell culture consisted of fibroblastoid, spindle-shaped morphology and another with epithelioid, polygonal morphology, only

the fibroblastoid population remained after enzymatic digestion and passaging until the third passage it appear pure homogenous population of MSCs. In their undifferentiated state MSCs are spindle-shaped and resemble fibroblasts.

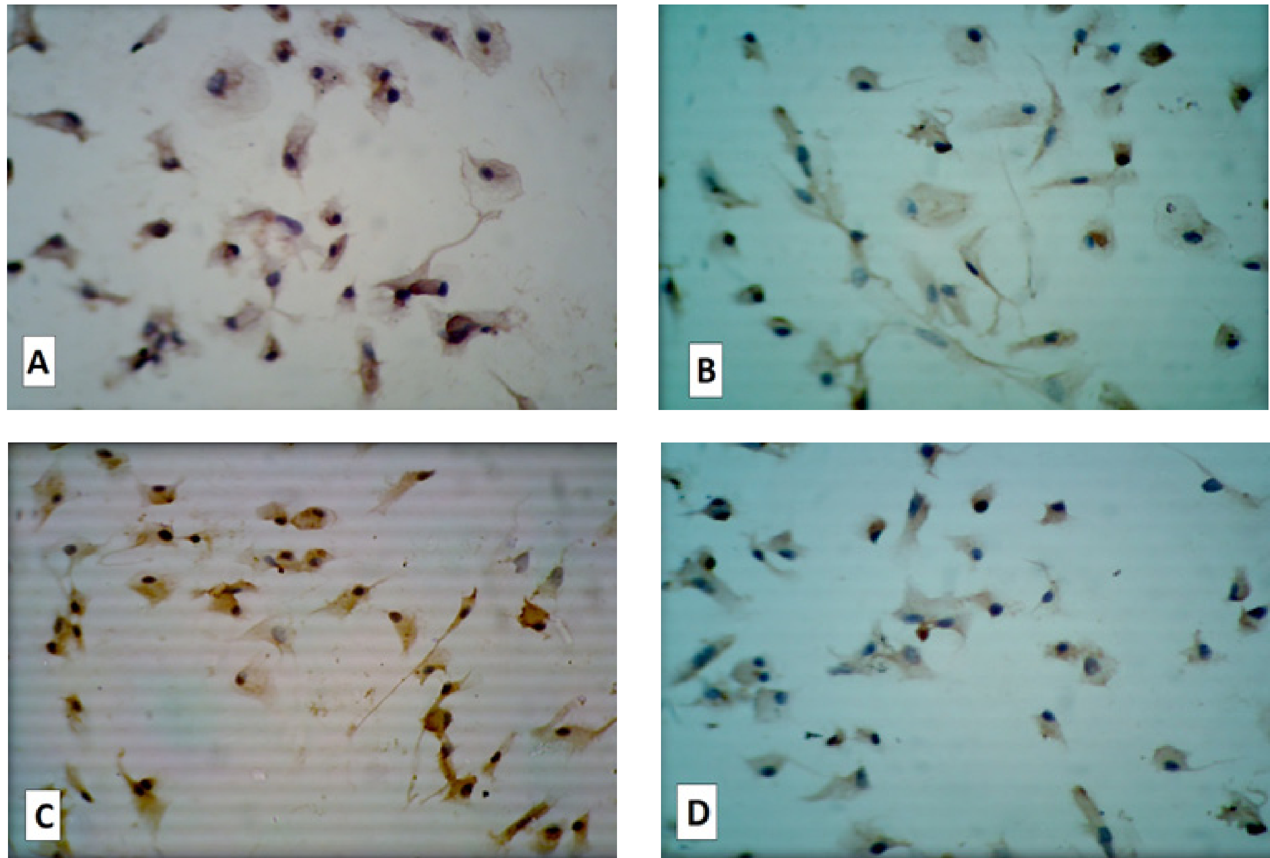


Figure (3): Immunophenotypic analysis of mouse BM-MSCs at the third passage of culturing on coverslips revealed by light microscope (X40). (A-B-C-D): the most of adherent MSCs were positive response for CD90, CD105, CD106 and CD44 marker respectively were stained with brown color DAB stain.

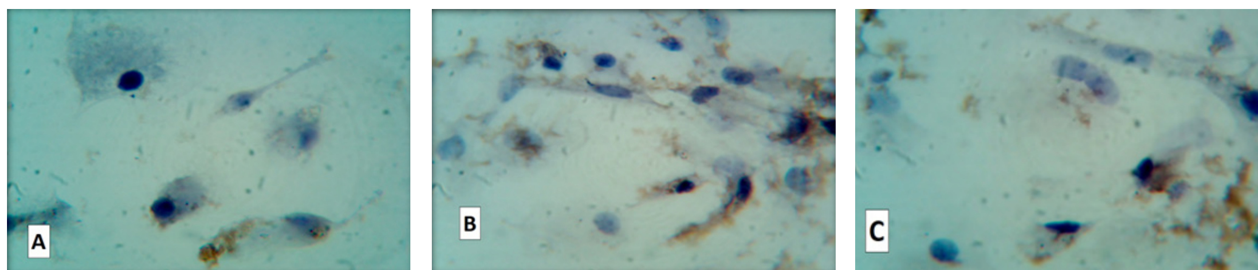


Figure (4): Immunophenotypic analysis of mouse MSCs at the third passage of culturing on coverslips revealed under inverted microscope (X100). (A, B, and C): the majority of adherent cells MSCs were negative response for CD34, CD45, and CD31 marker and this cell stained with blue color of counter stain Haematoxylin.

Discussion:

Based on morphology, fibroplastoid cells present within the colonies indicate a greater potential for expansion. On the other hand, as the cells are passaged, large flat cells with slow replicative ability appears in the monolayer cultures (18). Other studies have distinguished the presence of a third small round-cell subpopulation called 'rapidly self-renewing cells' (RS cells) (19,20). It has been proposed that RS cells represent particularly multipotent progenitors with the potential to differentiate into many lineages. It seems likely that the RS cells described in these studies may not represent

a pure progenitor fraction but represent a variation in the morphology of MSCs that have the potential to rapid expansion in culture (21). Based on the expression profile of these markers, BM- MSC behave as one sole cell population, as all the studied markers were homogeneously expressed compatible with agreed MSCs markers. Indicating that the cells isolated in present study had the characteristics of MSCs (22). In addition, the isolated MSCs of high purity at third passage were not contaminated by heamatopoietic or endothelial cells as immunocytochemical staining demonstrated that cell preparations were always negative for CD45, CD34, and CD31. Identifying the MSCs fraction in the BM will be a more formidable challenge, since there are no markers that specifically and uniquely identify MSCs and therefore they are defined by their Immunophenotypic profile. There has been a common opinion that CD105, CD90, and CD44 are highly specific for MSCs (23). In the BM, where the over whelming majority of cells are heamatopoietic, these markers may indeed be useful to explain that the phenotyped cells are non-heamatopoietic and stromal in origin. CD44, CD105 and CD90 were also reported to react with undifferentiated MSCs and thus seem suitable for isolation of more pure MSCs population (24). More recently, several studies showed that these markers were expressed on stromal cells from many locations as well as on skin fibroblasts (25). Since, the contamination of MSCs cultures with fibroblasts lead to run-down of the stem cell differentiation potential because fibroblasts undergo senescence and eventually die. Moreover, such MSCs preparations are potentially unsafe to use for clinical applications since, a few of fibroblasts survive and can become tumorigenic (26). The surface markers CD44, CD90, CD105 was not highly specific for BM-MSCs. fibroblasts were also positive for these markers. Both heamatopoietic cell markers (CD34, CD45) and the endothelial cell marker (CD31) were absent in MSCs and fibroblasts.

This study confirms the fact that CD106 marker expressed only on MSCs but not on fibroblasts. Hence, added to the proteomic analysis list, identification and elimination of fibroblasts from MSCs cultures could improve the MSCs yield and differentiation potential and prevent tumor formation after MSC transplantation. Most of these surface makers were consistent with the criteria of the International Society for Cellular Therapy (ISCT) (15). The ISCT proposed the minimal criteria to define human MSCs. First, MSCs must be plastic-adherent when maintained in the standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts invitro. Therefore, combinations of positive and negative markers are commonly used to identify and isolate MSCs. The advantages of MSCs are their minimal invasive isolation, ability to self-renew (proliferate while maintaining undifferentiated state without any phenotypic changes).

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عزل وتشخيص الخلايا الجذعية اللحمية المشتقة من نخاع عظم الفئران

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الخلاصه:

استخدمت الخلايا الجذعية اللحمية المشتقة من نخاع العظم بنجاح في الطب التجديدي. هدفت الدراسة الحالية الى عزل وتوصيف الخلايا الجذعية اللحمية من نخاع عظم الفأر لغرض استخدامها في المجالات البحثية.أوضحت معطيات البحوث السابقة بأن الخلايا الجذعية اللحمية المشتقة من نخاع العظم يمكن اغناؤها فقط بواسطة التصاقها بالمزارع البلاستيكية.وفصل الخلايا الليفية المولدة. ان تشخيص الخلايا الجذعية اللحميةيتم من خلال الشكل، والصفات الوراثية المظهرية، والسلوك البيولوجي لها. ويلعب الشكل الخلوي دورا مهما لتشخيص الخلايا الجذعة اللحمية في المزرعة. وبشكل عام، تظهر الخلايا الجذعية اللحمية الفتية صغيرة الحجم ومغزليه بينما تظهر الناضجة منها بشكل خلايا كبيرة الحجم، مسطحة ومتعددة الاضلع. وتميل الخلايا الى التجمع موضعيا والنمو في مستعمرات محددة. أظهرت نتائج تحليل الكيمياء النسيجية المناعية للخلايا الجذعية اللحمية بانها ذات استجابة موجبة لل CD106، CD105، CD90، CD44، وبنسبه منخفضه لل nestin. وذات استجابة سالبة للمعلمات المتخصصة بالخلايا الدميةCD45، CD34، والمعلم الظهاريCD31. ويمكن الاستنتاج بإمكانية عزل الخلايا الجذعية اللحمية وبنجاح من نخاع عظم الفئران البيض بالطريقة المباشرة والتي تعتبر مصدرمهم للخلايا الجذعية لتعويض الأعضاء المتضررة.