ISSN 2347-6893

Generation of hepatocyte from mouse mesenchymal stem cells in Vitro

Shahlaa M. Salih [1], Zahraa K. Zedan [1], Shalal M. Hussain [3]

[1]Al-Nahrain University, College of Science, Deparetemnt of Biotechnology, Baghdad, Iraq

shahlaam2006@yahoo.com

[2]Al-Nahrain University, College of Sience, Department of Biotechnology, Baghdad, Iraq

zahraa.alfahad@yahoo.com

[3] Al- mustansyria University , Faculty of Pharmecy, Baghdad, Iraq

dr_shalal@yahoo.com

ABSTRACT

This study was designed to generate hepatocyte cells from mesenchymal stem cells (MSCs) isolated from mouse bone marrow via using culturing protocols for this goal. Cells of MSCs were isolated by collecting the thigh bone of 50 albino mice, both femur and tibia were collected and cells were flushed from bones and MSCs have been isolated based on the ability of MSCs to adhere on plastic surfaces. MSCs reactivity to CD45, CD90, CD34 and CD105 were tested by immunocytochemistry. Isolated MSCs exhibited positive reactivity towars CD105 and CD90 cell surface markers. MSCs were negative for the hematopoietic surface markers including CD34 and CD45. Differentiation into hepatocyte was induced by adding hepatogenic specific growth factors (Hepatocytes growth factors (HGF), Fibroblast growth factor4 (FGF4), oncostatin M and dexamethasone) to the differentiation medium. Cells were examined after differentiation protocol and characterized using immunocytochemistry analysis for Albumin and alpha fetoprotein. Positive results were observed indicating the potential ability of the isolated bone marrow MSCs to differentiate into Hepatocytes.

.**Keywords:** mesenchymal stem cells; hepatocytes; hepatocytes growth factors.

Academic Discipline And Sub-Disciplines

Stem cell technology

SUBJECT CLASSIFICATION

Regenerative medicine Classification

TYPE (METHOD/APPROACH)

Immunocytochemistry analysis

Council for Innovative Research

Peer Review Research Publishing System

Journal of Advances in Biology

Vol. 7, No. 3

www.cirjab.com

editorsjab@gmail.com , editor@cirjab.com

ISSN 2347-6893

INTRODUCTION

Recently mesenchymal stem cells have received widespread attention because of their potential use in tissue engineering applications [1]. MSCs considered as the ideal cells in the therapeutic approaches. The differentiation of MSCs is regulated by various signals from microenvironment, which is consisting of many biological molecules and biomechanical forces. These factors play a vital role in determining the efficacy of MSCs biology [2].

After MSCs were first described by Fridenstein *et al* [3], in 1976 as clonal, plastic adherent cells, interest in MSCs has rapidly grown with expanding knowledge about their spesific properties and benefits in the field of clinical treatments by means of their differentiation potential under in vitro conditions [4,5].

MSCs was found to have the ability to differentiates in to all mesodermal cells like myocytes, chondrocytes and adipocytes[6], in addition to that MSCs were found to have the ability to differentiates to other cell lines using the proper growth factors that property made MSCs are referred as a multipotent adult stem cells [7].

New experiments were suggested that BM-MSCs are able to give rise to a more broad range of cells, like hepatocytes, neurons, epithelial cells and keratinocytes [8, 9]. This plasticity of bone marrow derived MSCs has attracted much clinical interest for their *invivo* new functions under either metabolic or pathologic conditions, and their clinical therapy for tissue repair. In fact, several studies in animal models have suggested that endogenous MSCs may “naturally” be involved in wound healing and tissue regeneration and the engrafted exogenous MSCs have beneficial effects in tissue repair, including that of bone, myocardial tissue, skin, kidney and liver [10-11].

These may encourage further studies on the new insight into MSCs biology and the differentiation mechanisms of MSCs which supposed to be hardly known at present time. According to previous studies many differentiation protocols were examined to induce hepatocytes from bone marrow derived MSCs, in this study a combination hepatocyte growth factor (HGF), fibroblast growth factor 4 (FGF4), oncostatin M and dexamethasone all are growth factors which had been found to a great role in guiding the hepatocytes differentiation strategy [12].

MATERIALS AND METHODS

Experimental animals

Four to eight weeks old, Swiss albino mice obtained from the Laboratory Animal Unit of the Iraqi center for cancer researches and medical genetics Baghdad, Iraq were used in this research. Animals were housed under specified pathogen- free enviroment with 12 hrs dark and night cycle coditions [3].

Isolation and culturing of bone marrow MSCs

The mouse bone marrow MSCs (mBM-MSCs) were isolated by collecting the thigh bones. The bone marrow was extruded by clipping of the epiphyseal ends of both femur and tibia bones and flushing with Minimum essential medium (MEM) (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (US Biological, USA), 1% penicillin/streptomycin (Medium A). After 24 hours, non-adherent cells and debris were removed, and the adherent cells were cultured continuously. At near confluence, the cells were replated at 5×10^4 cells/ml [7].

Characterization of mouse MSCs

Isolated MSCs were identified from the other bone marrow cells haematopoietic stem cells using immunocytochemical analysis, which based on the cell nature of expressing specific cell markers on their surface. These techniques are based on the immunoreactivity of antibodies and the chemical properties of enzymes or enzyme complexes which react with colorless substrate- chromogens to produce a colored end product. Initial immuno-enzymatic stains utilized the direct method, which conjugates enzymes directly to an antibody with known antigenic specificity (primary antibody). It allowed for the visualization of tissue antigens using a standard light microscope [13].

ISSN 2347-6893

Induction of hepatogenic differention

In hepatogenic differentiation the 3rd passage of (2 ×10^4) of mBM-MSCs were used. The differentiation strategy involved two main stages in first stage which lasted for a week the cells were cultured in a DMEM medium supplemented with 10% FBS, 20 ng/ml (Fetal bovine serum) HGF, 20 ng/ml FGF4 (Fibroblast growth factor) and 10^{-7} M/L dexamethasone. At the second stage which lasted for two weeks the same media where used as the first stage with the addition of 10 ng/ml of oncostatin M (Sigma, USA). As a negative control, mBM-MSCs were cultured in medium without differentiation stimuli along with the differentiation experiments in the same conditions. Cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37℃. Cultures were maintained by medium exchange every 3 d. The cell morphology was observed under inverted microscope [6].

Characterization of induced hepatocytes

After three weeks of differentiation, the cultured cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30-45 min at room temperature and permeabilized with 0.4% Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 10 min. After blocking with bovine serum albumin, the washed cells were incubated overnight at 4 °C with primary antibodies, including mouse anti-human albumin (1:1000) and mouse anti-human α-feto protein (AFP) (1:500) (Sigma-Aldrich, St. Louis, United States) [4]. The cells were then incubated with DAPI (4',6-diamidino-2-phenylindole; 1:1000) for nuclear staining, between each incubation, the samples were washed with PBS-0.05% Tween. Then the counter stain heamatotoxilin stain is added. The ratio of immunopositive cells to the total number of cell nuclei labeled with DAPI was recorded [7].

Results

Characterization ofBM- MSCsMSCs were isolated with perfect confluence form bone marrow flashing and cultured in MEM medium containing 20% of FBS in primary culture then after passaging the cells were cultured in MEM mediumwith 10% FBS. Results showed attachment of isolated MSCs to the surface of culture falcon after 24 hrs of primary culture while MSCs showed the spindle shape by after 80% confluence Figure1(A, B,C and D).

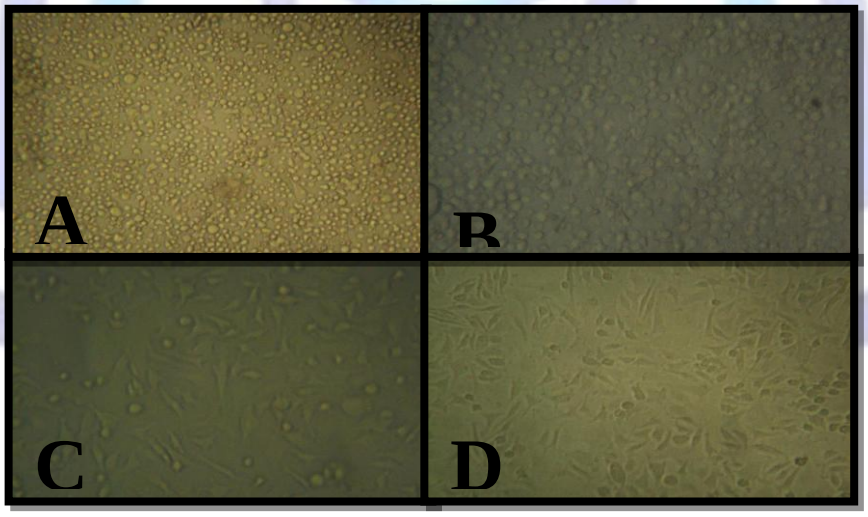

Figure 1: MSCs Isolated from mouse bone marrow cultured on a falcon containing DMEM 10% FBS viewed by inverted microscope (10X) (A) After 24 hrs. (B), after 48 hrs. (C) after 3 days and (D) after 5 days showing the 80% confluence of isolated BM MSCs with their distinct spindle elongated ends morphological characteristic.

Results showed the positive reactivity for CD 90 and CD105 in which the cells stained withDAPI stain while the negative reactivity was noticed for both hematopoietic stem cell markers CD34 and CD45 cells and stained with heamotoxilin stain Figure (2).

ISSN 2347-6893

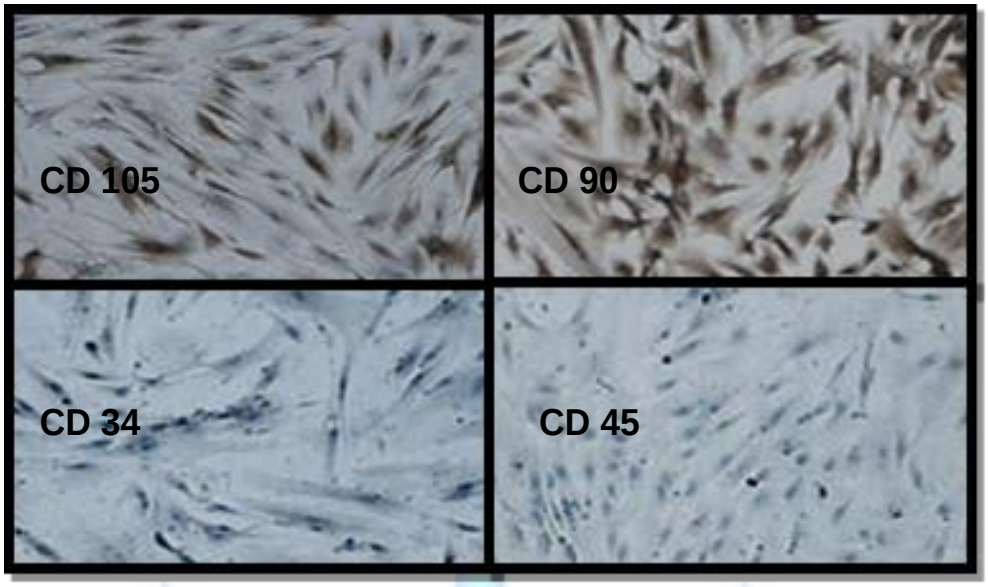

Figure (2): Immunocytochemistry analysis of isolated BM-MSCs: shows the positive reaction of cells towards CD105 and CD90 markers of cell surface and the negative reaction for CD34 and CD45 cell surface markers.

Hepatocytes differentiation

during the differentiation experiment the cultured cells were monitored via inverted microscope for morphological changes of MSCs during the stages of differentiation of to hepatocytes Figure 3 (A,B,C,D,E,F and G) and positive results to albumin and α-feto protein Figure(3 a and b).

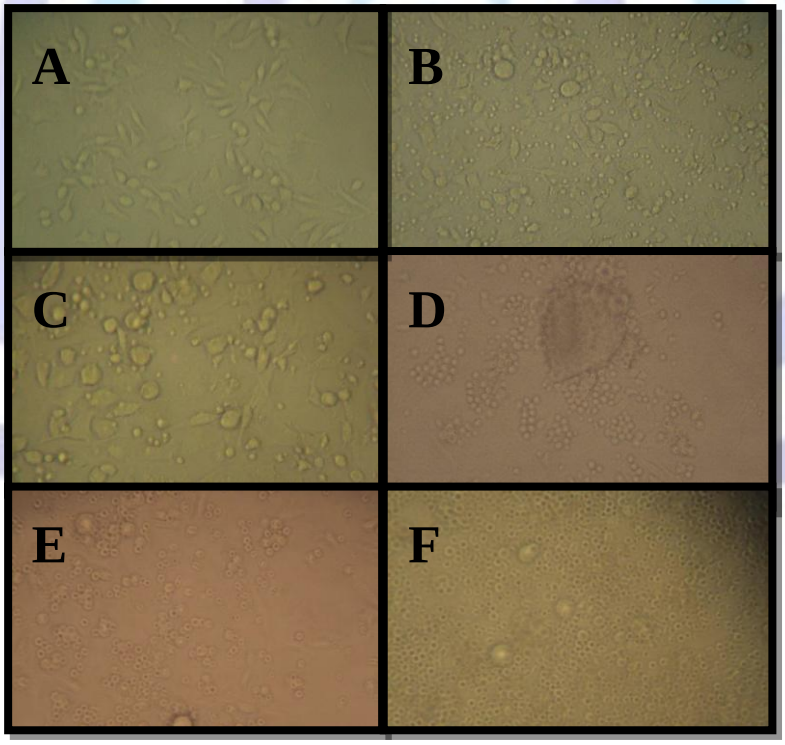

Figure (3): Different stages of morphological patterns of hepatogenic differentiation of Mouse MSCs. A: the mesenchymal stem cells isolated from mouse bone marrow; B: BM-MSCs under differentiation condition at day 10; C: BM-MSCs under differentiation condition at day 16; D, E: Differentiated cells at day 19 and 21 F: cells at the end of differentiation experiment.

ISSN 2347-6893

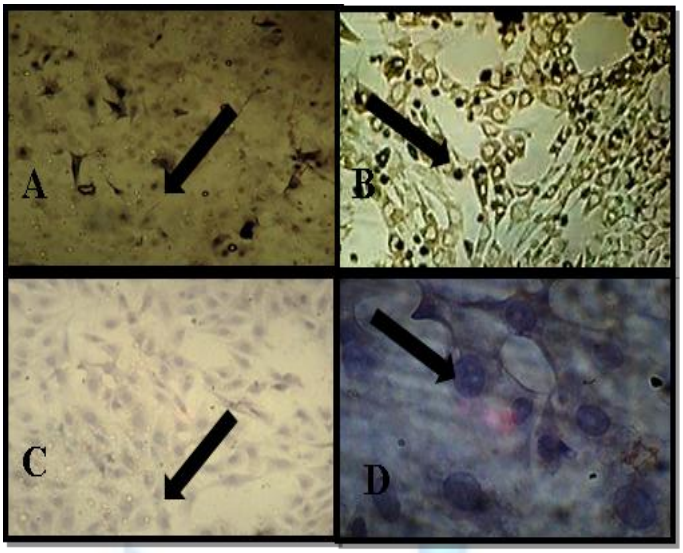

Figure (4): Immunocytochemical analysis of albumin and alpha-fetoprotein in hepatocytes after 21 days of differentiation. Homogeneous expression of albumin (A) and AFP (B) showed positive reactivity (brown color). Staining for both albumin and AFP was negative in non-hepatic cells (Cand D).

ACKNOWLEDGMENTS

This study was supported by the Coordination of Iraqi center of Cancer researches and medical genetics and the college of Science/department of biotechnology/Al-Nahrain Univ.

Discussion

For each stage of differentiation and growth of cells are strictly regulated by cell autonomous mechanisms and extracellular signals including cytokines and growth factors [14] .The induce hepatocyte protocol used in this study have been demonstrated to be efficient and convenient way to induce MSCs to hepatic lineage, the resultshowed that the differentiated cells displayed the hepatocyte like morphology, expressed of hepatic specific gene and protein as adult liver cells, in accordance with previous studies of both Wang et al., [15] and Van Rie et al., [16].Mesenchymal stem cells (MSCs) have emerged as a promising resource of functional hepatocytes for treatment of liver diseases because of its plasticity of multiple cell lineages. To date, many inducing systems for hepatic differentiation from MSCs have been developed [17, 18]. It is undoubted that exposition of MSCs to the inducing systems, resembling the conditions in liver development [19]. Previous studies have shown that mouse bone marrow mesenchymal stem cells(mBM-MSCs) could be induced to differentiate into hepatic cells by conditioned culture medium of hepatocytes [20]. Thus identification of the exact cytokines involved in liver-injury conditions for the mBM-MSCs differentiation needed further studies. This study provides such investigation, four growth factors related to liver growth, repair. It was found that three cytokines (FGF-4, HGF and oncostatin M) are play a crucial role in the conditioned medium-induced hepatic differentiation, In the present study provided a direct basis on the selection of cytokines for hepatic differentiation. However, besides these three key cytokines, some other factors involved in the liver injury need to be identified for improving the cytokine-based inducing system. FGF-4, HGF, Dexamethasone and Oncostatin M play important roles in liver regeneration, healing, initiation and development. FGF-4 was considered to be one of the most important fibroblast growth factor family members that can irritate the proliferation of mesodermal and endodermal cells and improve development of fetal liver [21]. HGF was found to be essential for the development of several epithelial organs and was one of the most well characterized cytokine for the stimulation of DNA synthesis in primary hepatocyte cultures, and for liver development [22]. The Oncostatin M, however, is a member of the interleukin-6 family produced by hematopoietic cells and induces differentiation of fetal hepatic cells.

These four factors used in this project are participating in different liver developmental stages. It suggested that HGF, FGF-4 and dexamethasone were essential for the initiation of hepatic differentiation, while oncostatin M is critical for the maturation of hepatocytes. In conclusion, the present study analyzed the potential growth for hepatic differentiation from mBM-MSCs. It was found that FGF-4, HGF, dexamethasone and oncostatin M might be the key cytokines which has various roles during hepatic differentiation, which is similar to their functions in liver development.

References

[1] Nambu, M.; Ishihara and M., Nakamura. "Enhansed healing of mitomycin C-treated wounds in rats using inbred adipose tissue-derived stromal cells within an atelocollagen matrix". Wound Repair. Regen. ;15, 505-510, 2007.

[2] Hwang NS, Zhang C, Hwang YS and Varghese S. "Mesenchymal stem cell differentiation and roles in regenerative medicine". Wiley Interdiscip Rev SystBiol Med. 1,97-106, 2009.

[3] Friedenstein AJ, Gorskaja JF and Kulagina NN."Fibroblast precursors in normal and irradiated mouse hematopoietic organs".Exp Hematol.;4:267–274, 2001

[4] Caplan AI." Why are MSCs therapeutic? New data: new insight". J Pathol.;217:318–324, 2009.

6] MiranvilleA,et al. "Improvement of postnatal neovascularization by human adipose tissue-derived stem cells". Circulation.;110:349–355, 2004.

[7] Schwartz RE, et al. "Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells". J Clin Invest. 109:1291–1302, 2002.

[8] Cheng Z, et al "Targeted migration of mesen- chymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance". MolTher, 16: 571-579, 2008.

[9] Wang Y, Deng Y and Zhou GQ. "SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model" Brain Res ,1195: 104-112, 2008.

[10] Zhao J. et al, "Recruitment of endogenous stem cells for tissue repair. Macromol- Biosci; 8: 836-842. 14, 2008.

[11] AyatollahiM,et al,. "Expansion of human marrow derived mesenchymal stem cells and their transdifferentiation potential". IRCMJ.;12:446–45. 2010.

[12] SnykersS,et al. "Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells". Toxicol Sci.;94:330–341, 2006.

[13] Farr AG and Nakane PK. "Immunohistochemistry with enzyme labeled antibodies: abrief review". J Immunol Methods; 47(2):129-44.1991.

[14] Anjos-Afonso F., Siapati E. K. and Bonnet D. "In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions". J. Cell. Sci. 117, 5655–5664.2004.

[15] Wang Y, Deng Y and Zhou GQ. "SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model". Brain Res; 1195: 104-112, 2008.

[16] Van Riet I and Rogiers V. "Chromatin remodeling agent tricho- statin A: a key-factor in the hepatic differentiation of human mesenchymal stem cells derived of adult bone marrow". BMC DevBiol; 7: 24, 2007.

[17] Pan RL,et al. "Fetal liver-conditioned medium induces hepatic specifica- tion from mouse bone marrow mesenchymal stromal cells: a novel strategy for hepatic transdifferentiation". Cytotherapy; 10: 668-675. 2008.

[18] Lee KD,et al. "In vitro hepatic differentia- tion of human mesenchymal stem cells. Hepatology "; 40: 1275-1284, 2004.

[19] Snykers S, et al. "Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells". ToxicolSci; 94: 330-341, 2006.

[20] Chen Y, et al. "In vitro differentiation of mouse bone marrow stromal stem cells into hepatocytes induced by conditioned culture medium of hepatocytes". J Cell Biochem; 102: 52-63, 2007.

[21] Rappolee DA, Basilico C and Patel Y. "Expression and function of FGF-4 in peri-implantation development in mouse embryos". Development; 120: 2259-2269, 2000.

[22] Schmidt C, et al. "Scatter factor/hepatocyte growth factor is essential for liver development". Nature; 373: 699-702 32. 2001.