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Identification of Cytochrmoe oxidase p450 in Heptocytes generated from in vitro differentiation of mouse mesenchymal stem cells

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ABSTRACT

This study was designed to identify the presence of cytochrome oxidase p450 (CYP3A4), in the hepatic cell lines which was generated from the in vitro differentiation of mouse bone marrow mesenchymal stem cells. Bone marrow meenchymal stem cells were first isolated by collecting both the femur and tibia of the mouse thigh bone, then exess tissue were removed and the cells were flushed from the bones and cultured under highly aseptic conditions. The isolated cells were characterized as a mesenchymal stem cells via using immunocytochemistry analysis. The characterized hepatocytes were subjected to a differentiation protocol in which the differentiation medium was contained a spesfic growth factor including (Hepatocytes growth factor (HGF), Fibroblast growth factor4 (FGF4), Oncostatin M and Dexamethasone, All theses growth factor was used for directing the BM-MSCs twards the hepatic line of cells. After three weeks of the differentiation protocol the diffrentation cells were examined for the presence of Cytochrome oxidase p450 (CYP3A4), which consider as one of the specific markers of hepatocytes. Identification eas done via two different methods first, via using the immunocytochemical analysis for the antihuman mouse CYP3A4 antibody and second by ELIZA test. The results showed a posotive reactivity of the differentiated cells towards the mouse antihuman CYP3A4 antibody as well as the eliza results showed a significant increase in the enzyme level of the cell lysate during the weeks of the differentiation experiment.

Keywords: Cytochrome oxidase p450; mesenchymal stem cells; hepatocytes;

Academic Discipline And Sub-Disciplines

Stem cell technology

SUBJECT CLASSIFICATION

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INTRODUCTION

Cytochrome P450 3A4 (abbreviated CYP3A4) is an important enzyme in the body, mainly found in the liver and in the intestine. Its purpose is to oxidize small foreign organic molecules (xenobiotics), such as toxins or drugs, so that they can be removed from the body [1]. The potential ability of CYP3A4 to oxidize molecules with widely diverse in size and chemical structure is due to its large and malleable active site of the detoxitative molecule [2]. Another intriguing feature of CYP3A4 is the ability to accommodate more than one molecule in the substrate-binding pocket, where one molecule serves as a substrate while another acts as a modulator of substrate metabolism. Among the substrates that exhibit binding cooperativity with CYP3A4 are testosterone, progesterone, diazepam, α-naphthoflavone, and others [3].While many drugs are deactivated by CYP3A4, there are also some drugs which are activated by the enzyme. Some substances, such as grapefruit juice and some drugs, interfere with the action of CYP3A4. These substances will therefore either amplify or weaken the action of those drugs that are modified by CYP3A4 [4].The enzyme CYP3A4 is a member of the cytochrome P450 family of oxidizing enzymes. Several other members of this family are also involved in drug metabolism, but CYP3A4 is the most common and the most versatile one. Like all members of this family, it is a hemoprotein, i.e. a protein containing a heme group with an iron atom. In humans, the CYP3A4 protein is encoded by the CYP3A4 gene, this gene is part of a cluster of cytochrome P450 genes on chromosome 7 [5].

MSCs are a type of multipotent adult stem cells that can be readily obtained from BM aspirates and expanded into large quantities in vitro. Furthermore, MSCs possess potent immunosuppressive activities. Therefore MSCs could be used in patients who are in need of immune modulation, as well as tissue repair, such as organ transplant recipients and patients with severe autoimmune diseases [6]. 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 [7].

A major area of research in transplantation medicine is the potential application of stem cells in liver regeneration. This would require well-defined and efficient protocols for directing the differentiation of stem cells into the hepatic lineage, followed by their selective purification and proliferation in vitro.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice of four to eight weeks old were obtained from the Laboratory Animal Unit of the Iraqi center for cancer researches and medical genetics Baghdad, Iraq were used in this research. The animal housing were performen under a clean conditions of healthy diet and acycle of 12 hrs day and light.

Isolation 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 × 104 cells/ml [8].

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].

Induction of hepatogenic differention

In hepatogenic differentiation the 3rd passage of (2 ×104) 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].

Identification of CYP3A4 in differentiated cells via immunocytochemical anlyais

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 antibody which is a mouse anti-human CYp3A4 (1:1000) [4]. The cells were then incubated with DAPI stain (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].

Identification of CYP3A4 in differentiated cells lysate via Elisa test

Cytochrome P450 3A4 (CYP3A4) BioAssay™ ELISA Kit (Mouse) utilizes the sandwich Enzyme Immunoassay technique for in vitro quantative measurement of CYP3A4 in mouse, the microtiter plate provided in the detection kit has been pre-coated with an antibody specific to CYP 3A4.Standers or samples are added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to CYP3A4. Next, avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain CYP3A4, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometrically at a wave length of 450nm ± 10nm. The concentration of CYP3A4 in samples is then determined by comparing the OD of the samples to the standard curve. The standard curve was drawn using curve expert 3.1 computer software by plotting on the horizontal axis the cytochrome p450 concentrations of the standards and on the vertical axis the corresponding average absorbance. To locate the concentration of cytochrome p450 in the samples, the average absorbance for each sample on the vertical axis was located and the corresponding cytochrome p450 concentration was located on the horizontal axis. (figure 1).

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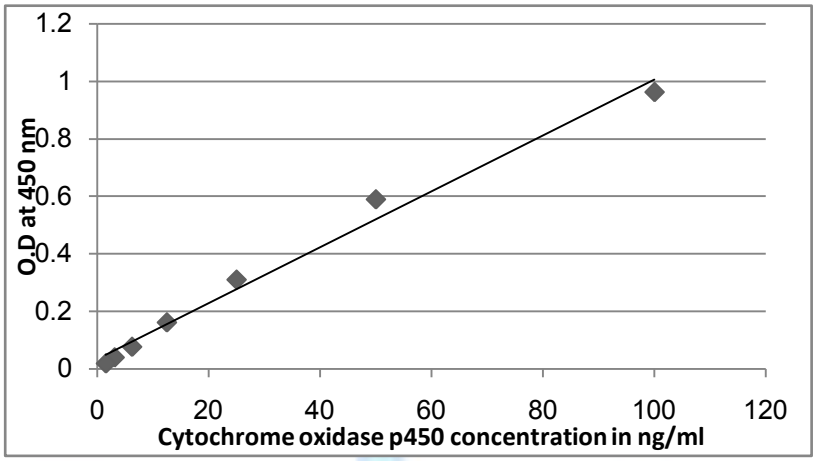

Figure (1): Standard curves for mouse cytochrome p450.

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. 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).

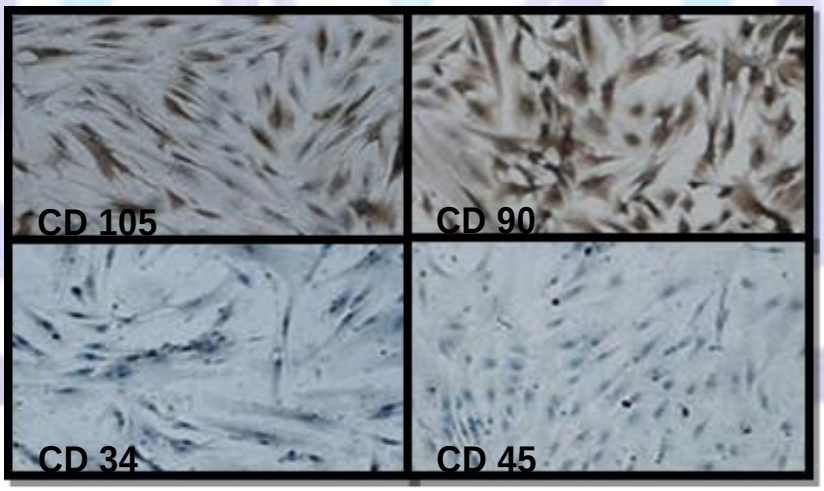

Figure (2): Immunocytochemistry analysis of isolated BM-MSCs: shows the positive reaction of cells towards CD105 and CD90 markers of cell surface and the negative reaction for CD34 and CD45 cell surface markers.

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 hepatocytes Figure 3 (A,B,C,D,E and F).

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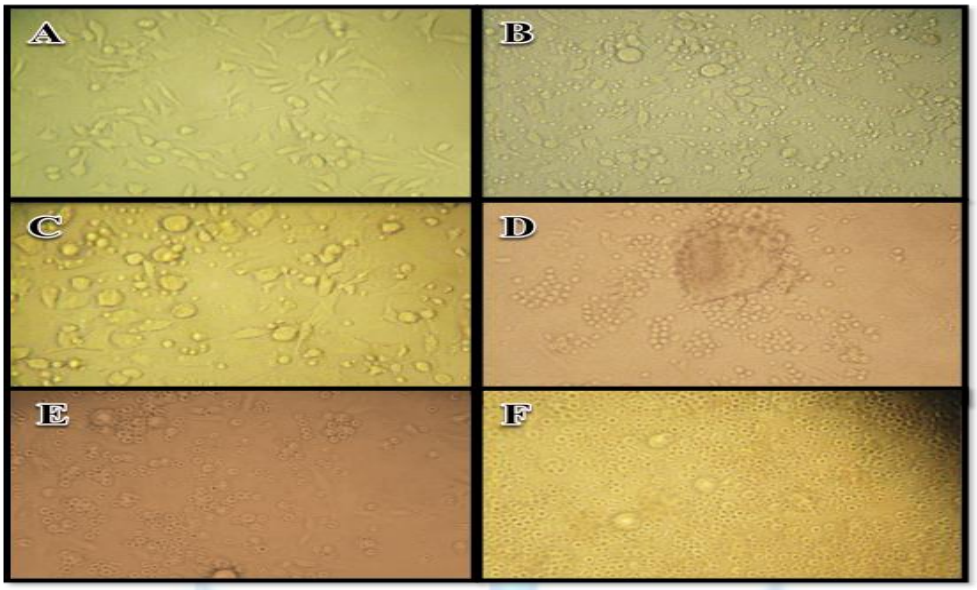

Figure (3): Different stages of morphological patterns of hepatogenic differentiation of Mouse MSCs. A: the mesenchymal stem cells isolated from mouse bone marrow; B: BM-MSCs under differentiation condition at day 10; C: BM-MSCs under differentiation condition at day 16; D, E: Differentiated cells at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

Immunocytochemistry analysis of CYP3A4 of the differentiated cells

At the end of the differentiation experiments the resulting hepatocytes like cells were tested for Cytochrome oxidase p450 (CYP3A4 p450) expression, results showed that the majority of the differentiated cells (about 70%) were stained with the brown DAP stain (Figure 3-5 A), means these cells are positively reacted towards CYP3A4 p450 mouse anti human primary antibody explain that the cells are positive for CYP3A4 enzyme expression, as well as some of these cells were shown to be negatively reacted to the CYP3A4 p450 suggested that these cells may be undifferentiated cells or are not completely differentiated to express the CYP3A4 p450 enzyme as a mature differentiated hepatocytes.

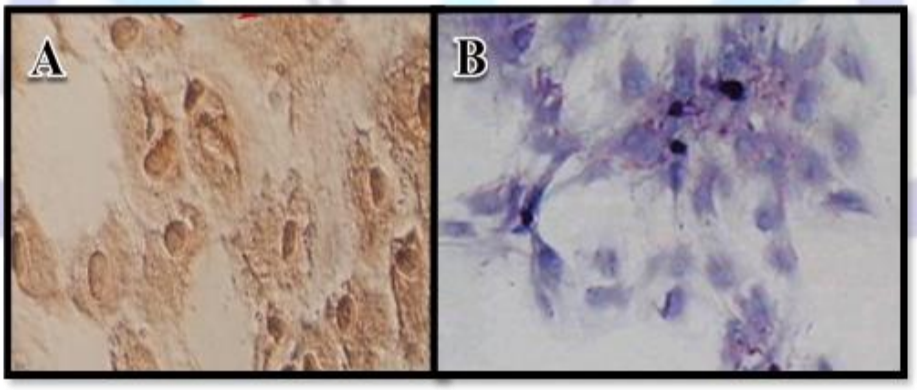

Figure (4): Immunocytochemical analysis of cytochrome oxidase p450 (CYP3A44 p450) in hepatocytes like cells. Positive reaction in (A) in induced hepatocytes like cells, while (B) shows the negative reaction of cells against mouse antihuman CYP3A4 p450.

Elisa assay of CYP3A4 in differentiated cells lysate

Results revealed that a significant increase in CYT3A4 concentration in hepatocyte lysate at 21 day (65.27± 0.52) ng/ml in comparison with 14 and 7 33.0 ±0.0.72 and 16.73±0.92) ng/ml respectively (Figure 5).

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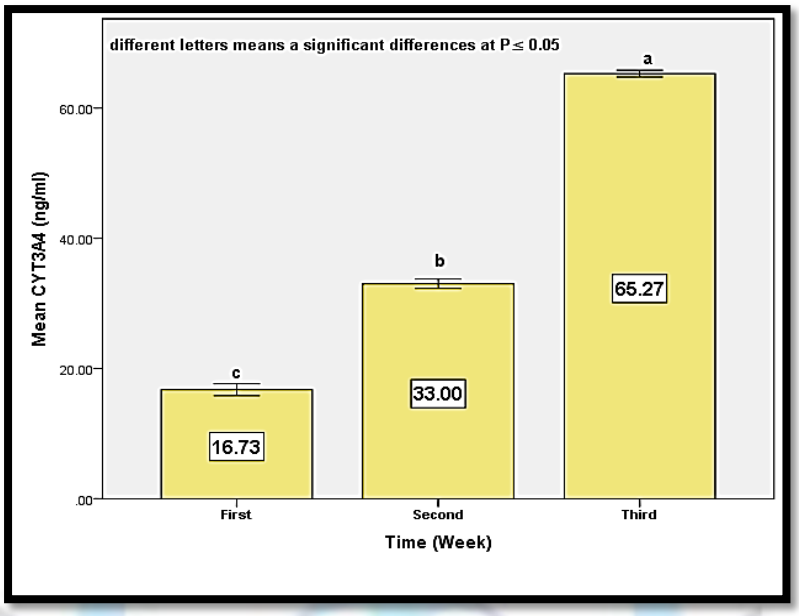

Figure (5): Level of CYT3A4 (ng/ml) in hepatocyte lysate after differentdifferentiation period.

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Discussion

The differentiation mechanism of mesenchymal stem cells is guided by a number of growth factors which affected the expression of specific genes which directed the cells towards the cell type of interest in vitro. [9].Mesenchymal stem cells (MSCs) have because of their plasticity and mulipotency had played a promising and reliable source for hepatic cell line for the treatment of liver diseases [10].

Although the stem cell differentiation mechanism remains unclear to date, transdifferentiation might either be induced by stimulating with suitable media/substrates/factors, or by genetic reprogramming in vitro [11]. Fibroblast growth factors (FGFs), are involved at an initial stage of endodermal patterning to induce hepatic fate [9] Oncostatin M, a member of the interleukin-6 cytokine family produced by hematopoietic cells, is required from the mid-fetal to the neonatal stages and apparently coordinates liver development and hematopoiesis in the fetus [12]. This is consistent with the fact that FGF-4 may play a role in endoderm specification, and that HGF induces differentiation of hepatocytes that are not actively proliferating [13]. Dexamethasone has been shown to have a specific differentiation-inducing effect on primary fetal hepatic cells towards mature hepatocytes [14].

The cytochrome P450 (P450) superfamily consists of a large number of haem-containing mono-oxygenases that play a pivotal role in the metabolism of many drugs and carcinogens [15]. Cytochrome P450 contain isoenzymes which catalyze he metabolism of a wide number of drugs in the liver [16]. The non -hepatic cells may also possess this kind of catabolic enzyme (CYp3A4) but in a ratio lower than in the hepatic cells in which these cells having a major role in drug metabolism in the liver, this why the Elisa results showed a significant increase in the level of CYP3A4 at the end of the differentiation protocol where the cells are fully differentiated in to hepatic cell line.

From all above we conclude that the bone marrow derived mesenchymal stem cells were able to differentiates into hepatic cell line and expressed a higher level of the catabolic enzyme CYP3A4 which is involved in the detoxification processes of many drugs achieved by the liver.

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