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ORIGINAL ARTICLE

In vitro mesenchymal stem cells differentiation into hepatocyte-like cells in the presence and absence of 3D microenvironment

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Abstract Our study was aimed to select the best growth factor cocktail for differentiation of mesenchymal stem cells (MSCs) into hepatocytes-like cells (HLCs) in absence and presence of three dimensional (3D) microenvironment. Ninety milliliters of bone marrow was aspirated from the iliac bone for separation of MSCs. This study was conducted on 20 patients with advanced liver cirrhosis. They were divided into two groups. Group I; MSCs were plated onto 96-well plates without microenvironment (control group). Group II; MSCs were plated onto 96-well plates with 3D microenvironment. Surface expression of CD271, CD29, and CD34 were analyzed using flow cytometry. MSCs were differentiated in vitro into HLCs by adding four growth factor cocktails in presence and absence of 3D microenvironment. Hepatogenesis was assessed by immunohistochemical analysis of OV6, α-fetoprotein, albumin, and cytokeratin 18 expressions. There was statistically significant increase in the expression of CD271 and CD29 after MSCs culture ($P<0.001$). Heterogeneous cell population composed of MSCs and differentiated HLCs were encountered (40 % hepatocytes and 60 % MSCs). Furthermore, our results showed that growth factor cocktails 1 and 2 gave the best result for differentiation of MSCs into HLCs. MSCs could be feasible, readily available, and novel source for differentiation of MSCs into HLCs for future therapeutic implication.

Keywords Differentiation · Growth factors · 3D microenvironment · Hepatocyte-like cells · Mesenchymal stem cells

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Introduction

Mesenchymal stem cells (MSCs) are bone marrow (BM) populating cells; they have generated a source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types including osteocytes, adipocytes, chondrocytes, myocytes, cardiomyocytes, and neurons (Christoforou and Gearhart 2007; Chop and Li 2002). Up to now, no single specific marker has been identified. MSCs express a large number of adhesion molecules, extracellular matrix proteins, and cytokines and growth factor receptors (Silva et al. 2003). The population of MSCs isolated from bone marrow express: CD44, CD271, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD166, CD29, CD73 (SH3 and SH4), CD90 (Thy-1), CD117, STRO-1, and stem cell antigen-1 (Silva et al. 2003; Boiret et al. 2005). In addition, MSCs do not possess markers typical for hematopoietic and endothelial cell lineages: CD11b, CD14, CD31, CD33, CD34, CD133, and CD45 (Baddoo et al. 2003). MSCs have generated a great deal of excitement and promise, as they home to sites of tissue injury and repair the tissues by several mechanisms; these mechanisms include transdifferentiation into a different type of cell, cell fusion between bone marrow-derived cells and hepatocytes (Wang et al. 2003), secretion of chemokines to enhance repair of damaged cells, and stimulation of proliferation of tissue endogenous stem cells (Stefania and Pietro 2007; Allen et al. 2005).

Cirrhosis is a progressive liver disease and is marked by the gradual destruction of liver tissue over time. Persistent injuries lead to hepatic scarring (fibrosis). Portal hypertension and hepatocyte dysfunction are the end results and give rise to major systemic complications and premature death (Forbes 2008; Svegliati-Baroni et al. 2008). The main causes of cirrhosis globally are hepatitis C and B and alcohol abuse. In Egypt, it is a health crisis of alarming proportions. Egypt has

the highest prevalence of hepatitis C virus (HCV) in the world, up to 20 % in some areas (Darwish et al. 2001).

To date, there are many in vitro and in vivo studies that have tried to demonstrate a role of bone marrow mesenchymal stem cells (BMSCs) in liver repair after injury. In patients with chronic hepatitis C, Libbrecht et al. (2000) was able to follow the differentiation of hepatic progenitor cells both into hepatocytes and cholangiocytes, suggesting that this stem cell compartment participates in the parenchymal regeneration associated with chronic viral liver disease. Further studies in acute and chronic liver disease of different etiology also demonstrated differentiation of progenitor cells into hepatocytes (Xiao et al. 2003). Our study aimed at selecting the best growth factor cocktail for differentiation of MSCs into hepatocytes like cells (HLCs) by comparing different growth factor cocktails in absence and presence of three dimensional (3D) microenvironment.

Subjects and methods

Subjects

The present work was conducted on a total number of 20 patients with advanced liver cirrhosis following HCV infection genotype 4 (RBV). HCV RNA was negative for all patients at time of study. According to the modified Child Pugh scoring, all patients were Child's C liver cirrhosis; MELD score was >12. They were divided into two groups. Group I; MSCs were plated onto 96-well plates without microenvironment. Group II; MSCs were plated onto 96-well plates with 3D microenvironment (Corning 96-well microplates with Ultra-Web Synthetic Surface). Their ages ranged between 39 and 60 years with mean value 51.6±7.2 years. They were 16 males (80 %) and 4 females (20 %). All patients were selected among cases referred from Gastroenterology Outpatient Clinic in Kasr EL-Aini hospitals; a written informed consent was taken from all patients.

Methods

Sampling

About ninety milliliters of bone marrow were aspirated under local anesthesia and placed in sterile tubes containing preservative-free heparin (Sigma-Aldrich, St. Louis, USA).

Mononuclear cells isolation

The bone marrow aspirate was diluted with phosphate buffer saline containing 2 mM EDTA (PBS/EDTA buffer). Then the mononuclear cells (MNCs) were isolated by density gradient centrifugation at 1,700 rpm for 35 min (density 1.077, GibcoBRL, Grand Island, NY, USA).

Mesenchymal stem cell separation as previously described by Bruno et al. (2009)

In brief, the MNCs were plated at concentration of 2×10^5 cells/ml in liquid culture media containing 45 ml Alpha modified Eagle's medium (GibcoBRL), 5 ml fetal calf serum, 100 μl Gentamicin, and 100 μl fungizone then incubated at 37 °C in 5 % CO_2. After 1 day, nonadherent cells were removed and adherent cells were cultured in the presence of mesenchymal media (Cambrex BioScience, Nottingham, UK) for 3 weeks. The media were changed every 4–5 days. After reaching 80 % confluence, the MSCs were recovered by incubation with trypsin/EDTA and counted on hemocytometer. The cell count of the isolated MSC is adjusted at the concentration of 500,000/ml.

Flow cytometry

Analysis of surface expression of MSCs using anti-CD271, anti-CD29, and anti-CD34 monoclonal antibodies was done. MSCs (2×10^5 cells) were suspended in PBS containing 1 % BSA and were stained with fluorochrome-conjugated mAbs for 20 min on ice (anti-mouse mAanti-CD271, mAanti-CD29, and mAanti-CD34; BD Biosciences, MN, USA). Each sample was analyzed in duplicate; 10,000 cells were passed in front of the laser for each sample. A cutoff value at 20 % was set to categorize samples as positive. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences) equipped with CellQuest Software. It revealed positive CD271 and CD29 expression and negative CD34 expression.

Hepatocytes differentiation

Two hundred microliters of MSCs were placed onto two plates; 96-well plates without microenvironment and onto another one with 3D microenvironment (Ultra-Web Synthetic Surface plate) (Sigma-Aldrich). These 96-well microplates provide cells with a synthetic 3D in vivo-like nanofibrillar substrate for better performance and functionality. Ultra-Web® Synthetic Surface is polyamine treated for a positively charged surface for enhanced cell attachment, binding, and covalently linking biomolecules. Four cocktails were added as mentioned below:

Cocktail 1 Days 0–7: basal medium + 1.6 μM fibroblast growth factor-4 (FGF-4) + 3.2 μM hepatocyte growth factor (HGF) + 0.4 μM epidermal growth factor (EGF).

Days 8–14: basal medium + 1.6 μM FGF + 3.2 μM HGF + 0.4 μM EGF.
Days 15–21: basal medium + 1.6 μM FGF + 3.2 μM HGF + 0.4 μM EGF.

Cocktail 2 Days 0–7: basal medium + 1.6 μM FGF + 3.2 μM HGF + 0.4 μM EGF.
Days 8–14: basal medium + 1.6 μM of dexamethasone.
Days 15–21: basal medium + 1.6 μM of dexamethasone.

Cocktail 3 Day s 0–7: basal medium + 1.6 μM FGF + 3.2 μM HGF + 0.4 μM EGF.
Days 8–14: basal medium + 1.6 μM of dexamethasone + 1.6 μM of oncostatin M.
Days 15–21: basal medium + 1.6 μM of dexamethasone + 1.6 μM of oncostatin M.

Cocktail 4 Days 0–7: basal medium +1.6 μM FGF + 3.2 μM HGF + 0.4 μM EGF.
Days 8–14: basal medium + 1.6 μM of dexamethasone + 1.6 μM of oncostatin M + 2 μM of nicotinamide.
Days 15–21: basal medium + 1.6 μM of dexamethasone + 1.6 μM of oncostatin M + 2 μM of nicotinamide (All from R&D System, Minneapolis, MN).

Immunohistochemistry

Staining using anti-CD29 and -CD34 was performed to assess MSCs expansion. Hepatogenesis was assessed by detection of OV6, α-fetoprotein (AFP), albumin, and cytokeratin 18 (CK18) antibodies. Primary monoclonal antibodies for different markers (R&D system), were diluted in PBS, and then placed on the slides that were primarily smeared with the cultured cells. Incubation at 4 °C to next day was done then diaminobenzidine chromagen was used for 10-20 min until desirable brown color (positive expression) was obtained. The slides were then washed in buffer, and nuclear counter staining was done by Mayer's hematoxylin HX. Our results revealed negative CD34 and positive CD29, OV6, α-fetoprotein, albumin, and cytokeratin 18 expressions. Positivity for these markers were heterogeneous; purity was approximately 40 % (40 % of total cellularity was HLCs and 60 % was MSCs), this pattern was achieved mainly by growth factor cocktails 1 and 2 (they gave the best results for differentiation of MSCs into HLCs).

Statistical analysis of data

Data were analyzed using SPSS with statistical package version 15 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. For quantitative data, comparison between two groups was done using paired *t* test. Comparison between >2 groups was done using ANOVA test. A p value < 0.05 was considered significant.

Results

On comparing each of CD271, CD29, and CD34 before and after MSCs culture, there was a highly statistically significant difference with increase of both levels of CD271 and CD29 at the end of the culture (P value < 0.001); meanwhile, CD34 did not show a similar effect (P value >0.05) (Table 1).

Expanded MSCs were differentiated in vitro into HLCs by plating them onto 96-well plates without and with 3D microenvironment; on comparing percentages of differentiated HLCs recovered from both wells, there was a statistically significant difference between the two groups with higher percentages of HLCs that were cultured onto the plate with 3D microenvironment on day 14 (1 week earlier than that were cultured onto the plate without microenvironment) (Table 2).

In addition, on comparing percentages of HLCs recovered after cultivation of expanded MSCs in the presence of different four growth factor cocktails, cocktails 1 and 2 gave the best results for differentiation.

Immunohistochemical staining was performed to assess MSCs expansion using anti-CD29 and -CD34 antibodies and to assess hepatogenesis by detection of OV6, AFP, albumin, and CK18 marker expression. Our results showed heterogeneous positivity of HLCs for these markers with higher percentages of positive cells that were cultivated in the presence of cocktails 1 and 2 (Table 2 and Fig. 1).

Table 1 Statistical comparison of percentages of both CD271 and CD29 before, after MSCs culture, and after MSCs differentiation ($P<0.05$ statistically significant)

Items	Before culture (no. 10)	After culture (no. 10)	After differentiation (no. 10)	P value
CD271 (%)				
• Range	1.10–5.00	55.00–89.00	26.00–53.00	0.001
• Mean ± SD	2.60±1.10	74.7±13.2	39.20±8.11	
CD29 (%)				
• Range	2.00–8.00	60.00–95.00	25.00–50.00	0.001
• Mean ± SD	5.4±1.8	85.80±10.20	38.80±7.81	

Table 2 Percentages of hepatocyte markers positive cells recovered after MSCs differentiation with and without 3D microenvironment on day 14 ($P<0.05$ statistically significant)

Hepatocyte marker (Mean ± SD)	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	*P* value
Growth factor cocktail with 3D microenvironment					
OV 6	12.00±2.00	11.50±1.99	6.60±2.27	7.50±2.40	<0.001
Albumin	25.60±4.43	26.60±4.41	12.20±2.30	12.50±2.70	<0.001
AFP	24.90±3.98	27.30±3.50	13.20±1.93	11.50±1.99	<0.001
CK18	35.30±4.60	35.00±4.55	18.70±1.95	17.80±2.30	<0.001
Growth factor cocktail without microenvironment					
OV 6	5.00±1.02	4.20±0.90	2.00±0.65	3.20±0.75	<0.001
Albumin	11.30±2.01	12.25±2.33	5.25±0.85	5.90±1.25	<0.001
AFP	10.30±1.89	11.75±1.78	5.50±0.95	4.75±1.01	<0.001
CK18	14.25±2.08	13.90±1.95	7.50±1.40	8.30±1.78	<0.001

Discussion

Human hepatocytes are available only in scanty number; this necessitates the development of new culture systems that allow improving cultivation and function of differentiated hepatocytes. In contrast to the more conventional two dimensional cultures, 3D nanofibrillar microenvironment was evaluated for prolonged hepatocyte function. These surfaces offer cells more in vivo-like fibrillar topography that, unlike biological coatings, are more stable, more consistent lot to lot, and animal component-free. They can be modified by linking cell attachment and growth factors to improve the culture environment (Schindler et al. 2006). It falls within the interest of our study to investigate effect of 3D microenvironments, mimicking the liver's stem cell niche on differentiation of expanded MSCs into HLCs and to choose the best growth factor cocktail for their differentiation.

Surface expression of expanded MSCs was assessed by flow cytometric analysis of CD271, CD29, and CD34 that showed a highly statistical significant difference before and after the culture and after differentiation with a higher level of each of CD271 and CD29 at the end of the culture ($P<0.01$). Meanwhile, CD34 did not elicit an equivalent expression (hematopoietic stem cell marker) (Fig. 2). This is consistent with the opinion of Bühring et al. (2007) who stated that, so far, CD271 is one of the most specific markers for BM-derived MSCs (Figs. 3 and 4).

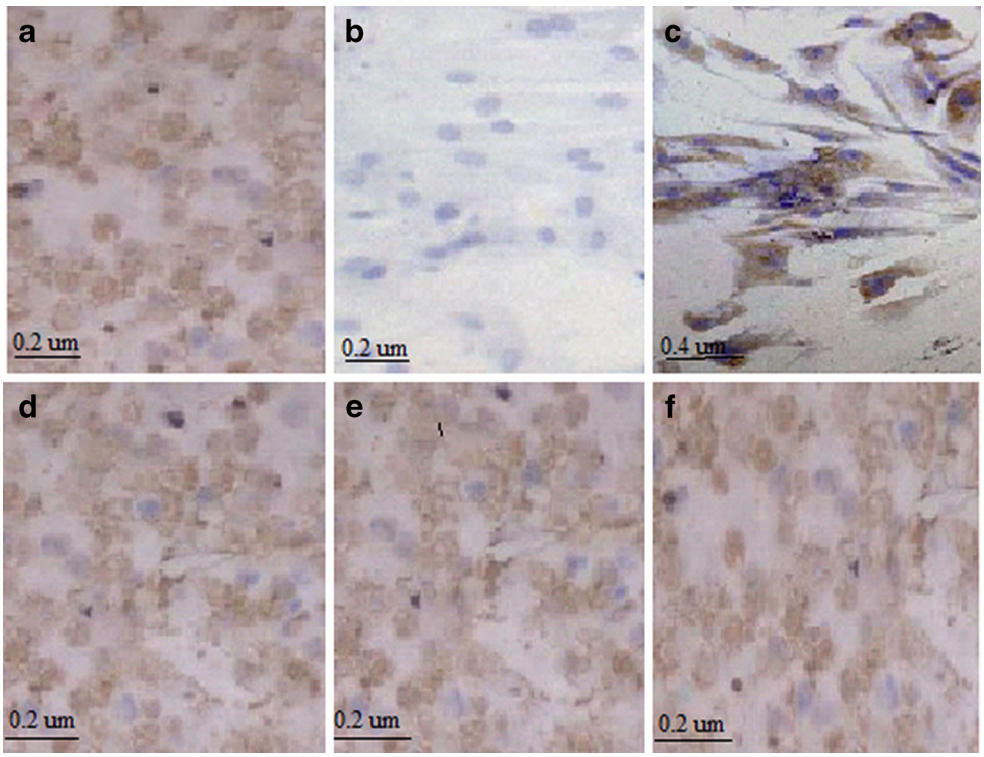

Fig. 1 Photography of MSCs (undifferentiated) and HLCs (differentiated MSCs). *1a* HLCs many of them are positive for AFP; *1b* HLCs are negative for CD29; *1c* MSCs are positive for CD29 (*brown color*); *1d* HLCs many of them are positive for albumin; *1e* HLCs many of them are positive for CK18; *1f* HLCs many of them are positive for OV6 (All X200 apart from 1c X400). *Scale bar* 200 nm for all images apart from *1c scale bar* 400 nm

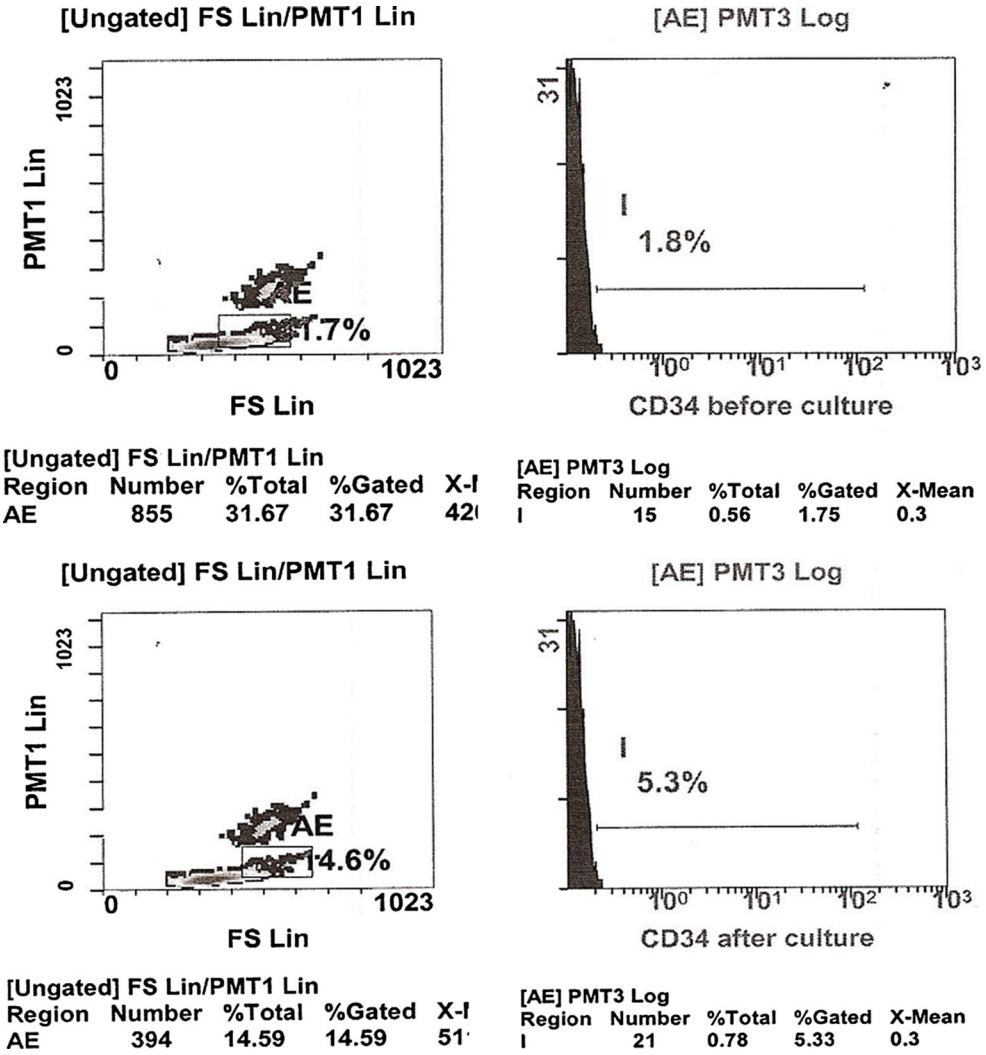

Fig. 2 CD34 expression before and after culture by flow cytometry, revealing negative expression after culture

On comparing differentiation of MSCs into HLCs with and without 3D microenvironment, there was a statistical significant difference between the two groups with a higher percentage of HLCs recovered from the plates with 3D microenvironment on day 14 than that were cultured onto the plates without microenvironment on day 21 ($P<0.001$). In addition to this, the detection of differentiated HLCs using OV6, albumin, AFP, and CK18 antibodies showed that most of the differentiated MSCs into HLCs cultured onto the plates with 3D microenvironment picked up these stains with heterogeneous positivity more and earlier than that cultured onto plates without microenvironment by 1 week. Our results agree with Piryaei et al. (2011), who implement nanofibers for better differentiation and to maintain the function and engraftment of differentiating MSCs. They investigated MSC differentiation into early (day 18) and late (day 36) HLCs in the presence or absence of ultra-web nanofibers. They found that hepatocyte markers-ALB and HNF4α were elevated in nano-positive group at day 18; however, those with similar levels or slightly decreased in the nano negative group was encountered from day 18 to 36. Also, Kazemnejad et al. (2008) performed differentiation of human BM-MSCs to HLCs on a constructed 3D nanofibrous biocompatible scaffold and reported that hepatocyte markers show progressive expression during the 3 weeks of differentiation on the 3D scaffold. Our results were further supported by the work studied by Li et al. (2010) who introduced bioscaffold for differentiation of rat BM-MSCs into hepatocytes, and their results showed that isolated cells possessed the basic features of BM-MSCs. Differentiated HLCs in the 3D scaffolds expressed hepatocyte-specific markers 1 week earlier than in the controls and differentiated HLCs grew more stably in the 3D scaffolds than that in controls.

In addition, statistical comparison was held between the effects of four growth factor cocktails on the differentiation of MSCs into HLCs. HGF was originally identified and cloned as a potent mitogen for hepatocytes, and it has been shown to play essential roles in the development and regeneration of the liver. Schwartz et al. (2002) reported that MSCs from adult murine marrow differentiated into HLCs in culture

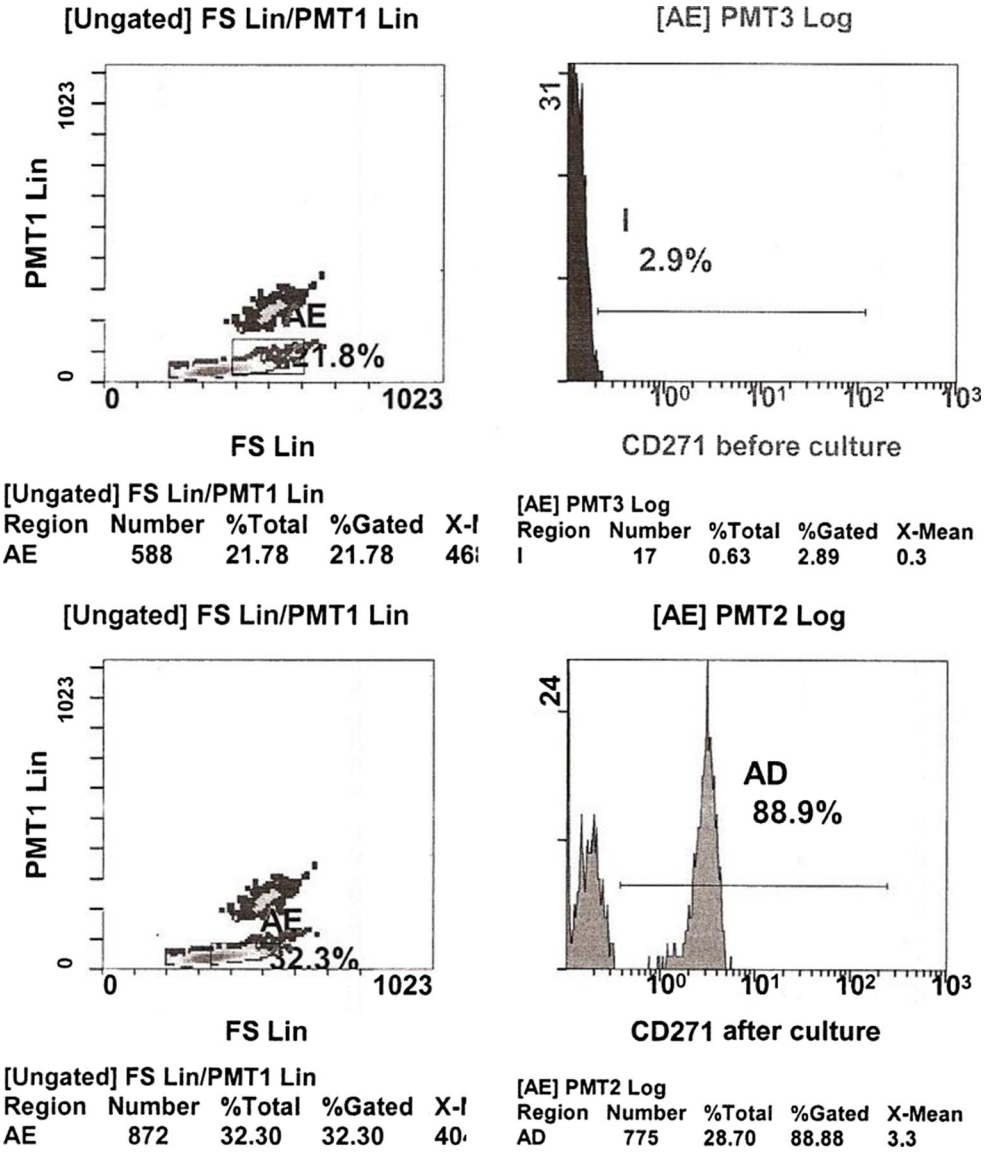

Fig. 3 CD271 expression before and after culture by flow cytometry, revealing positive expression after culture

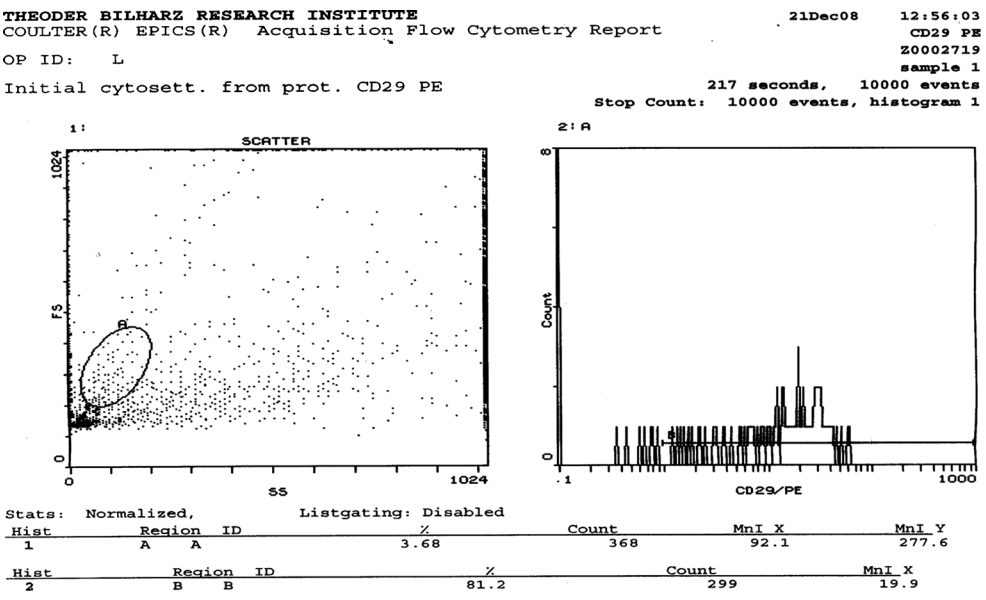

Fig. 4 CD29 expression before and after culture by flow cytometry, revealing positive expression after culture

necessitated the presence of FGF-4 and HGF. Also, Block et al. (1996) found that HGM medium contains dexamethasone which has a strong stimulatory effect on DNA synthesis of primary hepatocytes. Also, FGFs, oncostatin M, and several families of transcription factors, have been shown to be important components of liver development and differentiation processes (Darlington 1999). Our results revealed that cocktails no. 1 (EGF, HGF, and FGF-4) and 2 (EGF, HGF, FGF-4 and dexamethasone) gave the best results for differentiation of MSCs into HLCs ($P<0.001$). The data obtained in our study confirmed with those reported by Lin et al. (2010) who investigated the potential of BM-MSCs to differentiate into HLCs; they cultured BMSCs in 3D scaffolds in the presence of specific growth factors including HGF, EGF, and FGF-4. They found that differentiated HLCs displayed several liver-specific markers and functions. In addition, these cells produced both ALB and urea and expressed CK18, its percentage was 56.7 %. This matches the opinion of Xie et al. (2010) who reported that continuous exposure of BM-MSCs to cytokine cocktail FGF-4, EGF, and HGF promoted its differentiation into HLCs. These cells express hepatic markers, i.e., HNF-3β, GATA4, CK19, α-fetoprotein, albumin, and CK18. They also possess functional characteristics of hepatocytes, i.e., secreting urea and albumin, having phenobarbital-induced cytochrome p450, taking up LDL, and storing glycogen. In contrast with our data, Chivu et al. (2009) determined the differentiation efficacy of various liver-specific factors (HGF, insulin–transferrin–selenium, dexamethasone, and nicotinamide) for stem cell differentiation into HLCs, and they found that HGF and nicotinamide were the factors with the most hepatogenic potential as evidenced by their gene expression and functional testing. Also, Yagi et al. (2008) implemented exclusive use of HGF, and they stated that BMSCs appeared to differentiate into HLCs in response to the culture with HGF as demonstrated by RT-PCR and immunocytochemical analysis of albumin mRNA and the production of protein after cultivation with HGF for 2 weeks. Perusing the same subject, but on animal models, Oyagi et al. (2006) found that the transplantation of HGF-treated BM-MSCs significantly reduced the serum transaminase levels and liver fibrosis in CCl4-injured rats. Priming the culture with HGF before BM-MSC transplantation appeared to be effective for the suppression of liver inflammation and fibrosis. From the above data, it seems that cell differentiation and regeneration are controlled by growth factors especially HGF that is originally identified and cloned as a potent mitogen for hepatocytes, showing mitogenic and morphogenic activities for a wide variety of cells that express the HGF receptor c-Met. Moreover, HGF plays an essential role in the development and regeneration of the liver (Oh et al. 2000).

Furthermore, in our study, the detection of differentiated heptocytes using OV6, albumin, AFP, and CK18 antibodies showed that most of the differentiated MSCs into HLCs picked up these stains more with growth factor cocktails 1 and 2 than those with growth factor cocktail 3 and 4. Also, our results did not encourage the use of oncostatin M and nicotinamide as they did not help anymore in the differentiation of MSCs into HLCs.

Conclusion

The differentiation of MSCs into HLCs using the growth factor cocktail numbers 1 (EGF, HGF, FGF) and 2 (FGF, HGF, EGF, and dexamethasone) gave the best result for in vitro differentiation primarily in the presence of microenvironment and to a lesser extent without the microenvironment (3D microenvironment encourage growth of MSCs and their differentiation into HLCs essentially in the presence of growth factors). MSCs could be feasible, readily available, and novel source for differentiation of MSCs into HLCs, consequently being a prerequisite for potential future therapeutic use.

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Conflict of interest The authors declare no conflicts of interest.

Declaration of ethics This study was approved by the review board of Kasr El-Aini hospital (FWA 00010609), and written informed consent was obtained from all patients according to Helsinki guidelines of research ethics.

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