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Differentiation of Human Peripheral Blood Mononuclear Cells and Cd14+ Monocytes Into Hepatocyte-Like Cells Obtained From Liver Cirrhosis Patients

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Abstract

The current study has benefit of the potentiality of peripheral blood monocytes (MONs) and adherent peripheral blood mononuclear cells (MNCs) to differentiate into various progenitor cells according to growth factors added. Aim: the aim of this work was to investigate and compare the efficiency of PB monocytes (MONs), and adherent mononuclear cells (MNCs) obtained from cirrhotic patients to expand and differentiate into functioning hepatocyte-like cells in vitro. Material and methods: 20 patients suffering from chronic hepatitis and liver cirrhosis were included in the study. MNCs were isolated from PB using Ficoll Hypaque. MONs (CD14+), and adherent MNCs (contain CD34+Cs) were separately cultured for28 days in presence and absence of recombinant human hepatic growth factor (rh-HGF). The culture media were changed every 7 days and culture supernatants were harvested, allocated, and stored at – 80C^0. Hepatocyte-like cells were examined for morphology, function, and hepatocyte markers by: a) estimation of albumin secretion, urea production, and LDH release in the culture supernatants, b) qRT-PCR for cultured cells at end points of cultures to detect albumin gene expression, and c) detection of CK 18 and Hep Par-1 proteins in cultured cells using immunohistochemical technique.Results: Data obtained revealed: Monocyte % as detected by differential leukocyte count and flowcytometer showed highly significant increase in suspensions prepared using human Pan Monocyte isolation kit as compared to Ficoll separated MNCs, suspensions. Albumin, urea, and LDH showed highly significant differences between culture supernatants containing rh-HGF and those of control cultures depended on time points of estimations. Late specific liver markers; Hep par-1 and CK18 by immunocytochemistry showed increase in adherent MNCs cultured with rh-HGF compared to CD14+ MONs. Albumin gene expression increased significantly in differentiated cells derived

from MNCs cultured with rh-HGF compared to CD14+ MONs at end point of culture as detected by qRT-PCR. Conclusion: The obtained results showed successful in-vitro generation of functioning hepatocyte-like cells from easily accessible source of cells in presence of rh-HGF. So innovative regenerative cellular therapy indicates the possibility of autologous cell transfusion after hepatic differentiation for treatment of CLD and provide a promising alternative treatment for end stage liver disease.

Kea words: *Adherent PB MNCs, monocytes, rh- HGF, CK 18, Hep para-1, and hepatocyte differentiation.*

INTRODUCTION

The liver is one of the vital organs in the body and performs numerous important functions as well as homeostasis, production and storage of glucose and proteins, detoxification and immune defense [1]. Hepatocytes comprise the bulk of liver cells. Functional disorders of these cells are associated with many destructive diseases such as hepatitis, cirrhosis and hepatocellular carcinoma [2]. Cirrhosis is a permanent hepatocyte failure caused by long-standing infection with hepatitis viruses, alcohol misuse, autoimmune inflammation and exposure to metabolic metals like iron and copper [3].

Even though the liver is a naturally regenerative organ, at the end stage failure of liver disease such as fibrosis and cirrhosis, therapeutic intervention is required [4]. Liver transplantation is considered the most efficient therapeutic approach in liver failure and has been established as the most effective treatment modality for end-stage liver disease over the last few decades [5]. Yet, because of the shortage of proper donors, about 15% of waiting patients die [1]. In addition, risks associated with operation, immune suppression, immunological rejection, high costs, the need for high technology and a qualified team is clear limitations of liver transplantation. Because of these difficulties, cell therapy has been proposed as a new alternative technique for liver transplantation which has many advantages including overcoming the lack of liver donors, immunological rejection and a minimally invasive approach [6].

Stem cells, a unique source of self-renewing cells within the human body, in the adult are critical for self-renewal in tissues such as the hematopoietic system, the intestine and the skin that require a high cell turnover to maintain their homeostasis. Following injury, it has been suggested that repair of tissues involve the recruitment, proliferation and differentiation of stem cells [7].

Circulating monocytes are not yet fully differentiated cells and have the potential to transform into various other cell types [8]. For a long time, it was believed that monocytes could only differentiate into cell types possessing a phagocytic capacity. However, several recent studies indicate that CD14+ monocytes can express lineage markers other than their own when treated with specific additives [9]. It was reported that monocytes are capable of transdifferentiating into a progenitor cell population that is then able to differentiate into several mesenchymal cell types, including bone, fat, neurons and liver cells [10]. They could be expanded *in vitro* with reliability and reproducibility and stored in liquid nitrogen for long periods of time. It appears promising and valuable candidate for autologous cell therapy in liver disease [11].

SUBJECTS AND METHODS

Patients

Twenty patients (n=20) patients suffering from liver cirrhosis were enrolled from Gastroenterology Department, Theodor Bilharz Research Institute (TBRI). Written informed consent was obtained from all the patients. Inclusion criteria included age from 30 to 65 years, chronic hepatic failure due to hepatitis C or hepatitis B virus, Child C liver cirrhosis and Model of End stage Liver Diseases (MELD) score >12. On the other hand, patients aged below 30 or over 65 years, active bleeding, hepatocellular carcinoma, hepatic coma, history of autoimmune disease and /or presence of malignancy.

MNCs separation and Monocyte selection using magnetic cell sorting (MACS)

Venous blood samples obtained from liver cirrohsis patients were subjected to density gradient centrifugation using Ficoll Hypaque Plus to isolate MNCs. Moreover, Pan Monocyte Isolation Kit was used to isolate $CD14^+$ MONs from MNCs by depletion of non-monocytes (negative selection) using MiniMACS separator following manufaturer's protocol (Milteny Biotec GmbH, Germany CAT NO: 130-096-537).

Flow cytometer

Freshly isolated MNCs and selected CD14+ MONs were differentially counted using automated cell counter (DXH 500, Beckman Coulter, USA). $CD14^+$ expression was characterized by flow cytometry using phycoerythrin (PE)-conjugated anti-human CD14 (CD14 PE IOTest®) IMMUNOTECH Beckman Coulter, France CAT NO: A07764. Propidium Iodide (PI) staining was used to determine viability of MNCs and MONs before culture.

Culture of MNCs

Peripheral blood MNCs were cultured in RPMI 1640 (Cat. ECM2001D) containing 10% fetal bovine serum Euro- Clone, (Cat. ECS0180D), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin Euro- Clone (Cat. ECB3001D). Non adherent cells were removed by gentle washing with phosphate-buffered saline 2 hr later. Some adherent cells were then cultured in DMEM/F 12 (1:1) based medium CAT: ECM0095 Euro-Clone S.p.A. (MI) - Italy with 100 ng/ml recombinant human hepatocyte growth (r-HGF) R&D systems Catalog Number: 294-HGF factor for 28 days to induce direct reprogramming and hepatic differentiation. Some adherent cells were only cultured in DMEM/F 12(1:1) as a control. The culture medium was renewed every 7 days [12].

Culture of CD14+

Isolated Monocytes (CD 14+) were washed 2-3 times with RPMI 1640 medium. Cells were inoculated at $1X10^5$ cells per ml in 35 mm Petri dish and the medium was replaced every 7 days and treated with 50ng/ ml recombinant human macrophage-CSF(rhM-CSF) R&D systems, US CAT NO: 216-MC; 100ng/ml Phorbol 12- myristate 13-acetate (PMA) (Enzolife science) CAT NO: PE-160, and 1000 units/ml recombinant human LIF (rh-LIF) R&D systems, US CAT NO: 7734-LF. After 7 day of culture, in order to induce hepatocytic differentiation, 100 ng/ml of rh-HGF was added for 28 days. Control cells were cultured in DMEM/F 12(1:1) in absence of rh-HGF. After 28 days differentiated hepatocyte like cell suspensions were obtained by pipetting after incubation for 8 min with Trypsin EDTA 1X in PBS w/o Calcium w/o Magnesium w/

Phenol Red CAT NO: L0930 (Biowest) in PBS. The cell pellet was resuspended in fresh medium and counted [13].

Measurement of biochemical parameters

Supernatants of each culture were collected at the indicated time points (7,14,21&28) and stored in aliquots at -80o^{c} until further analysis. Albumin, urea and LDH were measured by cobas® 6000 analyzer.

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Immunocytochemistry

Hepatocyte-like cells (cultured with recombinant human hepatocyte growth factor for 28 days) were fixed with 4% paraformaldehyde at 48ºC for 15 min. The cells were then washed three times in PBS and permeabilized with 0.3% Triton X-100 for 10 min at room temperature. Following blocking in 10% preimmune goat serum in PBS for 2 hr, the cells were analyzed by standard immunocytochemistry methodology with primary antibodies including Hep-par1 (Clone OCH1E5) Mouse Monoclonal Antibody Zeta Corporation, CA, USACATNO: Z2087, CK18 Mouse Monoclonal Antibody Novocastra™ Leica Biosystems© Newcastle, UK CAT NO: NCL-CK1. Cells were washed three times and then stained with diaminobenzidine. Finally, the slides were counterstained with hematoxylin. For negative controls, an equivalent dilution of normal mouse immunoglobulin G or rabbit preimmune serum was used in place of the primary antibody.

Quantitative RT-PCR analysis for albumin gene mRNA expression in Hepatocyte like cells

In order to confirm the differentiation of hepatocyte-like cells, Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was performed to detect *ALB* gene mRNA expression. Total mRNA was purified from freshly isolated MNCs and Hepatocyte like cells after culture with mRNA isolation kit for blood/bone marrow RN easy R Mini Kit (Cat.No.74104, Qiagen USA). First-strand cDNA was synthesized using cDNA Quantitec R Reverse Transcription kit (Cat.No.205311, Qiagen USA) according to the manufacturer‘s instructions. The resulting cDNA was amplified by using real time instrument Step One R (Applied Biosystems) using QuantiFast multiplex PCR Kit, detecting ALB (Hs-ALB Fam) gene and ACTB (Hs-ACTB – max2) gene, according to the manufacturer‘s instructions.

Statistical analysis

The data were analyzed using Microsoft Excel 2010 and statistical package for social science (SPSS version 24.0) for windows (SPSS IBM., Chicago, IL). Results expressed as mean ± SE with 95% confidence interval using rang for quantitative variables and using the frequencies and percentage for qualitative ones; a p value < 0.05 was considered statistically significant. Spearman's rank correlation coefficient (r) was done to show the correlation between different parameters in this study. Diagnostic parameters of subjects were compared using the non-parametric Wilcoxon-Mann-Whitney U-test, whereas the parametric parameters were compared using the Paired samples (t) test. Also, Chi-square ($\chi 2$) test was used for comparison of categorical data. Whenever the expected values in one or more of the cells in a 2x2 tables was less than 5, Fisher exact test was used instead and used linear by linear association in larger than 2x2 cross-tables.

RESULTS

In order to compare expansion and differentiation potential of isolated unfractionated PB MNCs separated by Ficoll Hypaque and selected CD14+ MONs separated by magnetic cell sorting (MACS). Target cells were analyzed to evaluate WBC & differential leucocyte count using automated cell counter to estimate MON percent (%). Flow-cytometery techniques presented as mean ± S.E showed a highly significant statistical increase of CD14+ expression on suspensions prepared using MACS (38.0±4.0, 55.0±2.7 respectively) when compared to Ficoll Hypaque separated PB MNCs suspensions (11.0±0.8, 14.0±1.6 respectively) (*p* value: 0.001).

Impacts of culture time on biochemical parameters

Regarding albumin secretion our results showed highly significant increase of mean± S.E in both culture supernatants of adherent MNCs and selected CD14+ MONs cultured with rh-HGF when compared with the control cultures in absence of rh-HGF) which is progressive with elongation of incubation period (days 7,14,21&28) (*p* value: 0,001). On comparing Albumin secretion in culture with rh-HGF suspension of adherent MNCs with those of selected CD14+ MONs. We found significant increase of albumin secretion in adherent MNCs culture suspension with rh-HGF compared to those of selected CD14+ MONs with rh-HGF on all days of culture (7,14,21&28) (*p* value: 0.02, 0.02, 0.001, 0.0.001 respectively) (Fig 1).

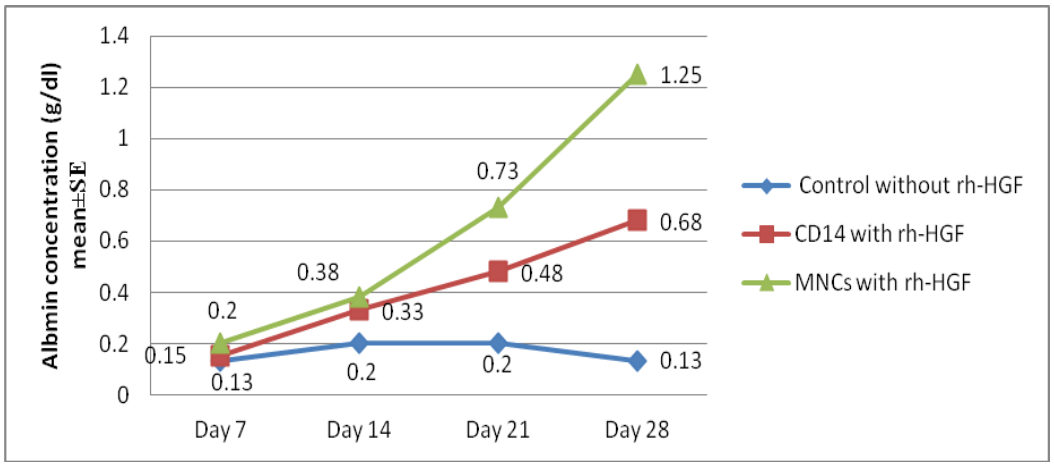

Figure 1: Changes in albumin concentration (g/dL) in MNCs and CD14 culture supernatants (with rh-HGF) and control culture supernatant (without rh-HGF)

Statistical analysis in our study revealed that urea production show no significant increase in culture supernatants of adherent MNCs cultured with rh-HGF On the other hand, selected CD14+ MONs cultured with rh-HGF showed an increase in the urea level at day 7, 28 (P value: 0.001, 0.1) when compared with the control (cells cultured without rh-HGF). On comparing urea production in supernatants of adherent MNCs and selected CD14+ MONs cultured with rh-HGF, significant increases in selected CD14+ MONs cells were noted on days 21 &28 (p value: 0.05, 0.04 respectively) (Fig 2).

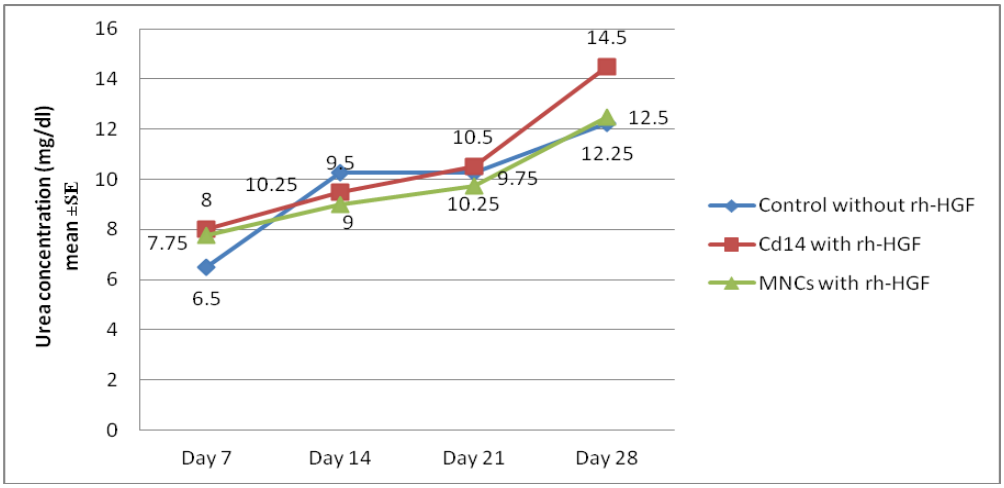

Figure 2: Changes in urea concentration (mg/dL) in MNCs and CD14 culture supernatants (with rh-HGF) and control culture supernatant (without rh-HGF)

Regarding LDH release in the supernatants of adherent MNCs cultured with rh-HG, it increased significantly on days 7& 21 (p value: 0.001, 0.02 respectively), but decreased significantly on day14 (p value: 0.001) and showed non-significant reduction on day 28 (p value: 0.7) when compared to control (culture without rh-HGF).

Selected CD14+ MONs cultured with rh-HGF showed an irregular pattern. It is decreased significantly on days 7& 21 (p value: 0.04, 0.001 respectively), but increased significantly on days 14 & 28 (p value: 0.01, 0.04 respectively) when compared to control (culture without rh-HGF)

On comparing LDH release in the 2 cell populations (adherent MNCs and monocytes; CD14+), it was significantly increased in supernatants of MNCs incubated with rh-HGF on days 7& 21(p value: 0.001, 0.02 respectively). On the other hand, CD14+ MONs cultured with rh-HGF showed significant increase in LDH level compared to adherent MNCs on day 28 (p value: 0.02) (fig 3).

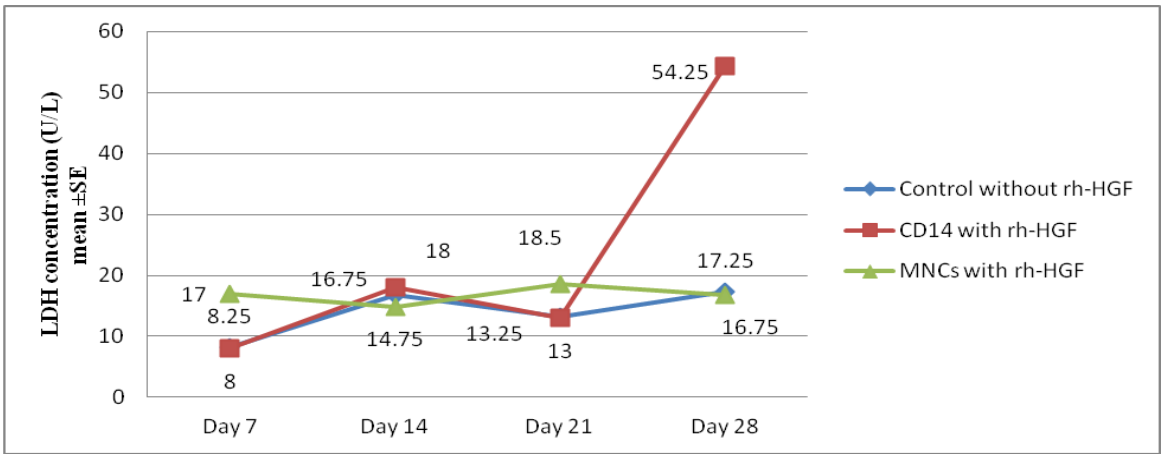

Figure 3: Changes in LDH concentration (U/L) in MNCs and CD14 culture supernatants (with rh-HGF) and control culture supernatant (without rh-HGF)

Immunohistochemical detection of hepatocytes markers

To further confirm the similarity of hepatocyte-like cells with the human normal hepatic cells, the former were stained immunohistochemically with antibodies against established hepatocyte marker proteins and we found that 75% of adherent MNCs cultured with rh-HGF showed CK18 expression and 50% in CD14+ MONs (table 1). Regarding Hep-par1 expression, only 45% of cultured MNCs incubated with rh-HGF and 25% of $CD14^{+}$ MONs showed positive expression (table 1) (fig 4,5,6,7).

Table 1: CK18 & Hep-par 1 % in adherent MNCs and selected CD14+ Monocytes cultured with rh-HGF

Culture with rh-HGF		**Negative**	**Positive**	**P. Value**
CK18	**Adherent MNCs**	5 (25.0%)	15 (75.0%)	0.01**
	Selected CD14+	10 (50.0%)	10 (50.0%)	0.9
Hep par-1	**Adherent MNCs**	11 (55.0%)	9 (45.0%)	0.1
	Selected CD14+	15 (75.0%)	5 (25.0%)	0.01**

Cytokeratin 18 and Hepatocyte Paraffin 1(Hep-par1) are represented as frequency and percent; the data were analyzed by x2 Test. *p. value <0.05 is significant, ** p. value <0.01 is highly significant.

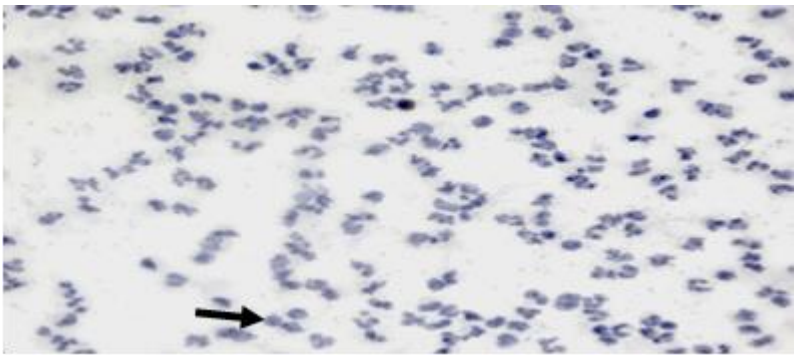

Figure 4: Smear from control case showing negative staining for Cytokeratin 18 (DAB, IHC, x400).

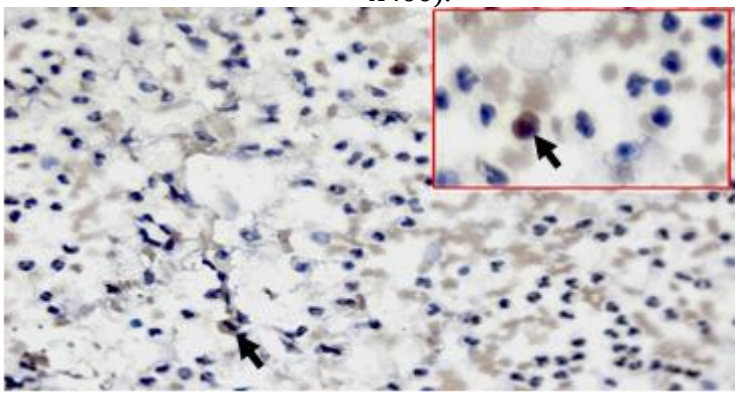

Figure 5: Smear from peripheral monocytes showing hepatocytes like cells with positive staining for Cytokeratin 18 as cytoplasmic staining (black arrow) (DAB, IHC, x400).

Figure 6: Smear from control case showing negative staining for Hep par-1 (DAB, IHC, x400).

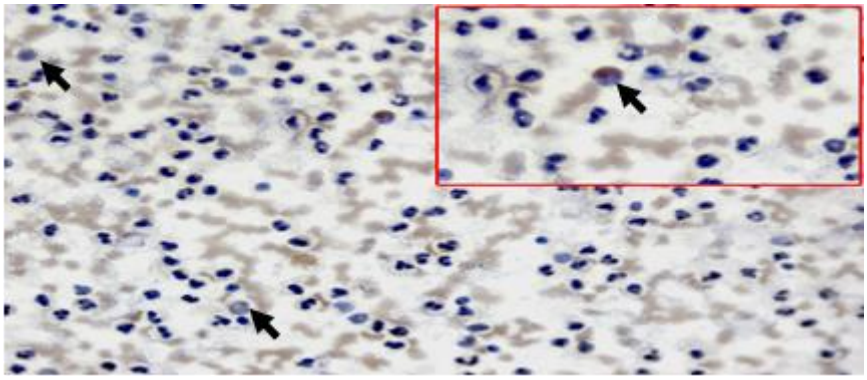

Figure 7: Smear from peripheral monocytes showing positive staining for Hep par-1 as cytoplasmic staining (black arrow) (DAB, IHC, x400).

Albumin gene expression by qRT-PCR

Hepatocyte like cells derived from adherent MNCs and MONs cultured with rh-HGF for 28 days showed highly significant increase in albumin gene expression compared to control culture (*p* value: 0.001). On comparing both MNCs and MONs cultured with rh-HGF, there was a significant increase of albumin gene expression in MNCs culture (*p* value: 0.04). (Table 2).

Table 2: Albumin gene expression in hepatocyte like cells derived from of MNCs and selected CD14+ MONs cultures with rh-HGF compared to control culture without rh-HGF.

Cell Populations	Control culture without rh-HGF At Day 28 n=20	Cell culture with rh-HGF At Day 28 n=20	p. value	p. value between two cell populations
MNCs	0	1.05 (0.2 - 1.68)	0.001**	0.04*
CD14+ MONs	0	0.025 (0.0125 - 0.03)	0.001**	

Albumin gene expression depending on the fold-change results x 10^{-3}.
All parameters are represented as Median with Interquartile range (25% -75%) of the fold change of the studied groups, the data were analyzed by Mann-Whitney U test. * p. value <0.05 is significant, ** p. value <0.01 is highly significant.

DISCUSSION

Cirrhosis, characterized by diffuse degeneration and the death of hepatocytes, followed by nodular regeneration, extensive fibrosis and the consequent collapse of the normal liver architecture, is the terminal stage of a variety of chronic liver diseases, resulting in irreversible impaired liver function, portal hypertension and the potential to develop into hepatocellular carcinoma [14]. Currently liver transplantation has been considered as the only managerial option [15]. During the last two decades there were many intensive and deep studies done to find a safe alternative treatment for liver cirrhosis and acute liver failure than liver transplantation [16]. One of the alternative approaches is stem cell therapy [17].

Cell therapy is a promising intervention for treating liver diseases and liver failure [18]. It is defined as cell transplantation to damaged organs for repair. The transplanted cells will replace or enhance the function of damaged tissues or organs. Research in the area of cell replacement therapy in the liver, particularly with stem cells, is at the beginning stages. The exciting capability of blood-derived stem cells that differentiate into multiple cell lineages raises the exciting opportunity of using these cells for tissue repair when the intrinsic pool of tissue stem cells is overwhelmed [19]. The development and evaluation of novel cell therapy for liver disease requires more investigations to identify an optimal strategy for long-term cell therapy treatment in liver failure [20]. However, stem cell therapy in liver failure is not standardized yet, as every center have their own methods [21]. Nevertheless, hepatocyte-based therapies are emerging as an alternative strategy to whole organ transplantation. Generating hepatocytes with complete liver function is still challenge and developing more functional hepatocytes is needed. Tell date works done to generate hepatocytes from monocytes are few. The large number of studies was performed using stem cells from different sources. Hepatocytes differentiation from stem cells depending on their potential to differentiate into any line of cells according to the growth factors added had developed a new therapeutic strategy for liver disease [22].

The cultured cells in our work were characterized by: (a) being obtained from an easily accessible source and by non-invasive technique, (b) their ability to increase in number in culture; their reliability and reproducibility, (c) their potential to differentiate into hepatocyte-like cells and (d) they can perform functions of liver cells after differentiation. Application of cell culture conditions described in the present work using samples obtained from patients suffering from chronic hepatitis and liver cirrhosis have succeeded to produce hepatoblast and hepatocyte-like cells. The morphological similarity of the progeny of the cultured adherent MNCs and selected PB MONs (CD14+) is not enough to prove that generated cells were mature hepatocytes.

In this work we compared between two methods of separation of PB monocytes for generation of hepatocyte like cells from the PB of patients suffering from chronic hepatitis and liver cirrhosis. Moreover, MNCs sorting was used to selectively isolate CD14+ MONs from MNCs. Monocytic count and CD14+ expression were performed by differential leucocytes count and flow cytometer using CD14 and PI to assess quality and viability of separated MNCs and MONs before culture.

MNCs sorted CD14+ MONs showed significantly higher CD14+ expression by flowcytometry. The proposed protocol yielded MONs with acceptable purity and viability from PB MNCs.

The numbers of separated monocytes by human Pan Monocyte Isolation Kit were significantly higher than those separated by Ficoll when counted by differential leukocyte count and flow cytometer. Culture studies were done on two populations; unfractionated adherent PB MNCs, and a MONs (CD 14+), both populations contained cells able to grow and differentiate to hepatocyte-like cells. Our work depends on the ability of monocytes to reprogram to stem cells which can differentiate to different types of cells *in vitro* according to growth factors added to cultures.

Various protocols for the differentiation of stem cells into hepatocytes have been reported in previous studies [23]. These protocols are different from each other in terms of their types of stem cells, the period for each stage of differentiation, used feeders for stem cells, and type and quantity of the used factors or chemical components. The used factors include hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF-4), oncostatin M (OSM, from interleukin 6 group) and chemical components including dexamethasone (Dex). These factors are signals influencing the differentiation of hepatocytes [24]. In our study we utilized differentiated PB monocytes to produce functioning hepatocyte like cells. Differentiation was going in a time dependent manner as we followed the cultures during the 28 days of cultures. Renewal of the media and measuring the concentration of albumin, urea and LDH in culture supernatants every 7 days, that indicated the presence of functioning hepatocyte- like cells. Indeed, during cells differentiation they began to perform their functions; including albumin secretion, urea production, and consumption of energy and LDH production. Results obtained proved that cultured cells differentiated into hepatocyte-like cells. Our results are in agreement with Ruhnke *et al*., [25] who stated that cultured PB MONs under conditions promoting generation of hepatocyte-like cells which resembled primary human hepatocytes regarding various secretory and metabolic functions such as; albumin secretion, urea production, lactate formation, lactate dehydrogenase and aspartate transaminase release. Also, with Yan *et al*., [12], who found that *in vitro* cultured PB MNCs with rh- HGF for a week, they differentiated to hepatocyte like cells which were quite similar to the human normal hepatic cell in several aspects such as release of albumin, LDH, and urea. In addition to, Jaafarpour *et al*., [26], who reported detection of albumin secretion when human-induced pluripotent stem cells were differentiated into hepatocyte-like cells.

There was also increased expression of albumin gene as detected by qRT-PCR in hepatocyte like cells derived from cultured MNCs and MONs compared to control cultures but MNCs derived hepatocytes showed significant increase in albumin gene expression compared to MONs derived hepatocytes. The expression of these hepatocyte markers suggests that at least some of the hepatocyte- like cells function properly.

Immunohistochemical markers of hepatocytes namely: Hep par-1 and CK 18 were detected in the generated cells. The hepatocyte like cells derived from MNCs differentiation cultured with rh-HGF showed increased expression of CK18 more than Hep Par-1. On the other hand, MONs differentiated hepatocyte like cells in presence of rh-HGF showed increased expression of Hep Par-1 more than CK18. Our results agree with Ruhnke *et al*., Yan *et al*., and Jaafarpour ***et al.,*** [5,12,26]**,** who confirmed increased immunocytochemical expression of CK18 protein and elevated mRNA level of albumin in hepatocyte-like cells.

Other authors documented that they were able to induce differentiation of stem cells to hepatocytes which displayed the functional characteristics of mature hepatocytes; including albumin secretion, glycogen storage and several detoxifying function as urea production [22]. Nasser *et al*., [27] and Jaafarpour *et al*., [26] reported, differentiation of human induced pluripotent stem cells into hepatocyte like cells using human adult bone marrow mesenchymal stem cells feeder and using hepatocyte differentiation culture media.

In conclusion neo-hepatocytes obtained resemble primary hepatocytes with respect to morphology, functions, and hepatocytes' markers. Some modifications in the methods to improve functions of the generated hepatocyte-like cells, and to increase number of the produced cells are recommended. However, our results, with mentioned modifications, may pave the way towards the generation of patient- specific autologous hepatocyte like-cells. Patients suffering from liver cirrhosis would benefit from the regenerative potential of this unlimited cell source (PB MNCs and MONs) and from this innovative technology which could be used to treat and improve the clinical stage of liver disease. Further investigations are necessary to assess the function and genetic stability of hepatocyte like cells in vivo. This opens the real possibility in the clinical application for cells of monocytic origin in tissue repair and organ regeneration.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
The institutional committee that has approved the experiments is Theodor Bilharz Research ethical committee.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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