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Bone Marrow-Derived Mononuclear Cells Differentiate into Hepatocyte-Like Cells within Few Hrs without Fusion

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Abstract

Background: Cell therapy using Bone Marrow Mononuclear Cells (BMMC) has been shown as a potential treatment for liver diseases. BMMC can act by fusion, differentiation into hepatocyte-like cells and/or secretion of paracrine factors. Here, we used encapsulated BMMC in a model of Carbon Tetrachloride (CCl_4)-induced acute liver injury to study in vivo and in vitro differentiation of BMMC.

Methods: Both in vitro and in vivo studies were conducted in Wistar rats submitted to CCl_4-induced acute liver injury. BMMC were isolated from Wistar rats and encapsulated in sodium alginate microcapsules. For in vivo experiments, animals received encapsulated BMMC 24 hrs after CCl_4 administration and capsules were collected within 6, 24 and 48 hrs ($tCCl_4$ group). For in vitro experiments, isolated hepatocytes from animals with CCl_4-induced liver injury were co-cultured with encapsulated BMMC for 6 h ($cCCl_4$ group). Control groups were not submitted to CCl_4 administration. The content of intracellular lipid droplets in hepatocytes was used to evaluate liver injury. BMMC differentiation was assessed by RT-PCR for hepatic genes and ability to produce and secrete urea.

Results: Liver damage was confirmed in CCl_4 treated animals by the presence of intracellular lipid droplets in hepatocytes and the characteristic nutmeg aspect of the liver. Retrieved encapsulated BMMC from $tCCl_4$ group expressed hepatocyte markers, such as Cytokeratin 18 and Albumin 48 hrs after treatment. On the other hand, BMMC from cCCl4 group showed Albumin expression 6 hrs after co-culture. Urea production was increased in BMMC from $cCCl_4$ group but not in cControl. BMMC from tControl or cControl groups did not express hepatocyte markers at any time point.

Conclusions: In this study we show that BMMC differentiate into hepatocyte-like cells in a short period of time both in vivo and in vitro. This differentiation is triggered by paracrine factors present only in injured liver.

Keywords: Bone marrow mononuclear cells; Cell differentiation; Paracrine effects; Hepatocyte-like cells; Acute liver failure

Introduction

Bone Marrow Mononuclear Cells (BMMC) have emerged as potential candidates for cell therapy due to their ease of use. In models of acute liver failure, transplantation of these cells increase the survival rate [1,2]. Furthermore, recent clinical trials have demonstrated that transplantation of these cells or their fractions improves the condition of patients with cirrhosis [3-5], as well as improve liver function in animal models of cirrhosis [6]. Moreover, BMMC are characterized by their ability to differentiate into several functional mature cell types both in vivo and in vitro, including cardiomyocytes [7,8], endothelial cells [9], neurons [10,11] and hepatocytes [12-18]. In addition, many groups have developed protocols for the in vitro differentiation of bone marrow-derived cells into hepatocyte-like cells [19-24]. Although bone marrow mononuclear fraction is used for in vivo transplantation, in vitro protocols usually work with Mesenchymal Stem Cells (MSC) [19,23-25].

Microencapsulation provides a vehicle for the discrete control of key parameters such as the diffusion of growth factors, metabolites, and wastes. It has been demonstrated that the alginate microenvironment maintains cell viability, is conducive to embryonic stem cell differentiation into hepatocytes, and maintains differentiated cellular function [26]. In addition, encapsulated bone marrow cells showed evidence of glycogen synthesis and expression of typical markers of hepatocytes, after transplantation in the 90% liver failure model [15,27].

In the present work we used semi-permeable alginate microcapsules to isolate BMMC in a model of Carbon Tetrachloride (CCl_4)-induced acute liver injury. Alginate microcapsules isolate BMMC and allow the study of in vivo and in vitro differentiation through paracrine factors. We also focused on investigating early reprogramming events that might take place during exposure to injury microenvironment.

Methods

Experimental design

Both in vitro and in vivo studies were conducted in Wistar rats submitted to CCl_4-induced acute liver injury. For in vivo studies, encapsulated BMMC were transplanted into animals with or without liver injury by CCl_4 and kept for 48 hrs. For in vitro studies the encapsulated BMMC were co-cultured with hepatocytes isolated from

rats with CCl_4-induced acute liver injury or without liver damage for 6 hrs (Figure 1).

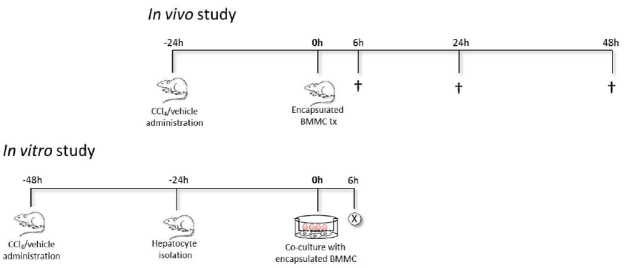

Figure 1: Experimental design of in vivo and in vitro studies. † Sacrifice of animals and retrieve of capsules; Ⓧ Interruption of co-culture and recovery of encapsulated BMMC

Animals

Adult male Wistar rats weighing 200 to 250 gr were kept under 24 hour light/dark cycles and fed standard chow and water ad *libitum.* Animals were sacrificed in the CO_2 chamber, either 6, 24 or 48 hrs after CCl_4 administration for *in vivo* studies (n=3/time point). For *in vitro* studies, animals (n=3) were submitted to hepatocyte isolation 24 hrs after CCl_4 administration. Control animals (n=12, 9 and 3 for *in vivo* and *in vitro* studies, respectively) were submitted to the same procedures but receiving vehicle instead of CCl_4. Bone marrow donors (n=9, 6 and 3 for *in vivo* and in vitro studies, respectively) were rats not submitted to any other procedure. This study was approved by the ethics research committee and national guidelines for animal care were followed.

Acute liver injury model

Acute liver injury was induced by a single CCl_4 (VETEC, Brazil) dose of 1.25 ml/kg diluted in olive oil [2]. A final volume of 1 ml was administered by gavage. Control animals received only olive oil by gavage.

Isolation of bone marrow mononuclear cells (BMMC)

BMMC were isolated from the femur and tibia of Wistar rats, as previously reported [1]. Briefly, bone marrow was flushed with Dulbecco's Modified Eagle Medium (DMEM-LGC, Brazil) supplemented with 10% Fetal Bovine Serum (FBS-Gibco, USA) and 1% Penicilin/Streptomicin (P/S-Gibco, USA) and BMMC were separated onto a Ficoll Histopaque (GE-Healthcare, USA) layer.

BMMC encapsulation

BMMC were encapsulated in sodium alginate microcapsules under sterile conditions, using the protocol described by our group [28,29]. BMMC were mixed with 1.5% sodium alginate (Sigma-Aldrich, USA) in DMEM and extruded through an encapsulation unit, type J1 (Nisco, Zurich, Switzerland), attached to a syringe pump (JMS, Singapore). Droplets were sheared off with an air flow of 5 L/min delivered to the tip of a 27-G needle and the rate of infusion was 40 mL/h. The droplets fell into a bath of 125 mM $CaCl_2$ and ionically cross-linked with $Ca2^+$ to form solid spherical hydrogel beads containing embedded cells. For in vitro experiment, in each well, beads were produced from a volume of 100 µL of alginate suspension, containing $1x10^5$ of BMMC. The resulting beads were maintained under normal tissue culture conditions: DMEM supplemented with 10% FBS and 1% P/S at 37°C and 5% CO_2 for 24 hrs prior to administration. For in vivo experiment, in each well, beads were produced from a volume of 2 mL of alginate suspension, containing $1x10^6$ BMMC/animal.

Capsules transplantation

For in vivo experiments, 24 hrs after CCl_4 administration animals were anesthetized with inhaled isoflurane and a small incision was made in the abdomen. A total of $1x10^6$ encapsulated BMMC suspended in 2 mL saline buffer was placed in the peritoneal cavity ($tCCl_4$ group, n=3/time). The same procedure was performed in animals without liver injury (tControl group, n=3/time). Sacrifice was performed in CO_2 chambers 6, 24 or 48 hrs after capsules transplantation. Capsules were retrieved by washing the peritoneal cavity with Phosphate Buffered Saline (PBS).

Hepatocyte isolation

For in vitro studies hepatocytes were isolated 24 hrs after CCl_4 using the modified 2-step perfusion method as previously described [30,31]. Hepatocytes from animals without liver injury were isolated by the same method. Cells were cultured in DMEM medium, supplemented with 10% FBS and 1% P/S (Invitrogen, USA) in tissue culture flasks for 24 hrs prior to co-culture experiments.

In vitro co-culture

Encapsulated BMMC ($1x10^5$) were co-cultured with hepatocytes ($3x10^6$) from rats with CCl_4-induced liver injury ($cCCl_4$ group, n=3) and controls (cControl group, n=3). In addition to the capsules, both cell types were separated by a semipermeable transwell membrane (70 µm filter; BD, USA) placing the hepatocytes in the lower chamber and the BMMC in the upper chamber. The cells were co-cultured in DMEM medium supplemented with 10% FBS and 1% P/S for 6 hrs.

Intracellular lipid droplets

To evaluate the liver injury, cultured hepatocytes were stained with Oil Red O (ORO, MP Biomedicals, USA) to identify intracellular lipid droplets (ILD). Hepatocytes were fixed with formalin. After washing, 1 mL of 100% PEG (Ineos, Germany) was added for 2 min, and ORO/PEG (0.5%) was added for 10 min. After that, cells were rinsed in 60% PEG for 1 min and washed. Hepatocytes were counter-stained with hematoxilin and observed under light microscope immediately [32].

Urea quantification

After co-culture, encapsulated BMMC were removed from hepatocyte contact, washed with PBS and placed in fresh medium for 2 h. The medium was collected and urea was quantified by Quanti ChromTM Urea Assay Kit (DIUR-500, BioAssay Systems, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Encapsulated BMMC recovered from in vivo and in vitro experiments were dissolved using a 100-mM sodium citrate (Labsynth, Brazil) solution prior to RNA extraction. Total RNA was extracted

using the RNeasy RNA isolation kit (Qiagen, Germany), and Reverse Transcription (RT) was carried out with 1 µg of RNA. Complementary DNA (cDNA) was synthesized using Superscript II RNA-reverse transcriptase (Invitrogen, USA).

For the RT-PCR, 2 µL cDNA-templates were mixed with 5 µL PCR-buffer, 1.5 mM µl $MgCl_2$, 10 mMol dNTPs, 20 pmol of each primer, and 2U Taq DNA polymerase in a total volume of 50 µL. All reagents were from Invitrogen (USA). PCR was carried out using primers and conditions showed in Table 1. Samples were analyzed on 1.5% agarose gels stained with ethidium bromide.

Primer name	Sequence	PCR condition	Fragment length
Alb	For: 5'GGTATGAATATGCAAGAAG3' Rev: 5'CACTCTTCCCAGGTTTCTTG3'	48°C	350 bp
Ck-18	For: 5'GGACCTCAGCAAGATCATGGC3' Rev: 5'CCAGGATCTTACGGGTAGTTG3'	50°C	518 bp
Afp	For: 5'CCCACCCTTCCACTTTCCAGA3' Rev: 5'GCTGGAACTGCCTTGTCATA3'	54°C	164 bp
Gapdh	For: 5'GAGTTGCTGTTGAAGTCACAGG3' Rev: 5'CAGCAATGCATCCTGCAC3'	42°C	429 bp
Alb- Albumin; Ck-18, Cytokeratin 18; Afp, Alpha-fetoprotein; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase			

Table 1: Primers and amplification conditions used to analyze gene expression of BMMC

Statistical analysis

Statistical comparison of urea levels of the cControl and $cCCl_4$ groups was carried out using Student's T-test, using the SPSS v.18. The level accepted for significance was $p<0.05$.

Results

In vivo experiments

In order to study BMMC differentiation, encapsulated cells were implanted in the peritoneal cavity of Wistar rats 24 hrs after CCl_4 or olive oil administration. In animals from $tCCl_4$ group, liver injury was confirmed at the time of surgery by the characteristic nutmeg aspect of the liver. Animals from tControl group didn't present this feature throughout the study. Animals were sacrificed in CO_2 chamber after 6, 24 or 48 hrs and capsules were retrieved from the peritoneum. Capsules were found freely disseminated in the peritoneal cavity and it was not observed liver tissue from the recipient attached to the capsules after explantation.

Gene expression pattern was markedly different in BMMC from $tCCl_4$ retrieved after 48 hrs, showing the expression of hepatocyte markers such as Albumin and Cytokeratin 18, but negative for Alfa-fetoprotein (Figure 2). On the other hand, BMMC retrieved at 6 or 24 hrs after injections were negative for all markers but Gapdh. Interestingly, BMMC from tControl group were also negative for hepatocyte markers at all time points, except for the internal control (Gapdh), showing an expression pattern similar to that of naïve BMMC.

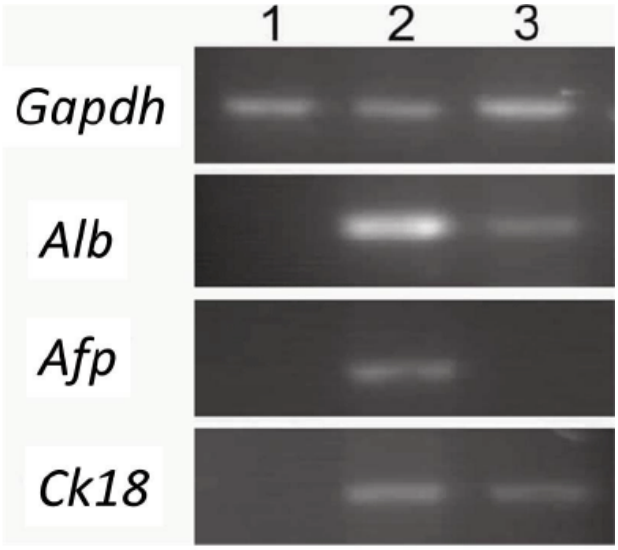

Figure 2: BMMC expression of hepatocyte markers 48 hrs after implantation in animals with CCl_4-induced acute liver injury. RT-PCR analysis of gene expression in encapsulated BMMC from tControl (lane 1) or $tCCl_4$ (lane 3) retrieved 48 hrs after implantation. Positive control (hepatocytes, lane 2)

In vitro experiments

Since encapsulated BMMC showed the expression of hepatocyte markers 48 hrs after implantation in animals with CCl_4-induced acute liver injury, we tried to replicate the same model in vitro in order to determine when this reprogramming takes place.

As a way to assess hepatocyte damage in this model, cells isolated from animals with or without liver injury were stained with oil red. In CCl_4 treated animals, esteatose could be observed with several intracellular lipid droplets, that were absent in control animals. Furthermore, we observed the macroscopic aspect of nutmeg liver by the time of hepatocyte isolation.

Encapsulated BMMC that were in co-culture with hepatocytes from CCl_4 treated animals for only six hrs showed Albumin expression, whereas those in contact with control hepatocytes were negative (Figure 3). No expression of Ck18 or Afp was detected.

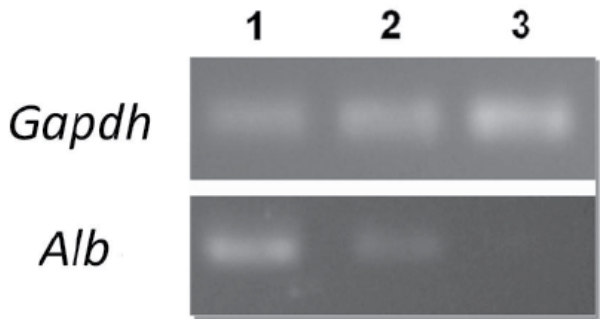

Figure 3: RT-PCR analysis of Alb gene expression in BMMC after 6 hour co-culture. Hepatocyte (positive control, lane 1). Encapsulated BMMC from $cCCl_4$ group (lane 2). Encapsulated BMMC from cControl group (lane 3). Gapdh: internal control

In order to determine if this reprogramming also had functional repercussion in BMMC we tested urea production by these cells. After co-culture with hepatocytes, encapsulated BMMC were kept for other 2 hrs in culture, with fresh medium. Urea production was greatly increased in BMMC from $cCCl_4$ group (Figure 4) as compared to cControl group. However, it was lower than the amount produced by hepatocytes in culture (5.85 mg/dL).

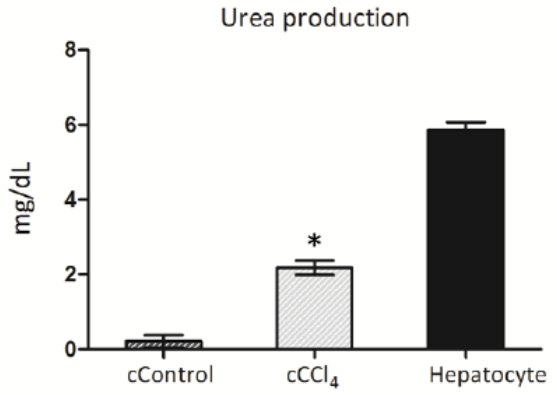

Figure 4: Quantification of urea production in the culture medium from encapsulated BMMC. Urea production was increased in BMMC from $cCCl_4$ group but not in cControl. Medium from hepatocyte culture was used as reference value. $^*p$=0.033 compared to cControl group (Student's T-test)

Discussion

The ability of BMMC to differentiate into hepatocyte-like cells has been shown both in vivo and in vitro by different groups. In the present study we have shown that BMMC are able to express hepatocyte-specific genes after 48 hrs of transplantation in CCl_4 treated animals. In vitro, these cells express Albumin and produce urea after only 6 hrs of co-culture with injured hepatocytes.

Several studies have shown the ability of BMMC to differentiate into hepatocyte-like in vivo. Most of these studies show the expression of Alb and Afp [12-15,17,33], but also Ck8 and Ck18 [15]. As in the previous studies, this differentiation occurs only when BMMC are transplanted into injured animals, suggesting that liver damage triggers the release of substances that modulate BMMC gene expression. However, unlike ours, other groups describe differentiation after 13 [34] and 60 days [12,13].

There is still debate in the literature if these cells convert into hepatocyte-like by means of fusion or differentiation [18]. Our results favor the differentiation hypothesis, in accordance with other study that used microencapsulated cells [15]. Liu and Chang [15] have shown that BMMC encapsulated with the APA (Alginate-Polylysine-Alginate) method improve survival in a 90% partial hepatectomy rat model of acute liver failure. In addition, they found that some BMMC express ALB, AFP, CK 8, CK 18 and were able to store glycogen, after 2 weeks of transplantation. In our study, we used similar alginate microcapsules to isolate BMMC and we observed differentiation signs in a shorter period of time (48 h). It is important to highlight that we assessed differentiation by gene expression analysis, whereas the previous study by Liu and Chang used immunocytochemistry. It was not possible to prove the expression of ALB, AFP nor CK18 by immunocytochemistry due to difficulties in capsule's histology. It cannot be ruled out, however, that fusion mechanisms do exist or play a role in cell-mediated tissue recovery after injury. Yet, in this work alginate microcapsules prevented any type of fusion between BMMC and hepatocytes, although allowing for the interchange of soluble compounds between these two cell types.

In vitro differentiation is usually obtained by the use of growth factors, especially Hepatocyte Growth Factor (HGF), although quite a number of differences exist between protocols [19,23-25]. Some authors have shown differentiation also with hepatocyte conditioned-medium [21] or serum from patients with hepatitis B virus-associated liver cirrhosis [35], co-culture with hepatocytes [36-38] or liver fragments [39] from healthy or injured animals. However, unlike the present study, the above mentioned groups use MSC and not BMMC. Even though MSC have a well known differentiation capacity, their isolation, culture and expansion are time consuming processes and changes in gene expression at early times like this are not reported by studies with MSC, which usually differentiate after 3 to 40 days in culture [22,36]. On the other hand, BMMC are readily available, and does not need a culture step prior to administration.

Our results showed that BMMC co-cultured with injured hepatocytes for six hrs showed expression of Albumin and urea production, both characteristics of hepatocyte activity. It has been reported that kidney tubular epithelium also produces urea, while extraembryonic cells express albumin, however, only hepatocytes can do both [40,41]. In contrast, when BMMC were co-cultured with hepatocytes isolated from healthy animals, no Albumin expression or urea production was detected after 6 hrs. Other reports have shown differentiation of MSC even after co-culture with healthy hepatocytes [36]. These differences may be reconciled by intrinsic characteristics of the cell types used in this study or by the short period of time analyzed. On the one hand, BMMC are composed of phenotypically and functionally different cell populations and we have preliminary data indicating that is the non-adherent fraction of bone marrow mononuclear cells that differentiates. However, further characterization of these cells is still pending. On the other hand, it is possible that injured hepatocytes secrete paracrine factors that lead to BMMC reprogramming sooner than healthy hepatocytes [42,43].

Interestingly, in vivo differentiation occurred only after 48 hrs. It is possible that in vitro hepatocytes secrete factors at higher concentrations, thus inducing differentiation in shorter times.

In summary, this work shows that BMMC are able to differentiate into hepatocyte-like cells in a short period of time both in vivo as in vitro. This differentiation occurs without fusion and is triggered by factors present only in injured liver. The identification of such factors and of the cells in the mononuclear fraction that respond to them is under further investigation.

Acknowledgements

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