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World J Gastroenterol 2004;10(15):2250-2253
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

Influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells

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Received: 2003-12-10 **Accepted:** 2004-01-07

Abstract

AIM: Recent studies in both rodents and humans indicated that bone marrow (BM)-derived stem cells were able to home to the liver after they were damaged and demonstrated plasticity in becoming hepatocytes. However, the question remains as to how these stem cells are activated and led to the liver and where the signals initiating the mechanisms of activation and differentiation of stem cells originate. The aim of this study was to investigate the influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells.

METHODS: Serum samples were collected from rats treated with a 2-acetylaminofluorene (2-AAF) /carbon tetrachloride (CCl_4) program for varying time points and then used as stimulators of cultured BM stem cells. Expression of M_2- and L-type isozymes of rat pyruvate kinase, albumin as well as integrin-β1 were then examined by reverse transcription polymerase chain reaction (RT-PCR) to estimate the differentiation state of BM stem cells.

RESULTS: Expression of M_2-type isozyme of pyruvate kinase (M_2-PK), a marker of immature hepatocytes, was detected in each group stimulated with experimental serum, but not in controls including mature hepatocytes, BM stem cells without serum stimulation, and BM stem cells stimulated with normal control serum. As a marker expressed in the development of liver, the expression signal of integrin-β1 was also detectable in each group stimulated with experimental serum. However, expression of L-type isozyme of pyruvate kinase (L-PK) and albumin, marker molecules of mature hepatocytes, was not detected in groups stimulated with experimental serum.

CONCLUSION: Under the influence of serum from rats with liver failure, BM stem cells begin to differentiate along a direction to hepatocyte lineage and to possess some features of immature hepatocytes.

Hong H, Chen JZ, Zhou F, Xue L, Zhao GQ. Influence of serum from liver-damaged rats on differentiation tendency of bone marrow-derived stem cells. *World J Gastroenterol* 2004; 10 (15): 2250-2253

http://www.wjgnet.com/1007-9327/10/2250.asp

INTRODUCTION

Recent studies in both rodents and humans indicated that bone marrow (BM) stem cells were able to home to the liver after they were damaged, and demonstrated plasticity in becoming hepatocytes[1-4]. Questions remain as to how BM stem cells are activated and led to the liver and where the signals initiating the mechanisms of activation and differentiation of stem cells originate. Transfused oval cells (hepatic stem cells) that had a selective tropism for the liver in an animal model of liver-damage suggested that "signal molecules" were present in serum of this animal model and played an important role in mediating both hepatic and non-hepatic stem cell activation[5]. However, the influence of these putative signal molecules in serum on the differentiation state of bone marrow-derived stem cells is yet unclear. The purposes of the present work were to confirm the existence of signaling molecules in serum of liver-damaged rats and to observe its effects on the differentiation of BM stem cells into hepatocytes.

MATERIALS AND METHODS

Establishment of animal model of liver-damage

Male Sprague–Dawley (SD) rats, 6-week-old, were used for the establishment of an animal model of liver-damage. The model was made by means of a 2-AAF/CCl_4 program according to Petersen[1]. In experimental group, 2-acetylaminofluorene (2-AAF, Sigma), 2.5 g/L in earthnut oil, was administered to stomach of rats everyday for 7 d. On the 7th d of 2-AAF administration, an Ld50 dose of CCl_4 was given by intraperitoneal injection. Animal blood was taken at the time points of 2, 4, 8, 12, and 24 h after CCl_4 injection. Experimental serum was prepared on standby. The serum from normal animals was used as control.

Isolation and culture of bone marrow stromal cells

The SD rats were sacrificed by means of ether asphyxia. Bone marrow was collected from tibiae and BM cells were suspended in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum. After centrifugation and re-suspension, the cells were seeded in a culture flask and cultured under a routine condition (37 ℃, 50 mL/L CO_2). The solution of medium was changed every 4 d while the cells floating on the medium were discarded. The cells adhering to bottom of the flask (so-called BM stromal cells) were cultured sequentially for 12-14 d. After 3 population doublings, the purified cells were harvested and used in the following stimulating culture with experimental serum.

Stimulating culture of BM stromal cells with experimental serum

BM stromal cells were cultured sequentially in a specialized medium (DMEM-F12) containing 3 ml/L experimental rat serum. In the control group, the culture medium contained 3 mL/L normal rat serum instead of experimental rat serum. Cultures were grown and submerged for 12 d. The cells were harvested on the 13th culture day for ribonucleic acid (RNA) isolation.

Table 1 Primer pairs for RT-PCR

Marker genes	Primer pairs	PCR fragments
M_2-PK	5'ccatctaccacttgcagttattcga3' / 5'tcatggtacaggcactacacgc3'	431 bp
L-PK	5'acctctgccttctggatactgact3' / 5'tgcaagactccggttcgtatct3'	322 bp
Albumin	5'gagcccgaaagaaacgagtgtt3' / 5'ggggaatctctggctcatacg3'	389 bp
INT-β1	5'tacttcagacttccgcattgg3' / 5'cagtgactgcaaaaatcgtctg3'	488 bp
GAPDH	5'ccatggagaaggctggg3' / 5'caaagttgtcatggatgacc3'	180 bp

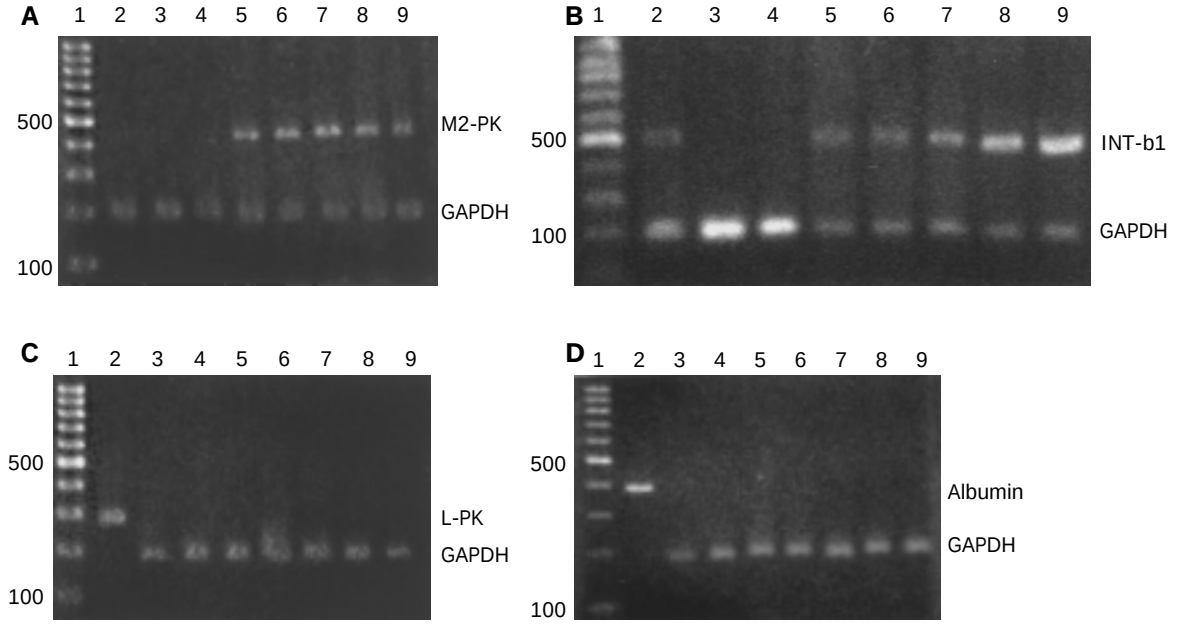

Figure 1 Expressions of M_2-PK, Integrin-β1, L-PK and albumin in BM stem cells. A: Expression of M_2-PK in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. B: Expression of Integrin-β1 in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. C: Expression of L-PK in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h. D: Expression of albumin in BM stem cells. Lane 1, 100 bp DNA marker; Lane 2, Hepatocytes; Lane 3, BM stem cells without stimulation; Lane 4, BM stem cells stimulated with control serum; Lane 5, BM stem cells stimulated with experimental serum for 2 h; Lane 6, BM stem cells stimulated with experimental serum for 4 h; Lane 7, BM stem cells stimulated with experimental serum for 8 h; Lane 8, BM stem cells stimulated with experimental serum for 12 h; Lane 9, BM stem cells stimulated with experimental serum for 24 h.

Isolation of RNA

RNA was extracted from the cells collected from the cultures described above, according to the protocol of QIAGEN RNA easy mini kit. RNA samples were then stored at -80 ℃.

Primers selection

Genes of M_2-type and L-type isozymes of rat pyruvate kinase (M_2-PK, L-PK), albumin and integrin-β1 (INT-β1) were selected as the markers representing different differentiation stages of hepatocyte lineage. The gene of glyceraldehyde-3-phosphate-dyhydrogenase (GAPDH) was used as an internal control for RT-PCR reactions. Primer pairs used for RT-PCR are shown in Table 1.

RT-PCR reactions

RNA samples were first reversely transcribed into cDNA, and then used as templates in the following PCR reactions. The reaction cocktails (containing 1 μg template cDNA, 50 μmol/L dNTPs, 400 μmol/L primers, 1×PCR buffer, 2.5 mmol/L $MgCl_2$, 1 U Taq-polymerase, add H_2O to 50 μL of total volume) were run on GeneAmp® PCR System 9600 (AB) with a combined program of program 1 (at 94 ℃ for 5 min), program 2 (at 95 ℃ for 1 min, at 60 ℃ for 1 min, at 72 ℃ for 1 min; 30 cycles), and program 3 (at 95 ℃ for 1 min, at 60 ℃ for 1 min, at 72 ℃ for 5 min). The PCR products were electrophoresed in 12 g/L agarose gel, stained with ethidium bromide, and photographed.

RESULTS

M_2-PK, as a marker of immature hepatocytes, was used to estimate the differentiation state of BM stem cells stimulated by the experimental serum. The results showed that the expression signals of M_2-PK were detected in each group

stimulated with experimental serum, but not in the control group of normal BM stem cells that had no stimulation, BM stem cells stimulated with control serum, and normal mature hepatocytes (Figure 1A). Integrin-β1 is a marker expressed during the development of liver. In this study, its expression signals were also detectable in each group stimulated with experimental serum as those observed in the positive control of hepatocytes (Figure 1B). L-PK and albumin, as marker molecules of mature hepatocytes, were used to estimate the terminal differentiation state of BM stem cells under the influence of experimental serum. However, no signals were detected in the experimental groups, except for the positive control of hepatocytes (Figure 1C, D).

DISCUSSION

The existence of liver stem cells had been widely proved in both rodents and humans[1-4,6-9]. By extension, liver stem cells could be divided into three groups: (1) mature hepatocytes that proliferate during normal liver tissue renewal and after less severe liver damage, (2) oval cells that are activated to proliferate when the liver damage is extensive and chronic, and (3) exogenous liver stem cells that may derived from bone marrow cells and respond to severe liver damage[10]. However, in a narrow sense, the concept of liver stem cells is usually limited to hepatic oval cells and non-hepatic bone marrow stem cells.

A phenomenon that was often observed both in experimental animal models and in clinics was that the proliferation of liver stem cells occurred most often in conditions of severe liver damages or chronic liver diseases[11-15]. Maybe it is the reason that stem cells were seldom detected in healthy livers. Thus it can be understood that liver-damage is an important prerequisite for activation of liver stem cells. This suggests that the signals initiating activation of hepatic or non-hepatic stem cells might originate from damaged livers. This hypothesis had been partially proved by our previous experimental work[5]. In our previous experiments, oval cells isolated from male SD rats were transfused, through caudal vein into the circulatory system of a female rat with liver damage. Sex-determining gene *sry* that was located on Y chromosome was then examined respectively by PCR and *in situ* hybridization technique in the liver, kidney and spleen of experimental animals. The results of cell-transplant experiments showed that *sry* gene was detectable only in the liver but not in the spleen and kidney of rats with liver damages and that no signals could be detected in control animals, neither in the liver, spleen nor in the kidney. It could be also morphologically observed that some exogenous cells with *sry* marker migrated into the parenchyma of liver and settled there, suggesting that transfused oval cells had a selective tropism for damaged liver. These results also suggested that signaling molecules existed in the serum of animals with liver damage and might play a role in mediating stem cell activation.

In the present study, an animal model of liver-damage was established with a 2-AAF/CCl_4 program. In this model, the capacity of hepatocyte self-regeneration was first impaired by 2-AAF and then the liver was damaged severely by CCl_4. In this status, the damaged liver would likely produce a signal to initiate the activation of stem cells in the bone marrow. The results of the present study showed that the expression of M_2-PK, a marker of immature hepatocyte[16-22], could be detected in each group stimulated with experimental serum, but not in any of the control groups. Integrin-β1 is a marker expressed during the development of liver. Its expression could be detected in fetal hepatocyte as early as at 8th wk of gestation[30]. In the present results, the expression signal of integrin-β1 was also detectable in each group stimulated with experimental serum. Thus, the functional state of BM-derived stem cells was changed under the influence of experimental serum, thus differentiating toward the direction of a hepatocyte lineage. Although the markers of a mature hepatocyte, L-PK and albumin were not detectable in stimulated BM stem cells, the leap from an undetermined state to a determined state was a marker of entry into the process of programmed differentiation. A variety of possibilities could account for the lack of detectable signals for L-PK and albumin in stimulated BM stem cells. Among the possibilities, one could be the deficiency in intensity and time of stimulation, while another could, by reasoning, be that the postulated "signal molecules" existing in the experimental serum were involved only in the early activation and determination of BM stem cells, while the terminal differentiation of the cells into hepatocytes might still need other signals.

The results in the present study indicated that the driving force promoting differentiation of BM stem cells to hepatocytes was certainly generated from the serum of rats treated by 2-AAF/CCl_4. It has been further testified that some "signal molecules" were existed in the circulation of rats treated by 2-AAF/CCl_4 and that they might play an important role in the initiation of activation of stem cell. It would be helpful for understanding the mechanisms of stem cell differentiation if the "signal molecules" could be further identified and isolated.

Recent studies have convincingly demonstrated that adult bone marrow contains cells capable of differentiating into hepatocyte-like cells. Nevertheless, what type of cell population are the ancestor cells for hepatocytes still remains a question. In the vast majority of reports, hematopoietic cells were considered to be capable of "transdifferentiating" into hepatocytes[2,4,23-28]. However, Wagers (2002) deemed that there was little evidence for transdifferentiation of adult hematopoietic stem cells[29]. The present study showed that bone marrow stromal cells demonstrated the plasticity in changing into hepatocytes. By this token, the debate about origin of liver stem cells will keep on.

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Edited by Wang XL and Qin D **Proofread by** Xu FM