Liver repopulation trial using bone marrow cells in a retrorsine-induced chronic hepatocellular injury model

Niaz KHOHNEH-SHAHRI (1), Jean-Marc REGIMBEAU (1), Benoît TERRIS (2), Valérie PARADIS (3), Marie-Pierre BRALET (4), William COLEMAN (5), Genelle BUTZ (5), Sandrine CHOUZENOUX (1), Didier HOUSSSIN (1), Olivier SOUBRANE (1)

(1) Laboratoire des Thérapeutiques Innovantes des Maladies du Foie (EA 1833), Université René Descartes Paris V ; (2) Service d’Anatomopathologie, Hôpital Cochin Port Royal, Paris ; (3) Service d’Anatomopathologie, Hôpital Beaujon, Clichy ; (4) Service d’Anatomopathologie, Hôpital Paul Brousse, Villejuif, France ; (5) Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, USA.

SUMMARY

Objectives — The aim of this study was to determine the potential of bone marrow derived cells to participate in liver repopulation. In this model, the injected cells had a “selective growth advantage” compared to the native hepatocytes whose proliferation was blocked by retrorsine.

Methods — Total bone marrow cells were isolated from male Fisher 344 rats not deficient in dipeptidyl peptidase activity (F344, DPP IV+). The animals were given an injection of retrorsine and were divided in 2 groups: 1/group R (N = 13): female F344 rats received 4.10⁶ male cells at day 0 (labeled by chromosome Y). 2/group RH (N = 19): Male F344 DPP IV- rats received 4.10⁶ male DPP IV+ cells after hepatectomy at day 0 (labelled by DPP IV activity).

Results — Group R: no male cell was detected by PCR at day 14, 28, 56 and 84. Group RH: isolated DPP IV+ transplanted cells were observed at days 14 and 28 in the periportal areas. Later, these cells were no longer visible. Liver regeneration occurred by proliferation of small clusters of hepatocytes.

Conclusions — In this experimental model the capacity of transplanted bone marrow cells to repopulate the liver was tested against the same capacity of native liver stem cells. Liver regeneration occurred via native liver cells seen as small hepatocytes. In this model the small hepatocytes may be considered as hepatic stem cells.

For certain indications, transplantation of isolated hepatocytes has been proposed as an alternative to whole organ transplantation [6]. Animal experiments [7-10] and clinical trials with isolated liver cell transplantation [11-13] have produced encouraging results. There have been however very few trials because of the technical problems involved: imperfect control of hepatocyte cryopreservation, small number of transplantable cells, requirement for immunosuppression.

Recent work has demonstrated that hematopoietic stem cells isolated from murine bone marrow can differentiate in vivo into different cell types including myocytes, endothelial cells, and hepatocytes [14-16]. In humans, cells coming from the donor bone marrow expressing characteristic features of hepatocytes have been demonstrated in the liver of patients with a liver or bone marrow graft [17, 18]. Several animal studies [19, 20] have also shown that bone marrow cells can differentiate into hepatocytes which can repopulate the liver partially. However, for these models, particular conditions had been created by sub-lethal radiation of the host and bone marrow graft or by hepatic lethal radiation of the host and bone marrow graft or by hepatic...
Methods

Recipient animals were wild 6-week-old female Fischer F344 rats (IFFA CREDO, France) (mean weight 120 g) and 6-week-old male Fischer 344 rats deficient in dipeptidyl peptidase (DPP IV-) (Charles River, Japan) (mean weight 100 g). DPP IV- rats have a point mutation blocking expression of dipeptidyl peptidase; this deficiency labels injected cells.

Bone marrow cells were harvested from donor animals and prepared ex tempore using total bone marrow extracted from the femurs and tibias of male Fischer F344 rats. Groups of three rats were then harvested from donor animals and prepared ex tempore.

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underwent two-thirds hepatectomy followed by intraportal injection of 4.10 bone marrow cells which had been harvested from wild male Fischer F344 rats (DPP IV+). These cells could be identified by their dipeptidyl peptidase activity. The animals were sacrificed at days 14, 28, 42, and 56 after surgery (one animal at each date because of the high mortality with this protocol, N = 11 rats). Transplanted cells were demonstrated by search for dipeptidyl peptidase activity using histochemistry methods on liver slices.

Control groups

Retrosinse protocol (group R)

Chromosome Y detected in hepatocytes and bone marrow cells in male rats was the positive control for this cell labeling method. The negative control consisted of the absence of chromosome Y in liver samples from four female rats which had received injections of bone marrow cells from syngenic female rats.

Retrosinse protocol and two-thirds hepatectomy (group RH)

DPP IV activity present in liver and bone marrow cells of DPP IV+ rats was the positive control for this labeling method. The absence of this activity in liver and bone marrow samples of DPP IV- rats was the negative control.

Polymerase chain reaction (PCR) in the presence of chromosome Y (group R)

Chromosome Y was detected by PCR of the sry fragment situated on the short arm of chromosome Y [32]. DNA was extracted with the DNeasy Tissue kit (Qiagen, France). The quantity of DNA was estimated by measuring the absorbance at 260 nm. The DNA obtained was preserved at -20°C until PCR amplification. Fifty ng DNA were mixed in 37.5 µL sterile water, 5 µL 10X buffer (Gibco, France), 1.5 µL MgCL2 (50 mM) (Gibco, France), 1.5 µL DNTP (2.5 mM), 1.5 µL oligo-sense at 100 ng/µL and 1.5 µL nonsense at 100 ng/µL (Genst Oligos, France) and 0.5 µL Taq polymerase (Promega, France). The oligo-sense and nonsense sequence used were: 5’ GATTTTTAGTGTTCAGCCCT 3’ and 5’ TGACGCTTCTACCTCAGCT 3’. The amplified sequence had 459 base pairs. Heat cycle conditions were: 1 min at 95°C, 30s hybridization at 53°C, and 1 min elongation at 72°C, followed by a final step of 1 min at 72°C.

Results

Retrosinse group (group R)

There were no deaths among the 13 rats in group R during the experimental protocol.

Intraoperative observations

The liver presented a congestive aspect but no signs of chronic disease (ascites, portal hypertension) at injection of bone marrow cells on day 0 then at sacrifice on days 7, 14, 28, 56 and 84. Inflammatory adhesions tightly connected the liver with neighboring organs. There were no detectable tumor formations at any of the sacrifice times. Mean weight of the liver was: 5 g on
day 7, 6.3 g on day 14, 6.7 g on day 28 and day 56 and 8 g on day 84 after injection of bone marrow cells.

**DETECTION OF TRANSPPLANTED BONE MARROW CELLS**

Despite technical improvements, no male cells could be detected with PCR at days 14, 28, 56, and 84 (figure 2b). Concomitant examination of the female rat liver which had been injected with bone marrow cells coming from syngenic animals of the same sex (negative controls), demonstrated the absence of contamination during the manipulations. Chromosome Y was detected in hepatocytes and in bone marrow cells of male rats.

**Retrorsine group and two-thirds hepatectomy (group RH)**

Mortality in the experimental group (N = 19 rats) was 58% (eight deaths during the first 48 postoperative hours and three deaths from the third to ninth postoperative day). Four rats were still alive at the end of the protocol.

**INTRAOPERATIVE OBSERVATIONS**

There were no signs of liver disease (ascites, collateral circulation) at hepatectomy and at injection on day 0; there were no adhesions. At sacrifice (after hepatectomy and injection of bone marrow cells) the following observations were made: ascites and collateral circulation on the abdominal wall as well as strong perihepatic adhesions on day 14; presence of collateral circulation without associated ascites and persistent strong perihepatic adhesions on day 28; presence of strong perihepatic adhesions on days 42 and 56. No tumor formations were observed at any of the sacrifice times. Mean weight of the liver at sacrifice was 5.7 g at day 28, 4.2 g and 4 g at days 42 and 56 respectively after hepatectomy and injection of bone marrow cells.

**DETECTION OF BONE MARROW CELLS**

Examination of the histological slides of specimens taken at hepatectomy and injection of bone marrow cells (day 0) demonstrated the presence of several apoptotic bodies and megalocytes as well as rare clusters of small hepatocytes (figure 3). At day 14, hepatocyte necrosis predominated with areas of inflammation and persistence of numerous apoptotic bodies. At this time, rare DPP IV+ cells were observed around the portal spaces (figure 4). At day 28, several DPP IV+ cells were observed in the hepatic parenchyma. On days 42 and 56, major hepatic regeneration had developed from several foci of small hepatocytes situated within pathological hepatic parenchyma (figure 5), but none of which expressed DDP IV+ activity. No DDP IV+ cells could be detected among the hepatocytes and the bone marrow cells of DDP IV- rats (negative controls). Hepatocytes and bone marrow cells from DDP IV+ rats strongly expressed DDP IV+ activity (positive controls).

**CHARACTERIZATION OF THE SMALL HEPATOCYTES**

Hepatic regeneration was observed in both groups of animals (R and RH) either as an increase in the hepatic mass (group R) or as the presence of regeneration foci (group RH). Our cell labeling technique did not allow identification of the origin of cells regenerating in group R but did in group RH. In group RH, hepatic regeneration did not originate from injected cells, but from small native hepatocytes. Secondary characterization of the small hepatocytes observed in group RH by Professor Coleman’s team enabled the demonstration that these small hepatocytes expressed certain markers of mature hepatocytes (albumin and transferrin), and of embryonic and fetal hepatocytes (HNF 1, 4, and 6). This co-expression of hepatocyte markers of different developmental stages, which produced a specific phenotype, enabled confirmation that the small hepatocytes observed in group RH were the same as previously described by this team [34-36] (figure 6).

**Discussion**

The purpose of this study was to test the capacity of bone marrow cells to colonize and repopulate rat livers in a model favorable for such repopulation. The experimental protocol for this study was designed after the protocol published by Laconi et al. [26, 27] but using bone marrow cells instead of hepato-
rare positive cells were detected in the liver lobes which might suggest the bone marrow cells had migrated into the hepatic parenchyma as has been described after transplantation of mature hepatocytes [27]. At six weeks, there were several clusters of regeneration composed of small hepatocytes. However, none of these clusters was labeled for DPP IV+ activity. Moreover, no DPP IV+ cell could be demonstrated in the liver at this time. We do not know what happened to the injected cells and thus cannot determine whether they integrated the bone marrow or circulating cells or whether they were simply eliminated. The hypothesis of elimination is not very likely since the injected cells were bone marrow cells coming from syngenic animals so immunological rejection would be unlikely.

For the two groups in this study, with and without hepatectomy, hepatic regeneration occurred either by increased hepatic mass (retorsine group) or by presence of several clusters of regeneration (retorsine hepatocyte group). This regeneration could be explained either by ineffective retorsine blockade of native hepatocytes or by hepatic regeneration from native liver stem cells or from injected bone marrow cells, or both. There is no reason to retain the hypothesis of retorsine inefficacy since this injury model has been used at the same dose and in similar conditions in experiments reported by several other teams. In addition, the presence of apoptotic bodies, megalocytes, and small clusters of hepatocytes (group RH) at the histological examination is on the contrary in favor of the efficacy of retorsine. Lacos et al. [27] were able to demonstrate preferential liver regeneration from transplanted mature hepatocytes compared with native liver stem cells, but there is no reason to extrapolate these results to transplantation of bone marrow cells. This is the main reason for using two labeling methods for injected cells to distinguish between the origin of the regeneration observed in this study.

It is known that complete liver regeneration from mature hepatocytes can occur after hepatectomy. Lacos et al. [26, 27] demonstrated that by blocking the capacity of native hepatocytes to divide, with or without hepatectomy, the largest part of the regeneration occurs from transplanted mature hepatocytes. In this experiment, and without irradiation the bone marrow, hepatic regeneration occurred exclusively from small native hepatocytes. This absence of irradiation is important because hepatic regeneration from small hepatocytes has not been observed after radiotherapy [22, 38]. Gordon et al. [34] observed the presence of small hepatocytes after two injections of retorsine and hepatectomy. These small hepatocytes were the source of complete hepatic regeneration with no cell therapy. The small hepatocytes observed in the present study are the same as studied by this team: they have the particular marking of fetal hepatocytes (EA 18 — EA 20), hepatoblasts (EA 14), oval cells (OCC2, OCC) and mature hepatocytes (antigen H, albumin and transferin) [35]. They do not express markers of bone marrow cells (CD34 and Thy1). They would also be resistant to retorsine due to the lack of or weak expression of their P450 hepatic cytochrome enzymes (CYP 2E1 and CYP 3A1) which transform retorsine into its active toxic form [34, 35]. The origin of these small hepatocytes is not known. However, given their site of emergence and their cell labeling characteristics, it can be assumed that they do not come from ovary cells (because they are OV6-) nor bone marrow stem cells (because they are CD34- and Thy1-). A recent study designated mature hepatocytes as the source of these small hepatocytes: labeling mature hepatocytes with the beta galactosidase gene enabled Avril et al. [39] to observe the expression of this gene by small hepatocytes after injection of retorsine and hepatectomy. Gordon et al. [36] successfully isolated these small hepatocytes and were able to maintain them in culture for 30 days. They then transplanted them into the hepatic parenchyma where they were capable of integration and division among other mature hepatocytes after hepatectomy. The expression of these small hepatocytes is not
constant since there was no hepatic regeneration in the work reported by Dahkle et al. [38], despite treatment with retrorsine but with radiation and after bone marrow grafting and CCL4 hepatic injury.

After the initial enthusiasm raised by the first experimental results on hepatic repopulation using transplanted bone marrow cells for fuaryl acetoacetate hydrolase deficient mice using the Lagasse et al. model [19], further work demonstrated that bone marrow cell hepatic repopulation is only a possible but rare event, even in the presence of very strong selection pressure [22]. Similarly, in humans, the chimerism of hepatocytes observed during bone marrow or hepatic transplantation demonstrated by sex-crossing appears to be an inconsistent phenomenon which regresses over the years [24]. This point is also being re-examined for other organisms, particularly for the myocardium: recent experimental work questioned the differentiation of bone marrow cells into myocytes during myocardial infarction [40, 41] in contrast with earlier encouraging results reported by Ferrari [15] and Orlic [16]. It would thus appear that bone marrow cells are less plastic than expected and that although they can differentiate, the phenomenon is rare, making it a difficult tool for exploitation with the current state of the art.

In this experimental model where transplanted bone marrow cells and native hepatic stem cells are in competition, only endogenous hepatic cells participated in liver regeneration seen as clusters of small hepatocytes. While these small hepatocytes arise from mature hepatocytes, as was demonstrated by Avril et al. [39], it is also possible, in the present model, to consider that hepatic regeneration occurred from a specific set of hepatocytes. This model again favors a very strong capacity of native hepatocytes for regeneration and the probable existence of certain subpopulations of hepatocytes which are less sensitive to chemical injury. One of the possible avenues for future research would be to isolate these specific subpopulations of hepatocytes and cultivate them for use with cell therapy strategies.

REFERENCES

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