Lack of evidence of sustained hematopoietic reconstitution after transplantation of unmanipulated adult liver stem cells in monkeys

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ABSTRACT

The aim of this study was to search for hematopoietic potential in the liver of non-human primates. Lethally irradiated (2×5 Gy γ) *Cynomolgus macaques* were given autologous hepatic mononuclear cells (HMNC) isolated from a liver lobe by perfusion and digestion with 0.1% collagenase. Two monkeys were given intramedullary injections of HMNC (18.6×10^6/kg, 20.4×10^6/kg) and two others were co-transplanted with HMNC (14.35×10^6/kg, 96.5×10^6/kg) and bone marrow mesenchymal stem cells (0.42×10^6/kg, 1.16×10^6/kg). All monkeys exhibited a transient neutrophil recovery from day 22 for 10 days, but failed to produce platelets and remained transfusion-dependent. In conclusion, adult liver stem cells from a monkey model show a low level of *in vivo* hematopoietic potential, suggesting *ex vivo* manipulation will be required before clinical use of such cells.

Key words: plasticity, liver, non-human primate, irradiation, mesenchymal stem cells.

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In recent years, grafting hematopoietic stem cells from solid organs has emerged as a promising concept in autologous transplantation settings in onco-hematology. The rationale is that contamination by malignant stem cells could be avoided provided that multipotent stem cells residing outside bone marrow are preserved from carcinogenic mutation(s) as a consequence of early embryonic divergence. Liver could be a valuable candidate in this context because hematopoiesis and hepatic development share common stages.[1] The hematopoietic potential of adult liver cells has been described by a few teams.[2-6] However, there is still controversy about the origin of the stem cells responsible and the underlying mechanisms. The hematopoietic activity of adult liver cells could be related to circulating hematopoietic stem cells, which reside in the liver but that have come from bone marrow (BM). Consistent with this hypothesis, Uchida *et al.*[5] reported that mouse liver contains a spectrum of hematopoietic cells that are phenotypically and functionally similar to those of marrow; Kotton *et al.* suggested that the hematopoietic activity is restricted to CD45-positive side population cells.[6] Alternatively, hematopoietic activity of adult liver could be explained by *adult stem cell plasticity*, a concept that has been introduced to explain the capacity of resident tissue stem cells to recapitulate the ontogeny of other tissues.[7] Unfortunately, as a general concern, initial enthusiastic descriptions of plasticity in mice have not been confirmed in large animal models. Thus, there is still a need to characterize human liver stem cells and to compare their functionality with that of BM hematopoietic stem cells (HSC). Our team has suggested a non-hematopoietic origin for liver stem cells with in vitro hematopoietic potential..[8] The present study was aimed at clarifying some of these points. Hepatic mononuclear cells (HMNC) were first transplanted alone. Then, based on studies from our group and others,[9,10] we tested the putative enhancing effect of co-grafting liver cells and bone marrow mesenchymal stem cells (MSC).

Design and Methods

Animals

Adult *Cynomolgus macaques* weighing 7.4±1.8 kg (n=7) were housed at the CRSSA-accredited animal facility in accordance with European guidelines on animal care after approval by the French Army Ethical Committee.

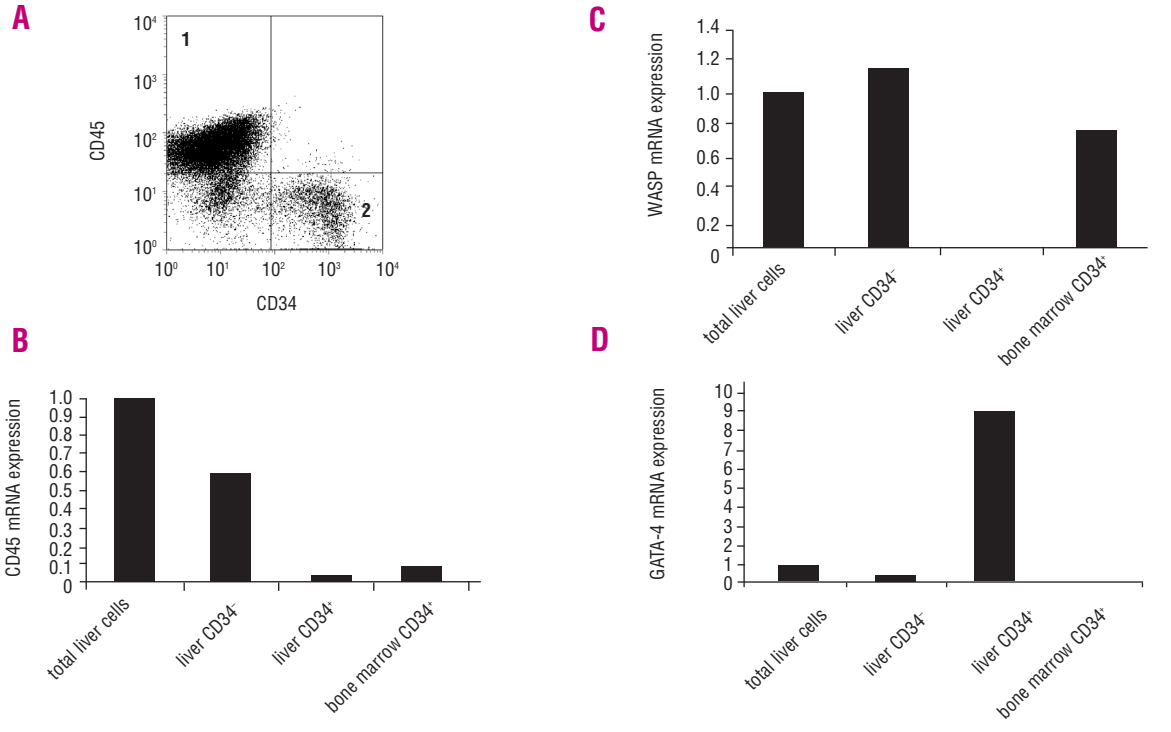

Figure 1. Characterization of liver mononuclear cells. (A) Flow cytometric analysis of CD45 and CD34 antigen expression: [1] CD34^{neg} CD45^{pos} liver-resident lymphocytes, [2] CD34^{pos} CD45^{neg} liver stem cells; (B-D) Reverse-transcription and real time quantitative PCR analysis: CD45 (B), WASP (C) and GATA-4 (D). The level of mRNA expression was quantified using geometric averaging of three internal control genes as a reference. For each gene, mRNA expression level was normalized using total liver cells as a standard which was arbitrarily attributed a value of one.

Liver cells

After left hepatic lobectomy under general anesthesia (Imalgene, Mérial, Lyon, France; 10 mg/kg intramuscularly), liver lobes were first depleted of blood cells by perfusion with HEPES (10 mM/L) solution (Life Technologies, Cergy-Pontoise, France) then liver cells were isolated by perfusion and digestion with 0.1% collagenase A (Roche Diagnostics, Meylan, France) diluted in HEPES with 0.075% $CaCl_2$. All liver cells, including HMNC, were then cryopreserved. Viable HMNC were enumerated according to morphological criteria, which allow easy separation from hepatocytes. For flow cytometric analysis, HMNC were labeled using phycoerythrin (PE) directly conjugated mouse anti-CD34 (clone 563, BD Biosciences, Le Pont-de-Claix France) and fluorescein isothiocyanate (FITC) directly conjugated mouse anti-CD45 (clone D058-1283, specifically raised against non-human primate CD45 antigen, BD Biosciences) monoclonal antibodies. Hematopoietic clonogenicity was assayed using a short-term assay in semi-solid medium (Methocult GF H4435; Stem Cell Technologies, Meylan, France) supplemented with 25 ng/mL of bone morphogenic protein 4 (BMP-4, R & D systems, Abingdon, UK).

Reverse-transcription and real time quantitative polymerase chain reaction (qRT-PCR) analysis

Messenger RNA was isolated from CD34$^+$ and CD34$^-$ cells after cell sorting (FACS Vantage option DiVA, BD Biosciences). qRT-PCR was performed as previously described.[11] Quantification was carried out using geometric averaging of three internal control genes (*ACTB, GAPDH, HPRT*) as reported by Vandesompele *et al.*[12]

Mesenchymal stem cells

As monkey MSC cannot be routinely cultured due to spuma virus infection and retrovirus-free human MSC can be detected after transplantation in non-human primates, we used a xenograft model.[10] Human MSC layers were pre-established by culturing BM cells from a healthy donor (after informed consent),[13] then cryopreserved in liquid nitrogen until use.

Transplantation study

After a 2-month rest, the anesthetized monkeys were globally exposed to a total dose of 2x5 Gy with a ^{60}Co γ radiation source (dose rate of 15 cGy/min); using frontal then dorsal irradiation on two consecutive days. This dose schedule is lethal without HSC transplantation.[10] HMNC±MSC were infused directly into the humerus on the day after the second fraction of irradiation. Two animals were given an autologous HMNC graft alone (monkeys S024 and 3537), two were co-grafted with autologous HMNC±MSC (monkeys 4157 and Z836) and two were left ungrafted as controls (monkeys 3648D and 10090).

Short-term reconstitution was defined as the time to achieve granulocyte recovery (absolute neutrophil count [ANC] >1x10^9/L) and platelet recovery (PLT >20x10^9/L).

Results and Discussion

Characterization of liver hematopoietic stem cells

We showed that liver CD34$^+$ cells, in contrast to BM CD34$^+$ cells, did not express the pan-leukocytic CD45 antigen (Figure 1A). Moreover, CD45 mRNA was almost undetectable (BM/liver ratio of 9) (Figure 1 B). Liver CD34$^+$ cells did not express WASP mRNA but strongly expressed GATA-4 mRNA, a non-hematopoietic marker (Figure 1C and 1D).

Graft cell content

On average, 662.6±184×10^6/L total liver cells were collected after lobectomy (Table 1). In fact HMNC were positively selected by the freezing/thawing step as trypan blue viability evaluation of thawed cells showed that almost all hepatocytes had died. Monkeys received 37.5±9.7×10^6/kg HMNC (range 14.35-96.5). Monkey S024, 3537 and 4157 were given 0.35, 1.22 and 1.19×10^6/L/kg CD34$^+$ cells, respectively. Clonogenicity of transplanted cells was 2.3±1.34 CFUs in 10^5 HMNC.

Hematopoietic recovery

HMNC transplantation

The ungrafted control monkeys died from BM aplasia and gastrointestinal distress on days 21 and 22 (Table 1). After grafting autologous HMNC, neutrophil recovery occurred from day 27 for monkeys S024 and 3537 (Table 1). The ANC recovered transiently above 1×10^9/L for 13 and 14 days respectively, before decreasing. No platelet recovery was observed and both animals remained transfusion-dependent until euthanasia.

HMNC and MSC co-transplantation

The neutrophil recovery of monkeys 4157 and Z836, co-grafted with HMNC and MSC, was similar to that of monkeys S024 and 3537 (Table 1 and Figure 2). Neutrophil recovery occurred from day 22 and day 25 and was shorter (4 and 8 days, respectively). Monkey 4157 appeared to exhibit platelet recovery on day 20 before dying from non-hematologic toxicity on day 25.

Extramedullary hematopoiesis is the subject of lively debate. Following the first observations, the involvement of resident/circulating BM-derived stem cells appeared a satisfactory hypothesis because it has been shown that HSC continuously enter/exit the circulation. This has been confirmed in mice for muscle HSC[14] but in the adult mouse liver the identification of hematopoietic-like stem cells appeared puzzling.[5,6] In this study we tested the feasibility of an autologous transplantation approach using liver stem cells, which could represent an alternative to BM-derived HSC in different types of leukemia. We have previously reported that HMNC are a mixture of cells mainly made of liver-resident lymphocytes and CD34$^+$ cells that contain the whole hematopoietic clonogenicity.[8] Here we found that CD34$^+$ cells did not express either the panleukocytic CD45 membrane antigen or the WASP marker (mRNA level),[15] which is restricted to hematopoietic cells, whereas the endodermic GATA-4 gene[16] was highly expressed. These findings differ from those of Crosbie *et al.*[2] who found that 49%±23% of human CD34$^+$ HMNC are CD34/CD45 double positive but the difference in cell sampling (biopsies including some cancer patients versus tissue from healthy monkeys) may account for this discrepancy. Our results suggest that liver HSC may be part of the oval cell populations. Oval cells are small lymphocyte-like cells located within the smallest branches of the intrahepatic biliary tree.[17] These cells may represent residing/circulating pluripotent stem cells. We are currently evaluating xeno-

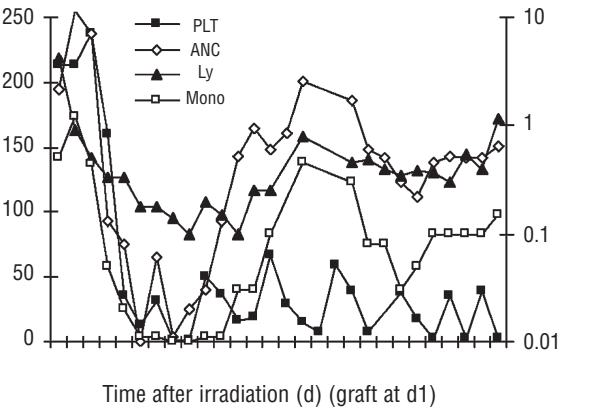

Figure 2. Hematopoietic parameters over 49 days of monkey Z836 co-grafted with autologous hepatic mononuclear cells and mesenchymal stem cells. Platelets (PLT), absolute neutrophil count (ANC), lymphocytes (Ly), monocytes (Mono). PLT peaks indicate blood cell transfusion.

Table 1. Graft cell content and hematopoietic parameters of the grafted monkeys.

Monkey	*3648D*	*10090*	*S024*	*3537*	*4157*	*Z836*
				×10^6/kg		
Collected cells			95.2	101.8	29.2	148.6
Grafted HMNC*		ungrafted	18.6	20.4	14.35	96.5
Co-gratfted MSC		controls	0	0	0.42	1.16
Liver CD34$^+$ cells			0.35	1.22	1.19	ND
Time to ANC >0.5×10^9/L		26	25	21	20	
Time to ANC >1×10^9/L			27	27	22	25
Duration ANC >1×10^9/L		NR	13	14	4	8
Time to PLT >20×10^9/L			NR	NR	20	NR
Time to PLT 5×10^9/L			NR	NR	NR	NR
Duration of anemia	day17-	0	day12-	day18-	day4-	day15-
Hb <10 g/dL	day21		day65	day41	day25	day49
Number of transfusions	2	2	8	10	4	7
Day of death	21	22	65	61	25	49

**After thawing, hepatocytes are mostly non-viable so that grafts consist of hepatic mononuclear cells (HMNC); MSC: mesenchymal stem cells; NR: no reconstitution.*

transplantation of purified subpopulations of liver MNC in the NOD-SCID model to validate the hypothesis of stem cell plasticity. The underlying question was how great the hematopoietic potential of liver stem cells is compared with that of BM HSC. Thus, we collected and transplanted the largest number of HMNC compatible with the animals' survival and liver function. Despite these high levels of autologous HMNC, we observed only a weak and transient hematopoietic recovery restricted to leukocytes. It is unlikely that this reconstitution resulted from rare contaminating circulating HSC as no clonogenicity was observed in aliquots of HMNC in the absence of BMP4. This profile of recovery differs from the multilineage reconstitution observed in mice, pointing out the discrepancy in stem cell plasticity between the two models.

In fact, a definitive comparison between liver and BM stem cells was not possible because of the difference in phenotype. An average of about two total colony-forming units (CFU) could be enumerated in 10^5 HMNC so that graft cell contents (about 0.1×10^4/kg CFU) were far under the cell threshold capable of ensuring stable hematopoietic recovery using BM-derived HSPC (7.9 and 8.6×10^4/kg CFU).[18] Clearly, the liver stem cell pool must be amplified to get a clinical benefit, for example by co-culturing stem cells on accessory/stromal cells.[9,10] Here no significant improvement in hematopoietic recovery was observed when the HMNC were co-grafted with MSC although monkey 4157 appeared to exhibit platelet recovery on day 20. Other strategies need to be explored. Indeed, so far HSC residing in non-hematopoietic organs have shown only a low potential in primate models.[19]

Authors' Contributions

All authors: conception and design, analysis and interpretation of data, drafting the article and final approval of the version to be published. We thank Philippe Garrigou and Jean-François Franetich for helpful collaboration, Nancy Grenier, Maud Fontenaud, Hervé Chaussard and Stephane Baugé for technical assistance.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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