Mice with humanized liver endothelium

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Chapter 3

Human fetal liver hematopoietic progenitor cells do not repair mouse liver endothelium

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Submitted

Abstract

Liver sinusoidal endothelial cells perform important functions in the secretion of plasma proteins, immunity and regulation of blood flow. Studies in animal models and patients that have undergone liver transplantation have shown that bone marrow derived hematopoietic progenitor cells are able to differentiate into liver endothelium. However, in other reports differentiation of bone marrow derived hematopoietic cells in liver endothelium was not observed and controversy thus surrounds the possible origin of liver endothelial cells.

We therefore examined whether human hematopoietic progenitor cells, isolated from fetal liver, were able to regenerate damaged mouse liver endothelium.

Hematopoietic progenitor cells from human fetal liver were used to reconstitute the immune system in immune deficient mice. Subsequently, the liver endothelium of these mice with humanized immune systems was damaged. In parallel, hematopoietic progenitor cells from human fetal liver were also directly transplanted in immune deficient mice with damaged liver endothelium. In neither of these conditions restoration of mouse liver endothelium with human cells was observed. In contrast, endothelial cells from human fetal liver, obtained after proteolytic digestion, were capable of rapid and abundant reconstitution of mouse liver endothelium.

We conclude that human hematopoietic progenitor cells have minor or no ability to regenerate damaged mouse liver endothelium *in vivo* suggesting that contribution of these cells in liver angiogenesis remains questionable and mainly occurs through outgrowth of (non-) circulating endothelial cells.

Introduction

Liver endothelial cells form the inner lining of the liver vasculature. They are a specialized type of endothelial cells characterized by the presence of fenestrae, the lack of a basement membrane and these cells play an important role in uptake of compounds such as hyaluronic acid and the regulation of the immune system (1-3). The liver has a remarkable capacity to regenerate following injury, with endothelial cells playing an important role in the regulation of this regeneration (4).

The cell source that contributes to regeneration of damaged liver endothelium is a matter of controversy. The presence of recipient endothelial cells in biopsies of liver transplant recipients raised the possibility that circulating (endothelial) progenitor cells contribute to repair of damaged liver endothelium (5, 6). This micro-chimerism is important, because it has been hypothesized that replacement of donor endothelium by recipient progenitor cells may play a role in decreasing graft rejection (6).

A great variety of studies have shown that bone marrow or peripheral blood derived endothelial progenitors can improve chronic liver injury in mice and rats (7-13). According to these studies, bone marrow cells or circulating progenitor cells can differentiate into endothelial cells, incorporate in the liver vasculature and mediate therapeutic effects. The relevance of these precursors remains uncertain, especially since several other groups have not been able to detect differentiation of hematopoietic bone marrow progenitors into endothelium. For instance, phenotypic correction of hemophilia A mice was obtained following transplantation of wild type murine bone marrow cells, but this correction was not due to differentiation of transplanted cells into liver endothelium (14). Transplantation experiments in other model systems also failed to detect bone marrow derived endothelium (15-17). The detection of donor derived endothelium in livers of hematopoietic transplant recipients has been questioned as the methodology to determine assignment of liver cells in biopsy material is not unequivocal (18). An elegant paper using parabiotic mice showed that circulating cells do not contribute to regenerating limb vasculature (19). These conflicting data require more scientific evidence, to test whether hematopoietic progenitor cells may play a role in regenerating damaged liver endothelium.

In embryology, hematopoiesis takes place in the fetal liver. Just before birth, hematopoiesis is established in the bone marrow, which remains the principal site of hematopoiesis postnatally. Until 25 weeks after gestation, the human liver is the main hematopoietic organ. In this period extensive proliferation of liver parenchymal cells and endothelium takes place with establishment of liver architecture completed after gestation week 25. Thus, the developing liver is a rich source of hematopoietic and endothelial precursors. Peichev *et al.* characterized a subpopulation of CD34 positive cells from human fetal liver that can differentiate into endothelial cells *in vitro* (20). It is however unknown whether these cells have the ability to differentiate into endothelium *in vivo*.

Endothelial progenitor cells are considered to be derived from the same precursor as hematopoietic progenitor cells, termed the hemangioblast (21). During development, the formation of hematopoietic and endothelial cells is closely linked. Furthermore, hematopoietic cells can be produced by a specialized type of endothelial cell, the hemogenic endothelium (22). In this light the formation of endothelium from bone marrow derived precursors is not surprising.

Most of the studies mentioned above have been performed in mouse and rat models, data on the differentiation of human progenitor cells *in vivo* are scarce. We have developed a model system in which repopulation of immune deficient mouse livers with human endothelial cells can be studied. This model is thus very well suited to establish the potential of human hematopoietic progenitor cells in the regeneration of damaged liver endothelium.

In previous experiments, we have shown that mature endothelial cells in fetal liver can engraft and repopulate the mouse liver (23). In the present study we examined whether human hematopoietic progenitor cells from fetal liver have the capacity to differentiate into endothelial cells *in vivo.* Two different approaches were used. Human hematopoietic progenitor cells (HPC) from fetal liver were transplanted in immune deficient mice to reconstitute a human immune system. Subsequently the liver endothelium was damaged and repair by human hematopoietic cells examined. In the second approach, human hematopoietic progenitor cells from fetal liver were directly transplanted in mice with damaged liver endothelium and repair by human cells was also examined.

Materials and Methods

Isolation of progenitor cells and endothelial cells from human fetal liver

Human fetal liver was obtained from elective abortions following informed consent. Gestational age ranged from 14-20 weeks.

The tissue was subjected to mild mechanical disruption and the supernatant cells were used to isolate $CD34^{+}CD38^{-}$ lineage-negative (CD3, CD11c, CD19, CD56 and BDCA2) hematopoietic progenitor cells (HPC) as described earlier, using the CD34 Progenitor Cell Isolation kit (Miltenyi Biotec) followed by a sorting enrichment step using a FACS-Aria (BD Biosciences) (24). Throughout the manuscript these cells are referred to as hematopoietic progenitor cells (HPC). HPC were used for flow cytometry analysis directly after isolation or plated out for overnight transduction followed by transplantation experiments the next day.

The larger tissue fragments were digested by collagenase (25) and CD31 positive (endothelial) cells were isolated as described earlier. In short, fetal liver tissue was digested in 0.05% collagenase IV (Sigma-Aldrich) in Hanks balanced salt solution (BioWhittaker) and seeded out on Primaria plates (BD Falcon). After 2-7 days in culture, CD31 positive cells were isolated from the human fetal liver cell suspension via magnetic separation using anti-human CD31 antibody conjugated magnetic beads (Miltenyi Biotec, Auburn CA, USA) according to the protocol provided by the manufacturer. These cells are referred to as liver sinusoidal endothelial cells (LSEC) throughout the manuscript. CD31 positive cells were used for flow cytometry analysis directly after isolation or plated out for phase contrast imaging 24 hours and 5 days later using a Leica DM IRE2 microscope.

Animals

Animal experiments were performed in accordance with the Animal Ethical committee guidelines of our institute. Male and female *Rag2*$^{-/-}$*yc*$^{-/-}$ (26) mice (own colony) with a mixed background and aged 3-6 weeks were used in the transplantation studies. Newborn male and female NOD/SCID IL-2Rγc$^{-/-}$ mice (Jackson Laboratory, Maine, USA) or BALB/c Rag2$^{-/-}$IL-2Rγc$^{-/-}$ (own colony) were used to generate HIS mice. The mice were fed *ad libitum* on standard laboratory chow.

Transplantation experiments

Rag2$^{-/-}$*yc*$^{-/-}$ (n=10) mice received an intraperitoneal injection of 200 mg/kg monocrotaline (27) (Sigma-Aldrich, St. Louis, MO, USA) in saline 7 days and 24 hours prior to cell transplantation in order to specifically damage the murine liver endothelium. The mice were anaesthetized with an intraperitoneal injection of FFM [2.5 mg Fluanisone/0.105 mg Fentanyl citrate (Hypnorm, Vetapharma, Leeds, UK) and 1.25 mg Midozalam HCl/kg in H_20 (Roche, Indianapolis, USA), 7 ml/kg]. Under anesthesia, the spleen was exposed after a sub costal incision at the left flank. The cells, 2 x 10^5 HPC in 100 µl PBS, were injected into the tip of the spleen with a 30-gauge insulin needle (Terumo, Somerset, USA). Control mice were injected with PBS (Frensius, Kabi, Bad Homburg, Germany). Homeostasis was secured by brief pressure for up to 5 minutes on the injection site. The animals were sutured and received the analgesic Temgesic (30-50 µl, 0.03 mg/ml; Reckitt Benckiser, Slough, UK) subcutaneously following recovery from FFM.

Generation of human immune system mice and injury of liver endothelium

Human immune system mice were generated as previously described (28, 29).
In short, 5 day old NOD/SCID IL-2Rγc$^{-/-}$ mice (n=8) or BALB/c Rag2$^{-/-}$IL-2Rγc$^{-/-}$ (n=6) were sub-lethally irradiated and injected focally in the liver with 1 x 10^4 HPC. Eight weeks after transplantation of HPC, human CD45, CD3 and CD19 (Biolegend) was measured in peripheral blood from HIS mice to determine the percentage of repopulation (30). HIS mice with a reconstitution of > 20% human CD45$^+$ cells received 1 x 200 mg/kg or 2 x 200 mg/kg intra-peritoneal injections of monocrotaline 7 days apart.

Tissue sampling

All mice were sacrificed 14-28 days after the second monocrotaline injection as described earlier (23). In short, blood was collected via a heart puncture and the animals were perfused intracardially with 20 ml PBS followed by perfusion with 20 ml 2% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in PBS. After perfusion, the liver, and spleen were removed and processed for immunohistochemistry as described (23) earlier.

Differentiation of hematopoietic progenitor cells (HPC) into endothelial cells

HPC were plated on collagen coated (1 µg/well in 60% ethanol) 24 wells plate at a density of 1 x 10^6 cells/well in EGM2-basal medium plus EGM-2 MV bulletkit

(Lonza, Walkersville, USA). After 5 days, the medium was refreshed every 3 days. Phase contrast images were taken using a Leica DM IRE2 microscope.

Lentiviral transduction

HPC and LSEC were transduced overnight with a codon-optimized mCD47-expressing pHEF lentiviral vector (31) 24 hours prior to transplantation as described earlier (32).

Flow cytometry analysis

Cells (n=3) were characterized by co-staining for CD31, CD34, CD133, CD146 (Miltenyi Biotec, Auburn CA, USA), CD45 (eBioscience) and CD309 (BD Pharmingen) (table 1; supplementary methods). Isotype matched nonspecific antibodies were used as controls (eBIoscience). Mean values are presented with ± SD.

Immunohistochemistry

Cryosections of 5-6 µm were affixed to poly-L-lysine-coated glass slides and kept at -20°C before use. Sections of fetal liver were fixed for 10 minutes in 2% paraformaldehyde before use. Sections were stained with antibodies specific for human proteins without cross reactivity to murine homologues. Monoclonal anti-human CD31, CD34 (1:100, DakoCytomation), CD45 (1:100, eBioscience) or polyclonal rabbit anti-human lymphatic vessel endothelial receptor 1 (LYVE-1) (Angio, Del, Mar, CA, USA) for 1 hour at room temperature (table 1; supplementary methods). Primary antibodies were detected with Alexa 594 or Alexa 488 conjugated goat anti-mouse or goat anti-rabbit antibodies (1:1000, Rockland Immunochemicals) followed by embedding in mounting medium containing DAPI (Vector labs, Burlingame). Images were taken with a Leica DM RA2 microscope or a Leica SP8-X Confocal microscope.

Results

Immunohistochemical characterisation of human fetal liver

Human fetal liver tissue, gestation week 14 to 16, was stained for LYVE-1 and CD31, CD34 and CD45 respectively (Figure 1). LYVE-1 has been shown to be expressed in human liver endothelium and was used to test colocalization with CD31, CD34 and CD45. Islands of hepatocytes surrounded by endothelial cells can be clearly distinguished. All LYVE-1 positive cells colocalize with CD31 and CD34. Cells

positive for CD31 and CD34 but negative for LYVE-1 are also observed. The LYVE-1 negative and CD31 or CD34 positive cells are round, do not have endothelial morphology and therefore likely represent cells of hematopoietic origin. In contrast, no colocalization of CD45 with LYVE-1 was observed. These data show that the endothelial cells surrounding the fetal liver sinusoid are positive for CD31, CD34 and LYVE-1 but negative for CD45.

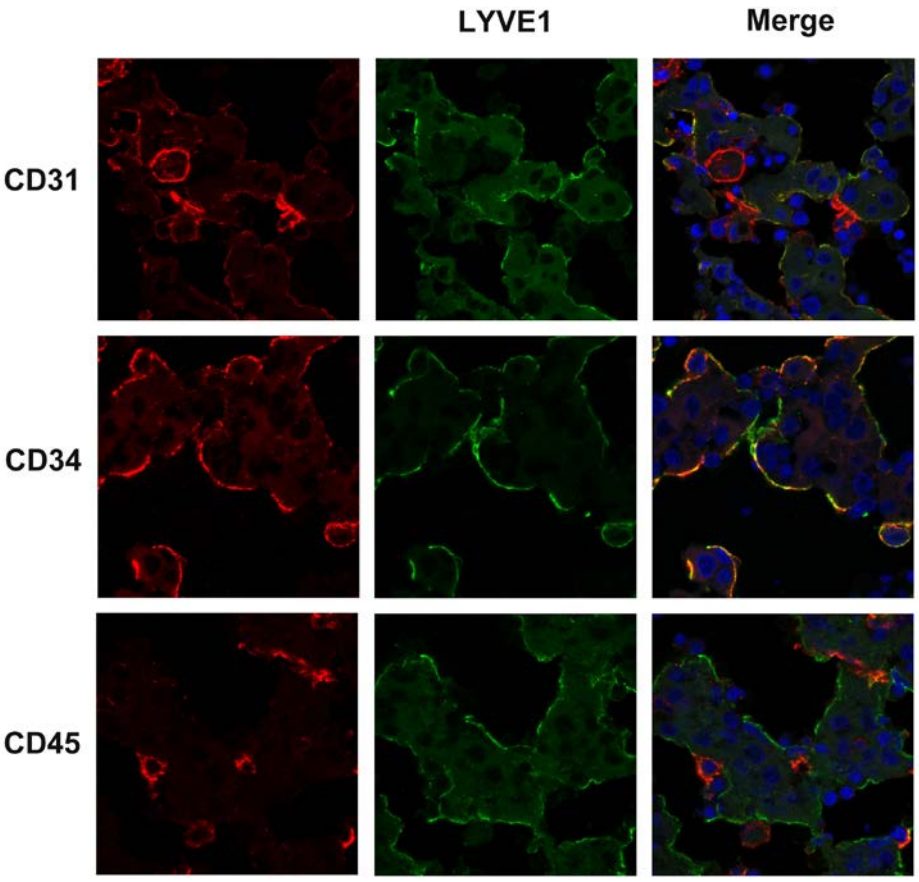

Figure 1. Immunohistochemical characterisation of human fetal liver
Human fetal liver tissue was stained for CD31 and LYVE-1, CD34 and LYVE-1 and CD45 and LYVE-1. Nuclei were stained with DAPI. Images were collected by confocal microscopy. LYVE-1 colocalizes with CD31 and CD34, these cells form the lining of the developing sinusoid and represent human fetal liver endothelium. No colocalization of LYVE-1 and CD45 was observed. Cells positive for CD31 or CD34 but negative for LYVE-1 are also observed. These cells are round, do not form a sinusoidal lining and represent hematopoietic cells. The panels represent sections of 125 µm squared.

Characterization of human fetal liver cells by flow cytometry

Hematopoietic progenitor cells (HPC) were isolated from human fetal liver by mild mechanical disruption and cell sorting for CD34. These cells show a phenotype consistent with hematopoietic stem cells as they are positive for CD31, CD34 , CD45 and CD133 with low expression of endothelial markers such as CD146 and CD309 (Figure 2). Throughout the manuscript these cells are referred to as hematopoietic progenitor cells (HPC).

Liver sinusoidal endothelial cells, which are obtained after enzymatic digestion of human fetal liver, are also highly positive for CD31 and CD34 as shown in figure 2. They are mostly negative for the hematopoietic marker CD45 and the stem cell marker CD133. In contrast, endothelial cell marker CD146 is expressed in a higher frequency and endothelial cell marker CD309 in a comparable frequency to HPC. This phenotype is consistent with endothelial cells and these cells are referred to as liver sinusoidal endothelial cells (LSEC) throughout the manuscript. This characterization shows that only enzymatic digestion of human fetal liver is able to liberate differentiated endothelial cells.

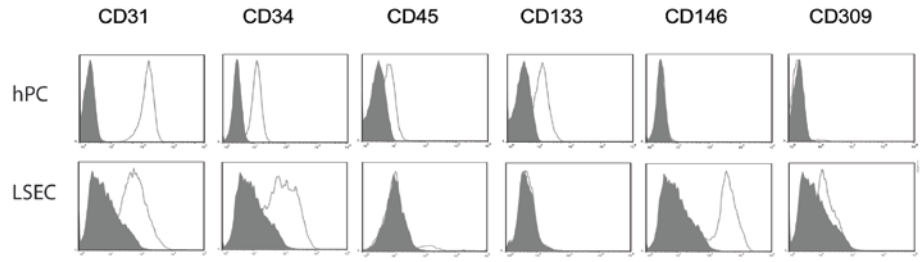

Figure 2. Characterisation of human fetal liver cells by flow cytometry

The phenotype of freshly isolated HPC from human fetal liver after mild mechanical disruption and CD34 sorting was analyzed by staining for CD31, CD34 (72±34%), CD45 (37±38%), CD133 (36±25%), CD146 (10±8%) and CD309 (23±36%) by flow cytometry. Upper panel, n=3. The corresponding isotype controls are shown as solid peaks. For comparison the same staining is shown for cells isolated from fetal liver after enzymatic disruption and magnetic CD31 isolation 2-7 days later (CD31 (84±6%), CD34 (37±35%), CD45 (21±22%), CD133 (0±0%), CD146 (36±25%), CD309 (23±9%)). Lower panel n=3.

Differentiation of human fetal liver-derived hematopoietic progenitor cells into endothelial cells

The CD34 positive HPC were cultured in endothelial cell specific medium on collagen coated wells. After 5 days the medium was refreshed. Cells had adhered and exhibited heterogeneity in morphology (Figure 3 bottom panel). Fourteen to 22 days after plating, several colonies of endothelial like cells displaying cobble stone morphology could be found. Phase contrast images taken from human liver endothelial cells (LSEC) purified using human CD31 conjugated magnetic beads are shown for comparison (Figure 3 bottom panel). After 14-22 days in culture, differentiated HPC were harvested and analyzed for the expression of several cell surface markers as depicted in the histograms of figure 3. Comparing the flow cytometry results of figure 2 and 3, it is shown that culture of HPC leads to complete loss of CD34 and partial loss of CD31. However, expression of CD45 remained confirming the heterogeneity of cells from the HPC pool that adhere after plating. More importantly, expression of the endothelial cell markers CD146 and CD309 was induced. These experiments show that some cells in the purified CD34 positive fraction that was obtained after mild mechanical digestion are capable of differentiating into endothelium. Our results thus confirm that cell populations with the potential for reconstituting a complete hematopoietic system also contain cells that can be differentiated into endothelium *in vitro*.

Human fetal liver-derived hematopoietic progenitor cells do not regenerate damaged liver mouse endothelium following intrasplenic transplantation

We next determined whether the population of human fetal liver hematopoietic progenitor cells, that should include cells with endothelial potential, are capable of restoring mouse liver endothelium *in vivo* by transplantation in immune deficient mice with damaged liver endothelium. The transplanted cells were previously transduced with a lentiviral vector encoding murine CD47 to protect the cells from mouse phagocytic activity and increase grafting. CD47 is a membrane protein, also known as integrin-associated protein, which prevents phagocytosis by macrophages through interaction with signal regulatory protein alpha (SIRPα) (33). Human hematopoietic reconstitution of HIS mice is improved following transplantation of mCD47-expressing HPC (34).

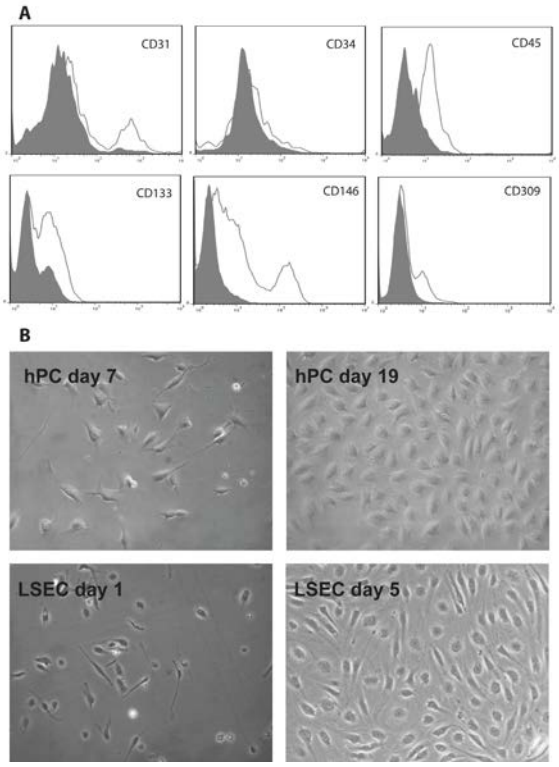

Figure 3. Differentiation of HPC into endothelial like cells.
A) Hematopoietic progenitor cells (HPC) were isolated after mild mechanical disruption of fetal liver and cultured for 14-22 days in endothelial cell medium. Flow cytometry was used to detect expression of CD31, CD34, CD45, AC133, CD146 and CD309 by differentiated HPC. Compared to freshly isolated HPC as shown in figure 2, expression of the endothelial specific markers CD146 and CD309 was increased after culture in endothelial cell medium (clear peak). In each panel, the solid peaks represent staining with an isotype control antibody. B) Adhered HPC show varying morphology. After 7 and 19 days in culture in endothelial cell medium, colonies of endothelial like cells displaying cobble stone morphology were present. As a comparison liver sinusoidal endothelial cells (LSEC) cultured in endothelial cell medium for 24 hours and 5 days after isolation from human fetal liver using magnetic separation are also shown (lower panels). Magnification: 10X.

For our experimental purpose we used monocrotaline that selectively damages liver endothelial cells and denudes the parenchymal plates (35, 36). The immune deficient mice received an intraperitoneal injection of monocrotaline 7 days and 24 hours prior to intrasplenic HPC transplantation. The mice did not show signs of adverse effects after monocrotaline treatment. Following transplantation of HPC (n=10) sporadic human CD45 positive cells could be detected in the liver, indicating that the transplantation was successful. The CD45 positive cells did not

resemble endothelium in morphology (Figure 4). No LYVE-1 positive cells were detected, thus showing that HPC obtained from human fetal liver are not capable of restoring damaged liver endothelium. Additionally, transplanted human cells were not observed in sections of the lungs and kidneys (data not shown).

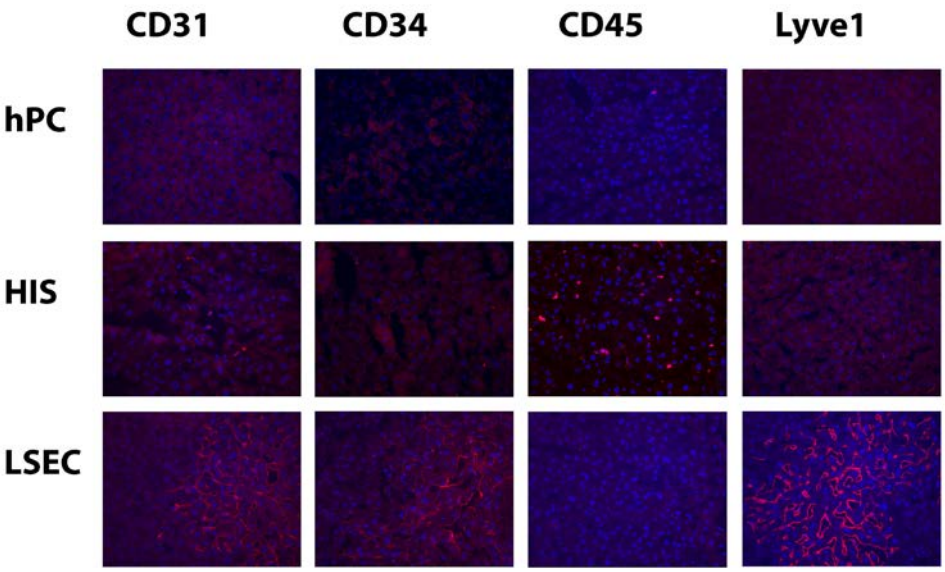

Figure 4. Immunohistochemistry shows that only human liver sinusoidal endothelial cells are capable of restoring damaged liver endothelium.

Livers from *Rag2*$^{-/-}$*yc*$^{-/-}$ mice transplanted with human hematopoietic progenitor cells (HPC), human immune system mice (HIS) (*NOD/SCID IL-2Rγc*$^{-/-}$ *mice* (n=8) or *BALB/c Rag2*$^{-/-}$*IL-2Rγc*$^{-/-}$ (n=6)) and mice transplanted with human liver sinusoidal endothelial cells (LSEC) (n=10) were stained for LYVE-1, CD31, CD34 and CD45. Nuclei are stained blue with DAPI. Only single CD45 positive cells were found in liver sections of mice transplanted with HPC (upper panels). Mice with reconstituted human immune systems (HIS) had an abundance of CD31 and CD45 cells present in the liver (middle panels). The morphology of these cells is consistent with a hematopoietic origin. No LYVE-1 positive cells were observed in any of liver sections of the mice transplanted with HPC and the HIS mice. In contrast, following transplantation of human liver sinusoidal endothelial cells (LSEC) (n=2), multiple clusters of cells could be found in both spleen and liver that co-stained with LYVE-1, CD31 and CD34. The morphology of these clusters is consistent with endothelium. GFP-signal is not displayed. Magnification: 20X.

Liver endothelium of mice with humanized immune systems is not regenerated from human hematopoietic cells.

Although the human fetal liver-derived hematopoietic progenitor cells were not directly able to restore damaged liver endothelium, it remained possible that the HPC need a differentiation step *in vivo* to be able to restore damaged liver endothelium.

We therefore used HPC to reconstitute a human immune system in immune deficient mice before damaging the liver endothelium. Mice with human immune systems had on average 35±10% human CD45 positive cells with differentiation in T and B cell lineages as indicated by positive human CD3 and CD19 staining respectively (Supplementary figure).

We damaged the liver endothelium of mice with human immune systems to determine if differentiated hematopoietic progenitor cells play a role in liver vascular regeneration.

Monocrotaline treatment did not change the percentage of human CD45 cells in peripheral blood two weeks after the second injection (data not shown). Immunohistochemistry showed abundant presence of human CD31 and CD45 positive cells in the liver but these cells did not resemble endothelium. LYVE-1 staining was negative, showing that, in this model, HPC are also not able to differentiate into liver endothelium (Figure 4). Sections of lungs and kidneys did not contain differentiated endothelial cells from human hematopoietic origin (data not shown).

These experiments show that differentiated human hematopoietic cells are not capable of restoring damaged liver endothelium.

Regeneration of damaged mouse liver with human liver sinusoidal endothelial cells.

As a positive control, immune deficient mice (n=2) were transplanted with mCD47-expressing human liver sinusoidal endothelial cells obtained after enzymatic digestion of human fetal liver. In contrast to the transplantation experiments with HPC, all transplanted mice had abundant clusters of CD31 and LYVE-1 positive cells that resembled endothelium in morphology. These results were consistent with an earlier study from our group (23). No human CD45 positive cells could be detected (Figure 4). These experiments confirm that our model of liver endothelial damage is sensitive and can be used to determine the liver endothelial regeneration potential of transplanted cells.

Discussion

The ability of progenitor cells from peripheral blood to repair damaged blood vessels has been demonstrated in a number of studies (10, 13, 37, 38). The aim of our study was to examine the capacity of hematopoietic progenitor cells isolated from human fetal liver to regenerate damaged liver vasculature.

We have examined the potential of human hematopoietic progenitor cells (HPC) for the repair of liver endothelium in two ways. In the first approach we transplanted HPC in immune deficient mice, which had been treated with monocrotaline to damage the liver endothelium. Histological examination of the mouse liver revealed only low amounts of human CD45 positive HPC or HPC derived cells. These cells likely are hematopoietic in origin. Incorporation of human cells in liver and spleen endothelium of transplanted mice was not observed. Staining for human LYVE-1 was negative. Thus, direct differentiation of HPC into liver endothelium does not appear to be an efficient process.

In the second approach, HPC were used to reconstitute a human immune system in immunodeficient mice before inducing liver endothelial damage. After good multi-lineage reconstitution of the murine immune system with human cells was observed, liver damage was induced by monocrotaline. In contrast to the mice directly transplanted with HPC, livers of the mice with human immune systems contained large amounts of CD31 and CD45 positive cells. These cells were not incorporated into the sinusoids, did not form a vascular network and were likely hematopoietic in origin. Because no LYVE-1 staining was observed, we conclude that no differentiation of human cells into liver endothelium occurred.

In contrast, transplantation of liver sinusoidal endothelial cells, isolated after digestion of human fetal liver with protease, gave rise to abundant repopulation of mouse liver endothelium, as evidenced by the presence of LYVE-1 positive clusters of human endothelium throughout the livers of transplanted mice. This result is consistent with our previous study in which we also showed that transplantation of human fetal liver-derived sinusoidal endothelial cells in immune deficient mice leads to grafting of differentiated and functional human endothelium (23). A crucial finding of this study therefore is that enzymatic digestion of fetal liver with collagenase followed by a culture step is required to obtain cells with endothelium regenerative properties. This strongly suggests that the only cells capable of

restoring damaged liver endothelium are differentiated liver sinusoidal endothelial cells.

Although our studies were performed using human cells, engraftment of human cells in murine liver is only an approximation of the situation in humans *in vivo*. Nevertheless, we are convinced that our results also describe the behavior of hematopoietic progenitor cells in humans *in vivo*. The HPC were transduced by a lentiviral vector expressing mouse CD47. CD47 is a membrane protein, also known as integrin-associated protein, which prevents phagocytosis by macrophages through interaction with signal regulatory protein alpha (SIRPα) (33). Human hematopoietic reconstitution of HIS mice is improved following transplantation of mCD47-expressing HPC (34). Thus, scavenging of HPC with endothelial potential by mouse macrophages is not likely to have occurred. In addition, both HPC and human liver sinusoidal endothelial cells were isolated from the same source, both transduced overnight under comparable circumstances and transplanted under similar conditions whereas only the human liver sinusoidal endothelial cells were shown to have endothelial regenerative potential *in vivo*.

Our results thus show that, in fetal liver, mechanical dissociation releases a population of CD31 and CD34 positive cells in which the hematopoietic stem cells reside. After enzymatic digestion of human fetal liver, a different population of CD31 and CD34 positive cells is released.. We have previously shown that CD31 negative cells obtained after enzymatic digestion of fetal liver are unable to regenerate damaged liver endothelium (23). Our study therefore conclusively shows that human fetal liver hematopoietic progenitor cells do not contribute significantly to restoration of damaged liver endothelium. However, in a previous study, we have also shown that liver endothelium can only be regenerated by fetal or adult liver endothelial cells. Microvascular endothelium from subcutaneous fat and macrovascular endothelium from umbilical cord is not able to restore damaged liver endothelium. Furthermore, examination of other tissues of mice transplanted with HPC or HIS mice showed that HPC are also incapable of restoring endothelia other than those in liver.

The HPC expressed cell surface markers CD31, CD34, CD45 and CD133, but did not express the endothelial cell markers CD309 and CD146. Recent studies have found that CD34+CD133+CD309+ cells are hematopoietic cells that do not yield endothelial cell progeny (39, 40). We confirmed that HPC could be differentiated

to endothelial cells *in vitro* as evidenced by the appearance of expression of endothelial cells markers CD146 and CD309. This could be differentiation of EPCs within the HPC suspension into endothelial cells. There remains however a possibility, that small amounts of CD34 positive liver sinusoidal endothelial cells were isolated from the human fetal liver along with the HPC and adhered and expanded following plating.

Several studies have been performed that examine the potential of endothelial progenitor cells for repair of damaged vasculature. The percentage of successful regeneration of damaged vasculature varies enormously from approximately 50% (7) to minimal (41, 42) or no incorporation at all (16, 17). Female rat liver endothelium was repaired very efficiently by transplanted male bone marrow derived stem cells following treatment with monocrotaline (8). However, in this study the engrafted endothelial cells were positive for CD45, a finding not reproduced by us and other groups (39, 40, 43, 44).

The role of hematopoietic progenitor cells in the regeneration of damaged endothelium is therefore controversial. A variety of studies has shown benefit of bone marrow derived stem cell transplantation in animal and human models of endothelial damage (8, 10, 45). However, other studies, including ours, show that the contribution of progenitor cells to endothelial regeneration is minimal (16, 46). A confounding factor that might explain these discrepancies is the lack of markers specific for progenitor cells and endothelium (18). Markers such as CD31 and CD34 are present on progenitor and differentiated endothelial cells (18). Thus, it is well possible that studies reporting endothelial grafting and repair by transplanted progenitor cells were in fact looking at the effects of dislodged differentiated endothelial cells. This hypothesis is strengthened by our observation that digestion of tissue with proteases is required for the liberation of cells with endothelial regenerative capacity. Only complete tissue breakdown liberates cells with CD146 and CD309 expression and morphology consistent with differentiated endothelial cells.

Conclusions

We could not confirm that hematopoietic progenitor cells from human fetal liver have the ability to differentiate in liver sinusoidal endothelial cells *in vivo* and regenerate damaged mouse liver endothelium. Our results suggest that the role of circulating HPC in postnatal neovascularization of liver sinusoids is limited.

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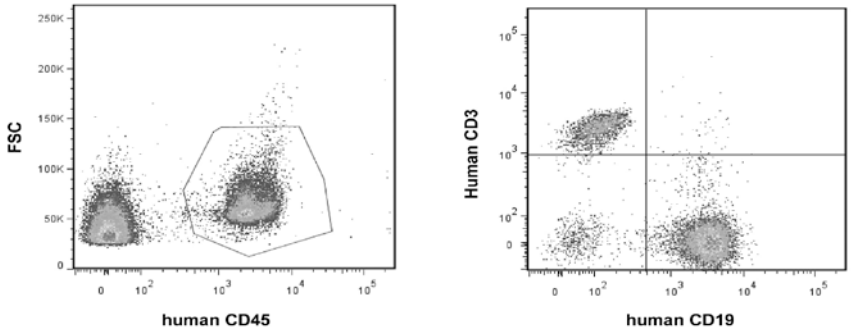

Supplementary figure. Generation of mice with human immune systems by transplantation of human fetal liver-derived hematopoietic progenitor cells.

Hematopoietic progenitor cells (HPC) were isolated after mild mechanical disruption of fetal liver and transplanted in neonatal irradiated (*NOD/SCID IL-2Rγc*$^{-/-}$ *mice* or *BALB/c Rag2*$^{-/-}$*IL-2Rγc*$^{-/-}$) immunodeficient mice. Grafting of human cells was determined by flow cytometry of peripheral blood eight weeks after transplantation. Panel A shows staining for human CD45, in panel B cells gated for human CD45 are shown to express human CD3 and CD19, indicating the presence of differentiated human B and T cells. A typical result is shown.

Table 1: Antibodies used in this study

Marker	Cells	Method
CD3	T-cells	Flow cytometry
CD19	B-cells	Flow cytometry
CD31	LSEC, HPC	Flow cytometry and immunofluorescence
CD34	LSEC, HPC	Flow cytometry and immunofluorescence
CD45	LSEC, HPC	Flow cytometry and immunofluorescence
CD133	LSEC, HPC	Flow cytometry
CD146	LSEC, HPC	Flow cytometry
CD309	LSEC, HPC	Flow cytometry
LYVE-1	LSEC	Immunofluorescence

Various anti-human antibodies were used in the present study. LSEC: liver sinusoidal endothelial cells. HPC: hematopoietic progenitor cells.

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