Mice with humanized liver endothelium

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Chapter 6

General Discussion and Conclusions

General Discussion

In search of potential treatment options for patients suffering from a liver disorder that requires transplantation, scientists have explored the possibilities of liver-directed cell and gene therapy. Although these studies have shown that liver-directed cell and gene therapy is feasible, long-term clinical outcome remains disappointing (1). Translating animal studies to the human situation remains problematic and hampers the outcome of clinical studies. In the present thesis, we sought out to solve this problem by developing a mouse with a "humanized liver". In the first transplantation experiments using human fetal liver cells, we came across a surprising result. Instead of finding human hepatocyte engraftment, we discovered clusters of human liver sinusoidal endothelium throughout the liver of transplanted mice. Apparently, the liver sinusoidal endothelial cells present in the human fetal liver were able to engraft and repopulate the liver following transplantation in the spleen of the immune deficient mice. This was a serendipitous finding and the starting point of the development of a mouse with a humanized liver endothelium. The results of subsequent transplantation experiments increased our knowledge about phenotypic plasticity of differentiated endothelial cells, the inability of human fetal liver-derived hematopoietic progenitor cells to give rise to differentiated endothelium, the capacity of human liver sinusoidal endothelial cells in long-term regulated gene therapy and the usefulness of mice with humanized liver endothelium in studies of vector targeting. However, they also gave rise to several questions. In this chapter our findings will be discussed and suggestions for future experiments given.

Most suitable cell type for mouse liver engraftment and repopulation

Liver-directed cell transplantation requires an unlimited source of healthy cells that are capable of restoring liver function. Many metabolic functions are performed by the hepatocytes, hence most liver-directed cell transplantation experiments have been performed, focusing on transplantation of hepatocytes. Healthy hepatocytes can be isolated from donor livers, but due to donor shortage and the fact that primary hepatocytes only divide once or twice *in vitro*, researchers have tried to create an unlimited source of hepatocytes by manipulating culture conditions and immortalizing hepatocytes using viral vectors. However, this approach has had limited *in vivo* success (2-5). Alternatively, human fetal liver cells represent a suitable source as they are able to expand in culture. Furthermore, fetal hepatoblasts, isolated from rats, are believed to have a higher

engraftment and repopulation potential compared to adult hepatocytes (6-8). We, however, could not reproduce similar results in this thesis as shown in **chapter four**. After transplanting human fetal liver cells intrasplenically in immune deficient mice, multiple clusters of human liver sinusoidal endothelium were present, but no hepatocytes could be detected, even though the human fetal liver contained hepatoblasts. In contrast, human adult hepatocytes were able to engraft the mouse liver using the same transplantation protocol. This surprising result not only emphasizes the potential of human liver sinusoidal endothelial cells in liver-directed cell and gene therapy, but also shows that the ability of transplanted human fetal liver hepatoblasts to graft and/or differentiate into mature hepatocytes within the host liver is limited.

In subsequent transplantation studies, we showed that sinusoidal endothelial cells from human adult liver were also able to engraft and repopulate the murine liver, following intrasplenic transplantation, in line with what we found for human fetal liver sinusoidal endothelial cells. The endothelial cells were kept in culture for a maximum of 5-7 days after isolation from either human adult or fetal liver and never passaged prior to transplantation. In order to serve as a suitable source of cells for liver-directed cell and gene therapy, it is important to know whether human liver sinusoidal endothelial cells keep their liver engraftment and repopulation potential after long-term culture conditions and passaging. This can be easily tested by using our established transplantation protocol as described in this thesis.

Liver sinusoidal endothelial cells and hepatocytes have to be isolated from normal donor liver or from the patient's own liver, before transplantation in the patient can be performed. Unfortunately, there is a donor shortage and the procedure needed to obtain cells from autologous liver remains invasive. With the unexpected discovery that human fetal liver endothelial cells are able to engraft and repopulate the mouse liver, doors were opened to look for an alternative cell source. We isolated endothelial cells from human umbilical vein (macrovascular endothelial cells) and human subcutaneous adipose tissue (microvascular endothelial cells) and compared their potential to engraft and repopulate damaged mouse liver endothelium. It was reported earlier that endothelial cells may exhibit phenotypic plasticity (9, 10). However, in our studies following transplantation of both macrovascular and microvascular endothelial cells, minimal liver engraftment and no liver repopulation was found. Our results show

that endothelial cells are not capable of transdifferentiation *in vivo*, which means they cannot adapt their phenotype depending on their micro-environment and thus do not exhibit phenotypic plasticity. Similar results were found after transplantation of GFP-expressing mouse lung endothelial cells in irradiated allogenic mice. GFP-expressing lung endothelial cells could be located in the lungs up to 24 hours after tail vein injection, but not in the spleen or liver of the transplanted mice (11).

Even though different types of endothelial cells share characteristics, phenotypic differences may play an important role in their ability to engraft in the mouse liver. Phenotypic differences may be due to differences in their extracellular environment, but also because of differences in genetics and epigenetics. This was demonstrated by *in vitro* studies, which used DNA microarrays to reveal differences in transcriptional profiles between endothelial cells isolated from different vascular beds (12). In another study, an increased expression of genes associated with susceptibility to atherosclerosis was found in human coronary arterial endothelial cells following stimulation with oxidized LDL compared to endothelial cells from human saphenous vein (13).

After intrasplenic transplantation several hurdles, such as entrapment of transplanted cells in the sinusoids and circumvention of clearance by Kupffer cells, need to be overcome, before liver engraftment can take place (14-17). A crucial step in liver engraftment is adhesion of transplanted cells to the extracellular matrix and resident liver cells. Cell attachment is mediated by adhesion proteins such as integrins. Integrins are cell surface molecules that bind to components of the extra-cellular matrix and are of great importance for cell invasion and migration (18-20). Since the different types of endothelial cells in our studies were transplanted under identical conditions, differences in their ability to engraft the mouse liver may be due to differences in expression of integrins and/or other cell adhesion molecules. In order to get an answer to this question further studies are needed using fast-developing techniques such as serial analysis of gene expression (SAGE) (21, 22). By comparing differences in gene expression between liver sinusoidal endothelial cells, macro- and microvascular endothelial cells, hopefully significant differences could be identified regarding adhesion molecules that will provide insight in our understanding of liver engraftment and perhaps lead to the development of an unlimited supply of endothelial cells for liver-directed cell and gene therapy.

Besides differentiated endothelial cells, stem cells and/or progenitor cells could also serve as an unlimited source of cells suitable for cell therapy. The liver has a remarkable capacity to regenerate following injury and, like hepatocytes, most of the regeneration of the liver vasculature is due to mitotic division of preexisting endothelial cells (23). However, the presence of recipient endothelial cells in donor liver biopsies several months after liver transplantation has raised the question whether circulating bone marrow-derived progenitor cells contribute to repair of damaged liver endothelium (24, 25). In the **third chapter** of this thesis experiments were performed trying to answer this question and this will be discussed in more detail below.

The role of human hematopoietic progenitor cells in liver regeneration

Stem cells have the capacity for self-renewal, are pluripotent and give rise to cells that differentiate further into multipotent tissue specific stem cells. These multipotent stem cells eventually give rise to cells that generate new tissue (26). Some of the stem cells must undergo cell division without differentiation so that there remains a pool of undifferentiated cells, while other cells proliferate and differentiate (27).

Transplantation of bone marrow-derived stem cells is an established therapy for patients suffering from hematologic diseases. Hematopoietic stem cell transplantation is used for patients suffering from hematologic cancers and inherited disorders such as, X linked SCID, thalassemia major and sickle cell anemia (28). Thanks to the progress made in the field of stem cell therapies, life expectancy and quality of life of these patients is improved. The success of clinical stem cell therapy has led researchers to expand the possibility of using hematopoietic stem cell therapy for other groups of diseases such as acute and chronic heart diseases (29-33), diabetes mellitus (34-36) and also liver diseases (37-40).

Endothelial progenitor cells are considered to be derived from the same precursor as hematopoietic progenitor cells, termed the hemangioblast (41-43). During embryonic development, 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 (44).

In the **third chapter** of this thesis we examined whether human hematopoietic progenitor cells from the fetal liver have the capacity to differentiate into endothelial cells and repair damaged mouse endothelium *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 progenitor 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. In both sets of experiments human fetal liver hematopoietic progenitor cells were not able to engraft and repair damaged mouse liver endothelium.

Several studies have been performed examining the potential of endothelial progenitor cells to repair damaged vasculature. The percentage of successful regeneration of damaged vasculature varies enormously between these studies with studies suggesting an important contribution of progenitor cells in repair of damaged blood vessels (45-48) up to approximately 50% (49). In one of these studies CD133/CD45 positive bone marrow-derived progenitor cells were able to replace up to 30% of damaged rat liver endothelium (50). Strikingly, the clusters of endothelial cells that were supposedly derived from differentiated bone marrow-progenitor cells were able to express the hematopoietic cell marker CD45, a result which could not be reproduced by us or other groups. The expression of CD45 by these clusters suggests that the liver was repopulated by bone marrow-derived monocytes/Kupffer cells rather than endothelial cells as suggested by the authors. In other studies, transplanted endothelial progenitor cells were able to ameliorate liver cirrhosis in rats that had previously been treated with carbon tetrachloride (51-53). In these studies, immunohistohemical analysis of the liver of transplanted rats, anti-CD31 antibody was used to demonstrate the presence of endothelial progenitor cells that had differentiated into liver endothelial cells. However, these stainings are insufficient as CD31 is also expressed by various other cell types as hematopoietic stem cells and/or monocytes. In fact, only indirect evidence was shown as proof for the ability of endothelial progenitor cells to differentiate into liver endothelial cells. We did not observe such cells in transplanted mice livers in the *in vivo* experiments conducted for this thesis and we were not able to reproduce these findings.

On the other hand, several other groups have found minimal (54, 55) to no incorporation at all (56, 57) comparable to our study. There are several reasons that may explain the large discrepancy between these studies such as the use of different mouse strains, the use of different antibodies to identify endothelial cells or differences in isolation and culture methods of progenitor cells, which may influence their capacity to integrate into target tissue. However, it has to be noted that under physiological conditions the amount of progenitor cells found in peripheral blood represents less than 1% of the total number of cells. In most of these studies, including ours, progenitor cells were isolated, cultured and transplanted in excessive amounts exceeding the amount of endogenous progenitors and still only limited to no incorporation was found. Based on these facts and our *in vivo* data, we conclude that hematopoietic progenitor cells derived from the human fetal liver do not give rise to endothelial progenitor cells capable of differentiating into liver sinusoidal endothelial cells.

A fast-growing field of study has been the development of induced pluripotent stem cells (iPS) (58-60). Induced pluripotent stem cells are mature somatic cells (most commonly fibroblasts) that have been reprogrammed to pluripotency following overexpression of a few transcription factors. Several studies thus far have been able to differentiate iPS cells into neurons (61), hematopoietic cells (62) and hepatocytes (63-65). In a recent study, phenotypic correction of murine hemophilia A was achieved using murine iPS cells that had been differentiated into CD31 and CD34 expressing endothelial cells and secreted factor VIII (66). Perhaps similar results can be achieved with human iPS cells. Our protocol for the development of a mouse with a humanized liver endothelium is especially suitable for evaluation of the potential of human iPS cells. Provided that iPS cells are able to differentiate into mature liver sinusoidal endothelial cells, they may be able to repair damaged liver endothelium. However, taking our results, regarding liver engraftment and repopulation potential of human fetal liver-derived stem cells and other endothelial cells into account, we have limited expectations from iPS cells.

Clinical applicability of liver sinusoidal endothelial cells in *ex vivo* gene therapy

Clinical implementation of liver sinusoidal endothelial cells in *ex vivo gene* therapy will require the ability to regulate the expression of genes to maintain expression levels within a therapeutic window (67). In **chapter four** of this thesis, we show

that human liver sinusoidal endothelial cells can be used in *ex vivo* regulated gene therapy. Following *in vitro* transduction using an auto-regulatory lentiviral vector (68), transplanted human liver sinusoidal endothelial cells were able to express the protein erythropoietin *in vivo,* only in the presence of doxycycline. Adding doxycycline to the drinking water was sufficient to regulate hematocrit levels in transplanted mice. Unfortunately, the immunogenicity of the rtTA protein has limited its clinical usefulness following *in vivo* administration (68, 69). By transducing the human liver sinusoidal endothelial cells *ex vivo*, the induction of a cellular and humoral immune response may be circumvented because no transduction of antigen presenting cells takes place (70, 71). Furthermore, liver sinusoidal endothelial cells are known to cross-present exogenous antigen on major histocompatibility class I (MHC I) molecules to CD8+ T cells. Instead of inducing immunity, cross-presentation by liver sinusoidal endothelial cells results in tolerance, (72-74) suggesting that liver sinusoidal endothelial cells could be used in *ex vivo* gene therapy to avoid an immune response to the transgene. Transplantation experiments in immune competent mice are necessary to investigate whether *ex vivo* transduction of transplanted mouse (or human) liver sinusoidal endothelial cells can prevent induction of a cytotoxic immune response.

Conditioning of the patient by inflicting damage to the liver endothelium before cell administration is an important aspect of successful cell engraftment and needs to be optimized before human liver sinusoidal endothelial cells can be considered for clinical application. In this thesis we used the drug monocrotaline to damage mouse liver endothelium in order to increase engraftment and repopulation of transplanted human liver sinusoidal endothelial cells. Unfortunately, monocrotaline is toxic, has oncogenic potential and so cannot be used in the clinic. Sorafenib, is a multikinase inhibitor that is widely used for the treatment of patients suffering from advanced renal cell carcinoma or unresectable hepatocellular carcinoma (75) and has been shown to inhibit neoangiogenesis through VEGF-mediated autophosphorylation of VEGFR-2 expressed by endothelial cells (76). Cyclophosphamide is an alkylating agent used for the treatment of patients suffering from cancer and as a conditioning regimen for bone marrow stem cell transplantation (77, 78). Hepatocyte transplantation experiments in rats have shown that it facilitates engraftment and repopulation by disrupting the hepatic endothelial cell barrier (79). In a pilot experiment we therefore performed transplantation experiments using sorafenib and cyclophosphamide as alternative conditioning agents. However, human liver

sinusoidal endothelial cell engraftment following intrasplenic transplantation and conditioning with either sorafenib or cyclophosphamide was low compared to mice that had been conditioned with monocrotaline (unpublished data). This may be due to suboptimal doses used in our experiments. Nevertheless, more experiments are required before the use of both substances can be excluded for application in liver-directed cell and gene therapy with human liver sinusoidal endothelial cells. Additionally, doxorubicin, which is also a clinically approved drug and has previously been shown to be effective at disrupting the liver endothelium (80), may be evaluated as an alternative to monocrotaline for clinical application of human liver sinusoidal endothelial cell and gene therapy.

The potential of a mouse model with a humanized liver endothelium

Studies into therapy for human diseases including inherited liver disorders have mostly been performed in rodents as experimental animal models. A major disadvantage of this approach is the considerable species difference in physiology between humans and rodents. For example, studies on drug metabolism are impaired by the large differences in cytochrome P450 enzyme expression between humans and rodents. In recent years, various types of humanized mice, transplanted with human cells or tissues (81, 82), have been developed to overcome this problem. Over the years, the development of humanized mice has progressed rapidly and it is now possible to achieve high levels of human chimerism in various organs and tissues such as the immune system (83) and liver (84-88). These mouse models provide new opportunities in the development of therapeutic agents for human use.

In the **fifth chapter** of this thesis our mouse model with a humanized liver endothelium has proven very useful in the evaluation of targeted gene therapy *in vivo*. Here we show that upon systemic administration of a human CD105 targeted lentiviral vector, human liver endothelial cells in mice with humanized liver endothelium were transduced with high specificity. Besides the usefulness in the development of *in vivo* gene therapy, a mouse with a humanized liver endothelium could also be very helpful in studies concerning liver drug metabolism and their role in human liver tumorigenesis (89).

Mouse models that combine several humanized organs and compartments will have even better clinical translational capacity. Human liver endothelial cells could for example be co-transplanted with human hepatocytes and lead to a more

potent mouse model that is able to better answer questions in the field of drug metabolism and liver pathophysiology.

As mentioned in the introduction, one of the remarkable characteristics of the liver is its capacity to regenerate following loss of cell mass (90, 91). Liver resections are performed regularly in the treatment of hepatic tumors. However, resections have to be kept to a minimum to preserve enough residual functional liver tissue and prevent fatal clinical course (92, 93). Clinical studies have shown that, unfortunately, incomplete liver regeneration forms an important unresolved problem (94). Therefore, therapeutic strategies that improve liver regeneration would be of great benefit, especially for those patients with impaired liver regenerative capacity due to cirrhosis or acute necrosis of the liver. Several studies have shown that erythropoietin stimulates liver regeneration (95-97). In these studies erythropoietin was administrated intravenously, subcutaneously or intraperitoneally. We have shown in **chapter four** that liver sinusoidal endothelial cells can be successfully transduced with a lentiviral vector expressing rat erythropoietin. Following transplantation, these cells were able to engraft in the mouse liver and express erythropoietin under the regulation of doxycycline. In order to examine whether liver sinusoidal endothelial cells can be used for stimulating liver regeneration, experiments in which mice are subjected to partial hepatectomy followed by an intrasplenic transplantation of erythropoietin-expressing liver sinusoidal endothelial cells the next day could be performed. In patients, liver endothelial cells could be isolated from the patient's own resected liver tissue and *in vitro* transduced by an erythropoietin expressing lentiviral vector.

Conclusions

Liver sinusoidal endothelial cells are a specific type of endothelial cell with a characteristic phenotype, and play an important role in multiple crucial functions of liver physiology (98). The studies performed in this thesis have shown that human liver sinusoidal endothelial cells have the unique ability to engraft and repopulate the mouse liver niche, while macrovascular and microvascular endothelial cells fail to do so.

Unexpectedly, differentiated human liver sinusoidal endothelial cells had a much higher liver engraftment and repopulation potential than human fetal liver-derived progenitor cells. This group of cells, which includes hepatoblastst as well as hematopoietic stem cells, failed to generate mature functional liver cells *in vivo* following transplantation.

Subsequently, we took advantage of the efficient repopulation capacity of human liver endothelial cells by developing *ex vivo* regulated erythropoietin gene therapy and used to mice with humanized liver endothelium as a model to study targeted *in vivo* gene therapy.

The results described in this thesis have set a basis for the use of human liver endothelial cells in cell transplantation and *ex vivo* gene therapy. However, they have also generated more questions about factors that play a role in liver engraftment and repopulation and emphasize the need for further experimentation. Before a step towards clinical application of human liver endothelial cells in cell transplantation and *ex vivo* gene therapy can be made, more work will need to be performed focusing on optimizing the repopulation success of transplanted human liver endothelial cells, elucidating the cause of differences in engraftment and repopulation capacity between different types of endothelial cells using fast developing techniques such as DEEP-SAGE and examining the role and effect of the immune system on long-term clinical effect of *ex vivo* gene therapy. Although a lot of challenges remain, the progress that has been made in the field of cell transplantation and *ex vivo* gene therapy so far could well lead to potential treatment options for patients that suffer from an inherited liver disorder.

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