Liver transplantation : chimerism, complications and matrix metalloproteinases

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Liver transplantation

Chimerism, complications and matrix metalloproteinases

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Contents

Chapter 1

Introduction

In December 1963 Dr. Thomas Starzl from Denver, Colorado, published his first three attempts of liver transplantation in humans.[1] The first patient he described was a three year old boy with biliary atresia. He bled to death during the procedure. The other two patients had cirrhosis and a malignant liver tumor and were 48 and 67 year old males. Technically the liver transplant procedure was successful. However, both patients died of pulmonary emboli, 22 and 7½ days after the procedure. In the decades that followed, liver transplantation evolved from an extremely hazardous into a standardized procedure with increasing survival rates and in 1983 the NIH declared liver transplantation an accepted therapy for end-stage liver disease.[2]

The first liver transplantation in the Netherlands was performed in 1966 in Leiden University Medical Center, but due to coagulopathy the patient did not survive the procedure. Years later, in 1979, a successful liver transplant was performed in Groningen University Medical Center and in the years that followed liver transplant programs were also started in Rotterdam (1986) en Leiden (1992).

Nowadays, well over 10.000 liver transplant procedures are performed each year worldwide and it is the treatment of choice for acute and chronic liver failure. One year and five year survival rates are around 90% and 85%, respectively.

In the early days surgical techniques and control of hemorrhage were of major concern. The use of cyclosporin A from 1983 on contributed enormously to successful immunosuppression and thus to improved graft and patient survival. Recently, research has shifted towards consequences of long-term survival, such as quality of life issues and recurrent disease within the liver graft. Although the outcome of liver transplantation has improved, the risk of serious complications still remains. Surgical complications, blood loss, rejection, biliary complications and infections all pose serious threats to the graft and its recipient.

The chapters 2, 3 and 7 of this thesis focus on chimerism, that is the coexistence of cells of different genetic origin within one organism. With organ transplantation, cells of two different organisms are brought together. Several questions arise: Do cells of recipient origin replace cells within the graft? Can transplanted blood stem cells develop into mature liver cells? Can cells of the transplanted organ be found elsewhere in the body? What is the clinical relevance of chimerism?

Chapters 4 and 6 focus on a fascinating group of proteolytic enzymes,

matrix metalloproteinases, in relation to complications after liver transplantation. Here questions are: Is the genetic make-up of these enzymes relevant? Is a different genotype of donor and recipient associated with the occurrence of complications like ischemia/ reperfusion injury, rejection and biliary strictures?
More everyday clinical tests are addressed in chapter 5: The value of routinely assessed liver enzymes and the liver ultrasound, for predicting biliary complications after liver transplantation is described using a time-dependent statistical model.
Chapter 7 addresses chimerism in liver tissue biopsies and in peripheral blood after liver transplantation, in those patients with a donor/acceptor mismatch for the studied matrix metalloproteinases.

Chimerism

Chimerism in medicine is defined as the coexistence of cells of donor and recipient origin within a single organism. This phenomenon was first described in autopsies on pregnant women who died from eclampsia, with fetus-derived cells in the maternal circulation.[3,4] Subsequently, chimerism has been described frequently in pregnant women with fetal cells present in maternal blood.[5,6] Similarly, dizygotic twins have shown to be chimeric for each other's blood group.[7,8] Potential sources for chimeric cells, other than pregnancy, are iatrogenic, namely blood transfusion and transplantation.[9]
The possible immunological consequences of chimerism are intriguing. Chimeric cells may be silently present, without interacting with the host's immune system, e.g., resulting from pregnancy. It has also been hypothesized that chimeric cells may induce autoimmune disease by instigating loss of tolerance to self-antigens. This is supported by observations that chimerism is present more often in patients with autoimmune diseases.[10-12] In transplantation medicine chimerism may enhance graft tolerance.
In the early days of solid organ transplantation it has been postulated that cells of the recipient could replace cells in a transplanted organ and that this could lead to graft tolerance. Many studies have addressed this phenomenon with disputing and even conflicting results, and the relevance of chimerism in transplantation is still quite unclear. [13-16]

We studied the existence of chimerism within the transplanted liver, looking at different lineages of non-lymphoid cells. A selection was made of male patients who had received a liver graft from a female

donor. In liver tissue biopsies cells of recipient origin were identified using in-situ hybridization for sex chromosomes. Findings of this study are described in **chapter 2**.
Extensive chimerism within transplanted livers can only be understood if circulating stem cells can develop into liver cells of mesenchymal phenotype. We used a different transplant model to study this. Female recipients were selected that had received allogeneic bone marrow transplantation from a male donor (for hematologic malignancies). Only if liver tissue was available the patients could be included in the study.

Again, sex chromosome identification was used to identify the origin of cells in liver specimens, as reported in **chapter 3**.
If chimerism is a persisting feature after liver transplantation, one would expect donor-derived cells even in peripheral blood samples late after transplantation. The study reported in **Chapter 7** not only focuses on chimerism in liver biopsies after transplantation, but also on chimerism in peripheral blood samples.

Matrix metalloproteinases and biliary complications

Matrix metalloproteinases
The matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that are important in many physiologic processes requiring matrix turnover. Basement membrane and matrix components like collagen, elastin, gelatin and casein are major components cleaved and degraded by these MMPs. The breakdown of these components is essential for many physiological processes such as embryonic development, growth, reproduction, tissue resorption and remodelling.[17-19] MMPs are also implicated in a variety of pathological processes such as arthritis, inflammatory bowel disease, cancer, and ischemic cardiovascular and neurological diseases.[20-22]
Among the different MMPs, the gelatinases MMP-2 and MMP-9 are of particular interest in liver pathophysiology. The main cellular source of MMP-2 is the hepatic stellate cell, whereas the principal sources of MMP-9 are the leukocytes and Kupffer cells. Expression of MMP-2 is increased in patients with chronic liver disease.[23,24] Different MMP genes have been shown to contain polymorphisms in their promoter region. These promoter polymorphisms have specific effects on the regulation of both MMP gene transcription and expression.

The donor liver graft is exposed to warm and cold ischemia with severe hypoxia before and during the transplant procedure. While ischemia

primes the cells for damage, the actual injury usually becomes manifest after the restoration of blood flow, i.e. the reperfusion. Many factors contribute to the extent of this ischemia-reperfusion (I/R) injury. **Chapters 4** and **6** address the genetic MMP make-up of both donor and recipient, in relation to clinical complications as I/R injury, rejection and the development of biliary complications.

Biliary strictures

Biliary complications are a significant cause of morbidity and even graft loss after liver transplantation. The most common biliary complications are biliary leakage and biliary tract strictures.[25-27] Anastomotic leakage occurs early after the transplantation procedure, whereas strictures occur later. Strictures can be divided into anastomotic and non-anastomotic. Anastomotic strictures occur at the anastomosis of the donor common bile duct and the recipient common bile duct (duct to duct anastomosis) or of the donor common bile duct with a recipient jejunal Roux-en-Y limb (hepaticojejunostomy). Strictures occurring at the anastomosis are usually due to surgical difficulties and/or local ischemia. Non-anastomotic strictures are thought to result from ischemia of the biliary epithelium by compromised arterial blood flow, hepatic artery thrombosis and/or ischemia/reperfusion injury. More complex immunologic factors and cytotoxic injury by bile salts may also contribute to non-anastomotic biliary strictures.[28,29]

We studied the relationship between MMP-2 and MMP-9 gene promoter polymorphisms in the donor and recipient DNA and the development of non-anastomotic biliary strictures after liver transplantation, the findings of which are described in **chapter 6**.

Biliary stricture formation is often insidious and typically first detected when biliary obstruction results in serum liver enzyme abnormalities, intrahepatic bile duct dilatation and/or infection. Imaging of the biliary tree is mandatory to make a definitive diagnosis. A cholangiography can be obtained endoscopically (ERCP), percutaneously (PTC) or by using magnetic resonance imaging (MRCP). Although ERCP and PTC are considered the golden standard to diagnose and treat strictures, they are invasive procedures. The predictive value of serum liver enzymes and abdominal ultrasonography for the development of non-anastomotic biliary strictures has been investigated before, but the results were not conclusive.[30,31] We performed a time-dependent statistical analysis to assess the predictive value of serum liver enzymes and abdominal

ultrasound as a first step in the diagnosis of biliary strictures after liver transplantation **(chapter 5**).

A summarizing discussion of the results obtained in the different studies as described in the separate chapters of this thesis is given in **chapter 8**. Finally, **chapter 9** provides a general discussion of the findings of this thesis in the Dutch language.

References

1. Starzl TE, Marchioro TL, Vonkaulla KN, Hermann G, Brittain RS, Waddell WR. Homotransplantation of the liver in humans. Surg Gynecol Obstet 1963;11:659-76.

2. National Institutes of Health Consensus Development Conference Statement: liver transplantation --June 20-23, 1983 Hepatology 1984:4:107S-10S.

3. Lapaire O, Holzgreve W, Oosterwijk JC, et al. Georg schmorl on trophoblasts in the maternal circulation. Placenta 2007;28:1-5.

4. Schmorl G: Patholigisch-anatomische Untersuchungen ueber Puerperal Eklampsie. Leipzig, Vogel, 1893.

5. Hamada H, Arinami T, Kubo T, et al. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet 1993:91:427-432.

6. Lo YM, Patel P, Wainscoat JS, et al. Prenatal sex determination by DNA amplification from maternal peripheral blood. Lancet 1989;2:1363-1365.

7. Booth PB, Plaut G, James JD, et al. Blood chimerism in a pair of twins. Br Med J 1957;1:1456-1458.

8. Nicholas JW, Jenkins WJ, Marsh WL. Human blood chimeras a study of surviving twins. Br Med J 1957;1:1458-1460.

9. Lee TH, Paglieroni T, Ohto H, et al. Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. Blood 1999;93:3127-3139.

10. Nelson JL, Maternal-fetal immunology and autoimmune disease: is some autoimmune disease auto-alloimmune or allo-autoimmune? Arthritis Rheum 1996;39:191-194.

11. Klintschar M, Schwaiger P, Mannweiler S, et al. Evidence of fetal microchimerism in Hashimoto's thyroiditis. J Clin Endocrinol Metab 2001;86:2494-2498.

12. Corpechot C, Barbu V, Chazouilleres O, et al. Fetal microchimerism in primary biliary cirrhosis. J Hepatol 2000;33:696-700.

13. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. Lancet 1992;339:1579-1582.

14. Demetris AJ, Murase N, Fujisaki S, Fung JJ, Rao AS, Starzl TE. Hematolymphoid cell trafficking, microchimerism, and GVH reactions after liver, bone marrow, and heart transplantation. Transplant Proc 1993;25:3337-2244.

15. Bishop GA, Sun J, Sheil AG, McCaughan GW. High-dose/ activation-associated tolerance: A mechanism for allograft tolerance. Transplantation 1997;64:1377-1382.

16. Meyer D, Loffeler S, Otto C, Czub S, Gassel HJ, Timmermann W, et al. Donor-derived alloantigen-presenting cells persist in the liver allograft during tolerance induction. Transpl Int 2000; 13:12-20.

17. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998;10:602-608.

18. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. Matrix metalloproteinases in chronic liver disease and liver transplantation. Lendeckel U, Hooper NM, editors. Dordrecht, the Netherlands, Springer. Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues. 2006:209-234.

19. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463-516.

20. Zhang B, Ye S, Herrmann SM, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999; 99:1788-1794.

21. Kubben FJ, Sier CF, Meijer MJ, et al. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. Br J Cancer. 2006;95:744-751.

22. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007;13:2960-2966.

23. Milani S, Herbst H, Schuppan D, et al. Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol 1994;144:528-537.

24. Kuyvenhoven JP, van Hoek B, Blom E, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003;89:718-725.

25. Qian YB, Liu CL, Lo CM, Fan ST. Risk factors for biliary complications after liver transplantation. Arch Surg 2004;139:1101-1105.

26. Rerknimitr R, Sherman S, Fogel EL, et al. Biliary tract complications after orthotopic liver transplantation with choledochocholedochostomy anastomosis: endoscopic findings and results of therapy. Gastrointest Endosc 2002;55:224- 231.

27. Turrion VS, Alvira LG, Jimenez M, et al. Management of the biliary complications associated with liver transplantation: 13 years of experience. Transplant Proc 1999;31:2392-2393.

28. Guichelaar MM, Benson JT, Malinchoc M, Krom RA, Wiesner RH, Charlton MR. Risk factors for and clinical course of non-anastomotic biliary strictures after liver transplantation. Am J Transplant 2003;3:885-890.

29. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13:517-524.

30. Zoepf T, Maldonado-Lopez EJ, Hilgard P, Dechene A, Malago M, Broelsch CE et al. Diagnosis of biliary strictures after liver transplantation: which is the best tool? World J Gastroenterol 2005; 11(19):2945-2948.

31. Hussaini SH, Sheridan MB, Davies M. The predictive value of transabdominal ultrasonography in the diagnosis of biliary tract complications after orthotopic liver transplantation. Gut 1999; 45(6):900-903.

Chapter 2

Extensive chimerism in liver transplants: Vascular endothelium, bile duct epithelium, and hepatocytes

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Liver Transplantation 2003;9:552-556.

Abstract

Background

The transplanted liver has been shown to be particularly capable of inducing tolerance. An explanation may be the presence of chimerism. Cells of donor origin have been found in recipient tissues after transplantation of any solid organ. Evidence for the presence of cells of recipient origin within the transplanted liver is very limited. We investigated whether nonlymphoid cells of recipient origin can be found within human liver allografts.

Methods

Five male patients who received a liver transplant from a female donor and 11 patients who received an HLA-I mismatched liver transplant were studied. We confirmed our observations with two different techniques in combination with double-staining techniques. To identify male cells in female liver transplants, we used in situ hybridiza- tion for sex chromosomes. To identify specific HLA class I antigens of recipient origin, we used immunohistochemistry with HLA class I–specific antibodies. Double staining was performed to discriminate different cell lineages and inflammatory cells.

Results

Endothelial cells of recipient origin were found in 14 of 16 donor livers. Bile duct epithelial cells of recipient origin were found in 5 of 16 cases. Hepatocytes of recipient origin were seen in only 1 of the 5 studied sex-mismatched donor livers.

Conclusion

Our study provides evidence that cells of recipient origin can replace biliary epithelial cells, endothelial cells, and hepatocytes within the human liver allograft.

This is consistent with the concept that circulating pluripotent progenitor cells exist, capable of differentiating into endothelial cells, epithelial cells, and hepatocytes.

Introduction

In the early days of solid-organ transplantation, it was postulated that the success of renal transplantation could be explained by the existence of chimerism within the graft. Especially chimerism of endothelial cells was thought to be relevant because endothelium is one of the major targets for graft rejection. Replacement of donor endothelial cells by recipient cells therefore would reduce the immunogenicity of the graft.[1] During the past four decades, several studies addressed the issue of intragraft chimerism in solid-organ transplants, with conflicting results.[2-7] Most studies did not find chimerism or found it only sporadically in poorly functioning grafts. Therefore, it became generally believed that non- lymphoid cells in organ grafts remain of donor origin. However, we found clear evidence of endothelial cell chimerism in renal allografts, and cardiac chimerism has also been described recently.[8,9]

The liver, in comparison to other transplanted organs, has been shown to be particularly capable of inducing tolerance.[10-13] A number of hypotheses have been put forward to explain this immune-privileged state. One of these is the presence of chimerism.[14-16] Other factors mentioned are regenerative capacity and the production of soluble major histocompatibility complex. Many studies addressed the issue of chimerism outside the graft resulting from donor-derived highly immunogenic passenger leukocytes, of which the liver is particularly rich. Donor-derived cells can be found in recipient peripheral tissues years and even decades after transplantation.[14,17] The clinical relevance of the persistence of donor leukocyte chimerism is still unclear.[18]

Little is known about chimerism within the human liver allograft. Only few have studied this human hepatic intragraft chimerism.[19,20] For decades, it has been the general belief that only Kupffer cells of recipient origin can be found within the transplant, whereas endothelial cells, bile duct epithelial cells, and hepatocytes remain of donor origin.[19] In animal studies, evidence is growing that bonemarrow–derived stem cells can differentiate into various hepatic cell types, such as hepatocytes and endothelial cells.[21-24] We therefore investigated whether nonlymphoid cells of recipient origin can be found within human liver allografts.

Patients and Methods

Patients and Biopsy Specimens

Five male patients who received a liver transplant from a female donor were selected. None of the female donors had had male offspring. In addition, 11 patients with HLA-I mismatching allografts for A2, A3, A9, or A11 were studied. All patients had undergone orthotopic liver transplantation at Leiden University Medical Center (Leiden, The Netherlands) between 1993 and 1998. Liver biopsy samples were obtained 1 year after transplantation, according to protocol. Additional biopsy specimens obtained early after transplantation were studied of one selected patient with evidence of extensive chimerism in the 1-year biopsy specimen. As per protocol, a part of every biopsy specimen had been stored at 80°C, whereas the other part was formalin fixed and stored in paraffin.

Approval by the Ethics Committee of Leiden University Medical Center was obtained.

HLA Typing

HLA typing for antigens of class I was performed using standard serological methods by complement-dependent microcytotoxicity on peripheral-blood leukocytes of recipients and splenocytes of donors, which are available as part of the routine pretransplantation workup.

In Situ Hybridization Sex Chromosomes

For sex-chromosome identification in the five sex-mismatched grafts, we performed in situ hybridization using repetitive DNA probes specific for X and Y chromosomes, as previously described.[25] Briefly, probes were biotinylated by nick translation and dissolved in a 60% formamide hybridization mixture. Paraffin sections 6-mm thick were cut and mounted on poly-L-lysine–coated slides. Predigestion steps consisted of incubation in 1 mol/L of sodium thiocyanate solution at 80°C, followed by 60 to 90 minutes of treatment with 0.5% pepsin in 0.1 mol/L of hydrochloric acid. Hybridization was performed overnight at 42°C. The hybridization reaction was visualized with avidin, biotinylated goat antiavidin, and avidin-peroxidase developed with diaminobenzidine. Positive and negative controls for in situ hybridization were biopsy specimens from normal male and female livers.

Immunohistochemical Analysis

For HLA class I antigens, immunohistochemical staining was performed

on cryostat sections from the 11 HLA-I–mismatched grafts, as previously described.[26] In short, sections were fixed in cold acetone and incubated with the primary antibody. Four monoclonal antibodies were used that recognize the HLA class I antigens A2, A3, A9, and A11 (American Type Culture Collection, Rockville, MD). A two-step immunoperoxidase technique was used with 3-amino-g-ethyl-carbazol as a coloring substrate. Each patient was tested with all antibodies, which provided many positive and negative controls. For additional negative controls, the second antibodies were replaced by phosphate-buffered saline. Recipient-derived graft-infiltrating cells stained positive for recipient major histocompatibility complex antigens and thus served as internal positive controls.

Additional Staining Techniques

For double staining, antibodies against endothelial cell–specific antigens (CD31, factor VIII; Dako, Carpinteria, CA) against lymphocytes, monocytes, and other inflammatory cells (CD45-LCA; Dako) and bile duct epithelial cells (keratin 18; LUMC, Leiden, The Netherlands) were combined with immunohistochemistry with HLA class I–specific antibodies and in situ hybridization with sex chromosomes. When possible, double staining was realized on the same slide. In other cases, different staining techniques were performed on consecutive slides.

Results

In Situ Hybridization for X and Y Chromosomes

Endothelial cells of recipient origin, i.e., Y chromosome positive, were found in all five sex-mismatched patients. A detail of a biopsy sample of a female liver transplanted into a male recipient can be seen in Figure 1A. This detail of a vessel wall shows an endothelial cell staining positive with the Y chromosome probe, indicating male (recipient) origin. Hepatocytes of recipient (male) origin were seen in only one of five studied patients, shown in Figure 1B. Most hepatocytes stain negative, indicating the donor origin of these cells, but some contain a Y chromosome. Hepatocytes can be tetraploid, which explains why two spots sometimes can be seen within one cell. Partial nuclear sampling in tissue sections may lead to undercounting of Y-positive nuclei. Recipient-derived bile duct epithelial cells were seen in three of five patients. Figure 1C shows a biopsy sample of a female liver graft transplanted into a male recipient 1-year posttransplantation, showing a bile duct. Epithelial cells containing a Y chromosome can be seen,

indicating the presence of male epithelial bile duct cells of recipient origin. Double staining with periodic acid–Schiff (PAS) makes the bile duct stand out because bile duct epithelial cells are PAS negative. Figure 1D shows the same bile duct in a consecutive slide with CD45 staining to confirm that the duct is free of inflammatory cells.

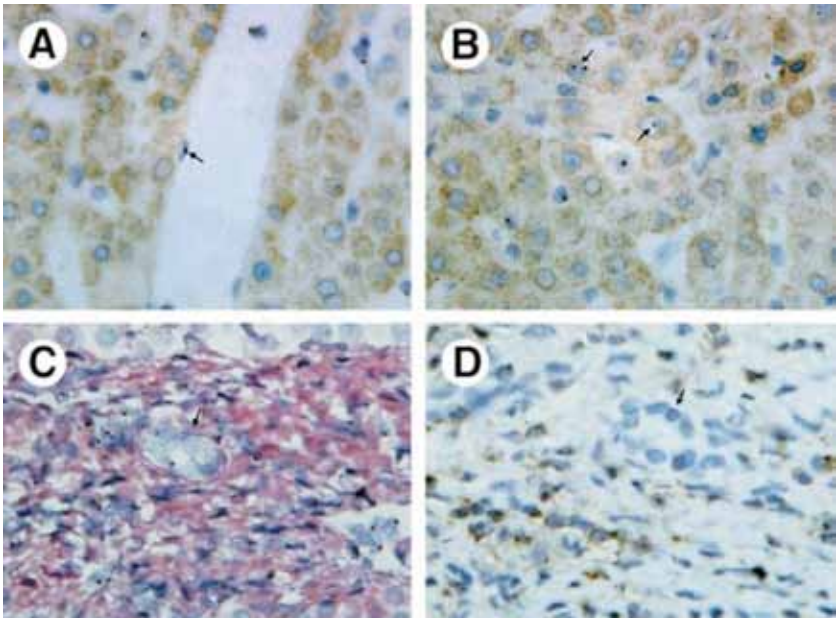

Figure 1.
In situ hybridization for Y chromosomes. A female donor liver was transplanted into a male recipient. In situ hybridization with a Y-specific centromeric DNA probe was performed on a posttransplantation biopsy sample. In (A) endothelial cells of middle large vessels, (B) hepatocytes, and (C, D) bile duct epithelial cells, clear signals can be appreciated after in situ hybridization with this probe, indicating that chimerism has taken place in all three cell types. (C) A PAS background that makes the bile duct stand out because cholangiocytes are PAS negative. (D) Staining on a consecutive slide with CD45 and no inflammatory cells can be seen in or near the bile duct. The female donor of this graft had no male offspring. The presence of endogenous biotin in hepatocytes causes some background staining. (Original magnification X400.)

Immunohistochemistry for HLA Class I Antigens

A biopsy sample of an HLA-A2–negative liver graft transplanted into an HLA-A2–positive recipient is shown in Figure 2. A bile duct with biliary epithelial cells staining positive for recipient type HLA-A2 is shown in Figure 2A. This chimerism of bile duct epithelial cells was observed in 2 of these 11 HLA-mismatched patients and could be seen in smaller, as well as larger, bile ducts. Replacement of donor type vascular endothelium by recipient type could be observed to a variable degree in 9 of the 11 studied patients. Sinusoidal endothelium staining positive for recipient-

type HLA-A2 is shown in detail in Figure 2B, with double staining with CD31, which stains endothelial cells irrespective of their origin. Hepatocytes were difficult to differentiate with certainty from inflammatory cells with this technique and therefore were not scored in this series.

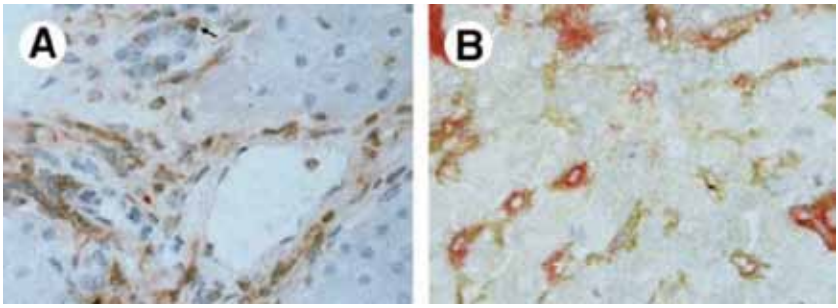

Figure 2.
Staining with recipient-type HLA antibody. An HLA-A2-negative liver was transplanted into an HLA-A2-positive recipient. Immunohistochemical staining against the HLA type of the recipient (HLA-A2) was performed on a biopsy sample obtained 1 year posttransplantation. (A) Bile duct epithelial cells stain positive for recipient type HLA-A2. (B) Sinusoidal endothelium staining positive for recipient type HLA-A2 is shown in red, and double staining against factor VIII is shown in brown. (Original magnification X 400.)

To investigate whether chimerism can develop earlier than 1 year after transplantation, we studied serial liver biopsy specimens by HLA staining from a patient in whose graft we found extensive endothelial cell chimerism 1 year after transplantation. We found no evidence for chimerism in the biopsy sample obtained 1 week after transplantation, whereas it became apparent in the specimens obtained 3 months after transplantation.
One male HLA-A3–negative patient received an HLA-A3–positive graft from a female donor. In this patient, we used both in situ hybridization for Y chromosomes and immunohistochemistry for HLA-A3. We observed endothelial cell chimerism, but no bile duct epithelial cell chimerism, with both techniques in this patient.

Discussion

Our study provides evidence that cells of recipient origin can replace biliary epithelial cells, endothelial cells, and hepatocytes within the human liver allograft. We confirmed our observations using two different techniques. Additional staining techniques were performed to distinguish different cell lineages from inflammatory cells of recipient

origin. Replacement of biliary duct epithelium was observed in one third of patients (5 of 16 patients with the two techniques combined). Endothelial cell chimerism was found to be very common (14 of 16 patients). Hepatocytes of recipient origin were found in only one of five donor livers studied with in situ hybridization for X and Y chromosomes (the HLA stain is not suitable for looking at hepatocyte chimerism). Chimerism within the human liver transplant to such extent that it involves endothelium, bile duct epithelium, and hepatocytes has not been reported previously. For decades, the general belief has been that lymphocytes and Kupffer cells of recipient origin are found in the liver, but graft bile duct epithelium, hepatocytes, and endothelium were considered to remain of donor origin.[19,27,28]

Gouw et al[19] reported in 1987, with the techniques available at that time, that endothelium, bile duct epithelium, and hepatocytes remained of donor origin. However, recent reports indicate that replacement of donor cells by recipient-derived cells occurs much more frequently than was generally assumed. For instance, Gao et al[20] found male endothelial cells in female liver graft recipients. Similarly, Theise et al[29] reported male hepatocytes and cholangiocytes in female liver grafts recipients; however, Fogt et al[7] recently did not find convincing evidence of stem-cell engraftment into transplanted liver tissue.

Baccarani et al[30] argued that the presence of male cells in female donor livers can result from previous male pregnancies of the donor because this is a verycommon phenomenon in women with male offspring. To date, all published studies mentioned did not provide information concerning pregnancies of the donor. In the present study, we observed Y chromosome–positive cells in grafts from female donors without male offspring (G. Persijn, Eurotransplant Foundation, Leiden, The Netherlands, personal communication, January 2003). In a graft from which serial biopsy specimens were obtained, we did not find endothelial chimerism 1 week after transplantation, whereas it was evident after 3 months. Furthermore, HLA antigens of the recipient are extremely unlikely to be identical to HLA antigens of the offspring of the donor. Our data therefore provide evidence that the observed endothelial cells, bile duct cells, and hepatocytes are of recipient origin and not derived from previous male pregnancies of the female donor.

In the present study, endothelial cell chimerism was much more common than chimerism involving bile duct epithelium. This observation is

consistent with the previously postulated concept that chimerism results from repair of damage, although other causes cannot be excluded.[31]

The liver is very susceptible to vascular damage and, to a lesser extent, bile duct damage caused by ischemia-reperfusion injury and acute cellular rejection.[32,33] This may explain the greater percentage of endothelial chimerism than bile duct epithelial chimerism in the present study. Possibly, apoptotic or necrotic hepatocytes may be replaced mainly by regeneration from a local pool of donor hepatocytes.

Serious damage to biliary ducts can be observed during chronic ductopenic rejection. This complication, which often leads to graft loss, has become a rare condition and was not present in our patients. Although unusual, repair of bile ducts has even been reported in this condition.[34,35] Our findings support the possibility of bile duct repair originating from circulating recipient precursor cells, although this does not exclude that it can occur next to repopulation from a local pool of donor oval cells.

In conclusion, our study provides evidence that recipient-derived cells can replace biliary epithelium, endothelium, and hepatocytes in liver transplants. This is consistent with the concept that circulating progenitor cells exist, capable of differentiating into endothelial and epithelial cells.[21,22]

Contributors

Rogier ten Hove, Malice Lagaaij, Han van Krieken, and Bart van Hoek planned and organized the study, analyzed the data, and wrote the manuscript.
Han van Krieken and Ingeborg Bajema helped design the study and supervised staining techniques. Bart van Hoek initiated collaboration and collected clinical data. Jan Ringers collected data.

References

1. Medawar PB. Transplantation of tissues and organs: Introduction. Br Med Bull 1965;21:97-99.

2. Sinclair RA. Origin of endothelium in human renal allografts. BMJ 1972;4:15-16.

3. Williams GM, ter Haar A, Parks LC, Krajewski CA. Endothelial changes associated with hyperacute, acute, and chronic renal allograft rejection in man. Transplant Proc 1973;5:819-822.

4. Bogman MJ, de Waal RM, Koene RA. Persistent expression of donor antigens in endothelium of long-standing skin xenografts and vulnerability to destruction by specific antibodies. Transplant Proc 1987;19:205-207.

5. Sedmak DD, Sharma HM, Czajak CM, Ferguson RM. Recipient endothelialization of renal allografts. An immunohistochemical study utilizing blood group antigens. Transplantation 1988; 46:907-910.

6. Bittmann I, Dose T, Baretton GB, Muller C, Schwaiblmair M, Kur F, Lohrs U. Cellular chimerism of the lung after transplantation. An interphase cytogenetic study. Am J Clin Pathol 2001; 115:525-533.

7. Fogt F, Beyser KH, Poremba C, Zimmerman RL, Khettry U, Ruschoff J. Recipient-derived hepatocytes in liver transplants: A rare event in sex-mismatched transplants. Hepatology 2002;36: 173-176.

8. Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ, van Es LA, Bruijn JA, van Krieken JH. Endothelial cell chimerism after renal transplantation and vascular rejection. Lancet 2001;357:33-37.

9. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, et al. Chimerism of the transplanted heart. N Engl J Med 2002;346:5-15.

10. Calne RY, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, et al. Induction of immunological tolerance by porcine liver allografts. Nature 1969;223:472-476.

11. Houssin D, Gigou M, Franco D, Bismuth H, Charpentier B, Lang P, Martin E. Specific transplantation tolerance induced by spontaneously tolerated liver allograft in inbred strains of rats. Transplantation 1980;29:418-419.

12. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. Lancet 1992;339:1579-1582.

13. Murase N, Starzl TE, Tanabe M, Fujisaki S, Miyazawa H, Ye Q et al. Variable chimerism, graft-versus-host disease, and tolerance after different kinds of cell and whole organ transplantation from Lewis to Brown Norway rats. Transplantation 1995;60:158-171.

14. Demetris AJ, Murase N, Fujisaki S, Fung JJ, Rao AS, Starzl TE. Hematolymphoid cell trafficking, microchimerism, and GVH reactions after liver, bone marrow, and heart transplantation. Transplant Proc 1993;25:3337-2244.

15. Bishop GA, Sun J, Sheil AG, McCaughan GW. High-dose/ activation-associated tolerance: A mechanism for allograft toler- ance. Transplantation 1997;64:1377-1382.

16. Meyer D, Loffeler S, Otto C, Czub S, Gassel HJ, Timmermann W, et al. Donor-derived alloantigen-presenting cells persist in the liver allograft during tolerance induction. Transpl Int 2000; 13:12-20.

17. Starzl TE, Demetris AJ, Trucco M, Zeevi A, Ramos H, Terasaki P, et al. Chimerism and donor-specific nonreactivity 27 to 29 years after kidney allotransplantation. Transplantation 1993;55: 1272-1277.

18. Wood K, Sachs DH. Chimerism and transplantation tolerance: Cause and effect. Immunol Today 1996;17:584-587.

19. Gouw AS, Houthoff HJ, Huitema S, Beelen JM, Gips CH, Poppema S. Expression of major histocompatibility complex antigens and replacement of donor cells by recipient ones in human liver grafts. Transplantation 1987;43:291-296.

20. Gao Z, McAlister VC, Williams GM. Repopulation of liver endothelium by bone-marrow-derived cells. Lancet 2001;357: 932-933.

21. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.

22. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168-1170.

23. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 2000;31:235-240.

24. Lagasse E, Connors H, Al Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6:1229-1234.

25. Kibbelaar RE, Leenheers-Binnendijk CF, Spaande PJ, Kluin PM. Biopsy specimen identification by detection of sex chromosomes: Application of in situ hybridisation. J Clin Pathol 1992; 45:149-150.

26. Lagaaij EL, Cramer-Knijnenburg GF, Van der Pijl JW, Bruijn JA, de Fijter JW, van Krieken JH. Rapid verification of the identity of questionable specimens using immunohistochemistry with monoclonal antibodies directed against HLA-class I antigens. Histopathology 1997;31:284-288.

27. Gassel HJ, Engemann R, Thiede A, Hamelmann H. Replacement of donor Kupffer cells by recipient cells after orthotopic rat liver transplantation. Transplant Proc 1987;19:351-353.

28. Gassel HJ, Otto C, Klein I, Steger U, Meyer D, Gassel AM et al. Persistence of stable intragraft cell chimerism in rat liver allo- grafts after drug-induced tolerance. Transplantation 2001;71: 1848-1852.

29. Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G. Teperman L, et al. Liver from bone marrow in humans. Hepa- tology 2000;32:11-16.

30. Baccarani U, Donini A, Risaliti A, Bresadola F. Replacement of liver venous endothelium. Lancet 2001;357:2137.

31. Pober JS. Is host endothelium a silver lining for allografts? Lancet 2001;357:2-3.

32. Wiesner RH, Ludwig J, Krom RA, Hay JE, vanHoek B. Hepatic allograft rejection: New developments in terminology, diagnosis, prevention, and treatment. Mayo Clin Proc 1993;68:69-79.

33. Natori S, Selzner M, Valentino KL, Fritz LC, Srinivasan A, Clavien PA, Gores GJ. Apoptosis of sinusoidal endothelial cells occurs during liver preservation injury by a caspase-dependent mechanism. Transplantation 1999;68:89-96.

34. Hubscher SG, Buckels JA, Elias E, McMaster P, Neuberger J. Vanishing bile-duct syndrome following liver transplantation—Is it reversible? Transplantation 1991;51:1004-1010.

35. Noack KB, Wiesner RH, Batts K, van Hoek B, Ludwig J. Severe ductopenic rejection with features of vanishing bile duct syndrome: Clinical, biochemical, and histologic evidence for spontaneous resolution. Transplant Proc 1991;23:1448-1451.

Chapter 3

Liver chimerism after allogeneic blood stem cell transplantation

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Abstract

Background

Blood stem cells can mature into elements of many different lineages. We investigated the presence and nature of donor-derived (chimeric) cells within the liver after allogeneic stem cell transplantation.

Methods

Liver biopsy autopsy specimens were examined from nine female patients who had undergone allogeneic bone marrow (n = 6) or peripheral stem cell (n = 3) transplantation from a male donor. To identify the male origin of cells within the liver, in-situ hybridization for Y-chromosomes was performed in conjunction with CD45 staining to identify leucocytes.

Results

Hematopoietic stem cell engraftment was confirmed in all nine recipients. Histologic examination of the liver tissue sections revealed 5.6-fold more Y-chromosome- positive than CD45-positive staining cells ($P < 0.02$), indicative of considerable nonleucocytic chimerism. This was particularly observed in patients who had developed graft-versus-host disease.

Conclusions

Donor-derived cells can be found in liver tissue specimens after allogeneic stem cell transplantation. A considerable fraction of chimeric (donor-derived) cells appeared to be of nonlymphohematopoietic origin. This finding supports the theory of blood stem cells developing into liver cells of mesenchymal origin.

Introduction

Chimerism is defined in transplantation medicine as the coexistence of cells of donor and recipient origin. We previously described chimerism after transplantation of the liver and found evidence that cells of recipient origin can replace biliary epithelial cells, endothelial cells, and hepatocytes within the human liver allograft.[1] This finding can be understood only by the existence of circulating hepatic progenitor cells. To study this phenomenon further we sought to evaluate liver chimerism after allogeneic blood stem cell transplantation.

In animal studies, abundant evidence exists that bone marrow gives rise to hematopoietic as well as mesenchymal stem cells. These elements can differentiate into various cell types within the liver, such as endothelial cells, hepatocytes and bile duct epithelial cells.[2–7] In humans, the existence of circulating hepatic progenitor cells is less well established, although various studies seem to support the concept.[8–10] Two recent studies found hepatocytic differentiation of recipient-derived cells within the transplanted liver to be a rare event.[11,12] Chimerism can be demonstrated after sex-mismatched organ transplantation with the use of in-situ hybridisation for sex chromosomes. When using a Y-chromosome specific probe, cells of donor and recipient origin can be readily identified to quantitatively estimate the number of donor-derived cells. In combination with other staining techniques cells can be further differentiated.

To assess hepatic chimerism in the present study we investigated liver tissue specimens from female recipients of allogeneic stem cell transplantation (male bone marrow or peripheral blood) for the presence of donor-derived cells.

Materials and methods

Patients

From January 1994 until March 2003, we performed 100 allogeneic bone marrow transplants and 162 allogeneic peripheral blood stem cell transplantations. Female patients were selected if they received a sex-mismatched (male) transplant and had liver tissue available after transplantation. Finally, only nine female patients were included in this retrospective study based upon adequate available liver tissue specimens.

All nine had been diagnosed with a hematologic malignancy. The treatment consisted of high-dose chemotherapy followed by allogeneic bone marrow transplantation (n = 6) or allogeneic peripheral blood stem cell transplantation (n = 3). All legal and ethical criteria set out by the ethical committee were met.

Donors

All allogeneic grafts were obtained from male, HLA-matched sibling donors. To obtain peripheral blood stem cells, the donors were pretreated with recombinant human granulocyte colony stimulating factor (G-CSF, dose 10 mg/kg per day SC for 4 or 5 days) and harvesting performed by apheresis on days 5 and 6 of G-CSF administration. A minimum of 4 x 10^6 CD34 cells/kg of recipient body weight was targeted for stem cell transplantation. The stem cell graft was T-cell depleted by adding Campath to the graft. Bone marrow was obtained from donors by standard methods and was harvested under general anesthesia.

Transplantation and Follow up

Patients were conditioned with a myeloablative regimen consisting mostly of cyclophosphamide at 60 mg/kg per day IV for 2 consecutive days followed by a single dose of total body irradiation at day 1. No posttransplant graft-versus-host-disease (GVHD) prophylaxis or hematopoietic growth factors were administered. Following incubation with Campath, the stem cell product was infused intravenously on day 0.[13]

After transplantation, peripheral blood, bone marrow, or both was collected at fixed time points for morphological examination and cytogenetic analysis.

Collection of Liver Tissue Specimens

Liver tissue specimens were available from all nine patients consisting of either needle biopsy specimens or autopsy tissue. Needle biopsy specimens were obtained from four patients (1, 3, 5, 9) for diagnostic purposes because GVHD was suspected. By the time the study began, six patients were deceased. In five cases, autopsy had been performed and liver tissue was available. From each tissue specimen, consecutive sections were obtained. One was used for in situ hybridization for the Y-chromosome in combination with an Alcian blue staining. A neighboring section was stained for CD45 to identify leucocytes.

In-situ Hybridization of Y-chromosomes

For sex chromosome identification, we used in-situ hybridization using repetitive DNA probes specific for the Y-chromosome as previously described.[1,14] Briefly, probes were biotinylated by nick translation and dissolved in a 60% formamide hybridization mix- ture. Paraffin sections (6 mm thick) were cut and mounted on poly-L-lysine coated slides. Predigestion steps consisted of incubation in 1 mol/L sodium thiocyanate solution at 80°C followed by 60 to 90 minutes of treatment with 0.5% pepsin 0.1 mol/L hydrochloric acid. Hybridization was done overnight at 42°C. The hybridization reaction was visualized with avidin, biotinylated goat-anti-avidin, and avidin-peroxidase developed with diaminobenzidine. Positive and negative controls for in-situ hybridization were tissue specimens from normal male and female livers, respectively.

Additional Staining

For consecutive staining an antibody against all known isotypes of the CD45 leucocyte common antigen family present on lymphocytes, monocytes, granulocytes, and other inflammatory cells (CD45-LCA; Dako, USA) was used, as described previously.[1]

Quantification of Y-chromosome Positive and CD45 Positive Cells

All counts were performed at a magnification of 400 and expressed as the mean number of positive dots or cells/high power field (HPF). Y-chromosome-positive cells as well as CD45-positive cells were counted in a median of 13 (range 8 –16) nonoverlapping fields per biopsy.

Fluorescent In-situ Hybridization Analysis on Bone Marrow and Peripheral Blood Samples

To determine hematopoietic stem cell engraftment, peripheral blood, bone marrow samples, or both were collected at fixed time points after transplantation. Fluorescent in situ hybridization analysis (FISH) was performed and the percentage of male, donor- derived nuclei was assessed on these samples.

Statistical Analyses

A paired nonparametric test was performed (Wilcoxon's signed ranks test) to compare the results of the Y-chromosome counts with the CD45 counts. This test was applied because a normal distribution of data was not expected. The differences in cell counts in relation to the presence of GVHD were assessed using the Kruskal-Wallis test.
$P < 0.05$ was considered significant.

Results

Characteristics of Transplant Recipients

All studied recipients were females receiving stem cells from a male HLA-matched donor. Clinical characteristics concerning underlying malignant disease are outlined in Table 1. Hematopoietic stem cell engraftment was con- firmed in all nine patients.

Donor-derived Cells in Liver Tissue Specimens

Liver tissue was available from all studied patients. Table 2 shows the characteristics of the liver tissue specimens, with the histological as well as the clinical diagnosis. In three cases (3, 5, 9) histological evidence of GVHD was observed, in one other case (1) a clinical diagnosis of GVHD was made even though the liver tissue histology was unremarkable. Autopsy showed recurrent hematologic malignancy in two cases and systemic infection in three cases. The number of Y-chromosome positive cells/HPF is shown in Table 2, as well as the number of CD45 positive cells/HPF, the latter indicating the presence of infiltrating leukocytes (of donor origin).

No relation was observed between the FISH engraftment scores (Table 1) and the results from liver histology or with clinical parameters such as GVHD.

All studied liver tissue specimens showed Y-chromosome-positive cells, indicating male (donor) origin. Many of these were likely to be of hematopoietic origin, representing infiltrating leukocytes. This is supported by the finding of CD45-positive staining cells in adjacent slides (Fig 1 A and B). The absence or presence of male offspring (Table 2) was not related to the number of Y-chromosome positive cells within the liver tissue. The extent of chimerism was not related to the time elapsed between transplantation and time of biopsy.

In some cases, donor-derived cells clearly appeared to be of nonlymphohematopoietic origin, supporting the presence of true tissue chimerism (Fig 1 C-F). Paired analysis of the median Y-chromosome and CD45 counts in the successive liver tissue sections revealed a statistically significant higher number of Y+ cells (median 19.5 vs 3.5, $P < 0.02$). Interestingly, the number of Y+ cells was particularly high in the patients who developed GVHD compared to those who did not (median 29.75 vs 5, $P < 0.04$), whereas the number of CD45 cells was found to not be increased (3 vs 4, NS). Accordingly, the Y/CD45 ratio was found to be significantly higher among patients who developed GVHD (7.9 vs 1.1, $P < 0.02$).

Table 1. Clinical characteristics of the female allogeneic stem cell transplant recipients

Recipients Number	Age at Transplantation *(Years)*	Diagnosis	Type of Transplant	Time to Histology *(Month)*	Time Elapsed Since Last Transplant* *(Months)*	FISH YInterval (Bone Marrow or Blood in %)	Interval FISH Y Bone Marrow-Liver *(Weeks)*
1	46	Acute myelogeneous leukemia (AML)	Allo-BMT	14	14	98	10
2	49	Multiple myeloma (MM)	Allo-BMT	12	12	88	5
3	29	Acute myelogenous leukemia	Allo-PSCT	12	3	100	3
4	45	Acute myelogenous leukemia	Allo-BMT	4	3	99	9
5	31	Chronic myelogenous leukemia (CML)	Allo-BMT	3	3	99	1
6	39	Acute myelogenous leukemia	Allo-PCST	16	16	23	6
7	43	Acute myelogenous leukemia	Allo-BMT	10	10	NA	NA
8	17	Chronic myelogenous leukemia	Allo-BMT	3	1	99	1
9	58	Non-Hodgkin's Lymphoma (NHL)	Allo-PCST	8	1	89	0

BMT: bone marrow transplant. PSCT: peripheral blood stem cell transplantation. NA = not available.
*Last transplant: either peripheral blood stem cell transplantation (PSCT)/bone marrow transplant (BMT) or donor lymphocyte infusion/retransplant.

Table 2. Characteristics of the Liver Tissue Specimens of the Female Allogeneic Stem Cell Recipients

No	Histological Diagnosis of Liver Specimens	Out-come*	Remarks/Cause of Death (Clinical or at Autopsy)	Male Offspring	Y+ Cells Median (Range) per HPF	CD-45+ Cells Median (Range) per HPF	Y/CD-45 Ratio
1	Normal	ANED	Clinical diagnosis of GVHD Complete remission of AML	+	19.5(11-46)	2.5 (0-7)	7.8
2	Normal	DWED	Recurrent multiple myeloma	+	23 (13-38)	13(6-35)	1.8
3	Consistent with acute GVHD	DNED	Histological confirmation of chronic GVHD, DLI 3 months prior to liver histology		35.5 (14-94)	3 (0 -12)	11.8
4	Congestion and cholestasis	DNED	Systemic candidiasis, myocardial infarction	+	6 (1-12)	4 (1-10)	1.5
5	Mild inflammation	ANED	Clinical confirmation of GVHD Complete remission of CML		24 (12-36)	3 (1-6)	8.0
6	Disseminated aspergillosis	DNED	Pneumonia, disseminated aspergillosis	+	3 (1-9)	3 (0 -8)	1.0
7	Normal	DWED	Intracranial bleed, recurrent AML	+	5 (0 -11)	5 (3-9)	1.0
8	Congestion, no recurrent disease	DNED	Merantic endocarditis, invasive aspergillosis re-PCST 1 month prior to liver histology		4 (0 -11)	3.5 (0-9)	1.1
9	Acute GVHD	DWED	GVHD by histology, complete remission of NHL DLI 1 month prior to liver histology	+	58 (37-90)	21 (15-65)	2.8

*ANED = alive, no evidence of recurrent disease, DNED = deceased, no evidence of recurrent disease, DWED = deceased with evidence of recurrent disease. Abbreviations; GVHD: graft versus host disease, DLI: donor lymphocyte infusion, AML: acute myelogeneous leukemia, CML: chronic myelogenous leukemia, PSCT: peripheral blood stem cell transplantation, NHL: Non-Hodgkin's lymphoma.

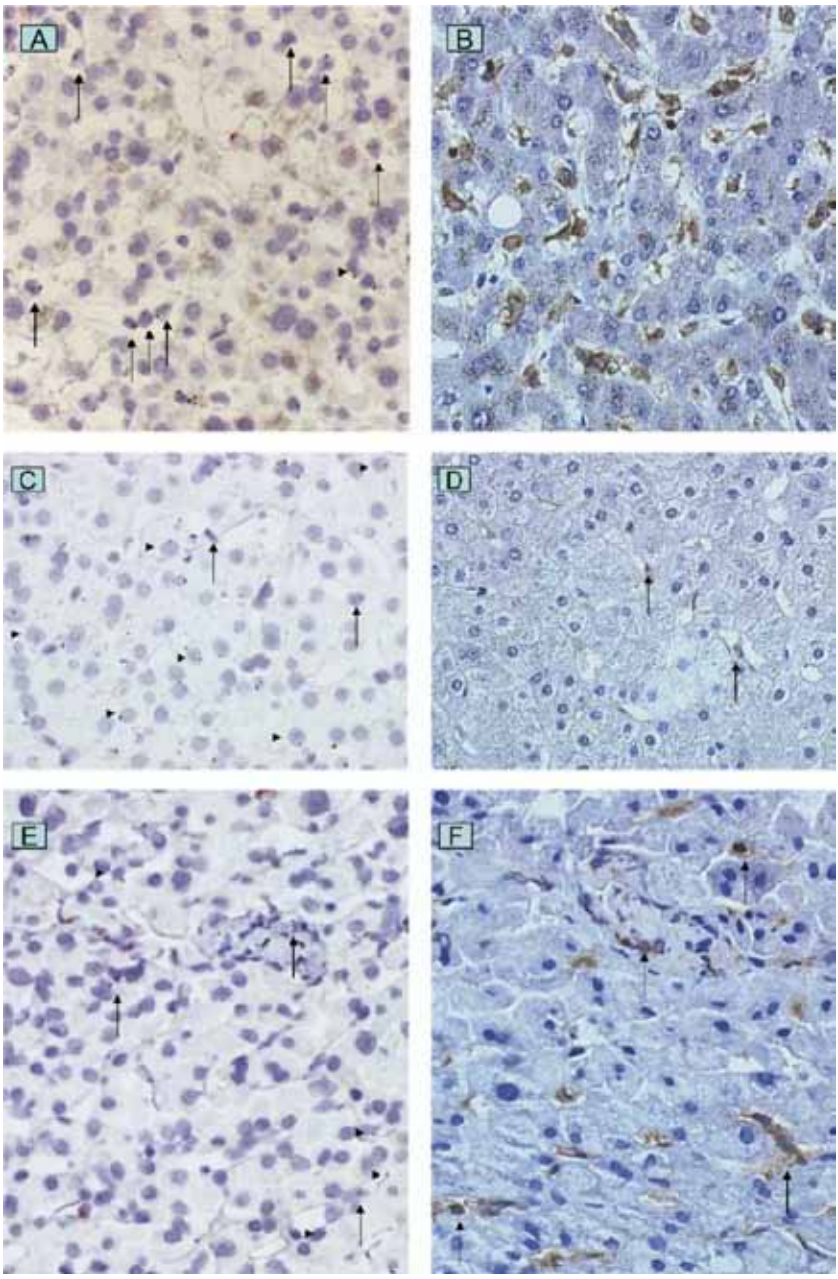

Fig 1.

In-situ hybridization for Y-chromosome (A, C, E) and parallel immunostaining for CD-45 common leucocyte antigen (B, D, F) of liver tissue sections from female patients receiving male bone marrow or blood stem cells. In several patients (example patient 2), most Y-positive cells (A, arrows) were also CD-45 positive (B, brown staining). In other patients (examples patients 1 and 5, respectively) many other cells (arrow heads), eg, hepatocytes (C and E) endothelial cells (E), were Y-positive and CD-45 negative (D and F).

Discussion

We identified liver chimerism by comparing the number of Y-chromosome-positive cells with the number of CD45-positive cells in consecutive thin tissue sections. Since the number of Y-chromosome-positive cells was significantly higher than the number of CD45-staining cells, the presence of Y-chromosomes cannot solely be attributed to infiltrating leucocytes. Furthermore, (immuno)histology confirmed Y-positive hepatocytes and endothelial cells within the liver biopsies. It strongly supports the presence of nonlymphohematopoietic cells of donor origin within the liver emerged from mesenchymal stem cells, similar to what we previously described after transplantation of the liver.[1] This indicates true chimerism within the liver. It has previously been postulated that male cells in females can result from male offspring.[15,16] In our study, however, the absence or presence of male offspring was not related to the number of Y-chromosome-positive cells.

Studies addressing chimerism in transplantation medicine mostly rely on a combination of standard histologic staining techniques and in-situ hybridization for sex chromosomes. These techniques cannot usually be applied sequentially on the same tissue section because this exposes the tissue to rough conditions, leading to loss of quality. This is especially the case in autopsy material, as in our study. Therefore, consecutive thin tissue sections are often used, as in this study. Consecutive slides are at best comparable, but never identical and therefore results need to be interpreted with some reservation.

The existence of a hepatic progenitor cell, capable of developing into hepatocytes and bile duct epithelial cells, has been investigated by many workers.[2, 9, 17-20] It has been the general belief that these hepatic stem cells (or 'oval cells') are located in the canals of Hering within the liver at the ductal plate. Fully differentiated hepatocytes themselves also possess great growth potential, and it is not known if stem cells are even required for hepatocyte regeneration. A recently postulated theory, derived from a mouse model, suggests that hepatocytes derived from bone marrow arise from cell fusion rather than by differentiation of hematopoietic stem cells.[21-24] In a study of sex-mismatched liver transplant recipients, Ng et al[12] report recipient cells constituted up to 50% of all cells in the liver allograft. Most cells showed macrophage/Kupffer cell differentiation, and only 1.6% showed hepatocytic differentiation. Again, no distinction could be made between

transdifferentiation and cell fusion. Körbling et al[8] studied recipients of peripheral blood stem cells and found donor-derived hepatocytes (up to 7%) in liver tissue specimens.

In our retrospective study, where needle biopsies were taken with a clinical suspicion of GVHD, it is remarkable that a high number of Y-chromosome positive cells was observed. One would expect this to result from a high number of infiltrating leucocytes, but this could not be confirmed because CD45 counts were not increased correspondingly. One could hypothesize that GVHD leads to hepatic tissue damage, inducing repair mechanisms,[25,26] leading to the influx of hepatic progenitor cells from the circulation and thereby to chimerism. A similar response to cellular injury may be observed in solid organ transplantation, where different types of injury, such as ischemia/reperfusion injury and rejection in liver transplantation, can initiate an immunological cascade leading to chimerism.

In conclusion, donor-derived cells may be observed in liver tissue specimens after allogeneic stem cell transplantation. A significant fraction of chimeric (donor-derived) cells appeared to be of nonlymphohematopoietic origin. This finding supports the theory of blood stem cells developing into liver cells of mesenchymal origin.

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References

1. ten Hove WR, van Hoek B, Bajema IM, et al: Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl 9:552, 2003

2. Petersen BE, Bowen WC, Patrene KD, et al: Bone marrow as a potential source of hepatic oval cells. Science 284:1168, 1999

3. Avital I, Feraresso C, Aoki T, et al: Bone marrow-derived liver stem cell and mature hepatocyte engraftment in livers under- going rejection. Surgery 132:384, 2002

4. Kanazawa Y, Verma IM: Little evidence of bone marrow- derived hepatocytes in the replacement of injured liver. Proc Natl Acad Sci USA 100 (suppl 1):11850, 2003

5. Lagasse E, Connors H, Al Dhalimy M, et al: Purified hema- topoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229, 2000

6. Fujii H, Hirose T, Oe S, et al: Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. J Hepatol 36:653, 2002

7. Holden C, Vogel G: Stem cell lines. 'Show us the cells' U.S. researchers say. Science 297:923, 2002

8. Körbling M, Katz RL, Khanna A, et al: Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med 346:738, 2002

9. Theise ND, Nimmakayalu M, Gardner R, et al: Liver from bone marrow in humans. Hepatology 32:11, 2000

10. Gao Z, McAlister VC, Williams GM: Repopulation of liver endothelium by bone-marrow-derived cells. Lancet 357:932, 2001

11. Fogt F, Beyser KH, Poremba C, et al: Recipient-derived hepatocytes in liver transplants: a rare event in sex-mismatched transplants. Hepatology 36:173, 2002

12. Ng IO, Chan KL, Shek WH, et al: High frequency of chimerism in transplanted livers. Hepatology 38:989, 2003

13. Barge RM, Brouwer RE, Beersma MF, et al: Comparison of allogeneic T cell-depleted peripheral blood stem cell and bone marrow transplantation: effect of stem cell source on short- and long-term outcome. Bone Marrow Transplant 27:1053, 2001

14. Kibbelaar RE, Leenheers-Binnendijk CF, Spaander PJ, et al: Biopsy specimen identification by detection of sex chromosomes: application of in situ hybridisation. J Clin Pathol 45:149, 1992

15. Bianchi DW, Zickwolf GK, Weil GJ, et al: Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA 93:705, 1996

16. Koopmans M, Kremer Hovinga ICL, Baelde HJ, et al: Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. Am J Transplant 5:1495, 2005

17. Theise ND, Badve S, Saxena R, et al: Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235, 2000

18. Theise ND, Saxena R, Portmann BC, et al: The canals of Hering and hepatic stem cells in humans. Hepatology 30:1425, 1999

19. Alison MR, Poulsom R, Jeffery R, et al: Hepatocytes from non-hepatic adult stem cells. Nature 406:257, 2000

20. Gale RP, Sparkes RS, Golde DW: Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. Science 201:937, 1978

21. Vassilopoulos G, Wang PR, Russell DW: Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901, 2003

22. Wang X, Willenbring H, Akkari Y, et al: Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422: 897, 2003

23. Alison MR, Vig P, Russo F, et al: Hepatic stem cells: from inside and outside the liver? Cell Prolif 37:1, 2004

24. Alison MR, Poulsom R, Otto WR, et al: Recipes for adult stem cell plasticity: fusion cuisine or readymade? J Clin Pathol 57:113, 2004

25. Abkowitz JL: Can human hematopoietic stem cells become skin, gut, or liver cells? N Engl J Med 346:770, 2002

26. DeWitt N, Knight J: Biologist question adult stem-cell versatility. Nature 416:354, 2002

Chapter 4

MMP-2 and MMP-9 Serum Levels Change but their Gene Promoter Polymorphisms are not Associated with Late Phase I/R Injury or Rejection after Orthotopic Liver Transplantation

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Abstract

Introduction
Matrix metalloproteinases (MMPs) are involved in connective tissue remodeling processes associated with chronic liver disease and complications after orthotopic liver transplantation (OLT). Genetic variations in the promoter region of the MMP-2 and MMP-9 genes are thought to contribute not only to their transcription rate but may also have predisposing clinical impact.

Methods
MMP-2 and MMP-9 gene promoter polymorphisms were analyzed in 109 patients who underwent an OLT. The relationship between these MMP polymorphisms in the donor and recipient DNA with the development of ischemia/reperfusion (I/R) injury and rejection after OLT was evaluated. In addition, serum MMP-2 and MMP-9 levels were determined to illustrate potential phenotypical consequences in these patients.

Results
The MMP-2 and -9 genotypes of the donor and recipient or a donor/recipient mismatch and chimerism were not associated with the development of late phase I/R injury or rejection in the OLT patients, although serological differences in the MMP levels did occur. The MMP-2 and -9 genotype distribution did also not have a major impact on the respective serum levels in patients that underwent an OLT.

Conclusions
MMP-2 and MMP-9 gene polymorphisms do not seem to contribute to late phase I/R injury or rejection after liver transplantation. Serological changes in the MMP-2 and MMP-9 levels appear to occur independent of the MMP genotype after transplantation of the liver.

Introduction

Matrix metalloproteinases (MMPs) comprise a large family of proteolytic enzymes that are important in physiological and disease-related extracellular matrix remodeling. The gelatinases MMP-2 and MMP-9 are capable of digesting components of the connective tissue matrix and type IV collagen within basement membranes. These MMPs are considered to play an important role in cancer development, vascular remodeling, fibrosis and inflammation [1,2]. In recent years it has become evident that also in the liver MMPs are involved in diverse (patho)physiological processes, like fibrosis, hepatocellular carcinoma development and liver transplantation [3].

MMP activity is transcriptionally regulated by various factors and controlled by tightly regulated activation of latent pro-enzymes and by interaction with endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs). Recently, several single nucleotide polymorphisms (SNP) in the gene promoter regions of MMPs have been found with a functional impact on the transcription rate. The C/T transition at position –1306 in the promoter of MMP-2, which abolishes the Sp 1 binding site, and the G/A transition at position –1575, which is located next to an estrogen receptor binding site, leads to decreased mRNA transcription [4,5].

In several studies an association was demonstrated between MMP-2 polymorphisms and the development of cancer [6,7]. In the MMP-9 gene a SNP at position –1562 is due to a C to T substitution in the promoter region [8]. *In vitro* studies have shown that this transition results in loss of binding of a nuclear repression protein in this region and an increase in transcriptional activity in macrophages. This functional effect on transcription was associated with the severity of coronary atherosclerosis [8]. In accordance with the increased activity of the –1562 T allele this allele was found to be associated with elevated MMP-9 plasma levels in patients with cardiovascular disease [9].

In the liver, the hepatic stellate cell is suggested to be the main cellular source of MMP-2. Liver fibrosis is a dynamic process in which activated stellate cells are involved in the synthesis of matrix proteins and in the regulation of matrix degradation. Increased mRNA expression of MMP-2 was reported in liver biopsy samples of patients with cirrhosis [10]. In addition, serum levels of MMP-2 are found to be increased in patients with chronic liver disease and to correlate with the severity of the liver function impairment [11]. MMP-9 is released predominantly from neutrophils and macrophages, but the principal source in the liver is

thought to be the Kupffer cell, the resident macrophage of the liver [12]. MMP-2 and MMP-9 are presumed to play a critical role in cold storage injury during preservation and in the subsequent reperfusion injury of liver grafts [13]. The extracellular matrix may also be an important target in the process of acute rejection after orthotopic liver transplantation (OLT). In a previous study we demonstrated elevated serum levels of MMP-9 at 1 week after OLT in patients with acute allograft rejection [14].

The aim of the present study was to establish the relationship between MMP-2 and MMP-9 gene promoter polymorphisms in the donor and recipient DNA and the respective MMP serum levels with the development of late phase ischemia/reperfusion (I/R) injury of rejection after OLT.

Patients And Methods

Study Design

Our study group consisted of 109 patients who underwent an OLT. All blood and tissue samples from these patients were collected prospectively and retrospectively analysed. Longitudinal MMP serum levels were measured in a representative subset of 27 patients. MMP genotype distribution in this subset was similar to the study group of 109 patients. Serum samples for MMP measurement were collected at 7 time points: before transplantation (I) and at 2 days (II), 1 week (III), 1 month (IV), 3 months (V), 6 months (VI) and 1 year (VII) after OLT. Serum samples were stored at –70 °C until use. MMP-2 and MMP-9 concentrations were determined using highly specific enzyme-linked immunosorbent assays, which measure the pro-enzyme, active- and inhibitor complexed forms, as described previously [15,16].

Ischemia and Reperfusion Injury

The degree of late phase hepatocellular injury was evaluated by measurement of aspartate aminotransferase (AST) during the first week after OLT. Patients were classified into 2 groups depending on whether the serum AST peak was lower than 1,500 IU/L (no or mild I/R injury) or higher than 1,500 IU/L (more severe I/R injury), respectively [17,18].

Rejection

Liver biopsies were taken according to our protocol at approximately 1 week after OLT or when there was a suspicion of rejection. Acute allograft rejection was graded according to the Banff scheme and the

histopathological severity was evaluated by three specific features (portal inflammation, bile duct inflammation/damage, venous endothelial inflammation) [19]. In this study patients were divided into 2 groups according to the presence or absence of acute allograft rejection at approximately 1 week. The rejection had to be clinically relevant, i.e. histologically confirmed and treated with additional immunosuppression.

Determination of SNPs of the MMPs

Genomic DNA was extracted by routine methods from peripheral blood leukocytes. In addition, DNA samples from the blood of the liver donor were obtained from the Eurotransplant Reference Laboratory and DNA was isolated from liver biopsy tissue of the allograft in the recipients obtainedmseveral months (median 17, range 5 to 48) after OLT.

The –1306 C/T MMP-2 gene promoter polymorphism was determined by tetra-primer amplification refractory mutation system- polymerase chain reaction (PCR) analysis, the principles of which are described elsewhere [20,21], and confirmed by direct sequence analysis of 4 patients. Briefly, the region flanking the SNP was amplified with outer primers 5′-ACCAGACAAGCCTGAACTTGTCTGA-3′ and 5′-TGTGACAACCGTCTCTGAGGAATG-3′ together with inner allelic specific primers 5′-ATATTCCCCACCCAGCACGCT- 3′ and 5′-GCTGAGACCTGAAGAGCTAAAGAGTTG- 3′. Genotypes CC, CT and TT (542+379; 542+379+ 211; 542+211 bp, respectively) are easily identified from the migration pattern on agarose gels. The other gene promoter polymorphisms were determined as described previously [20,22]. In brief: transition polymorphism G/A at –1575 of the MMP-2 promoter gene was determined by PCR amplification using outer primers also used for the –1306 polymorphism followed by restriction enzyme fragment length (RFLP) analysis with BspH I to produce 542, 542+458+83 or 458+83 bp fragments indicating the GG, GA and AA genotype, respectively. The SNP C/T at position –1562 of the MMP-9 gene promoter was determined by PCR-RFLP. The SNP flanking region was amplified using primers 5′- ATGGCTCATGCCCGTAATC-3′ and 5′-TCACCTTCTTCAAAGCCCTATT- 3′ followed by restriction analysis with Sph I to produce 352, 352+207+145 or 207+145 bp fragments in case of CC, CT and TT genotype, respectively.

Statistical Analysis

Genotype frequencies were analyzed by generating two-by-two contingency tables and statistical analysis was performed using the Chi-square test or Fischer's Exact test, when appropriate, using SPSS

software (SPSS Inc; Chicago, IL, USA). Differences in MMP levels according to the genotype and disease complications were assessed by the Mann-Whitney U or the Wilcoxon signed ranks test for nonparametric data [14,23]. MMP levels are expressed as mean ± S.E.M. Differences were considered to be significant at P-values of ≤ 0.05.

Results

Patients

Our study group consisted of 109 patients (70 male) who underwent an OLT. The median age was 47 years (range 16 to 67). 30 patients had chronic viral hepatitis, 26 patients had cholestatic liver disease, 21 patients had alcohol-related liver disease, and a miscellaneous group (n=32) was included, consisting of 7 patients with autoimmune hepatitis, 9 with cryptogenic liver cirrhosis, 4 with neoplastic disorders, 2 with azathioprine induced liver cirrhosis, 2 with polycystic liver disease, 1 with acute fatty liver of pregnancy, 4 with Budd Chiari Syndrome, two patients with Wilson's disease and one patient with alpha-1 antitrypsin deficiency.

The Role of MMP Polymorphisms in Liver Transplantation

The frequencies of the donor and recipient MMP-2 and MMP-9 genotypes in relation to the development of IR-injury and rejection after OLT are shown in Table 1. Because the gene polymorphisms at the -1306 and the -1575 position of the MMP-2 promoter were found to be in complete linkage disequilibrium only the data at the -1306 position are given. Late phase I/R injury was graded "more severe" in 33 of 109 patients after OLT, and rejection had occurred in 20 patients. The genotype frequencies for MMP-9 determined in the donor and recipient DNA of patients with or without late phase I/R injury, and with or without rejection, however, were not significantly different (Table 1A). Also, the MMP-2 genotype frequency of the donor and recipient were not significantly different according to the development of I/R injury or rejection, although the donors were found the have a significantly different genotype frequency distribution from the recipients (Table 1B). Therefore, we assessed the clinical impact of such a mismatch in allelic composition at the MMP-2 and MMP-9 SNP loci between the donor and recipient. However, no statistically significant differences between the presence or absence of a mismatch at –1306 C/T MMP-2 or –1562 C/T MMP-9 were found in relation to the development of I/R injury and to rejection after liver transplantation (Table 2). Obviously, due to the

Table 1. The Development of IR-Injury and Rejection after OLT Stratified According to Gene Promoter Polymorphisms at MMP-9 (A) and MMP-2 (B) SNP loci in Donor and Recipient

A		-1562 C/T MMP-9			
Complication	**Genotype**	**Donor**		**Recipient**	
		CC n (%)	**CT n (%)**	**CC n (%)**	**CT n (%)**
IR injury	No	53 (69.7)	23 (30.3)	59 (77.6)	17 (22.4)
	Yes	27 (81.8)	6 (18.2)	24 (72.7)	9 (27.3)
Statistical significance		X^2 = 1.7; P = 0.19		X^2 = 0.3; P = 0.58	
Rejection	No	66 (74.2)	23 (25.8)	67 (75.3)	22 (24.7)
	Yes	14 (70.0)	6 (30.0)	16 (80.0)	4 (20.0)
Statistical significance		X^2 = 0.1; P = 0.70		X^2 = 0.2; P = 0.66	
	Total	80 (73.4)	29 (26.6)	83 (76.1)	26 (23.9)
Statistical significance		X^2 = 0.1; P = 0.75			

B		-1306 C/T MMP-2			
Complication	**Genotype**	**Donor**		**Recipient**	
		CC n (%)	**CC & CT n (%)**	**CC n (%)**	**CC & CT n (%)**
IR injury	No	37 (48.7)	39 (51.3)	53 (69.7)	23 (30.3)
	Yes	13 (39.4)	20 (60.6)	21 (63.6)	12 (36.4)
Statistical significance		X^2 = 0.8; P = 0.37		X^2 = 0.4; P = 0.53	
Rejection	No	43 (48.3)	46 (51.7)	62 (69.7)	27 (30.3)
	Yes	7 (35.0)	13 (65.0)	12 (60.0)	8 (40.0)
Statistical significance		X^2 = 1.2; P = 0.28		X^2 = 0.7; P = 0.40	
	Total	50 (45.9)	59 (54.1)	74 (67.9)	35 (32.1)
Statistical significance		X^2 = 9.9; P = 0.002			

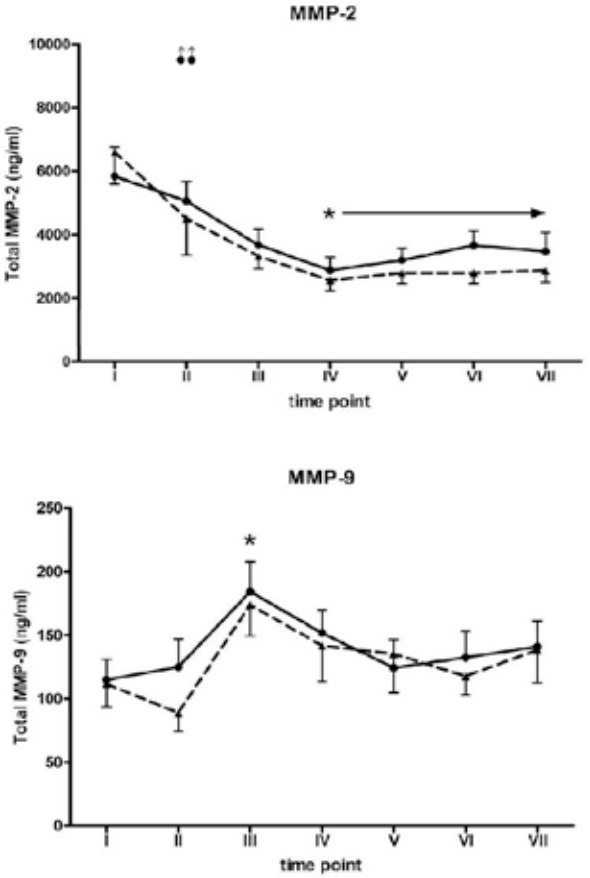
Table 2. The Development of IR-Injury and Rejection after OLT Stratified According to the Presence of a Mismatch in Allelic Composition at MMP-2 and MMP-9 SNP Loci between Donor and Recipient

		-1306 C/T MMP-2 Mismatch		-1562 C/T MMP-9 Mismatch	
Complication	**Genotype**	**No**	**Yes**	**No**	**Yes**
IR injury	No	33 (43.4)	43 (56.6)	48 (63.2)	28 (36.8)
	Yes	13 (39.4)	20 (60.6)	24 (72.7)	9 (27.3)
Statistical significance		$X^2 = 0.2$; P = 0.70		$X^2 = 0.9$; P = 0.33	
Rejection	No	39 (43.8)	50 (56.2)	58 (65.2)	31 (34.8)
	Yes	7 (35.0)	13 (65.0)	14 (70.0)	6 (30.0)
Statistical significance		$X^2 = 0.5$; P = 0.47		$X^2 = 0.2$; P = 0.68	
	Total	46 (42.2)	63 (57.8)	72 (66.1)	37 (33.9)
Statistical significance		$X^2 = 11.5$; P = 0.001			

donor-recipient differences, the frequency of a mismatch in MMP-2 was significantly higher than in MMP-9 (57.8% versus 33.9%, Table 2). Serum levels of MMP-2 are increased in patients with chronic liver disease and therefore a drop of MMP-2 levels was expected after OLT [11]. This decrease indeed was seen, as shown in Fig. (1), but occurred independent of the genotype. Serum levels of MMP-9 peak at 1 week after OLT, which is associated with acute allograft rejection, as previously reported [14]. Irrespective of a genotype mismatch between the donor and the recipient, we found a comparable serum MMP-9 peak and pattern over time (Fig. 1). In some patients with a mismatch evidence of chimerism was demonstrated in the DNA samples from the liver biopsy tissue of the allograft. The chimerism was identified by the presence of an MMP SNP signal from the recipient in addition to the strong signal of the donor in the DNA from the allograft liver biopsy (Fig. 2). These chimerisms were demonstrated in 37.5% of patients with a MMP-9mismatch and in 62.5% of patients with a MMP-2 mismatch, but were of no additional clinical significance.

Discussion

Our study shows that the –1306 C/T MMP-2 and the – 1562 C/T MMP-9 gene promoter polymorphisms, determined in the donor and the recipient, are not associated with rejection or late phase I/R injury after OLT. Moreover, the consistent changes in the serum MMP-2 and MMP-9 levels after OLT evolve independent of the genotypes and donorrecipient mismatches.



•: Patients without a mismatch between donor and recipient; ▲: Patients with a mismatch.
* MMP-2: P<0.01 for both groups compared to time point I, • two outliers.
* MMP-9: P<0.02 time point III compared to time point I, for both groups.

Fig. 1.
MMP-2 en MMP-9 serum concentrations before transplantation (I) and at 2 days (II), 1 week (III), 1 month (IV), 3 months (V), 6 months (VI) and 1 year (VII) after OLT. Data are expressed as mean ± SEM.

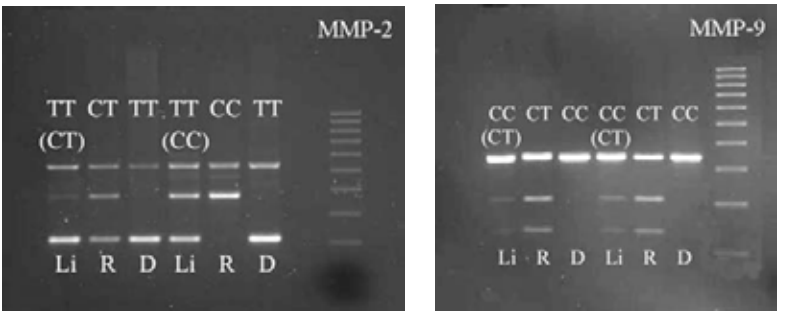


Fig. 2.
MMP genotype chimerism, indicated by genotypes between brackets, in OLT allografts based on the SNP analysis of the MMP-2 –1306 C/T locus and the MMP-9 –1562 C/T locus of sets of DNA from the transplanted liver (Li) and blood leukocytes of the recipient (R) and from blood leukocytes of the donor (D).

Polymorphisms in the promoter region of a number of MMP genes have been shown to influence MMP gene expression and/or were found to be associated with susceptibility of disease like coronary atherosclerosis, aneurysms, and cancer [24]. For example, a functional SNP in the promoter of the MMP-2 gene (–1306 C/T) leads to a diminished promoter activity and is principally studied in cancer [5]. In case-control studies from China it was demonstrated that the –1306 CC MMP-2 genotype may constitute a common susceptibility factor for cancer of the breast, lung, and stomach, whereas in hepatocellular carcinoma it was not [6,7,25,26]. Liver fibrosis is a highly dynamic process in which multiple genes may interact with environmental factors. Polymorphisms in genes encoding immunoregulatory proteins, proinflammatory cytokines, and fibrinogenic factors may influence disease progression [27]. MMPs play an important role in remodeling of the hepatic extracellular matrix and increased expression of MMP-2 was found in human liver fibrosis [10,28]. However, studies on MMP-2 promoter polymorphisms have never been reported in patients with chronic liver disease. In the present study the serum MMP-2 levels in patients with diverse chronic liver disease were increased pre-OLT and decreased significantly over time after OLT but unrelated to the –1306 C/T MMP-2 genotype and donor-recipient mismatch. Apparently, MMP-2 expression changes in liver disease processes and may even be indicative for disease progression, as reported for hepatic fibrosis [29] and recurrence of hepatocellular carcinoma [30], but this may occur unrelated to the genotypic constitution. Other studies similarly demonstrated that disease related changes in the hepatic mRNA MMP-3 expression was determined by the MMP-3 (–1171 5A/6A) promoter polymorphism, while the genotype distribution was not significantly different between controls, patients with chronic hepatitis, and patients with cirrhosis [31,32].

The expression of MMP-9 is regulated primarily at the transcription level in response to different regulators such as interleukin-1, tumor necrosis factor-alpha, and epidermal growth factor [2]. The functionally most important SNP at position –1562 in the MMP-9 gene leads to increased transcriptional activity in individuals with the T allele and is associated with severity of coronary atherosclerosis [8]. In another study, the T allele of the –1562 C/T polymorphism was associated with elevated MMP-9 serum levels, but no association with cardiovascular mortality was found [9]. Studies on functional polymorphisms of MMP-9 in patients with pulmonary emphysema [33], abdominal aneurysm [34], end-stage renal disease [35], multiple sclerosis [36], and intracranial aneurysms revealed contradictory results [37,38], In liver disease it has become obvious

that MMP-9 may have a very important impact, as illustrated by the predictive value for recurrence of hepatocellular carcinoma after liver transplantation [30], the protection against hepatic I/R injury in MMP-9 deficient/neutralized mice [39,40] and a contribution to the progression of chronic liver disease [41]. We found a peak in serum MMP-9 levels at 1 week after OLT in patients with rejection. The MMP-9 is most likely derived from infiltrating neutrophils in the portal triad of the liver or from Kuppfer cells activated by cytokines from the infiltrating cells [14]. However, the serological changes in MMP-9 were found to be unrelated to the –1562 C/T MMP-9 gene promoter polymorphism of the patients, donors and the presence of mismatch.

In previous studies we demonstrated that MMP-9, but not MMP-2, could play an important role in immediate I/R injury [23] and in acute allograft rejection after liver transplantation [14]. In the present study we assessed the impact of the donor MMP genotypes on the outcome and complications after liver transplantation but no associations were found. We did observe significant differences in the MMP-2 genotype distribution between donors and recipients but not in relation to clinical complications as I/R and rejection after OLT. Also the presence of a mismatch in the MMP genotype between the donor and the recipient and the evidence of chimerism in the liver biopsy tissue of the allograft were found not to contribute to the clinical complications after transplantation. The development of the chimerism we observed might in part be explained by the presence of lymphocytes and Kupffer cells of recipient origin, but recently it was demonstrated that recipient-derived cells can also replace biliary epithelium, endothelium, and even hepatocytes in liver transplants [42].

In conclusion, this study indicates that MMP-2 and MMP- 9 gene promoter polymorphisms are not associated with late phase I/R injury or rejection after liver transplantation. Furthermore, functionally and clinically relevant changes in the serum levels of MMP-2 and MMP-9 in patients who underwent OLT develop independent of the MMP promoter genotype. Although MMPs are involved in both the progression and resolution of disease processes in the liver [43], the exact contribution of the MMP genes still has to be elucidated.

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Abbreviations

MMP: matrix metalloproteinases; TIMP: tissue inhibitors of metalloproteinases; SNP: single nucleotide polymorphism; OLT: orthotopic liver transplantation; I/R: ischemia/reperfusion; AST: aspartate aminotransferase; PCR: polymerase chain reaction; RFLP: restriction enzyme fragment length polymorphism.

References

1. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998; 10(5): 602-8.

2. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001; 17: 463-516.

3. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. In: Lendeckel U, Hooper NM Eds. Matrix metalloproteinases in chronic liver disease and liver transplantation. Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues. Dordrecht, the Netherlands: Springer 2006; 209-34.

4. Harendza S, Lovett DH, Panzer U, Lukacs Z, Kuhnl P, Stahl RA. Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. J Biol Chem 2003; 278(23): 20490-9.

5. Price SJ, Greaves DR, Watkins H. Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. J Biol Chem 2001; 276(10): 7549-58.

6. Miao X, Yu C, Tan W, et al. A functional polymorphism in the matrix metalloproteinase-2 gene promoter (-1306C/T) is associated with risk of development but not metastasis of gastric cardia adenocarcinoma. Cancer Res 2003; 63(14): 3987-90.

7. Zhou Y, Yu C, Miao X, et al. Substantial reduction in risk of breast cancer associated with genetic polymorphisms in the promoters of the matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 genes. Carcinogenesis 2004; 25(3): 399-404.

8. Zhang B, Ye S, Herrmann SM, Eriksson P, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999; 99(14): 1788-94.

9. Blankenberg S, Rupprecht HJ, Poirier O, et al. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation 2003; 107(12): 1579-85.

10. Milani S, Herbst H, Schuppan D, et al. Differential expression of matrix metalloproteinase-1 and -2 genes in normal and fibrotic human liver. Am J Pathol 1994; 144(3): 528-37.

11. Kuyvenhoven JP, van Hoek B, Blom E, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003; 89(4): 718-25.

12. Winwood PJ, Schuppan D, Iredale JP, Kawser CA, Docherty AJ, Arthur MJ. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. Hepatology 1995; 22(1): 304-15.

13. Upadhya AG, Harvey RP, Howard TK, Lowell JA, Shenoy S, Strasberg SM. Evidence of a role for matrix metalloproteinases in cold preservation injury of the liver in humans and in the rat. Hepatology 1997; 26(4): 922-28.

14. Kuyvenhoven JP, Verspaget HW, Gao Q, et al. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. Transplantation 2004; 77(11): 1646-52.

15. Gveric D, Hanemaaijer R, Newcombe J, van Lent NA, Sier CF, Cuzner ML. Plasminogen activators in multiple sclerosis lesions: implications for the inflammatory response and axonal damage. Brain 2001; 124(Pt 10): 1978-88.

16. Hanemaaijer R, Visser H, Konttinen YT, Koolwijk P, Verheijen JH. A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjogren's syndrome contain increased latent and active gelatinase-B levels. Matrix Biol 1998; 17(8-9): 657-65.

17. Klar E, Angelescu M, Zapletal C, Kraus T, Bredt M, Herfarth C. Definition of maximum cold ischemia time without reduction of graft quality in clinical liver transplantation. Transplant Proc 1998; 30(7): 3683-5.

18. Strasberg SM, Howard TK, Molmenti EP, Hertl M. Selecting the donor liver: risk factors for poor function after orthotopic liver transplantation. Hepatology 1994; 20(4 Pt 1): 829-38.

19. Banff scheme for grading liver allograft rejection: an international consensus document. Hepatology 1997; 25(3): 658-63.

20. Kubben FJ, Sier CF, van Duijn W, et al. Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer. Br J Cancer2006; 94(7): 1035-40.

21. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 2001; 29(17): E88.

22. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007; 13(21): 2960-6.

23. Kuyvenhoven JP, Molenaar IQ, Verspaget HW, et al. Plasma MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 during human orthotopic liver transplantation. The effect of aprotinin and the relation to ischemia/reperfusion injury. Thromb Haemost 2004; 91(3): 506-13.

24. Ye S. Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. Matrix Biol 2000; 19(7): 623-9.

25. Yu C, Pan K, Xing D, et al. Correlation between a single nucleotide polymorphism in the matrix metalloproteinase-2 promoter and risk of lung cancer. Cancer Res 2002; 62(22): 6430-33.

26. Zhai Y, Qiu W, Dong XJ, et al. Functional polymorphisms in the promoters of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12 and MMP-13 are not associated with hepatocellular carcinoma risk. Gut 2007; 56(3): 445-7.

27. Bataller R, North KE, Brenner DA. Genetic polymorphisms and the progression of liver fibrosis: a critical appraisal. Hepatology 2003; 37(3): 493-503.

28. Benyon RC, Iredale JP, Goddard S, Winwood PJ, Arthur MJ. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. Gastroenterology 1996; 110(3): 821-31.

29. Larrousse M, Laguno M, Segarra M, et al. Noninvasive diagnosis of hepatic fibrosis in HIV/HCV-coinfected patients. J Acquir Immune Defic Syndr 2007; 46(3): 304-11.

30. Zhang Q, Chen X, Zhou J, et al. CD147, MMP-2, MMP-9 and MVD-CD34 are significant predictors of recurrence after liver transplantation in hepatocellular carcinoma patients. Cancer Biol Ther 2006; 5(7): 808-14.

31. Lichtinghagen R, Bahr MJ, Wehmeier M, et al. Expression and coordinated regulation of matrix metalloproteinases in chronic hepatitis C and hepatitis C virus-induced liver cirrhosis. Clin Sci (Lond) 2003; 105(3): 373-82.

32. Shin HP, Lee JI, Jung JH, et al. Matrix metalloproteinase (MMP)-3 polymorphism in patients with HBV related chronic liver disease. Dig Dis Sci 2008; 53(3): 823-9.

33. Minematsu N, Nakamura H, Tateno H, Nakajima T, Yamaguchi K. Genetic polymorphism in matrix metalloproteinase-9 and pulmonary emphysema. Biochem Biophys Res Commun 2001; 289(1): 116-9.

34. Jones GT, Phillips VL, Harris EL, Rossaak JI, van Rij AM. Functional matrix metalloproteinase-9 polymorphism (C-1562T) associated with abdominal aortic aneurysm. J Vasc Surg 2003; 38(6): 1363-7.

35. Hirakawa S, Lange EM, Colicigno CJ, Freedman BI, Rich SS, Bowden DW. Evaluation of genetic variation and association in the matrix metalloproteinase 9 (MMP9) gene in ESRD patients. Am J Kidney Dis 2003; 42(1): 133-42.

36. Nelissen I, Vandenbroeck K, Fiten P, et al. Polymorphism analysis suggests that the gelatinase B gene is not a susceptibility factor for multiple sclerosis. J Neuroimmunol 2000; 105(1): 58-63.

37 Krex D, Kotteck K, Konig IR, Ziegler A, Schackert HK, Schackert G. Matrix metalloproteinase-9 coding sequence single-nucleotide polymorphisms in caucasians with intracranial aneurysms. Neurosurgery 2004; 55(1): 207-12.

38. Peters DG, Kassam A, St Jean PL, Yonas H, Ferrell RE. Functional polymorphism in the matrix metalloproteinase-9 promoter as a potential risk factor for intracranial aneurysm. Stroke 1999; 30(12): 2612-6.

39. Fowell AJ, Benyon RC. Can matrix metalloproteinases be targeted in hepatic ischemia/reperfusion injury? Hepatology 2008; 47(1): 14-6.

40. Hamada T, Fondevila C, Busuttil RW, Coito AJ. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. Hepatology 2008; 47(1): 186-98.

41. Okamoto K, Mimura K, Murawaki Y, Yuasa I. Association of functional gene polymorphisms of matrix metalloproteinase (MMP)-1, MMP-3 and MMP-9 with the progression of chronic liver disease. J Gastroenterol Hepatol 2005; 20(7): 1102-8.

42. Hove WR, van Hoek B, Bajema IM, Ringers J, van Krieken JH, Lagaaij EL. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transplant 2003; 9(6): 552-6.

43. Han YP. Matrix metalloproteinases, the pros and cons, in liver fibrosis. J Gastroenterol Hepatol 2006; 21(Suppl 3): S88-S91.

Chapter 5

Sequential liver chemistry profiling and abdominal ultrasound assessments to predict biliary strictures after liver transplantation

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Abstract

Background

After orthotopic liver transplantation (OLT) early detection of biliary strictures is important. Our aim was to evaluate the predictive value of routine serum liver chemistry profiling and abdominal ultrasound as non-invasive diagnostic tools in detecting biliary strictures after OLT.

Methods

We performed a retrospective study in which 141 OLTs, performed between 1992 and 2007 with more than 1 year follow-up, were included. Routinely assessed serum levels of alkaline phosphatase, alanine-aminotransferase, aspartate-aminotransferase, gamma-glutamyl transpeptidase and bilirubin at 3, 6, 9 and 12 months, and abdominal ultrasounds performed at 3, 6 and 12 months after OLT were evaluated. Time-dependent Cox regression analysis was performed to identify predictive factors for the development of biliary strictures.

Results

Eighteen grafts developed non-anastomotic strictures (12.8%) and 18 grafts (12.8%) developed anastomotic strictures requiring intervention. An elevated gamma-glutamyl transpeptidase (HR 1.25 per 100 IU/L; $p = 0.04$) and dilated bile ducts on ultrasound (HR 3.54; $p < 0.01$) were found to have an independent predictive value for the development of biliary strictures requiring intervention. Bilirubin and the other studied liver enzymes were not independently predictive.

Conclusion

Dilated bile ducts on ultrasound and elevated gamma-glutamyltranspeptidase after OLT are independent predictive factors for the development of biliary strictures requiring intervention.

Introduction

Biliary complications are common after orthotopic liver transplantation (OLT), with a reported prevalence of 6% to 35%.[1-4] Biliary strictures occurring at the surgical anastomosis are classified as anastomotic strictures (AS), whereas strictures in the donor biliary tree are referred to as non-anastomotic strictures (NAS). Stricture formation is often insidious and usually only then detected when lead to clinical symptoms as cholestasis, with serum liver enzyme abnormalities, intrahepatic bile duct dilatation and/or infection.[5] The definite diagnosis is made with endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic cholangiography (PTC) or by magnetic resonance cholangiopancreatography (MRCP).[6,7] Although ERCP and PTC are considered the gold standard, they are invasive procedures and associated with considerable morbidity. ERCP and PTC are often immediately performed when clinical symptoms such as jaundice or cholangitis are present.[8] Several uncontrolled series evaluated the efficacy of ERCP in predicting biliary complications.[8,9] Most of these were evaluated in settings where patients present themselves with symptoms such as cholangitis due to a biliary stricture. However, in many liver transplant programs liver chemistry and enzymes and abdominal ultrasound are routinely assessed in an outpatient clinic at certain intervals as a screening tool, often long before patients develop symptoms such as fever or abdominal pain. It is remarkable that only few studies addressed the predictive value of routinely assessed serum liver chemistry profiles and abdominal ultrasonography (US) after OLT as predictors for the occurrence of biliary strictures.[10] Although some studies did evaluate the prognostic value of liver chemistry and US, the usefulness of routinely assessing these diagnostic tools in clinical setting remains unclear in a post-transplantation population.[10,11] The risk of developing biliary strictures varies over time, probably in association with the liver chemistry profile and US findings. The aim of the present study was to evaluate the predictive value of routinely assessed serum liver chemistry and abdominal ultrasound as non-invasive predictors for the development of biliary strictures requiring intervention after OLT.

Patients and Methods

Patients

We examined 141 consecutive OLTs with at least one year of follow-up and complete data on serum liver chemistry and enzymes and upper

abdominal ultrasonography (US) performed between September 1992 and April 2007 performed at the Leiden University Medical Centre. Re-transplantations (n=31) were excluded. Clinical data were obtained from the medical digital records, the hepatological and surgical patient charts, and endoscopy reports. Follow-up was up to August 2008 with a median of 5.2 years (range 1.0 -15.6).
OLT was performed according to standard procedures with cavo-caval, porto-portal, and hepatic artery to hepatic artery anastomosis. A duct-to-duct biliary anastomosis over an 8-12 Ch stent was performed, if possible. The biliary stent was removed after 6 weeks or removed earlier as indicated. In some cases the hepatic artery was anastomosed to the aorta via an iliac conduit. All patients received immunosuppressive agents according to protocol: cyclosporin A or tacrolimus, prednisone during the first half-year and patients with renal impairment received azathioprine before 2001 or mycophenolate mofetil from 2001 on. From 2001 on, basiliximab was given post OLT. In some cases sirolimus was used after month 3 in which case the calcineurin-inhibitor was lowered or discontinued. All patients received ursodeoxycholic acid in the first 3 months after transplantation.

Biochemical variables
Serum liver enzymes levels of alkaline phosphatase (ALP), alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT) and gamma-glutamyltranspeptidase (GGT) were determined daily during the first two weeks and weekly for two months, after that at 3, 6, 9, and 12 months post-operatively. The same was done for bilirubin. Only the latter 4 time points were included in the study because in the first three months after liver transplantation liver enzymes are very susceptible to change due to procedure-related causes such as ischemia-reperfusion damage, rejection and infections. Therefore, the first three months of liver chemistry assessments, ultrasounds and biliary strictures after transplantation were excluded.
The upper limit of normal serum level was for ALP 120 IU/L, for ASAT, ALAT and GGT the upper limits of normal were 40 IU/L, 40 IU/L and 51 IU/L, respectively. The upper limit for bilirubin (total) was 17 µmol/L.

Imaging variables
US was performed routinely on day 0, 1 and 7, and subsequently at 3, 6 and 12 months after OLT. The US performed at 3, 6 and 12 months were included in this study. These US were performed by different experienced radiologists. A bile duct of >7 mm on ultrasound was considered dilated

and prompted either direct intervention by ERCP or PTC or additional MRCP which in turn might prompt ERCP or PTC. Routine abdominal CT scan was performed after 3 to 7 days post-OLT and routine liver biopsies were performed at 6 months after transplantation. Additional liver biopsies were taken on indication. Virology monitoring, including CMV-DNA, was performed frequently in the first year.

Clinical variables

Presentation of a biliary stricture (anastomotic and non-anastomotic) was with clinical symptoms such as cholangitis, pruritus or jaundice and/or abnormal liver chemistry. Diagnostic tests to confirm the diagnosis were performed on indication but not included in the present analyses. Only strictures occurring more than three months after OLT that required intervention by ERCP, PTC or surgery were included in this study. From 2001 on, routine ERCP with stent-removal was performed at week 6 post-OLT in case of a duct-to-duct anastomosis. Strictures were treated endoscopically with ERCP and dilation and/or stenting, and percutaneously with percutaneous transhepatic cholangiodrainage (PTCD) or with surgical intervention.

Statistical analyses

We have used a time-dependent Cox regression model to evaluate the diagnostic value of liver enzymes and routine US assessments in predicting biliary strictures. The Cox proportional-hazards regression model for time-to-event data (in this case the development of biliary strictures) takes into account the changes of variables over time, in this study changes of liver enzymes and bile duct dilatation on US. Time dependent predictors (covariates) for stricture development in this study were liver chemistry variables ALP, GGT, ASAT, ALAT and bilirubin, obtained with an interval of three months at 3, 6, 9 and 12 months post OLT and US performed 3, 6 and 12 months post OLT. Recipient characteristics, variables on etiology of liver disease and procedure-related variables were baseline characteristics. Coefficients were considered significant when $p<0.05$. The reported hazard ratios and p-values are *per* 100 international unit elevation for the liver enzymes. Bilirubin levels are reported per 10 µmol/L elevation.

Ethical committee

All data were obtained as part of patient care according to a strict protocol after OLT. There was permission from the local ethics committee to use these data.

Results

Patients and biliary strictures

Baseline characteristics of recipients, etiology of liver disease and procedure-related variables are presented in Table 1. Non-anastomotic biliary strictures requiring intervention developed in 18 of the 141 grafts (12.8%). Median time from OLT to NAS was 8.5 months (range 3-29). Median follow-up after the diagnosis of NAS was 5.5 years (range 0.0 – 11.6). Anastomotic strictures developed in 18 out of 141 grafts (12.8%). Median time from OLT to AS was 5.5 months (range 3-72). Median follow-up after the diagnosis of an anastomotic stricture was 2.8 years (range 0.6-15.3). A total of twenty-one ERCPs and twelve PTCs for the management of strictures were performed in the included cases. In three cases a surgical intervention to resolve the stricture was necessary. There was no difference between the duct-to-duct type anastomosis and the Roux-en-Y anastomosis in the occurrence of biliary strictures ($p = 0.88$). Other potential risk factors were calculated and are listed in Table 2.

Liver chemistry and abdominal ultrasound

Elevation of serum liver enzymes ALP, GGT, ASAT and ALAT above the upper limit of normal occurred in respectively 57.4%, 71.6%, 53.9%, and 61.0% of the patients at 3, 6, 9 or 12 months after OLT. Bilirubin was elevated in 41.6% of the cases. There was a significant relationship between the level of GGT and the development of biliary strictures requiring intervention, both in the univariate and in the multivariate analysis (hazard ratios 1.35 and 1.25, $p < 0.001$ and $p = 0.04$, respectively), as presented in Tables 2 and 3. AF above the upper limit of normal was also found to be a significant indicator for the development of biliary strictures in the univariate analysis ($p < 0.001$), but not in the multivariate analysis ($p = 0.23$). Elevated ALAT and ASAT were not associated with biliary strictures in both univariate and multivariate analyses (ALAT; $p = 0.61$. and $p = 0.81$ respectively, ASAT; $p = 0.62$ and $p = 0.42$ respectively). Elevation of bilirubin was not significant in both univariate and multivariate analysis for the prediction of the development of biliary strictures ($p = 0.08$ and $p = 0.33$ respectively (Table 3) Regarding the US assessments a significant relationship was found between dilated bile ducts on abdominal ultrasound and the successive development of a biliary stricture requiring intervention in both the univariate (hazard ratio = 4.48, $p < 0.001$) and multivariate analysis (hazard ratio = 3.54, $p < 0.01$). (Table 3)

Table 1. Baseline characteristics of 141 orthotopic liver transplants.

Recipient data	
- Male/ Female	91/50
- Median age (years) (range)	50 (16 – 70)
Etiology of liver disease	
Hepatitis B/C cirrhosis	10/22
Biliary cirrhosis (PSC/PBC)	30 (22/8)
Alcoholic cirrhosis	25
Hepatocellular carcinoma	19
Other	35
Donor and OLT procedure data	
DBD / DCD donor	135/6
Choledochocholedochostomy (duct-to-duct)/ Roux-en-Y hepaticojejunostomy	132/9
Donor warm ischemic time (DCD donors) (minutes) (range)	17 (11 – 23)
Cold ischemic time (minutes) (range)	605 (268 – 1095)
Recipient warm ischemic time (minutes) (range)	35 (16-90)

Table 2.

Univariate analysis of potential predictors for the development of biliary strictures (BS). Time-dependent analysis was used to calculate the predictive value of routinely performed liver chemistry profile assessments and dilated bile ducts on abdominal ultrasound (US) for detecting BS requiring intervention after OLT (n=141). The hazard ratios for liver enzymes are shown per 100 IU/L increase. The hazard ratio for bilirubine is shown per 10 µmol/L increase.

Clinical Variables	**Hazard Ratio (95% CI)**	**P-Value**
Dilated bile ducts on US	4.48 (1.97 -10.12)	< 0.001
GGT	1.35 (1.22 - 1.49)	< 0.001
ALP	1.55 (1.22 -1.89)	< 0.001
ALAT	1.13 (0.74-1.82)	0.61
ASAT	1.19 (0.61 -2.45)	0.62
Bilirubin	1.07 (0.99 – 1.16)	0.08
Gender	0.64	0.23
Type of surgical anastomosis (duct-to-duct / Roux-en-Y)	0.90	0.88
Age (at OLT)	0.98	0.14

Table 3.

Multivariate time-dependent Cox regression analysis for liver enzymes and dilated bile ducts on abdominal ultrasound (US) for detecting presence of BS requiring intervention after OLT (n=141). Gamma-glutamyltranspeptidase (GGT) and US remain significant predictors for the development of BS. The Hazard ratio for GGT shows the risk of having a stricture with each 100 1U/l increase. The hazard ratio for bilirubin was calculated for each 10 µmol/L increase.

Clinical Variables	**Hazard Ratio (95% CI)**	**P- value**
Dilated bile ducts on US	3.54 (1.47 – 8.49)	< 0.01
GGT	1.25 (1.02 – 1.55)	0.04
ALP	1.46 (0.93 – 2.24)	0.10
ALAT	0.92 (0.46 – 1.85)	0.81
ASAT	0.55 (0.13 – 2.33)	0.42
Bilirubin	0.99 (0.80 – 1.08)	0.33

Discussion

Biliary strictures frequently complicate orthotopic liver transplantation and lead to significant morbidity, graft loss and mortality. Early diagnosis and prompt intervention is therefore of great clinical importance. Cholangiography remains the most sensitive and specific assessment in diagnosing biliary strictures but is invasive. The most commonly used and least invasive diagnostic modalities after OLT are routine serum liver chemistry profile determinations and abdominal ultrasound. Surprisingly, there are only few studies on the prognostic value of these routinely performed laboratory tests and ultrasound. Our aim was to evaluate the predictive value of these routine diagnostic tests, often performed in an outpatient clinic setting, for the development of clinically relevant biliary strictures in a time-dependent model. NAS and AS represent different entities and have different etiologies. However, the tools for diagnosing and treating NAS and AS are generally similar. Therefore, in our study, these data were pooled. We found that after OLT an elevated serum gamma-glutamyltranspeptidase level and bile duct dilatation on abdominal ultrasound are independently highly predictive for development of biliary strictures requiring intervention.

The current findings are in accordance with non-transplantation studies in which GGT corresponds with the presence of biliary strictures, while mixed data are reported on the predictive value of liver chemistry and ultrasound for the presence of biliary complications after OLT.[8,10,12,18] Some studies showed no relationship between an aberrant serum liver chemistry profile and biliary complications post-OLT, whereas other studies did.[5,7,10,11,18]

Abdominal ultrasound is a non-invasive, readily available and economic diagnostic tool. However, several studies observed that ultrasound is not very sensitive in detecting biliary strictures in a post transplant population, in contrast to a non-transplant population, whereas few studies reported the opposite.[3,8,11,13] We found bile duct dilatation on abdominal ultrasound to be a powerful predictor of subsequent development of biliary strictures requiring therapy, exemplified by the high hazard ratio of 3.54 in the multivariate analysis. The use of abdominal ultrasound remains one of the safest and cheapest diagnostic tools for early detection of biliary strictures.

Although it may be not as sensitive as MRCP, these data show that dilated bile ducts on US after OLT often precedes biliary strictures requiring intervention. Sensitivity may be even better if strictures are clinically suspected. Dilated bile ducts on abdominal US after OLT

should prompt cholangiography. Whether this should be a MRCP first or immediate ERCP or PTC is a matter of debate: With ERCP and PTC the contrast flow across the stenosis can be assessed dynamically (and the use of an air-inflated balloon can be of additional value). MRCP is more expensive than US and access to MRCP is often more difficult. Quick action is warranted to prevent cholangitis in the immunosuppressed patient. MRCP can be considered first if there is less urgency.

Apart from a dilated bile duct on ultrasound there was an independent association between the increased serum level of gamma-glutamytranspeptidase and the risk of a biliary stricture requiring intervention. The accompanying hazard ratio (HR=1.25) is given per 100 units increase of GGT above the upper limit of normal (i.e., 151 U/L). To put these figures in the appropriate perspective: a GGT of 151 U/l (100 U/l above normal range) would result in a 25% increased risk of having a stricture requiring therapy. In fact, the hazard ratio for any elevation of GGT can be calculated. The given hazard ratios in table 2 and table 3 were also calculated per 100 units of elevation for illustrating purposes. Time-dependent analysis calculates the hazard ratio per 1 IU/L of elevated GGT, which in our study was 1.0022. Thus, an elevation of 100 IU/L would result in $1.0022^{100} = 1.25$ or a 25% increased risk. In formula terms a GGT elevation results in a hazard ratio of $1.0022^{(\text{elevation above upper limit in IU/L})}$ for the development of a biliary stricture.

In clinical practice the calculated hazard ratios of both bile duct dilatation on US and the elevation of GGT are multiplied. In our example GGT levels 100 IU/L above the reference range together with dilated bile ducts on US result in a hazard ratio of 4.3 (3.54 x 1.25), which indicates that the risk of developing a biliary stricture requiring intervention in the next three months is 4.3 more likely compared to the standard risk.

It is remarkable that only few studies have addressed the usefulness of routinely assessing liver chemistry and performing ultrasound after OLT. Hussaini et al.[11], for example, showed that US was a valuable tool to diagnose biliary strictures with a sensitivity and specificity of 77% and 67%, respectively, whereas Zoepfi et al.[10] showed no significant relationship for both ultrasound and liver enzymes in the detection of biliary strictures. Que et al.[18] found US to detect biliary strictures with a sensitivity and specificity of 90% and 91% respectively, but reported GGT and ALP to be of poor diagnostic value even at 10-folds the upper limit of normal. However, these studies evaluated liver chemistry using sensitivity and specificity in relation to the presence of biliary strictures. Although this is a common way to evaluate the diagnostic value of clinical tools it has several limitations. Sensitivity and specificity are

used to determine the probability an aberrant result in case of present disease and vice versa. This method would applicable if the assessment of the liver chemistry profile and biliary strictures occur simultaneously as is the case in, for example, an emergency setting. However, routine assessments are often performed in an outpatient clinic setting where most patients have not yet developed symptoms due to biliary strictures. Routine assessments are used based on the idea that early detection of any aberrance of liver chemistry profile might prompt intervention to prevent complications due to biliary strictures such as cholangitis.

However, the time interval for occurrence of biliary strictures after routine assessment of liver chemistry varies for each patient, and the time for being at risk varies along. This means that the risk of developing a biliary stricture for each patient varies along with the changes in liver chemistry. Sensitivity and specificity are also based on dichotomized variables, that is: liver chemistry or enzymes are elevated or not. However, liver chemistry profiles are continuous variables and change over time. These data show that the level of elevation significantly impacts the hazard for development of biliary strictures.

We realize that our study has limitations. We decided to use a follow-up of one year after OLT, but excluded the first three months. The first three months postoperative months were not included because early after transplantation liver chemistry levels are influenced by many variables, such as ischemia-reperfusion damage, rejection and infection. A shorter follow-up would have been possible, but since liver chemistry and enzymes levels vary over time it is obligate to assess them over a longer period for their utility. A shorter follow-up would have weakened the statistical analysis. One might criticize that in this study patients were included over a long period of time and management of strictures may have changed over the years. Since we focused on the development of strictures rather than the treatment, this is not a relevant issue in interpreting our findings. Routine liver chemistry profiling and abdominal assessments were performed since our center started OLTs and strictures and their treatment were consistently reported. In a retrospective study such as this, one should be careful when using parameters that have not been defined beforehand, such as the ultrasound findings. All ultrasound procedures in our institute were performed by radiologists with expertise in liver transplantation and bile duct dilatation was reported using strict criteria. Other reported ultrasound findings of the biliary tree, such as sludge or a thickened biliary wall, were not taken into account in our analysis.

The evaluation of routinely assessed liver chemistry and abdominal

ultrasound in a time-dependent Cox regression model for predicting biliary strictures, as has been used for the first time in the present study, takes into account the variability of both changes of liver chemistry and bile duct dilatation on US, hence providing clinicians with common non-invasive tools for predicting biliary strictures requiring intervention. Based on these findings we conclude that routine assessments of gamma-glutamyltranspeptidase and abdominal ultrasound are useful for early detection of biliary strictures after OLT. We advocate that dilated bile ducts on ultrasound and elevated gamma-glutamyltranspeptidase more than three months after liver transplantation should prompt cholangiography for early diagnosis and therapeutic intervention of biliary strictures.

References

1 Verdonk RC, Buis CI, Porte RJ, van der Jagt EJ, Limburg AJ, van den Berg AP et al. Anastomotic biliary strictures after liver transplantation: causes and consequences. *Liver Transpl* 2006; 12(5):726-735.

2 Nishida S, Nakamura N, Kadono J, Komokata T, Sakata R, Madariaga JR et al. Intrahepatic biliary strictures after liver transplantation. *J Hepatobiliary Pancreat Surg* 2006; 13(6):511-516.

3 Barriga J, Thompson R, Shokouh-Amiri H, Davila R, Ismail MK, Waters B et al. Biliary strictures after liver transplantation. Predictive factors for response to endoscopic management and long-term outcome. *Am J Med Sci* 2008; 335(6):439-443.

4 Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005; 18(6):627-642.

5 Colonna JO, Shaked A, Gomes AS, Colquhoun SD, Jurim O, McDiarmid SV et al. Biliary strictures complicating liver transplantation. Incidence, pathogenesis, management, and outcome. Ann Surg 1992; 216(3):344-350.

6 Morelli J, Mulcahy HE, Willner IR, Cunningham JT, Draganov P. Long-term outcomes for patients with post-liver transplant anastomotic biliary strictures treated by endoscopic stent placement. Gastrointest Endosc 2003; 58(3):374-379.

7 Lee SH, Ryu JK, Woo SM, Park JK, Yoo JW, Kim YT et al. Optimal interventional treatment and long-term outcomes for biliary stricture after liver transplantation. Clin Transplant 2008; 22(4):484-493.

8 Shastri YM, Hoepffner NM, Akoglu B, Zapletal C, Bechstein WO, Caspary WF et al. Liver biochemistry profile, significance and endoscopic management of biliary tract complications post orthotopic liver transplantation. World J Gastroenterol 2007; 13(20):2819-2825.

9 Shah SR, Dooley J, Agarwal R, Patch D, Burroughs AK, Rolles K et al. Routine endoscopic retrograde cholangiography in the detection of early biliary complications after liver transplantation. Liver Transpl 2002; 8(5):491-494.

10 Zoepf T, Maldonado-Lopez EJ, Hilgard P, Dechene A, Malago M, Broelsch CE et al. Diagnosis of biliary strictures after liver transplantation: which is the best tool? World J Gastroenterol 2005; 11(19):2945-2948.

11 Hussaini SH, Sheridan MB, Davies M. The predictive value of transabdominal ultrasonography in the diagnosis of biliary tract complications after orthotopic liver transplantation. Gut 1999; 45(6):900-903.

12 Ben-Ari Z, Weiss-Schmilovitz H, Sulkes J, Brown M, Bar-Nathan N, Shaharabani E et al. Serum cholestasis markers as predictors of early outcome after liver transplantation. Clin Transplant 2004; 18(2):130-136.

13 Kok T, Van der Sluis A, Klein JP, van der Jagt EJ, Peeters PM, Slooff MJ et al. Ultrasound and cholangiography for the diagnosis of biliary complications after orthotopic liver transplantation: a comparative study. J Clin Ultrasound 1996; 24(3):103-115.

14 Uzochukwu LN, Bluth EI, Smetherman DH, Troxclair LA, Loss GE, Jr., Cohen A et al. Early postoperative hepatic sonography as a predictor of vascular and biliary complications in adult orthotopic liver transplant patients. Am J Roentgenol 2005; 185(6):1558-1570.

15 Li S, Stratta RJ, Langnas AN, Wood RP, Marujo W, Shaw BW, Jr. Diffuse biliary tract injury after orthotopic liver transplantation. Am J Surg 1992; 164(5):536-540.

16 Rull R, Garcia Valdecasas JC, Grande L, Fuster J, Lacy AM, Gonzalez FX et al. Intrahepatic biliary lesions after orthotopic liver transplantation. Transpl Int 2001; 14(3):129-134.

17 Zemel G, Zajko AB, Skolnick ML, Bron KM, Campbell WL. The role of sonography and transhepatic cholangiography in the diagnosis of biliary complications after liver transplantation. Am J Roentgenol 1988; 151(5):943-946.

18 Que Y, Kaneko J, Sugawara Y, Tamura S, Makuuchi M. Role of protocol ultrasonography for detecting biliary stricture in adult living donor liver transplantation recipients. Biosci Trends 2007; 1(1):62-65.

Chapter 6

Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation

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Abstract

Background

Nonanastomotic biliary strictures (NAS) are a serious complica-tion after orthotopic liver transplantation (OLT). Matrix metalloproteinases (MMPs) are involved in connective tissue remodelling in chronic liver disease and complications after OLT.

Aim

To evaluate the relationship between MMP-2 and MMP-9 gene polymorphisms and NAS.

Methods

MMP-2 (- 1306 C/T) and MMP-9 (- 1562 C/T) gene promoter polymorphisms were analysed in 314 recipient-donor combinations. Serum levels of these MMPs were determined in subgroups of patients as well. NAS were identified with various radiological imaging studies performed within 4 years after OLT and defined as any stricture, dilation or irregularity of the intra- or extrahepatic bile ducts of the liver graft followed by an intervention, after exclusion of hepatic artery thrombosis and anastomotic strictures.

Results

The average incidence of NAS was 15%. The major clinical risk factor for the development of NAS was PSC in the recipient. The presence of the MMP-2 CT genotype in donor and/or recipient was associated with a significantly higher incidence of NAS, up to 29% when both donor and recipient had the MMP-2 CT genotype (P = 0.003). In the multivariate analyses, pre-OLT PSC (hazard ratio 2.1, *P* = 0.02) and MMP-2 CT genotype (hazard ratio 3.5, *P* = 0.003) were found to be independent risk factors for the development of NAS after OLT. No obvious association was found between NAS and the MMP-9 genotype and serum levels of the MMPs.

Conclusion

MMP-2 CT genotype of donor and recipient is an independent risk factor, in addition to PSC, for the development of NAS after OLT.

Biliary complications are a common feature after orthotopic liver transplantation (OLT), with a reported in-cidence of up to 35%. Leaks and strictures are the most common complications, often requiring endoscopic, radiological or surgical intervention[1-9]. Anastomotic strictures result from surgical or local ischaemic causes. Main categories of risk factors for nonanastomotic biliary strictures (NAS) include ischaemia-related injury, immunologically induced injury and cytotoxic injury by bile salts. A higher incidence of NAS is reported in patients transplanted for primary sclerosing cholangitis (PSC) and patients who suffered from a postoperative CMV infection[10-17]. Donation after cardiac death (DCD) procedures are also reported to have an increased risk of NAS compared with donation after brain death procedures[18,19]. NAS are often referred to as ischae-mic-type biliary lesions, based on the resemblance with biliary abnormalities observed after hepatic artery thrombosis. The reported incidence of NAS varies in different publications from 1 to 19%[4,5,20-25]. If untreated, NAS may lead to cholestasis, severe graft dysfunction, septic complications, secondary cirrhosis and graft loss[7,23,26]. Matrix metalloproteinases (MMPs) comprise a large family of proteolytic enzymes that are important in physiological and disease-related extracellular matrix remodelling processes[27-30]. MMP-2 and MMP-9 are capable of digesting components of the connective tissue matrix and type IV collagen within basement mem-branes. These MMPs are considered to play an important role in cancer development, tissue remodelling, fibrosis and inflammation, including cirrhosis and liver transplantation[31-35]. We showed previously, for example, that serum MMP-2 levels increased, whereas MMP-9 levels decreased in relation to the severity of the cirrhosis[31]. These serum MMP levels were subsequently found to change irrespective of their gene polymorphisms in late phase injury or rejection (I/R) after liver transplantation[36]. The aim of the present study was to assess whether a relationship exists between MMP-2 and MMP-9 gene promoter polymorphisms in the donor and recipient DNAwith the development of NAS after OLT.

Patients and methods

Patients

All adult patients who received a liver transplant at the Leiden University Medical Center (LUMC) and University Medical Center Groningen (UMCG) in the Netherlands were eligible for inclusion. For this study, 202 patients were identified from the transplantation databases who underwent OLT at the LUMC between 1992 and 2005, of whom

we were able to include 147 patients whose DNAwas available from both donor and recipient, and who had at least 7 days of follow-up after liver transplantation. Also, patients who received OLT be-tween 2000 and 2005 at the UMCG were eligible for the study because data were available. Of the 224 available patients, 167 unselected patients could be included of whom we had DNA from both recipient and donor, and who had at least 7 days of follow-up after transplanta-tion. Genomic DNA was extracted routinely from per-ipheral blood and/or tissue samples without given preference to any explicit clinical variables. All patients received standard immunosuppressive therapy consisting ofcorticosteroids, a calcineurin inhibitor (i.e., cyclospor-ine or tacrolimus) with or without mycophenolate mofetil or azathioprine and/or basiliximab. Azathioprine was used until 2001, and thereafter mycophenolate mofetil was given in case of impaired renal function. Demographical and clinicopathological characteristics of the recipient at the time of OLT (age, gender, indication for liver transplantation, laboratory MELD score), donor information (age, gender and donor type), transplanta-tion procedure variables (warm and cold ischaemia time) and post-transplant follow-up data of up to 4 years were collected from the transplantation databases.

This study was performed with informed consent from the patients according to the guidelines of the Medical Ethics Committee of both participating centres and in compliance with the Helsinki Declaration.

Nonanastomotic strictures

In this study, only biliary strictures followed that by an intervention were included. If a biliary stricture was suspected from clinical findings, liver function tests or abdominal ultrasound, further imaging of the biliary tract was performed. In both centres, a biliary drain was placed routinely after OLT and cholangiography was performed if clinically indicated and in the LUMC, cholangiography was also performed routinely 6 weeks after OLT. All imaging studies of the biliary tree, performed within 4 years after OLT, were included [direct cholangiography via the biliary drain, percuta-neous transhepatic cholangiodrainage (PTCD), ERCP as well as MRCP]. For the purpose of this study, NAS were defined as follows: any stricture, dilation or irregularity of the intrahepatic or extrahepatic bile ducts of the liver graft, either with or without biliary sludge formation, at least 1 cm above the biliary anastomosis and treated endoscopically with ERCP and dilation and/or stenting, percutaneously with PTCD or by surgical intervention. Hepatic artery thrombosis by either Doppler ultrasound or conventional angiography

as well as isolated strictures/ stenoses at the bile duct anastomosis and related dila-tions were, by definition, excluded from this analysis.

Genotyping
Genomic DNA was extracted by routine methods from peripheral blood leucocytes and/or tissue samples. In addition, DNA samples from the blood or tissue of the liver donor were obtained from the Eurotransplant Reference Laboratory or freshly isolated.

MMP-2: high-resolution DNA melting analysis
MMP2 1306 C/T (rs243865) genotyping, as most relevant SNP, was performed with the use of high-resolution DNA melting assay[37]. Sequences of the polymerase chain reaction (PCR) primers were 5′-CCAGTGCCTC TTGCTGTTTT-3′ (forward) and 5′- GACTTCTGAGC TGAGACCTGA-3′ (reverse). The unlabelled probe was designed according to the wild-type (C) genotype and had the following sequence: 5′-CCACCCAGCACTCCACCTCTTTAGCTC-3′. The probe had a 3′-amino-C7 modification to prevent DNA polymerase extension during PCR. In brief, high-resolution melting analysis of PCR products amplified in the presence of a satu-rating double-stranded DNA dye (LCGreenPlus, Idaho Technology, Salt Lake City, Utah, USA) and a 3′-blocked probe, identified both heterozygous and homozygous sequence variants. Heterozygotes were identified by a change in melting curve shape, and different homozy-gotes are distinguished by a change in melting tempera-ture. In each experiment, sequence-verified control donors for each genotype were used.

MMP-9: PCR-RFLP genotyping
The MMP-9 SNP C/T at position - 1562 (rs3918242) was determined with PCR analysis followed by restric-tion enzyme fragment length polymorphisms (RFLP) analysis, the principles of which are described elsewhere[36], and confirmed by direct sequence analysis of four patients. Briefly, the region flanking the SNP was amplified with outer primers 5′-ATGGCTCATGCCCGTAATC-3′ and 5′-TCACCTTCTTCAAAGCCCTATT-3′ followed by RFLP analysis with *Sph*I to produce 352, 35212071145 or 2071145 bp fragments in case of CC, CT and TT genotype respectively. Genotypes CC, CT and TT are easily identified from the migration pattern on agarose gels[36,38-40].

Determination of serological MMP levels
From two subgroups of patients included in our study, we also

assessed the serological levels of MMP-2 and MMP-9 before and after transplantation. This pretrans-plantation group consisted of 47 patients (30 males) with chronic liver disease of various aetiologies, including 27 patients who eventually underwent an OLT. Their med-ian age was 46 years (range 16-68). Fourteen patients had chronic viral hepatitis, 14 patients had cholestatic liver disease, 10 patients had alcohol-related liver disease and the remaining nine patients had miscellaneous liver diseases. From the group of 27 OLT patients, serum samples 1 month after transplantation were evaluated. All serum samples had been stored at - 80 °C until use. MMP-2 and MMP-9 concentrations were determined using highly specific enzyme-linked immunosorbent assays, which measures the pro-enzyme, active- and inhibitor complexed forms, as described previously[31,36].

Statistical analysis

Data were analysed using spss 17.0 software (SPSS Inc.; Chicago, IL, USA). Characteristics of the liver transplant recipients, donors and post-transplant follow-up data with the risk of developing NAS were analysed using the log-rank and two-tailed Student's *t*-tests. Differences in the serological levels of MMP were analysed using ANOVA.

Genotype frequencies were analysed by generating two-by-three contingency tables and statistical analysis was performed using the X^2-test or the Fisher's exact test, where appropriate. Comparison of time with NAS was made using Kaplan-Meier statistics with a log-rank test. Univariate and multivariate analyses were performed using Cox's proportional hazards method. Variables associated with an increased risk of NAS at the $P \leq 0.15$ level in the univariate logistic regression analysis were included in the backward stepwise multivariate logistic regression model. *P*-values ≤ 0.05 were considered statistically significant.

Results

The study population consisted of 314 OLT donor/ recipient combinations of which 48 (15%) developed NAS within the first 4 years after transplantation.

MMP-2 genotype and NAS

The frequencies of MMP-2 and MMP-9 gene promoter polymorphisms in recipients and in donors vs the occurrence of NAS are given in Table 1. Evaluation whether the MMP genotype is reflected in the serum level indicated that in patients with liver disease no such relation exists.

Table 1. Frequencies of matrix metalloproteinase polymorphisms in orthotopic liver transplant recipients and donors (*n* = 314)

Genotype				Recipient		Donor	
dbSNP ID	SNP			NAS %(*n*)	NoNAS %(*n*)	NAS %(*n*)	No NAS %(*n*)
MMP-2	— 1306	C → T	CC	46 (22)	61 (162)	44 (21)	53 (142)
rs243865			CT	52 (25)	33 (87)	56 (27)	41 (108)
			TT	2 (1)	6 (17)	0 (0)	6 (16)
				$P < 0.03$, X^2 7.2		$P = 0.05$, X^2 5.9	
MMP-9	— 1562	C → T	CC	67 (32)	71 (185)	75 (36)	78 (206)
rs3918242			CT	33 (16)	27 (71)	23 (11)	21 (56)
			TT	0 (0)	2 (5)	2 (1)	1 (2)
				$P = 0.46$, X^2 1.6(n = 309)		$P = 0.66$, X^2 0.9(n = 312)	

Table 2. Comparison of donor, recipient and procedure variables between patients with and without nonanastomotic biliary strictures after orthotopic liver transplant (*n* = 314)

	Total	NAS (*n* = 48)	%	No NAS (*n* = 266)	%	*P*-value
Donor variables						
Age (in years, median, range)	44 (16–72)	43	(16–67)	44	(9–72)	0.80
Gender						
Female	153	29	60	124	47	0.08
Male	161	19	40	142	53	
Recipient variables						
Age (in years, median, range)	48 (16–70)	45	(16–61)	48	(17–70)	0.08
Gender						
Female	122	16	33	106	40	0.39
Male	192	32	67	160	60	
Primary liver disease						**0.04**
Post viral cirrhosis	59	4	8	55	21	
Alcoholic cirrhosis	46	5	10	41	15	
PSC	57	15	31	42	16	
Other cholestatic disease*	28	6	13	22	8	
Other disease†	124	18	38	106	40	
Laboratory MELD score (median, range)	15	15 (6–40)		15 (6–40)		0.98
OLT procedure variables						0.50
DCD	25	5	10	20	8	
DBD	289	43	90	246	92	
WIT in minutes (mean±SD)	44±13 *n* = 296	42±10 *n* = 46		44±13 *n* = 250		0.37
CIT in minutes (mean±SD)	573±188 *n* = 299	595±183 *n* = 46		561±189 *n* = 253		0.38
MMP-2 [rs243865] CT						**0.003**
No CT present	115	10	21	105	39	
CT in recipient or donor	151	24	50	127	48	
CT in recipient and donor	48	14	29	34	13	

Age, MELD scores, WIT and CIT differences were evaluated by Student's t-test; frequency distribution data were analysed by w2 or Fisher's exact tests, where appropriate.

CIT, cold ischaemia time; time between the start of cold perfusion of graft in the donor and the end of cold preservation of the liver graft; DBD, donation after brain death; DCD, donation after cardiac death; MELD, model for end-stage liver disease; MMP, matrix metalloproteinase; NAS, nonanastomotic biliary lesions; OLT, orthotopic liver transplantation; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SD, standard deviation; SSC, secondary sclerosing cholangitis; WIT, warm ischaemia time; time between the end of cold ischaemic preservation of the liver graft and portal vein reperfusion in the recipient.

*Other cholestatic disease comprise PBC and SSC.

†Other diseases include predominantly autoimmune hepatitis, cryptogenic cirrhosis and metabolic disorders.

Specifically, MMP-2 levels in the pre-OLT serum of recipients with a CC (n = 32) genotype was 5123 ± 553ng/ml, whereas for those with a CT or TT genotype (n = 15), these levels were 5347 ± 886 (NS). For MMP-9, these levels were 129 ± 16 (n = 36) vs 156 ± 28 (n = 11) respectively (NS). The presence of MMP-2 CT genotype in the recipient as well as in the donor was significantly associated with the development of NAS. Furthermore, the cumulative presence of MMP-2 CT genotype in both recipient and donor vs the occurrence ofNAS is shown in Tables 2 and 3. In the group of patients that developed NAS, the absence of a CT genotype was more frequent (21%) than in the patients that did not develop NAS (39%) and for CT in donor and recipient, exactly the opposite was observed (29% vs 13%, Table 2). If CT genotype was present in neither recipient nor donor, the risk of developing NAS was 9% (10/115). When MMP-2 CT genotype was present in either donor or recipient, NAS developed in 16% (24/151) of cases. The occurrence of NAS increased to 29% if MMP-2 CT genotype was present in both recipient and donor (14/48; $P < 0.003$, Table 3). Figure 1 shows the cumulative incidence ofNAS within 48 months after OLT related to the presence of MMP-2 CT genotype in recipient and donor. We also evaluated whether this association between genotype MMP-2 and NAS was reflected in the serum levels. One month after OLT, the MMP-2 level in patients with NAS was showed a trend to be lower [i.e., 1892 ± 431ng/ml (n = 5) vs 2869 ± 287 (n = 22), P = 0.06], compared with the patients without NAS. Interestingly, a similar trend was observed in relation to the MMP-2 genotype, i.e. lower in relation to the presence of CT [2969 ± 452 vs 2540 ± 349 vs 2396 ± 448 for no CT in donor or recipient (n = 10), CT in donor or recipient (n = 15) and CT in donor and recipient (n = 2), respectively, NS].

Further assessment of the impact of the MMP-2 genotypes and NAS-related morbidity by including re-OLTs showed a similar stepwise increase in relation to the MMP-2 genotype from 14% (16/115) to 20% (30/151) and 38% (18/48) respectively (X^2 11.66, P = 0.003). By including death in the follow-up, this increased to 26% (30/115), 29% (44/151) and 44% (21/48) respectively (X^2 5.18, P = 0.08).

In a similar manner, the MMP-9 genotype distribution of recipient and donor vs the occurrence of NAS was evaluated. However, no significant correlation was found between MMP-9 genotype and the development of NAS (Table 1) or with the serum levels of MMP-9 (data not shown).

Multivariate analysis of MMP-2 genotype and covariates

The development of NAS was significantly higher when PSC was the

Table 3. Univariate and multivariate analysis for the association of risk factors of nonanastomotic biliary lesions in orthotopic liver transplant patients

				Univariate analysis		Multivariate analysis (n = 299)	
Risk factor		**NAS**	**%**	**HR (95% CI)**	***P*-value**	**Adjusted HR (95% CI)**	***P*-value**
MMP-2 [rs243865] CT					0.01		0.008
Donor and recipient	Vs. none	14/48	29	3.48 (1.55–7.84)	0.003	3.48 (1.54–7.87)	0.003
Donor or recipient	Vs. none	24/151	16	1.84 (0.88–3.84)	0.11	1.64 (0.78–3.46)	0.20
None	Reference	10/115	9	1 (reference)		1 (reference)	
Age recipient	Continuous	48/314		0.98 (0.96–1.01)	0.13		
Age donor	Continuous	48/314		1.00 (0.98–1.02)	0.72		
Gender recipient	Female	16/122	13	0.74 (0.41–1.35)	0.32		
	Male	32/192	17	1 (reference)			
Gender donor	Female	29/153	19	1.53 (0.86–2.73)	0.15		
	Male	19/161	12	1 (reference)			
Primary liver disease	PSC	15/57	26	2.0 (1.11–3.76)	0.02	2.14 (1.13–4.06)	0.02
	Other	33/257	13	1 (reference)			
Laboratory MELD score	Continuous	48/314		1.00 (0.97–1.04)	0.84		
Procedure	DCD	5/25	20	1.50 (0.60–3.80)	0.39		
	DBD	43/289	15	1 (reference)			
CIT	Continuous	46/299		1.000 (1.000–1.002)	0.15	1.001 (1.000–1.003)	0.08
WIT	Continuous	46/296		0.99 (0.96–1.01)	0.38		

CI, confidence interval; CIT, cold ischaemia time; time between the start of cold perfusion of graft in the donor and the end of cold preservation of the liver graft; DBD, donation after brain death; DCD, donation after cardiac death; HR, hazard ratio; MELD, model for end-stage liver disease; MMP, matrix metalloproteinase; NAS, nonanastomotic biliary strictures; OLT, orthotopic liver transplantation; PSC, primary sclerosing cholangitis; WIT, warm ischaemia time; time between the end of cold ischaemic preservation of the liver graft and portal vein reperfusion in the recipient.
Univariate and backward multivariate analyses were performed using Cox's proportional hazards method.

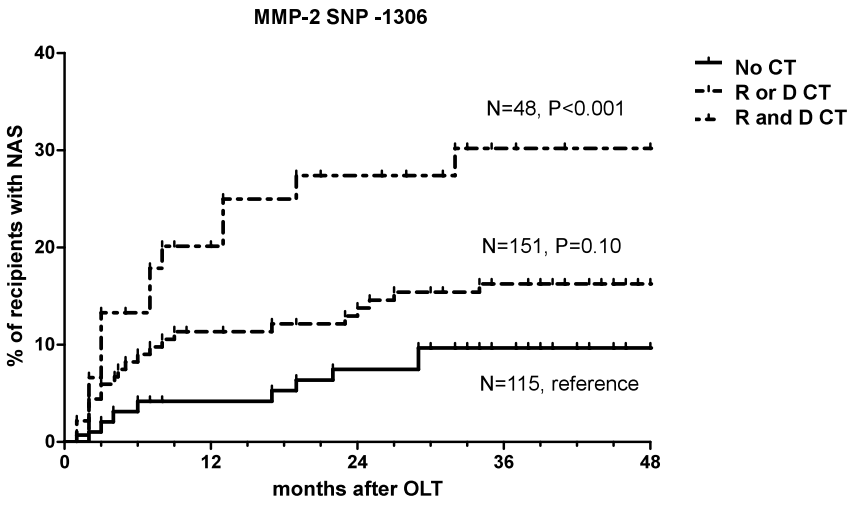

Fig. 1.
Cumulative incidence of NAS within 48 months after OLT related to the presence of MMP-2 CT genotype in recipient (R) and donor (D). MMP, matrix metalloproteinase; NAS, nonanastomotic biliary strictures; OLT, orthotopic liver transplantation.

indication for OLT [15/48 cases (31%) vs 33/ 257 cases (13%)], as expected. No significant association was found between the occurrence of NAS and other transplant characteristics, such as gender and age (both of recipient and donor), laboratory MELD score, length of warm or cold ischaemia time or DCD procedures. However, it should be noted that only 25 DCD procedures were included in this cohort. Also, in relation to immunosuppressive therapy, there was no association found with the development of NAS, i.e. patients on corticosteroids with a calcineurin inhibitor with or without basiliximab had a similar risk of developing NAS [13% (27/209) vs 20% (21/105), respectively, NS].

Early ($\leq$ 12 months) and late (12-48 months) onset of NAS was also looked at for all studied risk factors. NAS was diagnosed in 33 cases (10.5%) within the first year after OLT and in 15 cases (4.8%) from 12 to 48 months after OLT. Cold ischaemia time (CIT) was significantly longer for the group with early development of NAS (631 ± 179 vs 520± 174min; P =0.05). Interestingly, the effect of increased CIT and the incidence of NAS was particularly present in the first 12 months after OLT (hazard ratio 1.002; P = 0.03). Late occurrence of NAS was observed relatively more frequently when patients were transplanted for PSC. With PSC as the indication for liver transplantation, the occurrence of NAS within the first 12 months after OLT was 27% (9/33) as opposed to 40% (6/15) from the late onset NAS. A pre-OLT diagnosis of PSC was found to be accompanied particu-larly with an increased risk of late onset NAS (hazard ratio 3.1; P = 0.03).

Multivariate Cox regression analyses and the backward elimination procedure, taking all patient and transplant characteristics into account with an increased risk ofNAS (at the level of$P \leq 0.15$), indicated that the presence of MMP-2 CT genotype in donor and recipient was an independent risk factor for the development ofNAS with a higher hazard ratio than PSC as primary liver disease (3.5 vs 2.1, respectively; Table 3).

Discussion

In the present study, we report a strong association between the presence of MMP-2 CT genotype in donor and/or recipient and the development of NAS after OLT. In fact, MMP-2 genotype was a greater risk factor for NAS after OLT than PSC. The presence ofthe MMP-2 CT genotype in donor and/or recipient was found to increase the NAS incidence stepwise from 9% when absent, increasing to 16% when present in either recipient or donor, further increasing to 29% when present in both donor and recipient. In contrast, no association was

found between MMP-9 genotype and the development of NAS. Nonanastomotic strictures are considered to be the most troublesome biliary complication after OLT, associated with high retransplant rates in up to 20% of patients[5,7,25,41]. Interestingly, further assessment of the impact of the MMP-2 genotypes and NAS-related morbidity in our patients, by including re-OLTs, also revealed a stepwise increase in relation to the MMP-2 genotype from 14 to 38% and by including death in the follow-up, this increased even up from 26 to 44%. Apparently, the MMP-2 CT genotype also contributes to the morbidity accompanying NAS in the OLT patients.

Various risk factors for NAS have been identified, suggesting a multifactorial origin [9,14]. The main categories include ischaemia-related injury, immune-mediated injury such as ABO compatibility, pre-existing disease (especially PSC) and toxic injury by bile salts[42,43]. In addition to clinicopathological factors, we were also interested in the impact of the gelatinases MMP-2 and MMP-9 in the development of NAS.

Matrix metalloproteinases comprise a large family of proteolytic enzymes involved in physiological and dis-ease-related connective tissue remodelling processes and the gelatinases MMP-2 and MMP-9 are considered to play an important role in inflammation, degradation and remodelling processes in the liver[29,31,44]. MMP activity is regulated by various factors and controlled by activation of latent pro-enzymes and by interaction with endogenous inhibitors such as tissue inhibitors of metal-loproteinases (TIMPs). Recently, several single nucleotide polymorphisms (SNP) in the gene promoter regions of MMPs have been found with an impact on the transcription rate (30, 32). The C/T transition at posi-tion — 1306 in the promoter ofMMP-2, which abolishes the Sp 1 binding site, and leads to decreased mRNA transcription and protein expression, is generally ac-cepted to be the most relevant SNP for MMP-2. Other SNPs of MMP-2 have been reported as well, e.g. — 1575 G/A, — 790 C/T and — 735 C/T, but these are in almost complete linkage (dis)equilibrium with — 1306 C/Tand thus provide no additional information[30,32,33]. In several studies, an association was demonstrated between MMP-2 polymorphisms and the development of cancer. It has even been suggested that MMP-2 represents a potential target for tumour therapeutics[32,33].

In the MMP-9 gene, an SNP at position — 1562 is because of a C to T substitution in the promoter region. *In vitro* studies have shown that this transition results in loss of binding of a nuclear repression protein and increased transcriptional activity in macrophages, associated with the severity of coronary atherosclerosis.

Although other SNPs in the MMP-9 gene have been described, they were mainly nonsynonomous located in the exon part of the gene and found not to affect the activity or level of the enzyme[34,35] In contrast, in cardiovascular disease, for example, the — 1562 T allele was found to be associated with increased MMP-9 plasma levels[35].

In the liver, the hepatic stellate cell seems to be the main cellular source ofMMP-2 and when activated these cells are involved in the synthesis of matrix proteins and in the regulation of matrix degradation leading to liver fibrosis. Following liver injury, the stellate cells become activated and can express a wide range of MMPs and TIMPs, but in particular MMP-2[44-46]. Increased mRNA expression of MMP-2 was reported in liver biopsies of patients with cirrhosis[44]. We found pre-viously serum levels ofMMP-2 to be increased in patients with chronic liver disease and strongly correlated with serum markers indicative of a poor liver function[31]. After OLT, a gradual decrease of MMP-2 levels were found they remained higher, however, than found in healthy controls and increased with recurrent liver dis-ease. MMP-9 is released predominantly from neutrophils and macrophages, but the principal source in the liver is thought to be the Kupffer cell, the resident macrophage of the liver. Taking into account the different hepatic sources of MMP-2 (parenchymal stellate cells) and MMP-9 (Kupffer cells), one would expect a more long-standing effect of MMP-2 polymorphisms compared with MMP-9 polymorphisms. This is in line with our findings on the development of NAS within 4 years after OLT.

MMP-2 and MMP-9 seem to play a critical role in cold storage preservation injury and the reperfusion injury of liver grafts. We found previously elevated serum levels of MMP-9 at 1 week after OLT in patients with acute allograft rejection[45], illustrating that the extracellular matrix might be an important target in the process of acute rejection. In a recent study, however, we found serum levels to be affected but not in association with the MMP-2 and MMP-9 polymorphisms in late phase ischaemia/reperfusion I/R after OLT[36]. Also in the present study, we did not find that the genotype of the MMPs was reflected in the respective serum level of the patients before OLT. After transplantation, however, the MMP-2 levels showed a trend to be lower in patients that developed NAS and in association with the — 1306 CT genotype of recipient and donor. These observations might indicate a genetically determined reduced MMP-2 tissue remodelling as a cause of NAS after OLT, but the number of patients evaluated is too low to draw definite conclusions. With respect to MMP-9, no genotype (— 1562 CT)-phenotype (serum level) association was found in relation to NAS.

In the present study, early development of NAS (≤ 12 months) was associated with longer cold ischaemia times. It has been well described in previous studies that patients who are transplanted for PSC have a higher incidence of NAS after transplantation[7,20,21,47,48]. Our results confirm this finding and the multivariate analysis shows PSC to be a risk factor for the develop-ment of NAS, independent of MMP-2 genotype and occurring late after OLT (Table 3). The multivariate analysis shows MMP-2 genotype in donor and recipient to be a much stronger predictor for the development of NAS (hazard ratio 3.5) than a pre-OLT diagnosis of PSC (hazard ratio 2.1).

In summary, MMP-2 CT genotype in both donor and recipient is strongly and independently related to the development of NAS within 4 years after OLT. No association was found with MMP-9 CT genotype. These observations merit further studies on the influence of donor and recipient MMP-2 genotypes and on the MMP-related mechanisms involved in the development of NAS after OLT. As NAS leads to significant morbidity and graft loss in OLT, MMP-2 gene-based identification of these high-risk patients might be of great clinical importance.

References

1. Park JS, Kim MH, Lee SK, *et al.* Efficacy of endoscopic and percutaneous treatments for biliary complications after cadaveric and living donor liver transplantation. *Gastrointest Endosc* 2003; **57:** 78-85.

2. Qian YB, Liu CL, Lo CM, Fan ST. Risk factors for biliary complications after liver transplantation. *Arch Surg* 2004; **139:** 1101-5.

3. Rerknimitr R, Sherman S, Fogel EL, *et al.* Biliary tract complications after orthotopic liver transplantation with choledochocholedochostomy anastomosis: endoscopic findings and results of therapy. *Gastrointest Endosc* 2002; **55:** 224-31.

4. Sawyer RG, Punch JD. Incidence and management of biliary complications after 291 liver transplants following the introduction of transcystic stenting. *Transplantation* 1998; **66:** 1201-7.

5. Thethy S, Thomson BN, Pleass H, *et al.* Management of biliary tract complications after orthotopic liver transplan-tation. *Clin Transplant* 2004; **18:** 647-53.

6. Turrion VS, Alvira LG, Jimenez M, *et al.* Management of the biliary complications associated with liver transplanta-tion: 13 years of experience. *Transplant Proc* 1999; **31:** 2392-3.

7. Guichelaar MM, Benson JT, Malinchoc M, *et al.* Risk factors for and clinical course of non-anastomotic biliary strictures after liver transplantation. *Am J Transplant* 2003; **3:** 885-90.

8. Rizk RS, McVicar JP, Emond MJ, *et al.* Endoscopic management of biliary strictures in liver transplant recipients: effect on patient and graft survival. *Gastrointest Endosc* 1998; **47:** 128-35.

9. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. *J Hepatobiliary Pancreat Surg* 2006; **13:** 517-24.

10. Sheng R, Zajko AB, Campbell WL, Abu-Elmagd K. Biliary strictures in hepatic transplants: prevalence and types in patients with primary sclerosing cholangitis vs those with other liver diseases. *Am JRoentgenol* 1993; **161:** 297-300.

11. Distante V, Farouk M, Kurzawinski TR, *et al.* Duct-to-duct biliary reconstruction following liver transplantation for primary sclerosing cholangitis. *Transplint* 1996; **9:** 126-30.

12. Feith MP, Klompmaker IJ, Maring JK, *et al.* Biliary recon-struction during liver transplantation in patients with primary sclerosing cholangitis. *Transplant Proc* 1997; **29:** 560-1.

13. Schmitz V, Neumann UP, Puhl G, *et al.* Surgical complica-tions and long-term outcome of different biliary recon-structions in liver transplantation for primary sclerosing cholangitis-choledochoduodenostomy versus choledocho-jejunostomy. *Am JTransplant* 2006; **6:** 379-85.

14. Colonna JO, Shaked A, Gomes AS, *et al.* Biliary strictures complicating liver transplantation. Incidence, pathogen-esis, management, and outcome. *Ann Surg* 1992; **216:** 344-50.

15. Halme L, Hockerstedt K, Lautenschlager I. Cytomegalo-virus infection and development of biliary complications after liver transplantation. *Transplantation* 2003; **75:** 1853-8.

16. Kowdley KV, Fawaz KA, Kaplan MM. Extrahepatic biliary stricture associated with cytomegalovirus in a liver transplant recipient. *Transplint* 1996; **9:** 161-3.

17. Torras J, Llado L, Figueras J, *et al.* Biliary tract complica-tions after liver transplantation: type, management, and outcome. *Transplant Proc* 1999; **31:** 2406.

18. Maheshwari A, Maley W, Li Z, Thuluvath PJ. Biliary complications and outcomes of liver transplantation from donors after cardiac death. *Liver Transpl* 2007; **13:** 1645-53.

19. Dubbeld J, Hoekstra H, Farid W, *et al.* Similar liver transplantation survival with selected cardiac death donors and brain death donors. *Br J Surg* 2010; **97:** 744-53.

20. Campbell WL, Sheng R, Zajko AB, Abu-Elmagd K, Demetris AJ. Intrahepatic biliary strictures after liver transplanta-tion. *Radiology* 1994; **191:** 735-40.

21. Feller RB, Waugh RC, Selby WS, *et al.* Biliary strictures after liver transplantation: clinical picture, correlates and out-comes. *J Gastroenterol Hepatol* 1996; **11:** 21-5.

22. Ward EM, Kiely MJ, Maus TP, Wiesner RH, Krom RA. Hilar biliary strictures after liver transplantation: cholan-giography and percutaneous treatment. *Radiology* 1990; **177:** 259-63.

23. Sanchez-Urdazpal L, Gores GJ, Ward EM, *et al.* Diagnostic features and clinical outcome of ischemic-type biliary complications after liver transplantation. *Hepatology* 1993; **17:** 605-9.

24. Pascher A, Neuhaus P. Bile duct complications after liver transplantation. *Transpl int* 2005; **18:** 627-42.

25. Buis CI, Verdonk RC, van der Jagt EJ, *et al.* Non-anasto-motic biliary strictures after adult liver transplantation part one: radiological features and risk factors for early versus late presentation. *Liver Transpl* 2007; **13:** 708-1.

26. Rull R, Garcia Valdecasas JC, Grande L, *et al.* Intrahepatic biliary lesions after orthotopic liver transplantation. *Transplint* 2001; **14:** 129-34.

27. Shapiro SD. Matrix metalloproteinase degradation of ex-tracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998; **10:** 602-8.

28. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001; **17:** 463-516.

29. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. Matrix metalloproteinases in chronic liver disease and liver transplantation. In Lendeckel U, Hooper NM, eds *Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues.* Dordrecht, the Netherlands: Springer, 2006; 209-34.

30. Harendza S, Lovett DH, Panzer U, *et al.* Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. *J Biol Chem* 2003; **278:** 20490-9.

31. Kuyvenhoven JP, van Hoek B, Blom E, *et al.* Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. *Thromb Haemost* 2003; **89:** 718-25.

32. Price SJ, Greaves DR, Watkins H. Identification of novel, functional genetic variants in the human matrix metallo-proteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. *J Biol Chem* 2001; **276:** 7549-58.

33. Miao X, Yu C, Tan W, *et al.* A functional polymorphism in the matrix metalloproteinase-2 gene promoter (-1306C/T) is associated with risk ofdevelopment but not metastasis of gastric cardia adenocarcinoma. *Cancer Res* 2003; **63:** 3987-90.

34. Wu J, Zhang L, Luo H, *et al.* Association of matrix metalloproteinases-9 gene polymorphisms with genetic susceptibility to esophageal squamous cell carcinoma. *DNA Cell Biol* 2008; **27:** 553-7.

35. Zhang B, Ye S, Herrmann SM, *et al.* Functional polymorph-ism in the regulatory region of gelatinase B gene in relation to severity ofcoronary atherosclerosis. *Circulation* 1999; **99:** 1788-94.

36. Ten Hove WR, de Rooij BJF, van Hoek B, *et al.* MMP-2 and MMP-9 serum levels change but their gene promotor polymorphisms are not associated with late phase I/R injury or rejection after orthotopic liver transplantation. *Open Transplant Journal* 2008; **2:** 66-72.

37. Zhou LM, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous muta-tion scanning and genotyping in solution. *Clin Chem* 2005; **51:** 1770-7.

38. Kubben FJ, Sier CF, Meijer MJ, *et al.* Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. *Br J Cancer* 2006; **95:** 744-51.

39. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res* 2001; **29:** E88-8.

40. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, *et al.* Role of matrix metalloproteinase, tissue inhibitor of metal-loproteinase and tumor necrosis factor-alpha single nu-cleotide gene polymorphisms in inflammatory bowel disease. *World J Gastroenterol* 2007; **13:** 2960-6.

41. Sanchez-Urdazpal L, Gores GJ, Ward EM, *et al.* Ischemic-type biliary complications after orthotopic liver transplan-tation. *Hepatology* 1992; **16:** 49-53.

42. Buis CI, Geuken E, Visser DS, *et al.* Altered bile composi-tion after liver transplantation is associated with the devel-opment of nonanastomotic biliary strictures. *J Hepatol* 2009; **50:** 69-79.

43. Geuken E, Visser D, Kuipers F, *et al.* Rapid increase of bile salt secretion is associated with bile duct injury after human liver transplantation. *J Hepatol* 2004; **41:** 1017-25.

44. Milani S, Herbst H, Schuppan D, *et al.* Differential expression of matrix-metalloproteinase-1 and -2 genes in normal and fibrotic human liver. *Am J Pathol* 1994; **144:** 528-37.

45. Kuyvenhoven JP, Verspaget HW, Gao Q, *et al.* Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. *Transplantation* 2004; **77:** 1646-52.

46. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 2001; **21:** 373-84.

47. Sankary HN, McChesney L, Hart M, Foster P, Williams J. Identification of donor and recipient risk factors associated with nonanastomotic biliary strictures in human hepatic allografts. *Transplant Proc* 1993; **25:** 1964-7.

48. Brandsaeter B, Schrumpf E, Bentdal O, *et al.* Recurrent primary sclerosing cholangitis after liver transplantation: a magnetic resonance cholangiography study with analyses of predictive factors. *Liver Transpl* 2005; **11:** 1361-9.

Chapter 7

Chimerism as assessed by matrix metalloproteinase genotyping after orthotopic liver transplantation

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Abstract

Background

Chimerism in transplantation medicine refers to the coexistence of cells of donor and recipient origin. We used donor/recipient mismatches for matrix metalloproteinases (MMP) gene polymorphisms to study the presence of chimerism in liver biopsies and in blood after orthotopic liver transplantation (OLT).

Methods

MMP-2 and MMP-9 promoter polymorphism donor/recipient mismatches were determined in 147 OLT patients. The relationship between these MMP polymorphism mismatches in donor and recipient DNA with the development of ischemia/reperfusion injury and rejection after OLT was evaluated. Liver biopsy specimens and peripheral blood samples were subsequently evaluated for the presence of chimerism, also in relation to these complications.

Results

MMP polymorphism donor/recipient mismatches were found in 53.7% (MMP-2) and 35.5% (MMP-9) of the OLT patients but no relation was observed with I/R injury or rejection.

Chimerism in liver biopsy specimens was found to be present in 28.8% (MMP-2) and 16.2% (MMP-9) of the cases. Liver chimerism in MMP-2 was found to be significantly associated with rejection after OLT (Chi-square 6.4, P=0.01). In addition, evidence of donor chimerism was found in peripheral blood samples of the recipients in a few selected cases.

Conclusion

Chimerism after liver transplantation can be found in liver biopsy specimens and in peripheral blood using MMP polymorphism donor/recipient mismatches. Liver chimerism in MMP-2 was found to be significantly associated with rejection after OLT. The exact clinical and functional implications have still to be established.

Introduction

Chimerism after solid organ transplantation is a fascinating phenomenon. In the earliest days of organ transplantation, back in the 1960s, Medawar hypothesized that chimerism could lead to graft tolerance.[1] The existence of chimerism after transplantation have been studied by many, but the clinical significance remains unraveled.[2-5] Previously, we have addressed different aspects of chimerism within the liver, both after orthotopic liver transplantation (OLT) and after allogeneic bone marrow transplantation (BMT).[6,7] In these earlier studies we used in situ hybridization techniques targeting Y-chromosomes. A major drawback of this technique is that only a small fraction of sex mismatched donor/recipient combinations are relevant to be studied, i.e., male recipients receiving a female liver and female recipients receiving a male BMT.

In the present study we have used a different technique to investigate chimerism after OLT, which allowed us to include a larger number of liver transplant recipients. Gene polymorphisms of matrix metalloproteinases (MMP) 2 and 9 were analyzed. We previously reported on MMP polymorphisms in relation to different complications of liver transplantation and found preliminary indications that chimerism did occur.[8,9] Mismatches between donor and recipients were selected to study the presence of chimerism in liver biopsy specimens and in peripheral blood after liver transplantation.

The aim of the present study was to further explore the existence of chimerism in MMP-2 and MMP-9 promoter polymorphism donor/recipient mismatches in both liver biopsy specimens and in peripheral blood after OLT. The association between these MMP mismatches and the clinical occurrence of ischemia/reperfusion injury and rejection after OLT was also assessed.

Patients and Methods

Patients

All patients who received a liver transplant at Leiden University Medical Center (LUMC) between 1992 and 2005 were eligible for inclusion. Of these 202 patients, donor and recipient DNA was available of 147 patients with at least 7 days of follow-up after OLT. Demographical and clinicopathological characteristics of the study population were obtained from the transplantation database.

The study was performed according to the guidelines of the Medical

Ethics Committee of the Leiden University Medical Center and in compliance with the Helsinki Declaration.

Genotyping

Genomic DNA was extracted by routine methods from peripheral blood leukocytes and/or tissue samples. In addition, DNA samples from the blood of the liver donors was obtained from the Eurotransplant Reference Laboratory or freshy isolated from donor blood or spleen tissue. Also, DNA was isolated from liver biopsy tissue of the allograft in the recipients, obtained several months (median 17, range 5 to 48) after OLT.

MMP-2: high resolution DNA melting analysis

MMP-2 -1306 C/T promoter SNP was determined using a high resolution DNA melting assay with the 5′-CCACCCAGCACTCCACCTCTTTAGCTCF-3′ wild-type (C) gene probe, and the primers 5′-CCAGTGCCTCTTGCTGTTTT-3′ (forward) and 5′-GACTTCTGAG-CTGAGACCTGA-3′ (reverse).[9,10] This –1306 C/T MMP-2 gene promoter polymorphism was also determined by tetra-primer amplification refractory mutation system- polymerase chain reaction (PCR) analysis, the principles of which are described elsewhere[9], and confirmed by direct sequence analysis of 4 patients. Briefly, the region flanking the SNP was amplified with outer primers 5′-ACCAGACAAGCCTGAACTTGTCTGA-3′ and 5′-TGTGACAACCGTCTCTGAGGAATG-3′ together with inner allelic specific primers 5′-ATATTCCCCACCCAGCACGCT-3′ and 5′-GCTGAGACCTGAAGAGCTAAAGAG-TTG-3′. Genotypes CC, CT and TT (542+379; 542+379+211; 542+211 bp, respectively) are easily identified from the migration pattern on agarose gels.

This common functional polymorphism abolishes an Sp1 binding site within the promoter region of MMP-2. In brief, high-resolution melting analysis of PCR products amplified in the presence of a saturating double-stranded DNA dye (LCGreenPlus, Idaho Technology) and the 3′-blocked probe, identifies both heterozygous and homozygous sequence variants. Heterozygotes and homozygotes are distinguished by differences in the melting curve shape, due to differences in melting temperature. In each experiment, sequence-verified control donors for each genotype were used.

MMP-2 genotype distribution was as follows: in recipients CC 67.3% (n=99), CT 27.9% (n=41), TT 4.8% (n=7) and in donors CC 45.6% (n=67), CT 49.7% (n=73), TT 4.8% (n=7).

MMP-9: PCR-RFLP genotyping

The SNP C/T at position –1562 of the MMP-9 gene promoter was determined by PCR-RFLP. The SNP flanking region was amplified using primers 5′-ATGGCTCATG-CCCGTAATC-3′ and 5′-TCACCTTCTTCAAAGCCCTATT-3′ followed by restriction analysis with Sph I to produce 352, 352+207+145 or 207+145 bp fragments in case of CC, CT and TT genotype, respectively, which are easily identified from the migration patterns on agarose gels. [8,11-13]

MMP-9 genotype distribution was as follows: in recipients CC 75.9% (n=107), CT 23.4% (n=33), TT 0.7% (n=1) and in donors CC 73.0% (n=103), CT 27.0% (n=38) and none of the donors had TT genotype.

Assessment of MMP gene mismatch and chimerism in liver biopsy specimens and peripheral blood samples

Mismatch in the MMP-2 or -9 genes is defined as a non-identical genotype in OLT recipient and donor. Chimerism is defined as the presence of an MMP genotype signal in the DNA of the liver biopsy after OLT that originates from the recipient and in the blood DNA of the recipient after OLT when an MMP genotype signal appears from the donor.

Ischemia and reperfusion injury and rejection

The degree of I/R hepatocellular injury was evaluated by measurement of aspartate aminotransferase (AST) during the first week after OLT. Patients were classified into 2 groups depending on whether the serum AST peak was lower than 1,500 IU/L (no or mild I/R injury) or higher than 1,500 IU/L (more severe I/R injury), respectively.

Liver biopsies were taken according to our protocol at approximately 1 week, 3 months, 6 months and one year after OLT, or when there was a suspicion of rejection, and then each year. Acute allograft rejection was graded according to the Banff scheme. The rejection had to be clinically relevant for this study, i.e. histologically confirmed and treated with additional immunosuppression.

Statistical Analysis

Genotype frequencies were analyzed by generating two- by-two contingency tables. Statistical analysis was performed using the Chi-square test or Fischer's Exact test, where appropriate, using SPSS software (SPSS Inc; Chicago, IL, USA). Differences were considered to be significant at P- values of ≤ 0.05.

Results

Patients

Our study population consisted of 147 OLT donor/recipient combinations and MMP polymorphism mismatches were found in 53.7% for MMP-2 (79/147) and in 35.5% for MMP-9 (50/141). No statistically significant relation was found between the absence or presence of a mismatch at -1306 C/T MMP-2 or -1562 C/T MMP-9 in relation to the development of I/R injury or rejection after OLT, as illustrated in Table 1.

Chimerism in liver biopsy specimens

Of the 79 MMP-2 mismatches, liver biopsy specimens of 59 cases could be adequately studied for the presence of chimerism, which was found in 28.8% (17/59, Figure 1). For the MMP-9 mismatches, 50 in total, liver tissue of 37 patients could reliably be scored for chimerism, which was found in 16.2% (6/37).

The presence of chimerism in liver tissue was investigated in relation to I/R injury and rejection. A statistically significant association was found between chimerism for MMP-2 (but not MMP-9) and the occurrence of rejection, i.e., 41.2% versus 11.9% in the patients without chimerism (X^2=6.4; P=0.01). No association was found with I/R injury and MMP-2 or MMP-9 (Table 2)

Chimerism in peripheral blood

To assess chimerism in peripheral blood, donor/recipient combinations were selected that consisted of a MMP-2 genotype homozygote recipient and either a MMP-2 heterozygote donor or a donor of a different homozygote genotype. 27 patients could be included of whom blood DNA samples were available 3 to 12 years after OLT. Indications of blood chimerism was observed in 18.5% (5/27, Figure 2) of these patients, i.e., 6.3% (5/79) of the MMP-2 mismatches and 3.4% (5/147) of the total OLT population, where the donor MMP-2 gene signal was discernable in the recipient's blood DNA, in addition to the recipient's own MMP-2 gene signal. MMP-9 chimerism in blood of the recipients was not observed.

Table 1. Mismatch at -1306 C/T MMP-2 or -1562 C/T MMP-9 recipient-donor genotype in relation to the development of I/R injury or rejection after OLT

		Complication			
		IR-injury		Rejection	
Complication	**Genotype**	**No**	**Yes**	**No**	**Yes**
-1306 C/T MMP-2 recipient-donor	No	49 (72.1)	19 (27.9)	59 (86.8)	9 (13.2)
	Yes	51 (64.6)	28 (35.4)	64 (81.0)	15 (19.0)
Statistical significance		X^2 = 00.95; P=0.33		X^2 = 0.89; P=0.35	
-1562 C/T MMP-9 recipient-donor	No	60 (65.9)	31 (34.1)	77 (84.6)	14 (15.4)
	Yes	37 (74.0)	13 (26.0)	43 (86.0)	7 (14.0)
Statistical significance		X^2 = 0.98; P=0.32		X^2 = 0.05; P=0.83	

Table 2. MMP chimerism in liver tissue after OLT in relation to I/R injury and rejection.

		Complication			
		IR-injury		Rejection	
Complication	**Genotype**	**No**	**Yes**	**No**	**Yes**
MMP-2 in liver biopsy after OLT	No	31 (73.8)	11 (26.2)	37 (88.1)	5 (11.9)
	Yes	11 (64.7)	6 (35.3)	10 (58.8)	7 (41.2)
Statistical significance		X^2 = 0.49; P=0.48		X^2 = **6.4; P=0.01**	
MMP-9 in liver biopsy after OLT	No	22 (71.0)	9 (29.0)	26 (83.9)	5 (16.1)
	Yes	5 (83.3)	1 (16.7)	4 (66.7)	2 (33.3)
Statistical significance		X^2 = 0.39; P=0.48		X^2 = 0.97; P=0.32	

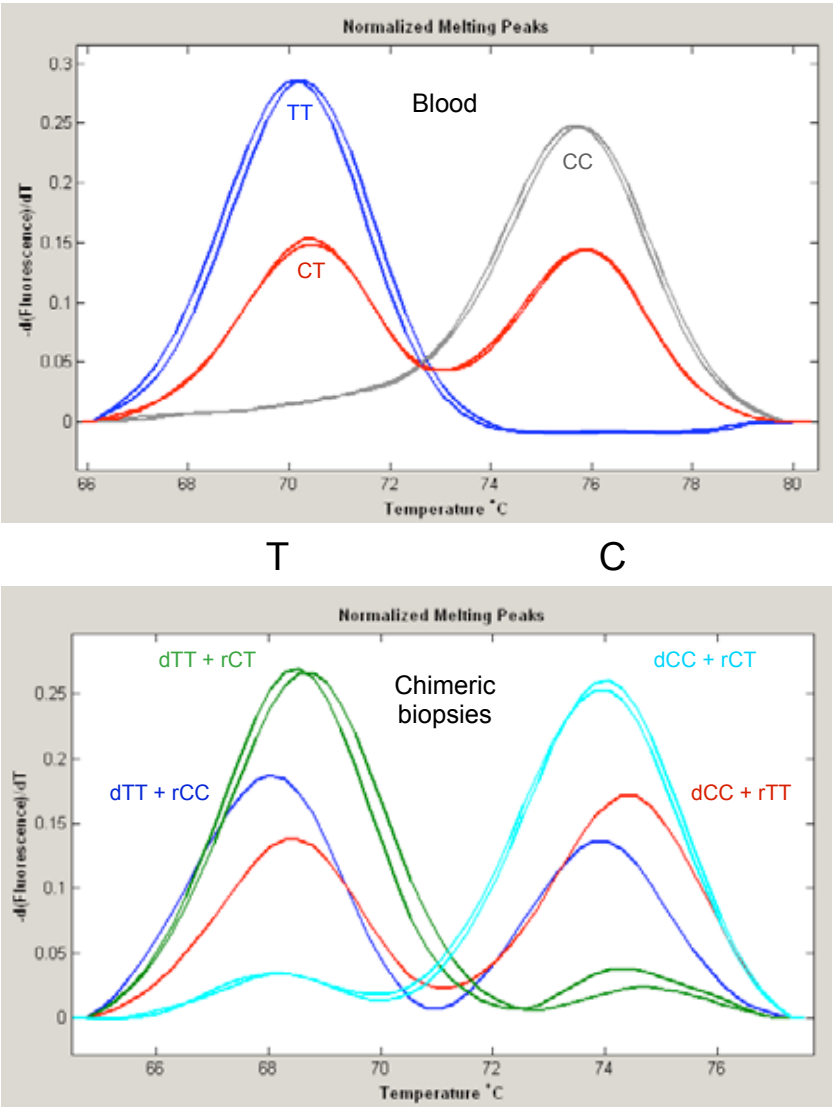

Figure 1:
Chimerism in liver biopsy specimens of OLT patients.

Above: Reference MMP-2 HMRA curves in blood: TT in blue, CC in grey and CT in red

Below: Chimeric MMP-2 HMRA curves in biopsies. Donor TT and recipient CC in dark blue; donor TT and recipient CT in green; donor CC and recipient CT in light blue; donor CC and recipient TT in red.

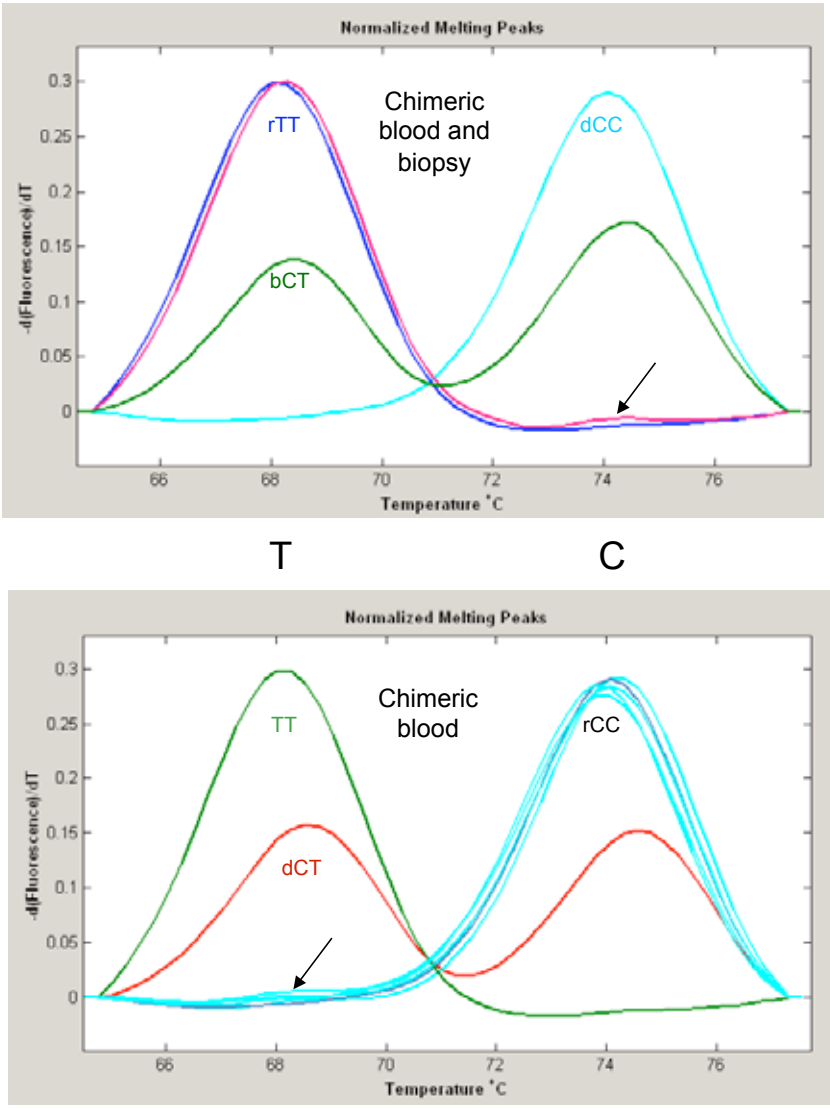

Figure 2:
Chimerism in biopsies and blood of OLT patients.

Above: MMP-2 HMRA curves of a TT recipient and CC donor in dark blue and light blue. Liver biopsy chimerism of the patient as shown by the CT curve in green. Blood chimerism is indicated by the arrow pointing at a minor peak at C in the purple TT curve of the recipient.

Below: Blood chimerism in CC recipients with CT donors in light blue, with the arrow pointing at minor chimeric T peaks; donor CT indicated in red, reference TT in green and recipient CC in grey.

Discussion

Assessment of the MMP-2 and MMP-9 genotypes in DNA of OLT patients show clear evidence of chimerism, both in liver tissue specimens and in peripheral blood, even years after transplantation.
In a previous study we already described liver chimerism in sex-mismatched donor/recipient mismatches.[6] Combinations selected were male transplant recipients and female donors in whom cells of recipient (male) origin could be readily identified with the use of an Y-chromosome specific in-situ hybridization technique.

In addition, patients who received an HLA-mismatched liver transplant were studied and chimerism was evaluated using immunohistochemistry with HLA class I-specific antibodies. To discriminate between cells of recipient and/or donor origin, double staining techniques were used with antibodies against specific cell types and subsets, i.e., endothelial and bile duct epithelial cells, lymphocytes, monocytes and other inflammatory cells. Endothelial cell chimerism was found to be quite common, whereas chimerism for biliary epithelial cells and hepatocytes could be shown only in a minority of cases. The limitation of using Y-chromosomes as a marker of chimerism is that only a small fraction of donor/recipient combinations (i.e., female/male) could be included. HLA staining could not differentiate hepatocytes from inflammatory cells with certainty and another limitation was the (sometimes poor) quality of liver biopsy samples for immunohistochemical analysis.
Matrix metalloproteinases are involved in connective tissue remodeling processes associated with chronic liver disease and complications after OLT.[8,14-16] In earlier studies we investigated, for example, MMP gene polymorphisms and their relation to ischemia/reperfusion injury, rejection and non-anastomotic biliary strictures (NAS) after OLT and found the MMP-2 CT genotype to be an independent risk factor for the development of NAS.[9]
Now we specifically studied several single nucleotide polymorphisms (SNP) in the gene promotor regions of MMP-2 and MMP-9, that were determined in DNA samples of peripheral blood leukocytes and/or liver tissue samples, for liver chimerism after OLT. For that purpose MMP gene mismatches were selected for the assessment of chimerism. Using this method we could include a far greater number than with the previously used methods (79/147 for MMP-2 and 50/141 for MMP-9). RFLP and HRMA for MMP-2 polymorphisms gave identical results and worked well, whereas for MMP-9 only the RFLP SNP analysis was found to be suitable, but this technique was not sensitive enough for

blood chimerism. Major advantage of the HRMA technique is that it was suitable for analyzing peripheral blood DNA samples of possible chimeric post-OLT patients. There are, however, also several limitations. The existence of chimerism can only be shown in a qualitative fashion. A quantification of chimerism would be very interesting, both in relation to donor/recipient and procedure-related variables and complications, and the follow-up of chimerism in time after the transplant procedure. Another limitation of this HRMA technique is that chimerism in liver tissue biopsies cannot be specified for different cell lineages, because tissue samples are processed for DNA extraction.

The MMP gene chimerism in liver tissue after OLT was assessed in relation to I/R injury and rejection, and an association was found between the chimeric MMP-2 genotype, but not for chimeric MMP-9 due to its low frequency, and the occurrence of rejection. This was not unexpected, since acute rejection is characterized by a portal mixed inflammatory infiltrate (of recipient origin), in combination with bile duct damage and endothelitis. A functional upregulation in acute rejection was previously described for MMP-9 and not for MMP-2.[16] In the present study, however, we analyzed MMP DNA polymorphisms as a gene marker and not as a functional MMP parameter. The relation of MMP-2 chimeric gene expression and rejection in the post OLT biopsies can therefore simply be explained by the influx of a mixed leukocyte infiltrate of recipient origin. However, although MMP-2 chimerism in the liver biopsies was found to be strongly associated with rejection it occurred in only 41.2% of cases where chimerism was observed. Nonetheless, in the majority of patients with signs of rejection this MMP-2 chimerism was discernable (58.3%; 7 out of 12 rejection cases). These observations imply that chimerism per se does not (have to) result in rejection. Furthermore, it cannot be excluded that biopsy sampling might have affected the chimerism assessment because all patients did have a MMP-2 gene mismatch but in 41.7% (5 out of 12 rejection cases) no expected chimerism was observed.
Indication of chimerism in peripheral blood samples after OLT was found in a minority of patients (18.5% of selected patients, i.e. 6.3% MMP-2 mismatches and 3.4% of the total OLT population) where the donor MMP-2 gene signal was detected in the recipient's blood DNA. As such an interesting observation was that a liver donor DNA signal is discernable in the DNA of circulating blood cells of the recipients. Further analyses need to be done in order to evaluate whether this is an indication of tolerance and where the signal is coming from, i.e., (re)

circulating liver (stem)cells or donor (stem)cells that have migrated to and recirculate from the recipients bone marrow.

In conclusion, this study indicates that chimerism after OLT persists both within the transplanted liver as well as in peripheral blood. There was a relationship between MMP-2 chimerism and rejection. The clinical relevance of chimerism in relation to graft tolerance, rejection or outcome remains unclear and requires further elucidation.

References

1. Medawar PB. Transplantation of tissues and organs: Introduction. Br Med Bull 1965;21:97-99.

2. Bogman MJ, de Waal RM, Koene RA. Persistent expression of donor antigens in endothelium of long-standing skin xenografts and vulnerability to destruction by specific antibodies. Transplant Proc 1987;19:205-207.

3. Sedmak DD, Sharma HM, Czajak CM, Ferguson RM. Recipient endothelialization of renal allografts. An immunohistochemical study utilizing blood group antigens. Transplantation 1988;46:907-910.

4. Fogt F, Beyser KH, Poremba C, Zimmerman RL, Khettry U, Ruschoff J. Recipient-derived hepatocytes in liver transplants: A rare event in sex-mismatched transplants. Hepatology 2002;36:173-176.

5. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. Lancet 1992;339:1579-1582.

6. Ten Hove WR, van Hoek B, Bajema IM, Ringers J, van Krieken JH, Lagaaij EL. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl. 2003;9:552-556.

7. Ten Hove WR, Verspaget HW, Barge R, Lamers CB, van Hoek B. Liver chimerism after allogeneic blood stem cell transplantation. Transplant Proc. 2007;39:231-236.

8. Ten Hove WR, de Rooij BJF, van Hoek B, Kuijvenhoven JP, Meijer MJ, van den Berg M et al. Serum levels change but their gene promoter polymorphisms are not associated with late phase I//R injury or rejection after orthotopic liver transplantation. Open Transplant Journal 2008;2:66-72.

9. Ten Hove WR, Korkmaz KS, op den Dries S, de Rooij BJF, van Hoek B, Porte RJ et al. Matrix metalloproteinase 2 genotype is associated with nonanastomotic biliary strictures after orthotopic liver transplantation. Liver International 2011; e-pub ahead of print.

10. Zhou LM, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. Clin Chem 2005;51:1770-1777.

11. Kubben FJ, Sier CF, van Duijn W, et al. Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer. Br J Cancer 2006;94:1035-1040.

12. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 2001;29:E88.

13. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Hommes DW, Verspaget HW. Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 2007;13:2960-2966.

14. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998;10:602-608.

15. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. Matrix metalloproteinases in chronic liver disease and liver transplantation. Lendeckel U, Hooper NM, editors. Dordrecht, the Netherlands, Springer. Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues. 2006:209-234.

16. Kuyvenhoven JP, Verspaget HW, Gao Q, Ringers J, Smit VT, Lamers CB et al. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. Transplantation 2004;77:1646-1652.

Chapter 8

Summarizing discussion

Introduction

Chimerism after orthotopic liver transplantation (OLT) is the main focus of the studies described in this thesis. The first study showed that chimerism of different cell lineages within the liver graft does occur after OLT. Subsequently, in allogeneic blood stem cell recipients, chimerism was demonstrated in liver tissue, providing evidence that circulating progenitor cells can indeed differentiate into parenchymal liver cells. The secondary focus of this thesis is on matrix metalloproteinases (MMPs). These proteolytic enzymes are involved in a wide variety of physiological and disease-related matrix remodeling processes. MMP-2 and MMP-9 gene promoter polymorphisms were assessed of OLT donors and recipients in relation to ischemia/reperfusion injury, acute rejection and non-anastomotic biliary strictures after transplantation. We performed a side study, in which we evaluated the value of serum liver chemistry profile and abdominal ultrasound for detecting clinically relevant biliary strictures using time-dependent multivariate regression analysis.

The two main themes, chimerism and MMPs, merge in the final chapter of this thesis. In liver transplant recipients with donor/recipient mismatches for MMP gene polymorphisms chimerism was studied, in both liver biopsies as well as peripheral blood after OLT.

Chimerism

Since the early days of organ transplantation the interaction between cells of donor and recipient origin has been studied by many in an attempt to understand rejection, graft tolerance and graft-versus-host disease.[1-3] Cell migration from the graft to the recipient results in systemic chimerism and cell migration from the host to the transplanted organ results in intragraft chimerism. Methods used to detect chimeric cells in transplanted organs are based on mismatches between donor and recipient.[4,5]

Liver chimerism was evaluated in sex-mismatched donor/recipient combinations, as described in **chapter 2**. This chimerism within the transplanted liver was studied after transplantation of a female donor liver into a male recipient. Cells of recipient (male) origin were identified with the use of a Y-chromosome specific in-situ hybridization technique. In the same study HLA-mismatched liver transplants were studied using immunohisto-chemistry with HLA class I-specific antibodies. Double staining techniques were used with antibodies against specific cell types

and subsets to differentiate between different cell lineages. Endothelial cell chimerism was found to be quite common. Chimerism for biliary epithelial cells and hepatocytes could be shown only in a minority of cases. HLA staining was found to be not adequate for differentiating hepatocytes from inflammatory cells with certainty. Findings were also limited by the (sometimes poor) quality of liver biopsy samples for immunohistochemical analysis. From these findings we postulated that circulating progenitor cells of recipient origin are probably capable of differentiating into endothelial and even epithelial cells within the transplanted liver.

In **chapter 3** a study is described in which the theory of blood stem cells developing into liver cells was further explored. Liver tissue specimens were examined from female patients who had received allogeneic blood stem cells from a male donor. All patients had been diagnosed with a hematologic malignancy, and treated with high-dose chemotherapy followed by allogeneic bone marrow transplantation or allogeneic peripheral blood stem cell transplantation. Fine-needle liver biopsy specimens were obtained because graft-versus-host-disease was suspected or from autopsy liver tissue. In this case, Y-chromosome identification was used to identify cells of donor origin. For consecutive staining an antibody against all known isotypes of the CD45 leucocyte common antigen family, present on lymphocytes, monocytes, granulocytes, and other inflammatory cells, was used. Y-chromosome-positive cells, indicating male (donor) origin, were found in all studied liver tissue samples. Many of these were likely to be of hematopoietic origin, representing infiltrating leukocytes but in some cases, donor-derived cells clearly appeared to be of non-lymphohematopoietic origin, supporting the presence of true tissue chimerism.

Matrix metalloproteinases and transplantation complications

Matrix metalloproteinases are proteolytic enzymes, involved in a wide variety of physiopathological tissue matrix remodeling processes.[6-9] We focused on MMP-2 and MMP-9 gene promotor polymorphisms in relation to clinical outcome of liver transplantation. **Chapter 4** describes the relationship of these polymorphisms with ischemia-reperfusion damage and rejection after OLT. Serum MMP-2 and MMP-9 levels were determined to study potential phenotypical consequences. No statistically significant differences between MMP gene promotor polymorphisms were found in relation to the development of I/R injury and to rejection after liver transplantation. Serum levels of MMP-2 were

found to be increased in patients with chronic liver disease and therefore a drop of MMP-2 levels was expected after OLT.[10,11] This decrease was indeed seen, but it occurred independent of the genotype. Serum levels of MMP-9 were found to peak at 1 week after OLT, which was associated with acute allograft rejection. Irrespective of a genotype mismatch between the donor and the recipient we found a comparable serum MMP-9 peak and pattern over time.

After liver transplantation the development of biliary strictures is a major clinical problem, due to its relatively high frequency, complications, morbidity and even mortality.[12-15] We were able to assess predictive factors of biliary strictures in a large cohort of liver transplant recipients, as described in **chapter 5**. This study focused on routine clinically diagnostic tests and their predictive value for the development of bile duct strictures, anastomotic as well as non-anastomotic (3 to 12 months after OLT). These common tests include liver chemistry profile and abdominal ultrasound (US) that are ubiquitously used to assess liver transplant recipient. The relationship between these tests and biliary complications is obvious, but very little is known on the predictive value of sequential profiling of these parameters. [16-18] We performed a time-dependent Cox regression analysis to identify predictive factors for the development of biliary strictures and found an elevated gamma-glutamyltranspeptidase and dilated bile ducts on ultrasound to have an independent predictive value for the development of biliary strictures requiring intervention. We advocate that dilated bile ducts on ultrasound and elevated gamma-glutamyltranspeptidase should prompt cholangiography for early diagnosis and therapeutic intervention of biliary strictures.

Given the fact that stricture formation in the liver bile ducts is accompanied by tissue remodeling and since MMP-2 and MMP-9 are considered to play a key role in connective tissue remodeling processes in the liver, we decided to study the relation of MMP-2 and 9 gene polymorphisms with non-anastomotic biliary strictures (NAS) after liver transplantation, as well. Specifically NAS was studied, because these strictures are a common and troublesome complication after OLT, leading to graft dysfunction, septic complications, secondary cirrhosis and even graft loss. The results of this study are described in **chapter 6**. The MMP-2 polymorphism was significantly associated with NAS, and donor and recipient genotypes had additive effects. Moreover, primary sclerosing cholangitis (PSC) and MMP-2 genotype polymorphism were independent risk factors for the development of NAS after OLT. No association was found with MMP-9 genotype. A higher risk for

developing (late) NAS was recently also described for graft recipients carrying specific *CCR5* alleles (encoding a chemokine receptor), especially in patients transplanted for PSC.[19] These and our findings strongly suggest that innate pathways contribute to NAS, the etiology of which involves multiple other mechanisms, such as ischemia, viral infection and bile salt-related injury.[15,20]

Matrix Metalloproteinase Chimerism

The last study of this thesis, **chapter 7**, merged our main focus areas, i.e., chimerism and MMPs. Donor/recipient mismatches for MMP gene polymorphisms were selected and evidence of chimerism was found in liver biopsy specimens for MMP-2 and MMP-9 polymorphism mismatches. An association between rejection and a specific MMP-2 genotype, in donors as well as in recipients, was found, but its role in pathophysiology remains to be established. In addition, evidence of donor chimerism was found in peripheral blood samples of liver transplant recipients in a few selected cases, in accordance with other recent studies.[21]

Discussion

In the chimerism studies described in this thesis a variety of techniques was used to study this intriguing phenomenon. Convincing evidence of chimerism is found in all three studies, but these studies also illustrate the difficulties encountered when studying this phenomenon. The selection of donor/recipient combinations depends on the technique which is chosen, often cutting down the number of cases that can be included (e.g., only the combinations of a female donor and a male recipient as in **chapter 2**). The next challenge was to further identify the chimeric cells. Circulating blood cells within the donor liver are without question from recipient origin and should be excluded since we are interested in true intragraft chimerism. To establish the functional relevance of chimerism it is essential to differentiate between different cell lineages. With a combination of techniques (determination of Y-chromosomes, HLA immunohistochemistry and double staining techniques with antibodies against specific cell types) the determination of chimeric cells in the liver graft was sometimes possible and at times even convincing, but often remained questionable. Poor quality of liver tissue samples made this an even more hazardous challenge, especially in autopsy specimens (**chapter 3**).

Donor/recipient mismatches for MMP gene polymorphisms (**chapter 7**) provided a larger study sample. In this study, MMP gene polymorphisms were solely used as a genetic association marker to explore chimerism and not as a functional parameter for disease progression as such. The disadvantage of this technique is that DNA is extracted from study specimens (whole liver tissue or peripheral blood samples), thus making differentiation of cell type impossible. However, the larger study sample made it possible to study the relation of chimerism in liver tissue to clinical outcome variables (I/R injury and rejection) and a statistically significant association was found between chimerism for MMP-2 (but not MMP-9) and the occurrence of rejection. This observation was consistent with earlier studies. However, the limitations in studying chimerism after liver transplantations with the described techniques are obvious. Indeed, we did find compelling evidence of chimerism, but quantification of chimerism could not be assessed and association of chimerism to some clinically important variables was difficult, due to lack of power. The relationship between the presence of chimerism and clinical outcome of organ transplantation thus remains largely unresolved. To establish this, we need techniques with a high(er) specificity to additionally distinguish between different cell types and the possibility to include a much larger cohort of patients for association studies with clinical outcome parameters.

Specific MMP-2 and MMP-9 gene promoter polymorphisms were studied in relation to clinical outcome parameters after liver transplantation. We found a strong association of a specific MMP-2 genotype with the development non-anastomotic biliary strictures (**chapter 6**). To assess changes in functional activity of MMPs, we studied changes in serum levels in relation to MMP gene polymorphisms. Before transplantation, we did not find an association between the genotype and the respective serum levels. After transplantation, however, MMP-2 levels tended to be lower in patients that developed NAS and in association with MMP-2 CT genotype. MMP-2 levels are increased in patients with chronic liver disease and therefore were expected to drop after OLT, as reported previously.[10] This decrease was indeed observed, but this occurred independent of the genotype or a MMP genotypic mismatch between donor and recipient. In addition, no association of the MMP polymorphisms and mismatches was observed with ischemia/reperfusion or rejection after OLT (**chapter 4**). Similarly, a peak in MMP-9 levels was found after OLT, as previously described by our group again independent of genotype and donor-recipient mismatch.

Concluding remarks

The studies described in this thesis provide compelling evidence of chimerism after orthotopic liver transplantation. Cells of recipient origin can indeed replace endothelial cells, biliary epithelial cells and hepatocytes. After blood stem cell transplantation, donor-derived cells can be found in liver tissue specimens. These findings strongly support the theory of blood stem cells developing into liver cells of mesenchymal origin. We found an association between MMP-2 chimerism and acute rejection, but many questions on the clinical relevance of chimerism remain unanswered, due to limitations of available techniques. Matrix metalloproteinases are important in connective tissue processes after liver transplantation. In our liver transplant series, we did not find a relationship between MMP genotype polymorphisms and ischemia/reperfusion damage or rejection after OLT. We did find a strong relationship, however, between MMP-2 CT genotype in donor and recipient and the development of non-anastomotic biliary strictures. In fact, in predicting the development of biliary strictures after OLT, we found MMP-2 CT genotype and dilated bile ducts on abdominal ultrasound to have a high and comparable hazard ratio. An elevated gamma-glutamyltranspeptidase appeared to be a much weaker, but nevertheless independent and statistically significant, predictor of these biliary strictures.

References

1. Starzl TE, Demetris AJ, Trucco M, Murase N, et al. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. Hepatology 1993;17:1127–1152.

2. Bishop GA, Sun J, Sheil AG, McCaughan GW. High-dose/ activation-associated tolerance: A mechanism for allograft tolerance. Transplantation 1997;64:1377-1382.

3. Orlando G, Soker S, Wood K. Operational tolerance after liver transplantation. J Hepatol 2009;50:1247-1257.

4. Ng IO, Chan KL, Shek WH, Lee JM, et al. High frequency of chimerism in transplanted livers. Hepatology 2003;38:989-998.

5. Pujal JM, Gallardo D. PCR-based methodology for molecular microchimerism detection and quantification. Exp Biol Med (Maywood) 2008;233:1161-1170.

6. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463-516.

7. Verspaget HW, Kuyvenhoven JP, Sier CF, van Hoek B. Matrix metalloproteinases in chronic liver disease and liver transplantation. Lendeckel U, Hooper NM, editors. Dordrecht, the Netherlands, Springer. Proteases in Biology and Disease 5: Proteases in Gastrointestinal Tissues 2006:209-234.

8. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. Semin Liver Dis 2001;21:373-384.

9. Hamada T, Fondevila C, Busuttil RW, Coito AJ. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. Hepatology 2008;47:186-198.

10. Kuyvenhoven JP, van Hoek B, Blom E, van Duijn W, et al. Assessment of the clinical significance of serum matrix metalloproteinases MMP-2 and MMP-9 in patients with various chronic liver diseases and hepatocellular carcinoma. Thromb Haemost 2003;89:718-725.

11. Kirimlioglu H, Kirimlioglu V, Yilmaz S. Expression of matrix metalloproteinases 2 and 9 in donor liver, cirrhotic liver, and acute rejection after human liver transplantation. Transpl Proc 2008;40:3574-3577.

12. Buis CI, Hoekstra H, Verdonk RC, Porte RJ. Causes and consequences of ischemic-type biliary lesions after liver transplantation. J Hepatobiliary Pancreat Surg 2006;13:517-524.

13. Graziadei IW, Schwaighofer H, Koch R, et al. Long-term outcome of endoscopic treatment of biliary strictures after liver transplantation. Liver Transpl 2006;12:718-725.

14. Heidenhain C, Pratschke J, Puhl G, et al. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int 2010;23:14-22.

15. Buis CI, Geuken E, Visser DS, Kuipers F, et al. Altered bile composition after liver transplantation is associated with the development of nonanastomotic biliary strictures. J Hepatol 2009;50:69-79.

16. Hussaini SH, Sheridan MB, Davies M. The predictive value of transabdominal ultrasonography in the diagnosis of biliary tract complications after orthotopic liver transplantation. Gut 1999;45:900-903.

17. Que Y, Kaneko J, Sugawara Y, Tamura S, Makuuchi M. Role of protocol ultrasonography for detecting biliary stricture in adult living donor liver transplantation recipients. Biosci Trends 2007;1:62-65.

18. Zoepf T, Maldonado-Lopez EJ, Hilgard P, et al. Diagnosis of biliary strictures after liver transplantation: which is the best tool? World J Gastroenterol 2005;11:2945-2948.

19. op den Dries S, Buis CI, Adelmeijer J, et al. Combination of PSC and CCR5- 32 in recipients is strongly associated with the development of nonanastomotic biliary strictures after liver transplantation. Liver Int 2011;DOI:10.1111/j.1478-3231-2010.02422.x.

20. Koch RO, Kaser A. Genetic insights into non-anastomotic biliary strictures after orthotopic liver transplantation. Liver Int 2011;DOI :10.1111/j.1478-3231-2011.02563.x.

21. Ayala R, Grande S, Albizua E, Crooke A, et al. Long-term follow up of donor chimerism and tolerance after human liver transplantation. Liver Transpl 2009;15:581-591.

Chapter 9

Nederlandse samenvatting

Introductie

In december 1963 verscheen de eerste publicatie over levertransplantatie van dr. Thomas Starzl. Hij beschreef hierin drie transplantatieprocedures. Een jongen van 3 jaar oud overleed tijdens de operatie door massaal bloedverlies. Twee volwassen mannen ondergingen een technisch succesvolle procedure, maar beiden overleden binnen enkele weken aan longembolieën.

De eerste levertransplantatie in Nederland werd in 1966 uitgevoerd in Leiden, maar de patiënt overleed tijdens de procedure door stollingsproblemen. Het duurde nog vele jaren tot levertransplantatie een erkende, volwaardige behandeling werd bij zeer ernstig leverfalen. Nu worden levertransplantaties in Nederland uitgevoerd in drie centra: Groningen (sinds 1979), Rotterdam (sinds 1986) en Leiden (sinds 1992). Aanvankelijk gingen de meeste zorgen uit naar verbetering van chirurgische technieken en het controleren van de bloedstolling. Met het verbeteren van de directe overleving werd het controleren van de afstotingsreactie belangrijk en inmiddels wordt veel onderzoek verricht naar de consequenties van langetermijnoverleving. Ook al is de 5-jaarsoverleving inmiddels zo'n 85%, het risico van chirurgische complicaties, van afstoting, van galwegcomplicaties en van infecties is onverminderd aanwezig.

Chimerisme

Verschillende aspecten van levertransplantatie komen in dit proefschrift aan de orde. Het centrale thema is chimerisme. Deze term stamt uit de Griekse mythologie, waarin de Chimaera een monsterlijk wezen was met het hoofd van een leeuw, het lichaam van een geit en de staart van een slang. Meer recent zijn verschillende fantastische chimere wezens beschreven in de (tevens verfilmde) boeken van Joanne Kathleen Rowling. In de wetenschap spreken we van chimerisme als cellen met verschillende genetische achtergronden voorkomen in één individu.

Na transplantatie zijn er twee vormen van chimerisme: ten eerste kunnen in het getransplanteerde orgaan cellen van de ontvanger zich nestelen en ten tweede kunnen vanuit het transplantaat cellen elders in de ontvanger terecht komen.

Over de klinische betekenis van chimerisme wordt al decennialang

gespeculeerd. Wanneer in het transplantaat schade ontstaat, bijvoorbeeld door ischemie (zuurstoftekort) of afstoting, zou dit door de ontvanger gerepareerd kunnen worden, wat leidt tot chimerisme. Een tweede hypothese is dat chimerisme kan leiden tot tolerantie voor het getransplanteerde orgaan. Meer cellen zijn dan immers afkomstig van de ontvanger. Als de ontvanger het orgaan beter tolereert, kan wellicht minder medicatie worden gegeven om afstoting tegen te gaan. Chimerisme als proces is dus intrigerend en bovendien mogelijk van belang voor de klinische praktijk.

De technieken om chimerisme te bestuderen, berusten op verschillen tussen donor en ontvanger. Dit noemen we mismatches. Wanneer donor en ontvanger niet hetzelfde geslacht hebben, kan het mannelijke Y-chromosoom worden gebruikt om de oorsprong van de cellen vast te stellen. De studies die worden beschreven in de hoofdstukken 2 en 3 zijn op dit principe gebaseerd.

In **hoofdstuk 2** hebben we mannelijke ontvangers geselecteerd die een lever ontvingen van een vrouwelijke donor. In de getransplanteerde lever werd gezocht naar cellen met een Y-chromosoom, afkomstig van de ontvanger. Dubbelkleuringen met verschillende antilichamen werden gebruikt om vast te stellen wat voor soort cellen dit nu waren. Het ligt voor de hand dat circulerende bloedcellen van de ontvanger afkomstig zijn, maar het bleek dat ook endotheelcellen, die de bloedvaten bekleden, afkomstig kunnen zijn van de ontvanger. Dit was al eerder beschreven. Tevens werd gevonden dat galwegepitheel, bekleding van de galwegen en zelfs hepatocyten, de levercellen zelf, afkomstig kunnen zijn van de ontvanger. Dit kan eigenlijk alleen worden verklaard door circulerende stamcellen van de ontvanger, die zich nestelen in de getransplanteerde lever en uitgroeien tot verschillende celtypes. In deze studie werd dus chimerisme aangetoond na levertransplantatie.

De studie beschreven in **hoofdstuk 3** betreft hetzelfde onderwerp, maar vanuit een ander perspectief. Nu werd gekeken naar patiënten die waren behandeld met een beenmerg-stamceltransplantatie, meestal voor een hematologische maligniteit zoals leukemie of een lymfoom. Vrouwelijke patiënten werden onderzocht die beenmerg ontvingen van een mannelijke donor en bij wie nadien leverweefsel was onderzocht. Ook nu werd gekeken naar de aanwezigheid van een Y-chromosoom, in dit geval passend bij cellen van donor-origine. In de lever bleken inderdaad cellen aantoonbaar afkomstig van de donor. Vaak ging het

om verschillende soorten bloedcellen, maar dubbelkleuringen toonden aan dat het soms echt om levercellen ging. Zo werd chimerisme aangetoond in de lever na beenmerg-stamceltransplantatie.

Matrix metalloproteinasen en complicaties na levertransplantatie

Het tweede thema van dit proefschrift, naast chimerisme, betreft matrix metalloproteinasen (MMP's). MMP's zijn belangrijke enzymen bij de opbouw en afbraak van bindweefsel. Ze spelen een rol bij groei en wondgenezing, maar ook bijvoorbeeld bij aderverkalking, longemfyseem en kwaadaardige ziekten. In de lever werden met name MMP-2 en MMP-9 onderzocht. We hebben de genetische variatie, polymorfismen, van deze MMP's bestudeerd in relatie tot verschillende processen na levertransplantatie. Ook werd gekeken naar serumspiegels van deze MMP's.

De studie beschreven in **hoofdstuk 4** behandelt de relatie van deze MMP's met afstoting en ischemie-reperfusieschade. Wanneer de donorlever enige tijd niet doorbloed is geweest, ontstaat schade door zuurstoftekort (ischemie). Het opnieuw doorbloeden van de lever kan die schade juist verergeren. Dit proces wordt ischemie-reperfusieschade genoemd. Er werd geen duidelijke relatie gevonden tussen genetische MMP polymorfismen en afstoting of ischemie-reperfusieschade. Wel werd, zoals we na eerdere studies al verwachtten, een daling van de MMP-2 en een stijging van MMP-9 spiegels gezien na transplantatie, onafhankelijk van het genotype.

In **hoofdstuk 6** (hoofdstuk 5 komt later aan de orde) werd opnieuw gekeken naar MMP-2 en MMP-9 genetische polymorfismen, maar nu in relatie tot galwegstricturen na levertransplantatie. Lekkage van gal en galwegvernauwingen (stricturen) zijn de meest voorkomende complicaties van de galwegen na levertransplantatie. Ze worden onderverdeeld in stricturen bij de aanhechting van de donorgalweg met de ontvangergalweg (anastomotische stricturen) en vernauwingen op andere plaatsen in de galwegen, de niet-anastomotische stricturen (NAS). Deze hebben we bestudeerd en het bleek dat een bepaald MMP-2 genotype van zowel de donor als de ontvanger significant geassocieerd was met NAS. Naast eerder beschreven oorzaken van deze galwegstricturen, zoals ischemie, schade door galzouten, eerdere chronische leverziekte of een virusinfectie, blijkt de genetische aanleg van donor en ontvanger dus van belang.

Nu we zoveel informatie hadden verzameld over galwegstricturen (314 levertransplantaties uit het LUMC-Leiden en het UMCG-Groningen), wilden we nagaan of het ontstaan van deze stricturen voorspeld kon worden met alledaagse klinische testen. De uitkomsten hiervan zijn beschreven in **hoofdstuk 5**. Leverchemie en echografie van de lever worden standaard toegepast na levertransplantatie en er is een overduidelijke relatie met de aanwezigheid van galwegstricturen. Maar de voorspellende waarde van deze onderzoeken voor het ontstaan van galwegstricturen is nauwelijks onderzocht. Een ingewikkeld statistisch model, een tijd-afhankelijke Cox regressie analyse, wees uit dat een verhoogd gamma-glutamyltranspeptidase (gamma-GT) en verwijde galwegen bij echo-onderzoek een onafhankelijk voorspellende waarde hebben voor het ontstaan van galwegstricturen.

Chimerisme en matrix metalloproteinasen

De belangrijke thema's van dit proefschrift, chimerisme en MMP's, komen samen in hoofdstuk 7. Donor/ontvanger mismatches voor MMP polymorfismen werden geselecteerd en chimerisme na levertransplantatie kon worden aangetoond in zowel leverweefsel als in bloedmonsters. Ook werd een associatie gevonden tussen rejectie en MMP-2 chimerisme in de lever. De pathofysiologische betekenis van deze associatie is overigens nog onduidelijk.

Conclusie

In de diverse studies van dit proefschrift werd chimerisme overtuigend aangetoond. Endotheelcellen, galwegepitheelcellen en levercellen in de getransplanteerde lever blijken afkomstig te kunnen zijn van de ontvanger. Na beenmerg-stamceltransplantatie kunnen gedifferentieerde cellen van de donor gevonden worden in leverweefsel. Maar wat is nu de klinische betekenis van dit fenomeen? Decennialang is gespeculeerd over een relatie tussen chimerisme en tolerantie voor het getransplanteerde orgaan. Om dit te bestuderen is het belangrijk de aard en het aantal chimere cellen precies te kunnen bepalen in grote groepen patiënten. Het identificeren van chimere cellen bleek soms mogelijk, maar soms ook niet, afhankelijk van de gebruikte technieken. Het kwantificeren van chimerisme bleek bijzonder lastig. Vaak was maar weinig leverweefsel beschikbaar, soms bleek het van matige kwaliteit en afhankelijk van de gebruikte techniek kon maar een beperkte selectie van donor/ontvanger combinaties worden bestudeerd. Selectie op

donor/ontvanger MMP genotype leverde een relatief groot aantal mismatches op, maar met deze techniek was differentiëren naar celtype juist weer niet mogelijk. Chimerisme is dus overtuigend aanwezig, maar de klinische relevantie blijft vooralsnog onduidelijk.

Matrix metalloproteinasen spelen een belangrijke rol in bindweefselprocessen, ook na levertransplantatie. We vonden geen relatie tussen MMP polymorfismen en ischemie-reperfusieschade of afstoting. Wel vonden we een sterke relatie tussen een specifiek MMP-2 genotype, van donor én ontvanger, en niet-anastomotische galwegstricturen. Op deze wijze kunnen patiënten worden geïdentificeerd die een hoog risico hebben op het ontwikkelingen van dit type galwegstricturen.

De voorspellende waarde van routine leverchemie en echografie werd onderzocht voor de vroegdetectie van galwegvernauwingen. Verwijde galwegen bij lever echografie en een verhoogd gamma-glutamyltranspeptidase (gamma-GT) bleken een onafhankelijk voorspellende waarde te hebben voor het ontstaan van deze galwegstricturen. Dit is van belang om deze belangrijke galwegcomplicaties vroeg te diagnostiseren en te behandelen.

Concluderend kan worden gesteld dat chimerisme na levertransplantatie, zowel vanuit de nieuwe lever als vanuit het beenmerg van de ontvanger, overtuigend is aangetoond, maar dat de klinische betekenis onopgehelderd blijft. Het sequentieel verrichten van lever echografie en het bepalen van gamma-glutamyltranspeptidase in serum is van klinische waarde bij het vroegtijdig opsporen van galwegstricturen, zodat deze tijdig kunnen worden behandeld. Daarnaast heeft het onderzoek naar matrix metalloproteinasen aangetoond dat er een duidelijke relatie is van een specifiek MMP-2 genotype met het ontstaan van galwegstricturen, hetgeen ook kan helpen hoog-risicopatiënten te identificeren. Daarnaast zijn er ook functionele veranderingen in de MMP spiegels tijdens afstoting en ischemie-reperfusieschade na levertransplantie, maar die zijn niet erfelijk bepaald.

List of abbreviations

ALAT	Alanine aminotransferase
AML	Acute myelogeneous leukemia
ANED	Alive, no evidence of recurrent disease
AP	Alkaline phosphatase
AS	Anastomotic strictures
AST	Aspartate aminotransferase
CIT	Cold ischemia time
CML	Chronic myelogeneous leukemia
CMV	Cytomegalovirus
CT	Computed tomography
DBD	Donation after brain death
DCD	Donation after cardiac death
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
DNED	Deceased, no evidence of recurrent disease
DWED	Deceased with evidence of recurrent disease
ERCP	Endoscopic retrograde cholangiopancreatogram
FISH	Fluorescence in situ hybridization
G-CSF	Granulocyte colony stimulating factor
GGT	Gamma glutamyltranspeptidase
GVHD	Graft versus host disease
HE	Hemotoxylin and eosin
HLA	Human leukocyte antigen
HPF	High power field

I/R	Ischemia/reperfusion
ISH	In situ hybridization
MELD	Model for end-stage liver disease
MM	Multiple myeloma
MMP	Matrix metalloproteinases
MRCP	Magnetic resonance cholangiopancreatogram
NAS	Non-anastomotic strictures
NHL	Non-Hodkgin's lymphoma
OLT	Orthotopic liver transplantation
PAS	Periodic acid–Schiff
PBC	Primary biliary cirrhosis
PCR	polymerase chain reaction
PSC	Primary sclerosing cholangitis
PSCT	Peripheral blood stem cell transplantation
PTCD	Percutaneous transhepatic cholangiodrainage
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SSC	Secondary sclerosing cholangitis
TIMP	Tissue inhibitors of metalloproteinases
US	Ultrasonography
WIT	Warm ischemia time

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List of publications

Highly variable anticoagulant response after subcutaneous administration of high-dose (12,500 IU) heparin in patients with myocardial infarction and healthy volunteers. Kroon C, ten Hove WR, de Boer A, Kroon JM, van der Pol JM, Harthoorn-Lasthuizen EJ, et al. Circulation. 1992;86:1370-5.

A comparison of amodiaquine and chloroquine in the treatment therapy of falciparum malaria in Kenya. Nevill CG, Verhoeff FH, Munafu CG, ten Hove WR, van der Kaay HJ, Were JB. East Afr Med J. 1994;71:167-70.

"Colorectal Cancer in Ashkenazi Jews: Is Aggressive Screening Indicated?". Rogier ten Hove W, Burke C, Church JM, van Stolk R. ACG. 1998;93;1690.

Very-low-calorie diet in treatment of morbidly obese patients with diabetes mellitus. ten Hove WR, de Meijer PH, Meinders AE. Ned Tijdschr Geneeskd. 2000;144:1089-92.

Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Hove WR, van Hoek B, Bajema IM, Ringers J, van Krieken JH, Lagaaij EL. Liver Transpl. 2003;9:552-6.

Switching monitoring of emulsified cyclosporine from trough level to 2-hour level in stable liver transplant patients. Langers P, Cremers SC, den Hartigh J, Veenendaal RA, ten Hove WR, Ringers J, et al. Liver Transpl. 2004;10:183-9.

Liver chimerism after allogeneic blood stem cell transplantation. Ten Hove WR, Verspaget HW, Barge R, Lamers CB, van Hoek B. Transplant Proc. 2007;39:231-6.

Spontaneous bacterial peritonitis, a severe complication in patients with liver cirrhosis. Plokker HM, ten Hove WR, van der Heyden JT. Ned Tijdschr Geneeskd. 2007;151:1258.

Dysphagia and an oesophageal stricture; not always cancer. Woittiez KJ, Ten Hove WR, van der Heyden JT. Gut. 2008;57:1032-40.

MMP-2 and MMP-9 serum levels change but their gene promoter polymorphisms are not associated with late phase I/R injury or rejection after orthotopic liver transplantation. ten Hove WR, de Rooij BJF, van Hoek B, Kuyvenhoven JPh, Meijer MJW, van den Berg M, et al. The Open Transplant J. 2008;2:66-72.

Acute portal vein thrombosis unrelated to cirrhosis: a prospective multicenter follow-up study. Plessier A, Darwish-Murad S, Hernandez-Guerra M, Consigny Y, Fabris F, Trebicka J, et al. Hepatology. 2010;51:210-8.

Surgical gastrojejunostomy or endoscopic stent placement for the palliation of malignant gastric outlet obstruction (SUSTENT study): a multicenter randomized trial. Jeurnink SM, Steyerberg EW, van Hooft JE, van Eijck CH, Schwartz MP, Vleggaar FP, et al. Gastrointest Endosc. 2010;71:490-9.

Lectin complement pathway gene profile of donor and recipient determine the risk of bacterial infections after orthotopic liver transplantation. de Rooij BJ, van Hoek B, ten Hove WR, Roos A, Bouwman LH, Schaapherder AF, et al. Hepatology. 2010;52:1100-10.

High detection rate of adenomas in familial colorectal cancer.,van der Meulen-de Jong AE, Morreau H, Becx MC, Crobach LF, van Haastert M, ten Hove WR, et al. Gut. 2011;60:73-6.

Matrix metalloproteinase 2 genotype is associated with non-anastomotic biliary strictures after orthotopic liver transplantation. ten Hove WR, de Rooij BJ, op den Dries S, Korkmaz KS, van Hoek B, Porte RJ, et al. Liver International 2011;31:1110-1117; doi: 10.1111/j.1478-3231.2011.02459.x.

Mannose-binding lectin and ficolin-2 gene polymorphisms predispose to cytomegalovirus (re)infection after orthotopic liver transplantation. de Rooij BJ, van der Beek MT, van Hoek B, Vossen AC, ten Hove WR, Roos A, et al. J Hepatol. 2011; doi:10.1016/j.jhep.2011.01.039.

Curriculum vitae

De auteur van dit proefschrift werd op 1 mei 1965 geboren in Oegstgeest. Hij deed in 1983 eindexamen aan het Stedelijk Gymnasium Leiden en vertrok vervolgens voor een jaar naar de Verenigde Staten waar hij in 1984 eindexamen deed aan de Wissahickon Senior High School in Ambler, Pennsylvania. Terug in Leiden ging hij geneeskunde studeren. In 1988 bezocht hij opnieuw de Verenigde Staten en fietste 7000 kilometer van de oost- naar de westkust. In 1992 behaalde hij het artsexamen aan de Rijksuniversiteit Leiden.

Na een jaar te hebben gewerkt als arts-onderzoeker bij het Centre for Human Drug Research, onder leiding van Prof. dr. A.F. Cohen, begon hij in 1994 met de opleiding voor internist, eerst in het Rijnland Ziekenhuis te Leiderdorp (opleider: dr. W.J. van Amstel), vervolgens in het Academisch Ziekenhuis Leiden (opleider: Prof. dr. A.E. Meinders) en één jaar in de Cleveland Clinic, Cleveland, Verenigde Staten (opleider: dr. B.J. Hoogwerf). De registratie als internist volgde op 1 januari 2000. De opleiding tot maag-, darm- en leverarts (MDL arts) in het LUMC (opleider: Prof. dr. C.B.H.W. Lamers) werd afgerond op 1 januari 2003. In die periode werd een voorzichtige aanvang gemaakt met het onderzoek, waarvan de resultaten beschreven zijn in dit proefschrift, onder leiding van Prof. dr. B. Van Hoek en Dr. ir. H.W. Verspaget met ondersteuning van analisten en onderzoekers van het MDL researchlaboratorium.

Sinds 2003 is hij werkzaam als MDL arts en internist. Eerst in het Groene Hart Ziekenhuis te Gouda en sinds 2009 weer in zijn woonplaats, in het Diaconessenhuis Leiden.

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