Liver transplantation : chimerism, complications and matrix metalloproteinases

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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	

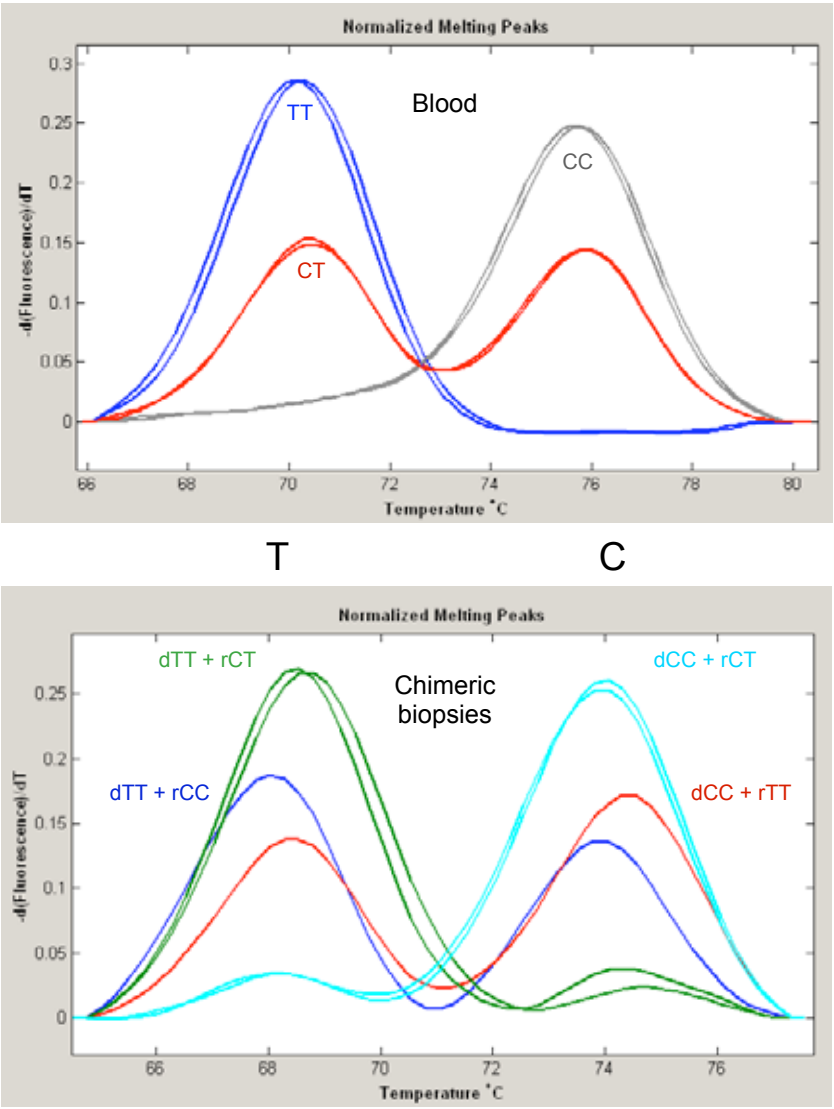

Figure 1:
Chimerism in liver biopsy specimens of OLT patients.

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.

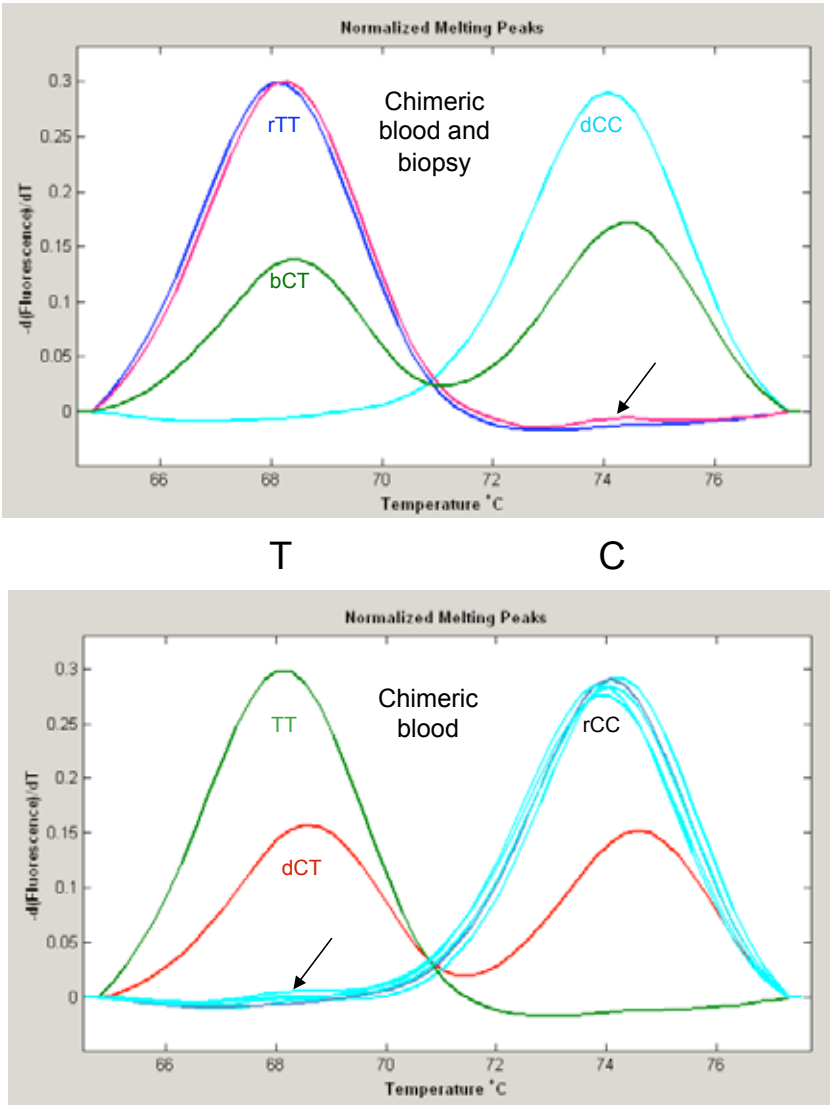

Figure 2:
Chimerism in biopsies and blood of OLT patients.

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.

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