Gelatinases in chronic liver disease. The clinical relevance of MMP-2 and MMP-9 in orthotopic liver transplantation

Kuyvenhoven, J.P.

Chapter 7

Matrix metalloproteinase (MMP)-2 and MMP-9 gene promoter polymorphisms in chronic liver disease – relation to ischemia/reperfusion injury and rejection after orthotopic liver transplantation.

Johan Ph. Kuyvenhoven, W. Rogier ten Hove, Bart van Hoek, Martin J.W. Meijer, Marlies van den Berg, Johan J. van der Reijden, Willem Verduyn, Cornelis B.H.W. Lamers, Hein W. Verspaget.

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List of 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.

Matrix metalloproteinase (MMP)-2 and MMP-9 gene promoter polymorphisms in chronic liver disease – relation to ischemia/reperfusion injury and rejection after orthotopic liver transplantation.

Johan Ph. Kuyvenhoven[1], W. Rogier ten Hove[1], Bart van Hoek[1], Martin J.W. Meijer[1], Marlies van den Berg[1], Johan J. van der Reijden[1], Willem Verduyn[2], Cornelis B.H.W. Lamers[1], Hein W. Verspaget[1].

[1]Department of Gastroenterology and Hepatology, [2]Department of Immunohematology and Blood Transfusion / Eurotransplant Reference Laboratory, Leiden University Medical Center, Leiden, The Netherlands.

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 gene have been found to have functional impact on the gene transcription rate.
Methods. Serum MMP-2 and MMP-9 concentrations were measured and the –1306 C/T MMP-2 and –1562 C/T MMP-9 gene promoter polymorphisms were analysed in 47 patients with chronic liver disease. In 27 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.
Results. Serum MMP-2 and MMP-9 levels in all chronic liver disease patients combined were not affected by the allelic composition at the promoter region of their respective genes. In patients with cirrhosis, however, the serum MMP-2 level showed an increase with the stage of cirrhosis in accordance with an increase in frequency of the wild type CC of the MMP-2 gene. In patients without cirrhosis the higher serum level of MMP-9 was accompanied by a higher CT genotype frequency of the –1562 C/T MMP-9 gene.
In contrast to the serum MMP-9 level, the MMP-9 genotype frequency of the donor and recipient or a MMP-9 gene donor/recipient mismatch was not associated with the development of late phase I/R injury or rejection in the OLT patients.
Conclusions. Serum MMP-2 and MMP-9 levels appear to be independent of the MMP genotype in chronic liver disease patients, although in relation to cirrhosis strong indications of genotypic impact on serum levels are discernable. In contrast to the increased MMP-9 serum level, the –1562 C/T MMP-9 polymorphism is not associated with late phase I/R injury or rejection after liver transplantation.

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, liver fibrosis and inflammation.(1;2)

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.(3;4) In several studies an association was demonstrated between MMP-2 polymorphisms and the development of cancer.(5-7) 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 investigate whether serum MMP-2 and MMP-9 levels in patients with chronic liver disease are influenced by the –1306 C/T MMP-2 and the –1562 C/T MMP-9 gene promoter polymorphism. Furthermore, we examined the relationship between these polymorphisms in the recipient and donor DNA with the development of ischaemia/reperfusion (I/R) injury or rejection after OLT.

Patients and methods

Patients

Our study group consisted of 47 patients (30 male) with chronic liver disease of various etiologies, including 27 patients who eventually underwent an OLT. The median age was 46 years (range 16 to 68). Fourteen patients had chronic viral hepatitis, 14 patients had cholestatic liver disease, 10 patients had alcohol-related liver disease, and a miscellaneous group was included, consisting of 5 patients with autoimmune hepatitis, one patient with a fibrolamellar hepatocellular carcinoma without underlying liver disease, one patient with a neuroendocrine tumor, one patient with Wilson's disease and one patient with alpha-1 antitrypsin deficiency.

In the group of 27 patients who underwent an OLT 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 measures 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 to 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 additional immunosuppressive treatment was required.

Determination of SNPs of the MMPs

Genomic DNA was extracted by routine methods from peripheral blood leukocytes in most of the patients with chronic liver disease. 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 obtained several 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), and confirmed by direct sequence analysis of 4 patients. Briefly, the region flanking the SNP was amplified with outer primers 5'-ACCA-GACAAGCCTGAACTTGTCTGA-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. 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 analysed 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 were assessed by the Mann-Whitney U test for non-parametric data. MMP levels are expressed as mean ± S.E.M. Differences were considered to be significant at *p*-values of ≤ 0.05.

Results

MMP allele frequencies in patients with various liver diseases.

The frequency of the MMP-2 and MMP-9 genotypes at loci –1306 and –1562, respectively, are shown in Table 1. Subgroup analysis according to the etiology of the liver disease demonstrated no significant differences in allele frequencies at both the –1306 C/T position of the MMP-2 promoter and the –1562 C/T position of the MMP-9 promoter. RFLP analysis of the MMP-2 promoter at the –1575 G/A position in these patients confirmed complete linkage disequilibrium between loci –1575 and –1306 (data not shown), as reported previously.(3) The expression of MMP-2 protein in the serum was not affected by the allelic composition at SNP locus –1306 C/T (CC: 5123 ± 553 ng/ml, CT & TT: 5347 ± 86; not significantly different). The MMP-9 level of patients with genotype CT at –1562 of MMP-9 was higher compared to the CC genotype, although not statistically significant (CC: 156 ± 28 ng/ml, CT: 129 ± 16; $p = 0.175$; Table 1).

Table 1. MMP genotype distribution (number, percentage of patients) and serum level (mean ± SEM; in ng/ml) stratified according to allelic composition in 47 patients with various chronic liver diseases. No significant differences were discernable.

Genotype / Disease	-1306 C/T MMP-2		-1562 C/T MMP-9	
	CC	*CT & TT**	*CC*	*CT*
Viral hepatitis	10	4	12	2
Alcoholic liver disease	7	3	7	3
Cholestatic liver disease	7	7	12	2
Miscellaneous	8	1	5	4
Total number (percentage)	32 (68.1%)	15 (31.9%)	36 (76.6%)	11 (23.4%)
Serum MMP level	5123 ± 553	5347 ± 866	129 ± 16	156 ± 28

* TT n = 2 (4.3%)

In accordance with our previous study, the serum MMP-2 level in patients with cirrhosis was significantly higher compared to that of the patients without cirrhosis.(11) However, the genotype distribution of the MMP-2 gene at SNP locus –1306 C/T did not differ between both groups (Table 2). The 33 patients with cirrhosis could be divided according to the Child-Pugh classification. The serum MMP-2 levels showed a step-wise increase with stage of cirrhosis, i.e., from Child A cirrhosis (4947 ± 882) to Child B cirrhosis (5540 ± 791) and Child C cirrhosis (8154 ± 967), all n=11. Remarkably, the genotype distribution of the MMP-2 gene promoter also showed an increase in the frequency of the wild type CC of the MMP-2 gene from 5 of the 11 patients with Child A, to 7 of the 11 patients with Child B, and 9 of the 11 patients with Child C cirrhosis. The serum MMP-9 levels in patients without cirrhosis was significantly higher compared to that of the patients with cirrhosis (Table 2).

Table 2. Genotype frequencies of MMP promoter polymorphism [number of patients (percentage)] and serum MMP level (mean ± SEM; in ng/ml) in 14 patients with chronic liver disease without cirrhosis and 33 patients with cirrhosis (Child A: 11; Child B: 11; Child C: 11).

Disease phenotype / Parameter		No cirrhosis	Cirrhosis Child A-C
-1306 C/T MMP-2	*CC*	11	21
	CT & TT	3 (21.4%)	12 (36.4%)
Serum MMP-2 level		2792 ± 372	6214 ± 552 *
-1562 C/T MMP-9	*CC*	8	28
	CT	6 (42.9%[a])	5 (15.2%)
Serum MMP-9 level		189 ± 26 *	113 ± 15

*$p < 0.005$, [a] $p = 0.06$

This higher serum MMP-9 level in the patients without cirrhosis was accompanied by a higher CT genotype frequency (6/14 = 42.9%) of the MMP-9 gene promoter at SNP locus –1562 C/T compared to the patients with cirrhosis (5/33 = 15.2%; Chi-square: 4,21; p = 0.06). Among the patients with cirrhosis the serum MMP-9 level, however, did not differ according to the Child stage (Child A: 108 ± 21; Child B: 109 ± 21; Child B: 121 ± 31) and the MMP-9 genotype distribution was also comparable. The wild type CC of the MMP-9 gene promoter was present in 10 of the 11 patients with Child A, in 10 of the 11 patients with Child B, and in 8 of the 11 patients with Child C cirrhosis.

The role of MMP polymorphisms in liver transplantation.

Late phase I/R injury was graded "more severe" (AST higher than 1,500 IU/L) in 9 of 27 patients after OLT, and rejection occurred in 11 patients. In previous studies it was demonstrated that MMP-9, but not MMP-2, could play an important role in immediate I/R injury (21) and in acute allograft rejection after liver transplantation.(14) 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 3). 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.

Table 3. The development of IR-injury and rejection after OLT (number of patients) stratified according to allelic composition at MMP SNP loci in donor and recipient. No significant differences were discernable.

Genotype		-1306 C/T MMP-2				-1562 C/T MMP-9			
		donor		recipient		donor		recipient	
Complication		*CC*	*CT & TT*	*CC*	*CT & TT*	*CC*	*CT*	*CC*	*CT*
IR-injury	*no*	10	8	12	6	15	3	16	2
	yes	4	5	5	4	6	3	7	2
Rejection	*no*	8	8	12	4	14	2	14	2
	yes	6	5	5	6	7	4	9	2

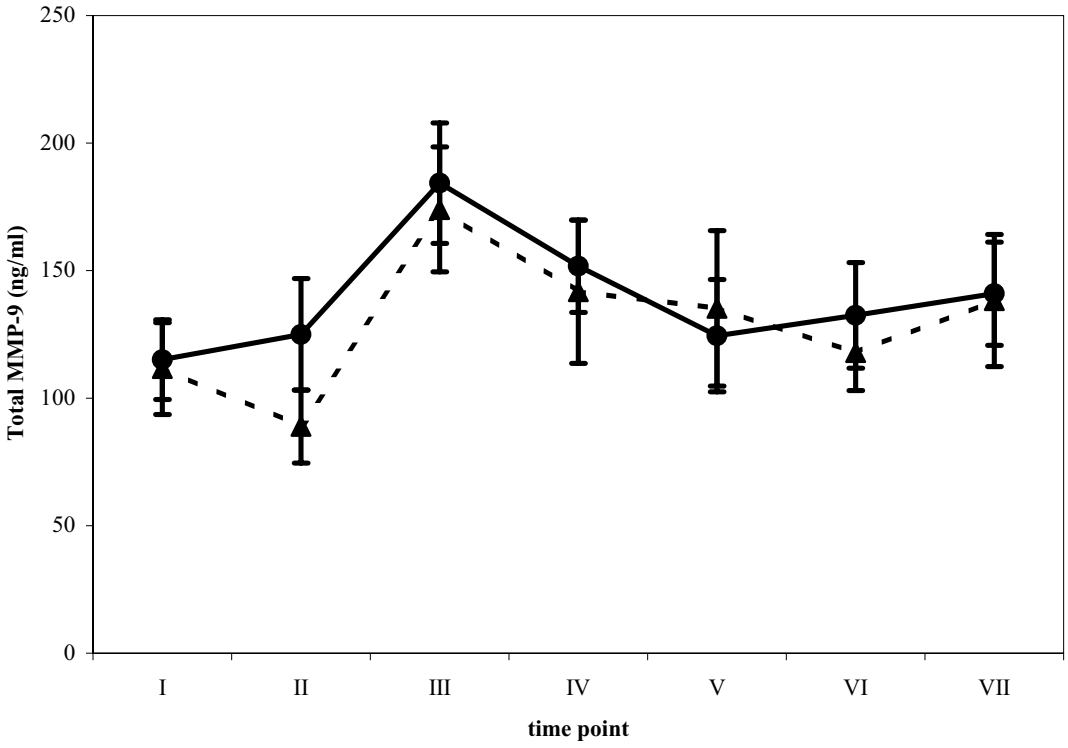
In table 4 the clinical impact of a mismatch in allelic composition at the MMP-2 and MMP-9 SNP loci between the donor and recipient is reported. No statistically significant differences between the presence or absence of a mismatch at –1306 C/T MMP-2 or –1562 C/T MMP-9 were found with regard to I/R injury and to rejection after liver transplantation.

Table 4. The development of IR-injury and rejection after OLT (number of patients) stratified according to the presence of a mismatch in allelic composition at MMP-2 and MMP-9 SNP loci between donor and recipient. No significant differences were discernable.

Genotype / Complication		-1306 C/T MMP-2 mismatch		-1562 C/T MMP-9 mismatch	
		no	*yes*	*no*	*yes*
IR-injury	*no*	9	9	13	5
	yes	2	7	6	3
Rejection	*no*	7	9	12	4
	yes	4	7	7	4

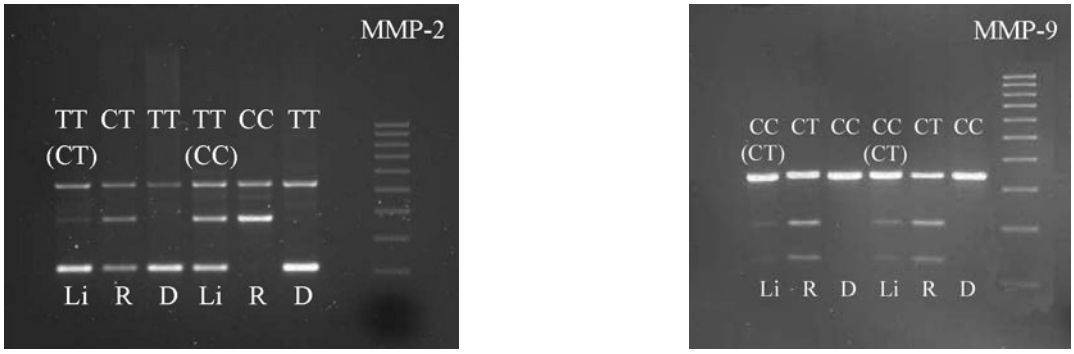
Serum levels of MMP-9 showed a peak at 1 week after OLT, which is associated with acute allograft rejection, as previously reported.(14) Patients with or without a mismatch at –1562 C/T MMP-9 between the donor and the recipient showed a comparable serum MMP-9 pattern over time (Figure 1). Moreover, the peak MMP-9 serum level at 1 week after OLT was similar in both groups (174 ± 25 and 184 ± 24, respectively).

Figure 1. 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. ●: Patients without a mismatch in allelic composition at MMP-9 SNP locus -1562 C/T between donor and recipient; ▲: Patients with a mismatch.



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 (Figure 2). These chimerisms were demonstrated in 3 of the 8 patients with a MMP-9 mismatch and in 10 of the 16 patients with a MMP-2 mismatch.

Figure 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).



Discussion

DNA polymorphisms have been estimated to occur on the average of one in every 1,000 base pairs throughout the human genome. Approximately 90 % are SNPs due to single base substitution.(22) Although the majority of DNA polymorphisms are functionally neutral a proportion of them can exert allele-specific effects on regulation of gene expression or function of the coded protein, thus underlying differences in susceptibility to disease. Polymorphisms in the promoter of a number of MMP genes have been shown to influence MMP gene expression and be associated with susceptibility of coronary atherosclerosis, aneurysms, and cancer.(23)

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.(4) 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.(5-7) 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.(24) 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;25) However, MMP-2 promoter polymorphisms were never determined in patients with chronic liver disease. In the present study the serum MMP-2 levels in patients with diverse chronic liver disease were not significantly different with respect to the –1306 C/T genotype. Yet, within patients with cirrhosis there seems to be a clear relationship between the higher serum MMP-2 levels with advanced Child stage and a more frequent wild-type –1306 CC genotype of MMP-2.

There are a number of regulatory mechanisms that can influence the ultimate impact of an MMP on extracellular matrix degradation. It appears, however, that the key step is transcriptional regulation because most MMPs are expressed only when active physiological or pathological remodeling takes place. An exception is MMP-2 which is widely expressed in

apparently quiescent tissues at significant levels, and therefore other levels of regulation, such as activation of the latent enzyme or inhibition by TIMPs might also be important.(1;23) These functional interactions may contribute to the increased serum MMP-2 level associated with the CC MMP-2 genotype in patients with cirrhosis, while the genotype shows a similar distribution in chronic liver disease patients with or without cirrhosis. Lichtinghagen et al. similarly demonstrated that the hepatic mRNA MMP-3 expression was determined by the MMP-3 (–1171 5A/6A) promoter polymorphism but the genotype distribution was not significantly different between controls, patients with chronic hepatitis C, and patients with cirrhosis.(26)

The expression of MMP-9 is regulated primarily at the transcription level in response to different regulators such as interleukine-1, tumor necrosis factor-alpha, and epidermal growth factor.(1) In 1999 a functionally important SNP at position –1562 in the MMP-9 gene was described which leads to increased transcriptional activity, and is associated with severity of coronary atherosclerosis.(8) Because MMP-9 possesses proteolytic activity against type IV collagen, a major component of basement membranes, it was suggested that this association may be explained by enhanced smooth muscle cell migration and proliferation in individuals with the T allele.(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 intracranial aneurysms revealed contradictory results.(27;28) Results of several other case-control studies are mixed, with associations seen with pulmonary emphysema(29) and abdominal aneurysm(30), but no association with end-stage renal disease(31) and multiple sclerosis.(32)

Our study revealed that chronic liver disease patients without cirrhosis have a higher serum MMP-9 level in association with a higher frequency of the –1562 C/T genotype and that the –1562 C/T MMP-9 polymorphism (determined in the donor and the recipient) is not associated with rejection or late phase I/R injury after OLT. The peak of serum MMP-9 at 1 week after OLT in patients with rejection 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) In cardiovascular disease, in which most of the functional polymorphisms of MMP-9 were studied, MMP-9 is mainly expressed in atherosclerotic plaques and circulating concentration may reflect vessel wall expression.(9) A number of polymorphisms in the MMP-9 have been reported but only two of them in the promoter gene have been shown to be functionally important.(33) We determined only the –1562 C/T polymorphism and did not study the (CA)n microsatellite polymorphism at position –90 nor evaluated the possibility that multiple SNPs in combination might contribute to liver disease susceptibility. Furthermore, it could be argued that our study population is too small thus affecting the power of our analyses.

In about half of our patients with a mismatch in the MMP genotype between the donor and the recipient, evidence of chimerism was found in the liver biopsy tissue of the allograft. This might in part be explained by the presence of lymphocytes and Kupffer cells of recipient origen, but recently it was demonstrated that recipient-derived cells can also replace biliary epithelium, endothelium, and even hepatocytes in liver transplants.(34)

In conclusion, serum MMP-2 and MMP-9 levels in general appear to be independent of the MMP genotype in chronic liver disease patients, although in relation to cirrhosis strong indications of genotypic impact on serum levels are discernable. In contrast to the increased MMP-9 serum level, the –1562 C/T MMP-9 polymorphism is not associated with late phase I/R injury or rejection after liver transplantation. These observations merit further studies on the MMP geno- and phenotypes in larger (sub)groups of chronic liver disease patients and after OLT.

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