A Novel MCP-1 Gene Polymorphism Is Associated With Hepatic MCP-1 Expression and Severity of HCV-Related Liver Disease

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Background & Aims: **Factors influencing the progression of chronic hepatitis C virus (HCV) infection are poorly understood. Monocyte chemotactic protein-1 (MCP-1) is a potent chemokine, and its hepatic expression is upregulated during chronic HCV infection mainly in activated hepatic stellate cells (HSC). In this study, we investigated the correlation of the functional −2518 MCP-1 promoter polymorphism with hepatic MCP-1 expression and the disease outcome in patients with HCV.** ***Methods:*** **MCP-1 genotyping was performed in 206 patients and 139 healthy controls. Hepatic MCP-1 messenger RNA (mRNA) expression was quantified by real-time PCR in 58 HCV patients. Cytokine-induced MCP-1 secretion of activated human HSC (n = 13) was determined by enzyme-linked immunosorbent assay (ELISA). Mobility-shift assays were performed using probes corresponding to the MCP-1 promoter sequence (−2511 to −2528) with or without the A to G mutation at −2518.** ***Results:*** **Frequency of MCP-1 genotypes did not differ between HCV patients and controls. However, carriers of the G allele were significantly more frequent in HCV patients with more advanced fibrosis and severe inflammation. In accordance, hepatic MCP-1 mRNA levels were significantly higher in patients with more advanced fibrosis and in patients carrying the G allele. Furthermore, cytokine-induced MCP-1 secretion of HSC isolated from carriers of the G allele was significantly higher, and there was binding activity in nuclear extracts from activated HSC specifically to the G allele, providing a potential mechanism for the differences seen.** ***Conclusions:*** **Inheritance of the −2518 MCP-1 G allele, which appears to affect hepatic MCP-1 expression, may predispose HCV patients to more severe hepatic inflammation and fibrosis.**

Chronic infection with hepatitis C virus (HCV) is a global problem, and the WHO estimates a worldwide prevalence of up to 3%. The course of chronic infection shows a strong variation among individuals. Although the majority of cases show a chronic infection without progression, 20%–30% of patients with chronic hepatitis C infection develop progressive fibrosis and cirrhosis.[1]

The pathologic consequences of chronic HCV infection are liver fibrosis and inflammation. The exact mechanisms by which the hepatocellular damage caused by hepatitis viruses leads to fibrosis and cirrhosis in some patients and less severe liver injury in others are not well understood.

Environmental and viral factors are likely to act in concert with individual susceptibility to induce liver damage. Some host factors such as alcohol consumption, duration of infection, older age at infection, coinfection with immunodeficiency virus (HIV), or male gender as well as increased body mass index and steatosis have been shown as independent risk factors.[2–8]

Furthermore, genetic variability in factors influencing fibrogenesis and inflammation may be responsible for some of the variability in disease progression seen in patients with chronic HCV infection. The identification of genetic determinants of susceptibility to HCV-induced chronic liver damage would assist in predicting individual risks of disease progression and would help to clarify pathophysiologic mechanisms involved in HCV-mediated tissue injury.

Chronic viral hepatitis is histologically characterized by predominantly periportal infiltration of mononuclear cells, including lymphocytes and monocytes/macrophages. Intralobular infiltration of these inflammatory cells is an ominous sign of deterioration and a criterion for disease activity. Therefore, it is of particular interest to identify the mechanism underlying the recruitment and activation of macrophages/monocytes. Chemotactic cytokines are known to be critical mediators of inflam-

Abbreviations used in this paper: ECM, extracellular matrix; HCV, hepatitis C virus; HSC, hepatic stellate cells; MCP-1, monocyte chemotactic protein-1.

0016-5085/03/$30.00
doi:10.1053/S0016-5085(03)01213-7

matory cell trafficking into sites of injury, modulating of tissue injury, inflammation, and repair.

Monocyte chemotactic protein-1 (MCP-1) is one of the most potent chemokines identified for monocytes and macrophages.[9] It belongs to the group of CC chemokines, with 2 conserved cystein residues adjacent.

Several lines of evidence indicate that MCP-1 may play a role in the recruitment and maintenance of the inflammatory infiltrate during liver injury. MCP-1 secretion is up-regulated during chronic hepatitis and correlates with the number of cells infiltrating the portal tract.[10]

There is growing evidence that nonparenchymal cells, predominantly activated hepatic stellate cells (HSC), and only to lesser extent Kupffer-cells and endothelial cells, are responsible for MCP-1 production in chronic liver disease.[10–12] Following hepatic injury, HSC undergo an activation process and transform to an activated myofibroblast-like phenotype and produce excessively extracellular matrix (ECM) in the liver, and their activity is recognized to be a central event in the development of hepatic fibrosis.[8,13]

Previous reports demonstrated considerable interindividual variability in MCP-1 expression in response to stimuli such as cytokines,[14–16] suggesting that inherited factors influence the expression of this chemokine. Recently, a mutation in the distal regulatory region of the MCP-1 gene at position –2518 relatively to the transcription start site (based on the published sequence (Gene Bank accession number D26087) was identified.[14] Monocytes from individuals carrying a G allele at −2518 produce more MCP-1 after treatment with interleukin 1β compared with cells from A/A homozygous subjects.[14]

Prevalence of these high MCP-1-producing genotypes has been shown to be associated with the presence and severity of bronchial asthma,[17] the risk for premature kidney graft failure,[15] the onset and disease behavior of Crohn's disease,[18] and the frequency of cutaneus vasculitis.[19] Furthermore, it has been postulated to be a genetic risk factor for severe coronary artery disease.[20] However, the role of this genetic polymorphism in liver disease has not yet been evaluated.

In this study we investigate the hypothesis that inheritance of the high MCP-1-producing G allele may predispose to more severe fibrosis and inflammation in patients with chronic hepatitis C. The aims of this study were (1) to examine the prevalence of this polymorphism in patients with chronic hepatitis C and the association with the stage of fibrosis and the grade of inflammation, (2) to investigate a genotype/phenotype correlation by analyzing MCP-1 expression in HCV-infected liver tissue and in vitro in activated HSC of donors with different MCP-1 genotype, and (3) to investigate the molecular mechanisms responsible for differences among individuals.

Materials and Methods

Patients and Controls

We studied retrospectively 206 patients (135 male, 71 female; mean age: 38.1 ± 13.0 years) with chronic hepatitis C (positive for HCV-RNA and anti-HCV) consecutively admitted to the hospital of the University of Regensburg. All patients were negative for hepatitis B surface antigen or antibodies to human immunodeficiency virus, and none of them had evidence of other types of liver disease. Risk factors for acquisition of hepatitis C infection were previous intravenous drug abuse in 28.2% of patients, receipt of blood or blood products before the introduction of donor screening in 18.9% of patients, and other factors or unknown in 52.9% of patients. Most patients received treatment with interferon α + ribavirin. For 157 patients, data of qualitative HVC-PCR analysis 3 months after initiation of antiviral therapy were available. Informed consent was obtained from all patients, and the study was approved by the local ethics committee. Frequency of MCP-1 genotypes was compared with 139 healthy controls from the same geographic region. Patients and controls were white.

Histologic Staging and Grading

Liver biopsy specimens were obtained from 185 patients before initiation of antiviral therapy (naive patients) by percutaneous Menghini-needle biopsy. In these patients, fibrosis and inflammation were graded and staged numerically according to a score proposed by Desmet et al.[21] by a single pathologist (A.H.).

Rate of Fibrosis Progression

According to Poynard et al., fibrosis progression per year was estimated as the stage of liver fibrosis divided by the duration of infection (years), or, in the cases in which 2 liver biopsy specimens—obtained several years apart—were available as difference of the stage of fibrosis between the first and second biopsy divided by the time interval between the 2 biopsies (years).[22] Time of infection was known or could be estimated in 84 cases. However, for calculation of fibrosis progression based on the duration of infection, only patients with fibrosis stage 2 or higher were included (n = 49) because the lowest fibrosis score (stage 1) did not differentiate between no and minimal hepatic fibrosis. In addition, 2 liver biopsy specimens obtained more than 5 years apart were available from 2 patients. The estimated time to develop cirrhosis (i.e., stage 4) was calculated as the following: 4 divided by fibrosis progression per year. In accordance to the definition by Poy-

nard et al., high-risk patients, or very rapid fibrosers, were defined as patients who developed cirrhosis (stage 4) before the age of 50 years.[22]

Within the group of patients with known or estimated time of infection and liver histology revealing fibrosis stage 1 (n = 35), duration of infection lasted 10 years or longer in 16 cases. Those patients were defined as slow fibrosers.

DNA Isolation and MCP-1 Genotyping

Genomic DNA specimens were prepared from 200 μL blood using the QIAamp blood kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The G to A polymorphisms at position −2518 of the MCP-1 gene was analyzed by performing PCR and subsequent restriction fragment length polymorphism (RFLP) analysis. PCR was performed under standard conditions (35 cycles, annealing temperature: 55°C) in a total reaction volume of 50 μL containing 2 μL of diluted genomic DNA, using the following pair of primers: forward: 5′-CCGAGATGTTCCCAGCACAG-3′ and reverse: 5′-CTGCTTTGCTTGTGCCTCTT-3′. PCR products were digested by *Pvu*II, and the resulting fragments were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. With the −2518 A polymorphic base, the recognition sequence 5′-CAG/CTG-3′ is modified to 5′-CAG/CTA-3′, which is not cut by *Pvu*II.

Isolation and Stimulation of Activated HSC

Tissue samples from human liver resections (n = 13) were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the local Ethics Committee, University Hospital of Regensburg, with informed patient's contest.

Only those liver tissues judged as noncancerous by local pathologists were used for cell preparation. Further exclusion criteria were known liver disease or histologic evidence for liver fibrosis or inflammation in surrounding nontumorous liver tissue.

Cells were isolated using a modified 2-step EGTA/collagenase perfusion procedure and sequential incubation of the cell suspension with pronase as described previously.[23,24] HSC were separated from other nonparenchymal liver cells by arabinogalactan gradient ultracentrifugation, as previously described for rat HSC.[24] This procedure yielded HSC that were more than 85% pure and 90% viable, as determined by phase contrast- and UV-excited fluorescence microscopy and Trypan blue exclusion, respectively. HSC were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 95% air/5% CO_2-humidified atmosphere. Growth medium was changed on a daily basis for the first 4 days in culture then every second day thereafter. HSC were cultured on plastic dishes for 20 days and then plated in 24-well plates and serum starved for 12 hours before stimulation.

MCP-1 and IL-8 Secretion of Activated HSC

Activated HSC were stimulated with recombinant human TNF-α (10 ng/mL; R&D Systems, Wiesbaden–Nordenstadt, Germany) for 16 hours. Subsequently, supernatants were collected and centrifuged to remove cellular debris, and MCP-1 and IL-8 concentrations were analyzed by sandwich enzyme-linked immunosorbent assays (ELISA) following the instructor's manuals (for MCP-1: Biosource; for IL-8: R&D Systems).

Isolation of Hepatic RNA and Quantification of MCP-1 Expression

From 58 patients with chronic hepatitis C, a small part of the liver biopsy specimen (obtained by percutaneous needle biopsy for histologic examination, before initiation of antiviral therapy) was snap frozen immediately after collection and stored in liquid nitrogen until used for later RNA isolation. Total liver RNA was isolated using the RNeasy Mini Kit including a RNase free DNase step following the instructor's manual (Qiagen). RNA amounts were analyzed by using a fluorescence microplate reader and following the instructors' manual of the RiboGreen RNA Quantitation Reagent and Kit (MoBiTec, Göttingen, Germany). Integrity of the RNA was verified by agarose gel electrophoresis and by visualization of ribosomal bands with ethidium bromide staining. First-strand cDNA was synthesized using 1 μg of total RNA and the AMV reverse transcription reaction (Promega, Madison, WI). MCP-1 mRNA expression was quantified using real-time PCR technology (Lightcycler, Roche) with specific sets of primers for human MCP-1 and β-actin based on published sequences: β-actin forward: 5′-CTA CGT CGC CCT GGA CTT CGA GC-3′ and β-actin reverse: 5′-GAT GGA GCC GCC GAT CCA CAC G-3′; MCP-1 forward: 5′-GCG GAG CTA TAG AAG AAT CAC-3′ and MCP-1 reverse: 5′-TTG GGT TGT GGA GTG AGT GT-3′.

Gel Mobility-Shift Assays

Complementary synthetic oligonucleotides corresponding to the promoter sequence −2511 to −2528 of the MCP-1 gene were hybridized and phospholabeled (for the A allele, −2518A and for the G allele, −2518G, respectively): MCP-1/G allele forward: GAG GAC AGC TGT CAC TT; MCP-1/G allele reverse: AAG TGA CAG CTG TCT GC; MCP-1/A-allele forward: GCA GAC AGC TAT CAC TT, MCP-1/A allele reverse: AAG TGA TAG CTG TCT GC.

Nuclear extracts were prepared from human activated HSC from donors with different MCP-1 genotypes, and gel mobility-shift assays were performed as described previously.[25] Competition experiments were performed using a 50-fold excess of the same binding site, the mutated binding site, or an unrelated binding site.

Statistical Analysis

Results are expressed as mean ± SD (range), median, or percentage. Genotype frequencies are reported with their

group percentages. Comparisons between clinical subgroups were made using the Student unpaired t test (continuous measures) or Fisher exact test (categoric measures). A 2-sided χ^2 test was used for comparison of qualitative variables. A P value <0.05 was considered statistically significant. All computations were performed by using the SPSS-10 for Windows statistical computer package (SSPS, Inc., Chicago, IL).

Results

Frequency of the −2518 MCP-1 Polymorphism in Patients With Chronic HCV Infection

The frequencies of the different MCP-1 genotypes A/A homozygotes, A/G heterozygotes, and G/G homozygotes are summarized in Table 1, revealing no significant differences between 206 patients with chronic hepatitis C infection and 139 healthy controls. Frequencies of individual genotypes were similar to those previously reported in other white control populations.[15,17,19,20]

Because most previous in vitro and epidemiologic studies reported functional and disease-related differences mainly between −2518 MCP-1 genotypes AA and non-AA (i.e., AG or GG), we continued this differentiation throughout the following analysis, focused on the comparison of HCV-infected patients with no G allele (AA homozygotes) and carriers of the G allele (genotypes AG or GG).

Comparing carriers and noncarriers of the G allele, we found neither differences in demographic features such as age and distribution of sexes nor in the frequencies of potential routes of HCV infection, serum HCV-RNA levels, or frequencies of different HCV genotypes (Table 2). The number of initial virologic responders to interferon therapy (no HCV-RNA detectable in the serum 3 months after initiation of therapy) was slightly lower in carriers than in noncarriers of the G allele, but this difference was not statistically significant (38 of 65 [58.5%] vs. 32 of 45 [71.1%], respectively [$P = 0.18$]).

Table 1. Frequency of the −2518 MCP-1 Polymorphism in Patients With Chronic Hepatitis C Infection and Healthy Controls

−2518 MCP-1 genotypes	HepC patients, n = 206 (%)	Controls, n = 139 (%)
A/A	93 (45.1)	71 (51.1)
A/G	91 (44.2)	59 (43.1)
G/G	22 (10.7)	9 (6.6)

HepC, chronic hepatitis C infection.

Table 2. Clinical and Biologic Characteristics of Patients With Chronic Hepatitis C Infection According to MCP-1 −2518 Genotypes (G-carriers or noncarriers)

Clinical and biologic characteristics	All patients	MCP-1 −2518 genotype	
		A/A	A/G or G/G
Age, *yr; mean ± SD*	38.1±13.0	37.8±12.5	38.4±13.4
Male gender, %	63.4	67.3	65.5
HCV transmission routes, %			
IV drug use	28.2	24.7	31.0
Posttransfusion	18.9	23.7	15.0
Other factors or unknown	52.9	40.9	54.0
HCV genotypes, %			
Type 1	65.6	66.3	64.3
Type 3	24.8	23.2	28.4
Other than type 1 or 3	9.6	10.5	7.3
Viral load, *10^6 copies/mL*; median	1.5	1.4	1.6
Initial virologic response to anti-HCV treatment, %[a]	63.6	71.1	58.5

[a]HCV-RNA negative, 3 months after start of antiviral therapy; data available from 110/206 patients.

Frequency of the −2518 MCP-1 Polymorphism in HCV Patients With Different Staging of Fibrosis

One hundred eighty-five of the 206 patients with chronic HCV infection underwent liver biopsy and histopathologic staging and grading was performed. As summarized in Table 3, in patients with chronic HCV infection, the frequency of the A/A genotype decreased with the severity of fibrosis. The difference was most apparent comparing patients with no or only mild periportal fibrosis (staging 1 or 2) to patients with more advanced septal fibrosis or cirrhosis (staging 3 or 4) (Figure 1*A*). The A/A genotype was significantly more frequent in patients with stage 1 or 2 compared with stage 3 or 4: 70 of 138 (50.7%) vs. 10 of 47 (21.3%), respectively ($P = 0.0004$).

Frequency of the −2518 MCP-1 Polymorphism in HCV Patients With Different Rates of Progression of Fibrosis

Progression of liver fibrosis was estimated based on the duration of HCV infection (n = 49) or the changes in the stage of fibrosis in follow-up biopsies (n = 2), similarly as proposed by Poynard et al.[22] Based on that calculation, the estimated time to develop cirrhosis (stage 4) was significantly shorter for carriers of the G allele than for AA homozygotes: 6.8 ± 1.2 years vs. 14.4 ± 4.6 years, respectively ($P = 0.038$).

Furthermore, patients who developed cirrhosis before the age of 50 years were defined as rapid fibrosers,

Table 3. Frequency of the −2518 MCP-1 Genotype A/A in Patients With Chronic Hepatitis C Infection

		Total number of patients	Patients with MCP-1 genotype AA	
			n	%
Staging[a]	1	86	47	54.7
	2	52	23	44.2
	3	24	6	25.0
	4	23	4	17.4
Portal grading[a]	0 or 1	91	55	60.4
	2	63	17	27.0
	3	29	8	27.6
	4	2	—	0.0
Lobular grading[a]	0	78	38	48.7
	1	86	38	44.2
	2	21	4	19.0
Total grading[a] (portal + lobular)	1	55	32	58.2
	2	59	31	52.5
	3	33	7	21.2
	4	25	8	32.0
	5 or 6	13	2	15.4

NOTE. Comparison between patients with different histologic staging and grading.
[a]According to the score proposed by Desmet et al.[21]

whereas patients with staging 1 and a duration of infection of 10 years or longer were considered as slow fibrosers. Out of 14 patients defined as rapid fibrosers, 12 (86%) were carriers of the G allele, whereas only 3 of 13 (19%) slow fibrosers carried the G allele.

Frequency of the −2518 MCP-1 Polymorphism in HCV Patients With Different Degree of Inflammation

The prevalence of the A/A genotype is decreased in patients with more severe portal as well as lobular inflammation, compared with patients with no or only minimal portal or lobular inflammation, respectively (Table 3). Differences were most apparent when comparing patients with no or only minimal portal inflammation without piecemeal necrosis (portal grade 0 or 1) to patients with more severe portal inflammation (portal grading >1): 55 of 91 (60.4%) vs. 25 of 94 (26.6%), respectively ($P < 0.0001$). Similarly, comparison between patients with no or only mild lobular inflammation (lobular grading 0 or 1) and patients with more severe lobular inflammation or necrosis (lobular grading >1) revealed a significantly lower prevalence of the genotype A/A in the latter group of patients (76 of 164 [46.3%] vs. 4 of 21 [19.0%], respectively [$P = 0.0175$]). Combination of portal and lobular inflammation in total grade (21) showed the same differences in frequency of the A/A genotype. Patients with mild or only minimal hepatic inflammation (grading 1 or 2) were significantly more often carriers of the MCP-1 genotype A/A, compared with patients with more severe hepatic inflammation or necrosis (grading >2): 63 of 114 (55.3%) vs. 17 of 71 (23.9%), respectively ($P < 0.0001$) (Figure 1*B*).

Phenotypic Manifestation of the Functional −2518 MCP-1 Polymorphism in the Liver

MCP-1 mRNA levels in hepatic specimens of HCV-infected patients were significantly lower in individuals with the MCP-1 genotype A/A (n = 28) compared with patients with the genotypes A/G or G/G (n = 30): 4.1 ± 0.9 vs. 13.5 ± 3.3, respectively ($P = 0.010$) (Figure 2). To confirm that this difference was not caused by different hepatic inflammatory activity, we repeated the analysis in the subgroup of HCV patients with none or only mild hepatic inflammation (grading 1-2) (n = 43). Similar as in the whole cohort, also in this subgroup of HCV patients, carriers of the G allele (n = 21) had significantly higher hepatic mRNA levels than AA homozygotes (n = 22): 10.3 ± 3.3 vs. 2.9 ± 0.9, respectively ($P = 0.032$).

In accordance to the correlation found between MCP-1 genotypes and hepatic fibrosis, mean intrahepatic MCP-1 mRNA levels were significantly lower in patients with no or only minimal fibrosis (staging 1; n = 28) compared with HCV-infected patients with more advanced fibrosis and cirrhosis (staging ≥2; n = 30): 4.9 ± 1.6 vs. 12.9 ± 3.2, respectively ($P = 0.031$) (Figure 3*A*).

Similarly, intrahepatic MCP-1 mRNA expression tended to be lower in HCV-infected patients with no or only minimal hepatic inflammation (grading 1; n = 19) compared with HCV patients with more severe inflammation or necrosis (grading ≥2; n = 39): 3.9 ± 1.8 vs.

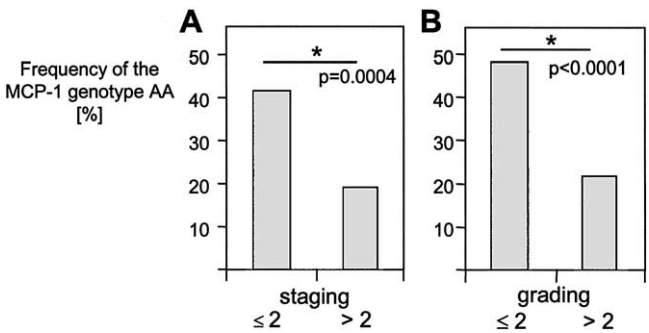

Figure 1. Frequency of the AA genotype at position −2518 of the MCP-1 gene in patients with chronic hepatitis C. Comparison between patients with (*A*) no or only mild fibrosis (stage 1 or 2) and patients with prominent fibrosis or cirrhosis (stage 3 or 4): 70 of 138 (50.7%) vs. 10 of 47 (21.3%), respectively ($P = 0.0004$). (*B*) No or only mild inflammation (grade 1 or 2) and patients with severe inflammation or necrosis (grade >2): 63 of 114 (55.3%) vs. 17 of 71 (23.9%), respectively ($P < 0.0001$).

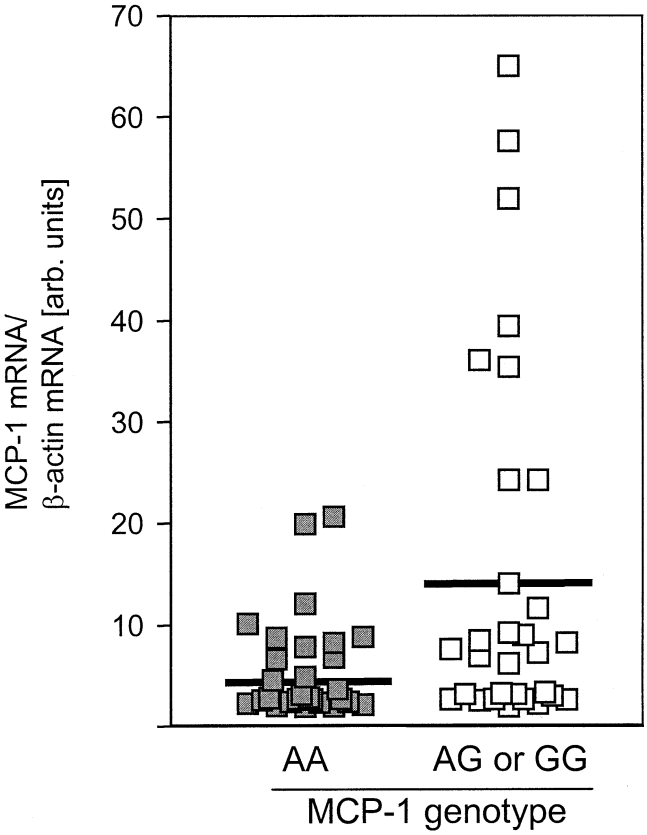

Figure 2. Hepatic MCP-1 mRNA expression in liver tissue of hepatitis C patients. Comparison between carriers of the G allele (non-AA) and noncarriers (AA) at position −2518 of the MCP-1 gene. Mean intrahepatic MCP-1 mRNA were significantly lower in patients with the MCP-1 genotype AA (n = 28) than in patients with the genotypes AG or GG (n = 30): 4.1 ± 0.9 vs. 13.5 ± 3.3, respectively ($P = 0.010$). *Bars* represent mean.

11.5 ± 2.6, respectively (Figure 3*B*). However, because of a wide variation of values, this difference did not reach statistical significance ($P = 0.055$).

MCP-1 Expression in Activated HSC Isolated From Individuals With Different MCP-1 Genotypes

To analyze the functional relevance of the MCP-1 promoter polymorphism in chronic liver disease, we investigated the in vitro MCP-1 expression of activated HSC isolated from donors with different MCP-1 genotypes. Activated HSC were selected for this analysis because it has been shown previously that, during chronic hepatitis, MCP-1 is predominantly expressed by these nonparenchymal liver cells.[10–12] After stimulation with TNF-α (10 ng/mL), HSC from A/A homozygote donors (n = 5) secreted significantly less MCP-1 than HSC with genotypes A/G or G/G (n = 8): 2.0- ± 0.3- vs. 4.0- ± 0.7-fold induction, respectively ($P = 0.044$) (Figure 4).

In addition, IL-8 secretion was measured to ensure that the TNF pathway was intact in the HSC of all donors and to confirm that the differences found in MCP-1 secretion are actually caused by the MCP-1 genotypes. It has already been shown that TNF induced IL-8 expression in activated HSC.[26,27] TNF-α-induced IL-8 secretion did not differ significantly between HSC from A/A homozygote donors and donors carrying the G allele, respectively (9.9- ± 5.8- vs. 10.9- ± 2.6-fold induction; $P = 0.863$).

Molecular Mechanism Underlying Differences Between the A Allele and the G Allele of the −2518 MCP-1 Polymorphism

To investigate the molecular mechanisms responsible for the effect of the (A −2518G) MCP-1 promoter polymorphism on transcription, the specific binding of transcription factors to the region of the polymorphism was analyzed. We performed gel shift assays using probes corresponding to the promoter sequence (−2511 to −2527) of the MCP-1 gene, with either the nucleotide G or A at position −2518 (representing the G allele or the A allele, respectively).

Using nuclear extracts from activated HSC, we found a clear band in the presence of the G probe (Figure 5). However, when using the A probe, no band was visible, indicating nuclear-binding activity to the promoter region (−2511 to −2528) of the MCP-1 gene specific for the −2518 G allele in activated HSC. This specific binding activity could be completely abolished in com-

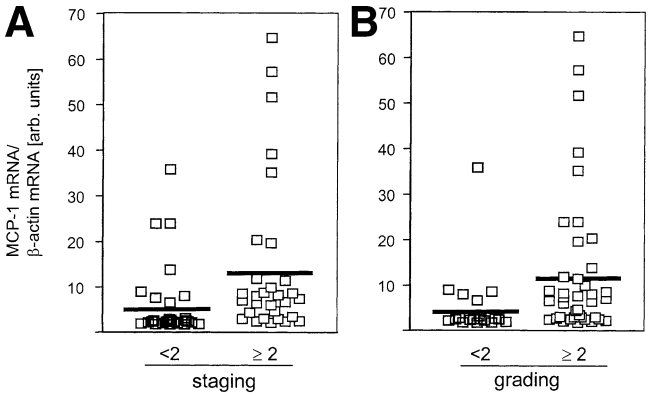

Figure 3. Hepatic MCP-1 mRNA expression in liver tissue of hepatitis C patients. Comparison between patients with (*A*) no or only minimal fibrosis (stage 1) and patients with more advanced fibrosis or cirrhosis (staging ≥2) and (*B*) no or only mild inflammation (grade 1) and patients with severe inflammation or necrosis (grade ≥2). Mean intrahepatic MCP-1 mRNA levels were significantly lower in patients with staging 1 (n = 28) compared with staging ≥2 (n = 30): 4.9 ± 1.6 vs. 12.9 ± 3.2 ($P = 0.031$) and tended to be lower in patients with grading 1 (n = 19) compared with grading ≥2 (n = 39): 3.9 ± 1.8 vs. 11.5 ± 2.6 (ns), respectively. *Bars* represent mean.

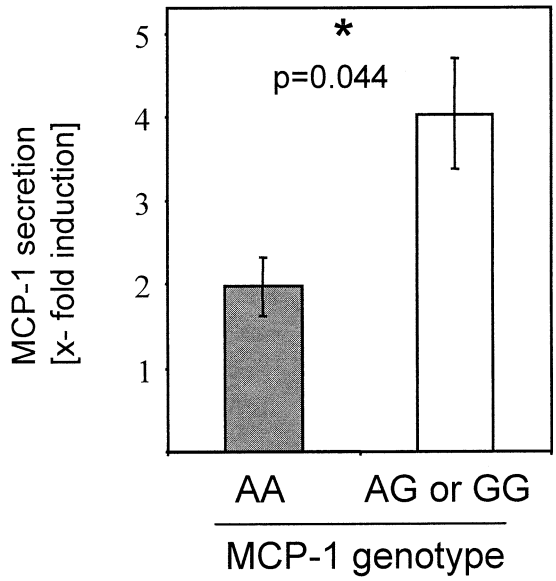

Figure 4. MCP-1 secretion of activated hepatic stellate cells (HSC) after stimulation with TNF-α. Comparison between HSC from donors with the genotype AA and non-AA (AG or GG) at position −2518 of the MCP-1 gene. MCP-1 levels were measured in the supernatant of TNF-α-treated HSC from individuals with nondiseased livers and were stratified according to the genotype at position –2518. *Bars* indicate the mean MCP-1 induction for each group (x-fold induction compared with basal expression). After stimulation with TNF-α, G allele carriers (genotypes: AG or GG; n = 8) secreted significantly more MCP-1 than noncarriers (AA homozygotes; n = 5) ($P = 0.044$).

petition experiments using 50-fold excess of cold G probe. Excess of cold A probe or an unrelated binding site could not compete the specific binding activity. The specificity of this binding activity was independent of the genotype of the donors of the HSC.

To identify possible transcription factors, putative binding sites on the A allele and the G allele were compared, using the following data bases of known transcription factors: TF search, Mathinpector and TESS (http://www.cbil.upenn.edu/tess; http://transfac.gbf.de/TRANSFAC; http://www.epd.isb-sib.ch). The analysis revealed potential binding sites of the transcription factors MyoD and AP-4 to the promoter region surrounding the mutation in the presence of the nucleotide G. However, no binding affinity of either of the 2 factors was recorded on the same relative position of the promoter in the presence of the nucleotide A at position −2581 relative to the transcription start site.

Discussion

The results of this study demonstrate an association between different MCP-1 genotypes and hepatic fibrosis and inflammation in patients with chronic hepatitis C, confirming the initial hypothesis that host genetic factors may account for some of the variability in the rate of disease progression seen in these patients. This relationship between MCP-1 genotypes and hepatic fibrosis and inflammation is in accordance with the well-documented role of MCP-1 in the pathophysiology of chronic liver disease.[10–12]

Here, in chronically HCV infected patients grouped according to the extent of fibrosis or inflammatory activity, an overrepresentation of carriers of the G allele (A/G heterozygotes or G/G homozygotes) was found in those whose livers were more severely affected, judged by each criterion of damage. We found carriers of the G allele more frequently in patients with more severe portal or lobular inflammation, suggesting that this genotype is associated with higher MCP-1 expression and successively more attraction of monocytes.

This hypothesis was confirmed by higher hepatic MCP-1 mRNA levels in carriers of the G allele in comparison with A/A homozygotes and is in line with findings from Marra et al., who observed a positive linear correlation between MCP-1 expression and the number of monocytes/macrophages in the portal tract.[10]

The same authors have shown that activated α-smooth muscle actin-positive myofibroblasts expressing MCP-1 are present in the active fibrous septa originating from the portal tract and around regenerating nodules in patients with chronic hepatitis and active cirrhosis,[10] suggesting that inflammation and fibrogenesis during chronic hepatitis are 2 strictly connected processes. Our study with an association of the MCP-1 polymorphism not only to the extent of inflammation but also to fibrosis adds to this evidence.

Several previous studies identified activated HSC to be the major source of MCP-1 in the liver of patients with

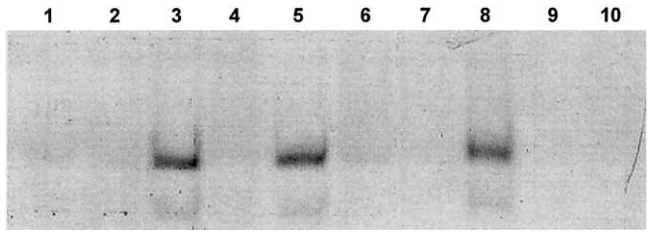

Figure 5. Gel shift assay using nuclear extracts (NE) from activated HSC of donors with either the genotype G/G (*lanes 3, 4, 7,* and *8*) or A/A (*lanes 5* and *6, 9* and *10*) and probes corresponding to the promoter sequence (−2511 to −2527) of the MCP-1 gene, with either the nucleotide G (G probe; *lanes 3* and *5*) or A (A probe; *lanes 4* and *6*) at position −2518. Competition experiments were performed using NE of the GG homozygous donor and a 50-fold excess of unlabeled A-probe (*lanes 8* and *10*) or G probe (*lanes 7* and *9*), respectively (*lanes 1* and *2*: probes alone). NE of both HSC reveal a clear band in the presence of the G probe (*lane 3* and *5*) but not the A probe (*lanes 4* and *6*). This binding activity can be competed away with an excess of unlabeled G-probe (*lane 7*) but not with the A probe (*lane 8*).

chronic hepatitis C infection.[10–12] This prompted us to investigate in vitro culture activated HSC from donors with different −2518 MCP-1 genotypes to compare their MCP-1 expression. In line with the above-mentioned findings, we found a higher expression of MCP-1 in HSC from donors carrying at least 1 G allele compared with A/A homozygotes. This provides further evidence for the functional relevancy of the MCP-1 polymorphism and its potential role in liver disease.

Functional in vitro studies comparing cells from individuals with different genotypes had been performed so far only with peripheral blood mononuclear cells. The 2.1-fold higher expression of HSC from carriers compared with noncarriers of the G allele found in our study is well in the range detected in 2 previous studies using peripheral mononuclear cells (1.7- and 2.5-fold, respectively).[14,15]

There are few studies investigating the molecular mechanisms responsible for the effect of the (A −2518G) MCP-1 promoter polymorphism on MCP-1 transcription. Rovin et al. performed in vitro studies using appropriate reporter plasmids and provided evidence that the region of the polymorphism is influencing the transcriptional activity of the MCP-1 gene.[14] To further elucidate these effects of the MCP-1 genotype on transcription, we tested whether they may be linked in part to altered protein-DNA interaction. Indeed, we found binding activity in nuclear extracts from activated HSC specifically to the G allele, providing a potential mechanism for the differences seen.

By performing database search, we identified potential binding sites of the transcription factors MyoD and AP-4. Binding of both transcription factors was indicated only in the presence of nucleotide G, not in the presence of nucleotide A at position −2518 relative to the transcription start site. No data are available concerning the presence of AP-4 in activated HSC, whereas it has been reported recently, that MyoD protein expression and DNA binding activity is increased during in vivo or in vitro activation of the cells.[28] The myogenic transcription factor MyoD is a class B bHLH protein, and the expression of MyoD DNA binding activity in activated HSC implicates the transcription factor in the regulation of genes controlling myogenic properties of these cells. However, so far, no functional role for MyoD in the regulation of HSC activation or the molecular pathology of liver fibrosis or inflammation was shown. Here, we describe the E-Box displayed on the G allele of the MCP-1 gene as a potential target for MyoD binding.

The polymorphism at position −2518 of the MCP-1 gene has been implicated before in the progression of coronary artery disease, asthma, cutaneus vasculitis, and kidney graft failure and the onset of Crohn's disease.[15,17–20] Considering the known pathophysiologic role of the chemokine MCP-1 in these diseases, these examples further suggest that the G allele may be the higher expressing allele in certain inflammatory conditions. However, in these studies, the phenotypic manifestation of the −2518 MCP-1 polymorphism had neither been investigated in affected tissues nor in parenchymal or mesenchymal cells.

Here, we could demonstrate for the first time an association of the −2581 MCP-1 polymorphism with MCP-1 tissue levels in patients with chronic hepatitis C in vivo and with MCP-1 expression of activated HSC in vitro. The demonstration of these genotype/phenotype correlations distinguishes our study also from previous studies investigating the influence of different other polymorphisms on disease progression in chronic HCV. Polymorphisms of several genes, including tumor growth factor (TGF)-β1, angiotensinogen,[29] TNF-α,[30] myeloperoxidase,[31] microsomal epoxide hydrolase,[32] and apolipoprotein E and B[33] were associated with disease severity of chronic liver disease. However, none of these studies investigated the phenotypic manifestation of the individual polymorphism in the hepatic tissue of patients.

In summary, our findings indicate that HCV patients who are genetically predisposed to produce greater amounts of MCP-1 protein seem to be more prone to hepatic inflammation and fibrogenesis. The functional relevance of the −2581 MCP-1 promoter polymorphism was additionally confirmed by in vitro studies revealing for the first time a molecular mechanism that could be responsible for the transcriptional differences between the individual MCP-1 genotypes.

If confirmed by other studies, these findings may have implications for the prediction of the natural course of chronic hepatitis C infection, and knowledge of this MCP-1 polymorphism may direct more aggressive therapy toward those patients with an increased risk of disease progression.

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Received March 14, 2003. Accepted July 10, 2003.

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Supported by grants from the Deutsche Forschungsgemeinschaft (DFG; to A.B. and C.H.) and by the Else Kröner Fresenius-Stiftung (to C.H. and H.H.).