Original Contribution**SUPEROXIDE DISMUTASE IN PATIENTS WITH CHRONIC
HEPATITIS C VIRUS INFECTION**

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(Received 8 July 1997; Revised 7 October 1997; Accepted 13 November 1997)

Abstract—It has been reported that hepatitis C virus (HCV) may cause oxidative stress in infected cells. Patients with chronic hepatitis C exhibit an increased production of tumor necrosis factor- α (TNF α), a cytokine that can produce oxidative stress by stimulating the generation of reactive oxygen species (ROS). Cell defense against ROS includes overexpression of Mn-superoxide dismutase (SOD), an inducible mitochondrial enzyme. To investigate cell defense against oxidative stress in HCV infection, we analyzed Mn-SOD mRNA in liver and in peripheral blood mononuclear cells (PBMC) from patients with chronic hepatitis C. Mn-SOD expression in PBMC was significantly increased in patients with HCV infection. Patients with sustained virological and biochemical response after therapy showed significantly lower Mn-SOD than patients with positive viremia. By contrast, Mn-SOD expression was not enhanced in the liver of patients with chronic hepatitis C. The values of Mn-SOD mRNA did not correlate with TNF α mRNA expression, viral load, or liver disease activity. Our results indicate that in HCV infection an induction of Mn-SOD was present in PBMC but absent in the liver, suggesting that this organ could be less protected against oxidative damage. Oxidative stress could participate in the pathogenesis of HCV infection. © 1998 Elsevier Science Inc.

Keywords—Superoxide-dismutase, PCR, Oxidative stress, TNF α , Hepatitis C virus genotypes, Viral load, Free radicals

INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic and lymphotropic agent¹ with a strong tendency to establish chronic infections. The pathogenesis of HCV infection is not well understood. It has been suggested that HCV may cause oxidative stress in infected cells. Several lines of evidence support this contention, including the existence of an activated glutathione turnover, the presence of increased levels of lipid peroxidation products and augmented iron stores in the liver,^{2–4} and the finding of diminished reduced glutathione values in peripheral blood mononuclear cells (PBMC) and erythrocytes.^{5,6} Moreover, it has been shown that patients with chronic hepatitis C exhibit an increased production of tumor necrosis factor- α (TNF α),⁷ a cytokine that can produce oxidative stress by stimulating the generation of reactive oxygen species (ROS) such as superoxide ion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).⁸

Reactive oxygen metabolites are mainly produced in mitochondria during electron transport^{9,10} but also, to a lesser extent, in endoplasmic reticulum, peroxisomes, and nuclear and plasma membrane.¹⁰ ROS can damage cells by causing lipid peroxidation and oxidative damage of DNA and proteins¹¹ and by depleting ATP stores.¹² In the presence of metals (such as Fe^{3+}), $O_2^{\cdot-}$ can react with H_2O_2 to generate a hydroxyl radical that is even more reactive and cytotoxic than $O_2^{\cdot-}$ or H_2O_2 .¹⁰

Cells are protected against oxidative insults by natural antioxidant products, notably glutathione, and by diverse antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 , whereas glutathione peroxidase and catalase scavenge H_2O_2 .^{13,14} By rapidly eliminating $O_2^{\cdot-}$, SOD reduces the production of hydroxyl radical, thus attenuating oxidative damage of cellular constituents.

Eukaryotic cells have an extracellular SOD (EC-SOD) and two forms of intracellular SOD: one is found in the mitochondrial matrix, the manganese SOD (Mn-SOD), and another is predominantly present in cytosol,

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the copper-zinc SOD (CuZn-SOD).^{15,16} Synthesis of CuZn-SOD is constitutive, whereas Mn-SOD is inducible. Mn-SOD expression is enhanced by TNF α and other inflammatory mediators that cause intracellular release of oxidant compounds.^{17,18} Also, other agents and treatments that generate oxidative insult such as x-ray irradiation and hyperoxia^{19,20} can induce directly or indirectly the synthesis of Mn-SOD. These observations together with the strategic localization of Mn-SOD in mitochondria, a main site of ROS production, suggest that Mn-SOD is important in the protection against oxidative stress.

Because in chronic HCV infection there is an increased production of TNF α and there are data indicating the presence of oxidative stress, in the present work we have analyzed the levels of Mn-SOD mRNA in PBMC and in the liver in this condition.

MATERIALS AND METHODS

Patients

We have studied Mn-SOD and TNF α mRNA levels in 56 PBMC samples from 44 patients with chronic hepatitis C; 36 showed positive serum HCV-RNA (28 male, 8 female, age range from 22–71), and 20 corresponded to patients with sustained complete response to interferon (IFN) treatment (persistent normal serum transaminases and negative HCV-RNA in serum for at least 6 months after completion of 1 year IFN treatment) (13 male, 7 female, age range from 17–51). In 12 patients, who showed sustained response to the therapy, the study was performed on two occasions—before and after completion IFN therapy. Twenty healthy subjects (7 male, 13 female, age range from 25–38) were studied as controls.

A fragment of the liver biopsy obtained for diagnostic purposes was used for analysis of transcriptional expression of Mn-SOD and TNF α in 16 patients with chronic hepatitis C (9 males and 7 females; age range 26–64). In addition, in 12 controls (7 males and 5 females; age range 41–66 years) liver samples were taken at laparotomy for diagnostic purposes (in 5 cases because of gallstones and in 7 because of pancreatic cancer or cancer of the rectum without apparent liver metastasis).

Serum levels of Mn-SOD protein were determined in 19 healthy controls, 19 serum HCV-RNA-positive and 18 serum HCV-RNA-negative patients included in the study.

Diagnosis of chronic hepatitis C was based on elevation of serum transaminases for more than 6 months, positivity for anti-HCV antibodies (ELISA second generation, Ortho Diagnostic Systems, Raritan, NJ), presence of HCV-RNA (reverse transcription-PCR) in serum

and histological evidence of chronic hepatitis. Causes of chronic liver disease other than HCV were excluded: in particular, alcoholic patients were not included in this study.

Preparation of PBMC, liver specimens, and serum samples

For isolation of PBMC, fresh blood diluted with 1 volume of 0.9% NaCl was overlaid on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) with a density of 1.077 g/ml and centrifuged $600 \times g$ for 30 min. The mononuclear cell layer was harvested at the interphase and washed twice in 0.9% NaCl. PBMC were lysed in 4 mol/l guanidinium thiocyanate and stored at -80°C until extraction of total RNA, which was performed according to the method of Chomczynski and Sacchi.²¹ To obtain serum samples, venous blood was taken into sterile vacuum blood collection tubes and then centrifuged. Serum was stored at -70°C until used. Liver specimens were obtained with Tru-Cut biopsy needles (Baxter, Deerfield, IL). One-third of the biopsy was immediately frozen in liquid nitrogen and stored at -80°C until the extraction of RNA, and the remaining was used for histological study.

Analysis of mRNA levels of Mn-SOD and TNF α in PBMC and in the liver

Estimation of TNF α mRNA levels was carried out using a quantitative reverse transcription-PCR procedure as previously described.⁷ To determine Mn-SOD mRNA levels we used a similar procedure. Either PBMC or liver total RNA (1.25 or 0.75 μg , respectively) was reverse-transcribed (60 min at 37°C) with 200 U of M-MuLV reverse transcriptase (BRL, Gaithersburg, MD) in 25 μl volume of $5\times$ RT buffer (0.25 mol/l Tris-HCl pH 8.3, 0.375 mol/l KCl, 15 mmol/l MgCl_2), supplemented with 5 mmol/l DTT, 0.4 mmol/l deoxynucleoside triphosphate, ribonuclease inhibitor (30 U), and random hexamers (250 ng) (Boehringer-Mannheim, Mannheim, Germany). After heating (95°C , 5 min) and quick chilling on ice, an aliquot of 10 μl (0.5 or 0.3 μg for PBMC and liver, respectively) of the cDNA pool was used for PCR amplification of Mn-SOD in 50 μl of $10\times$ PCR buffer (100 mmol/l Tris-HCl pH 9.0, 500 mmol/l KCl, and 1% Triton X-100) containing 70 $\mu\text{Ci/ml}$ of (α - ^{32}P)-deoxycytidine triphosphate (Amersham, Buckinghamshire, UK), upstream and downstream primers (40 ng each), 1.5 mmol/l MgCl_2 and 2 U of Taq DNA polymerase (Promega Corporation, MD). Blank reactions with no RNA were performed in all experiments. As internal

Fig. 1. Relationship between input of total RNA in the reaction and cpm of the amplification band after reverse transcription-PCR of Mn-SOD and β -actin gene products in PBMC and in the liver.

control for each sample, PCR amplification of a fragment of β -actin cDNA (using 10 μ l aliquot of the cDNA pool) was performed as previously described.⁷ Mn-SOD cDNA fragments were amplified by 24 or 23 cycles (PBMC or liver, respectively) (94, 60, and 72°C, 1 min each step), and β -actin was amplified by 20 cycles (94, 55, and 72°C, 1 min each step), a protocol that avoided interference of the plateau effect. Oligonucleotides (5'-3') d(TGCACCACAGCAAGCACCAC) and d(AAACCAAGCCAACCCCAACC) were the upstream and downstream primers, respectively, used for amplification of 310 bp fragment from human Mn-SOD cDNA.²² After PCR amplifications, 20 μ l aliquots of the PCR reactions were electrophoresed in 2% agarose gel and bands were visualized using ethidium bromide. Agarose fragments of the same size containing these bands were excised and radioactivities were determined. Obtained values were corrected for background radioactivity from blank reactions with no RNA. Finally, values corresponding to Mn-SOD mRNA were normalized to those of β -actin mRNA, and results were expressed as cpm ratio of Mn-SOD to β -actin. Mn-SOD mRNA values were normalized to those of β -actin mRNA because we had previously demonstrated that β -actin mRNA was constantly expressed in patients with chronic hepatitis C with or without IFN treatment.⁷

Validation experiments of PCR assays using known quantities of total RNA (from 0 to 1 μ g) were carried out. As shown in Fig. 1, resultant cpm values corresponding to the amount of Mn-SOD or β -actin mRNA used (0.5 and 0.3 μ g in PBMC and liver, respectively) were in the linear part of the curve. To analyze whether changes of Mn-SOD mRNA levels relative to those of β -actin resulted in changes in the ratio of Mn-SOD PCR

Fig. 2. (A) Relationship between the amount of Mn-SOD riboprobe added to RNA extracted from normal PBMC and the ratio Mn-SOD PCR product cpm/ β -actin PCR product cpm. (B,C) The gel photographs show increasing intensity of the Mn-SOD amplification band (B) but constant intensity of the β -actin amplification band (C), when increasing amounts of a Mn-SOD riboprobe (0, 0.16, 0.31, 0.62, 1.25, 2.5, 5, 10, 20, and 40 pg, lines 2 to 11, respectively) were added to the RNA extracted from normal PBMC. Line 1, molecular weight.

product cpm/ β -actin PCR product cpm, we performed a reverse transcription-PCR with 0.2 or 0.5 μ g of total PBMC RNA from a healthy control (for Mn-SOD or β -actin, respectively) and serial dilutions of known quantities of Mn-SOD riboprobe (from 40 to 0.16 pg) were added. Mn-SOD riboprobe was obtained by run-off transcription of a plasmid that contained a 310 bp insert from the human Mn-SOD cDNA. As shown in Fig. 2, with increasing amounts of Mn-SOD mRNA used in the reaction, we obtained a progressive increase of the Mn-SOD/ β -actin ratio (the increase was linear up to 1.25 pg of the Mn-SOD riboprobe).

The coefficient of interassay variation for Mn-SOD/ β -actin was 16.05%. The identity of the PCR product from Mn-SOD cDNA amplification was verified by digestion with Nva I and SacI. The first enzyme yielded the predicted restriction fragments, while SacI did not digest the amplified PCR product (no restriction site for this endonuclease is present in the amplified region).

Fig. 3. Transcriptional expression of Mn-SOD gene (as ratio to that of β -actin) in PBMC (A) and in the liver (B) in healthy controls (c), patients with detectable hepatitis C virus RNA in serum (HCV-RNA+) and patients who cleared hepatitis C virus RNA after IFN therapy (HCV-RNA-). (In B, $p = .18$.)

Analysis of serum Mn-SOD levels

Serum Mn-SOD concentration was analyzed by means of an ELISA kit (NOF corporation, Tokio, Japan) using specific antihuman Mn-SOD antibodies. The assay was performed following the instructions of the manufacturers. The sensitivity limit of the assay was 2.5 ng/ml.

Detection and quantification of HCV-RNA and HCV genotyping

Serum HCV-RNA was studied by reverse transcription-PCR as previously described.⁷ Two sets of primers specific for the 5'untranslated region of HCV genome were used (5'-CCTGTGAGGAAGTACTGTCT-3' and 5'-CTATCAGGCAGTACCACAAG-3' for outer primers; 5'-ACTGTCTTCACGCAGAAAGC-3' and 5'-GACCAACACTACTCGGCTA-3' for inner primers). HCV-RNA was quantitated using a competitive PCR technique as previously reported.⁷ HCV genotypes were determined following the method of Viazov *et al.*²³ as described elsewhere.^{7,24} For genotype 4 the probe 5' G(A,G)CCGTCTTGGGGCC(A,C)AAATGAT was used.

Statistical analysis

Results of Mn-SOD and $\text{TNF}\alpha$ are presented as mean $\pm$ standard deviation. Normality was assessed with the Kolmogorov-Smirnov test. Statistical analysis of Mn-SOD protein was performed using parametric tests (Anova and Scheffe *F*-test). The statistical evaluation of Mn-SOD and $\text{TNF}\alpha$ mRNA levels in PBMC and in the

liver was made using nonparametric tests (Kruskal-Wallis and Mann-Whitney *U*-tests). The Wilcoxon signed-rank test was used to compare the levels of Mn-SOD mRNA before and after therapy in the same patients. Associations between quantitative variables were studied with Pearson's correlation coefficient. All *p*-values were two tailed.

RESULTS

As shown in Fig. 3A, Mn-SOD mRNA levels in PBMC were higher in patients with chronic HCV infection than in normal controls (1.14 ± 0.69 vs. 0.50 ± 0.25 , $p < .01$). Patients who had cleared the virus and normalized serum transaminases after IFN therapy showed values of Mn-SOD mRNA in PBMC (0.76 ± 0.23), that were significantly lower than subjects with detectable viremia ($p < .05$) but still higher than healthy controls ($p < .01$). However, Mn-SOD mRNA levels in liver were similar in chronic hepatitis C and in normal livers (1.28 ± 0.82 vs. 0.84 ± 0.38 , $p = .18$, Fig. 3B). The transcriptional expression of Mn-SOD in PBMC was analyzed sequentially (before and after completion IFN therapy) in 12 patients who exhibited complete sustained response (both virological and biochemical) to treatment. As shown in Fig. 4, the disappearance of viremia was associated with a significant reduction in Mn-SOD mRNA values.

Serum Mn-SOD levels were also higher in patients with positive viremia than in normal controls (120.45 ± 33.53 vs. 76.19 ± 17.38 , $p < .001$). Patients who had eliminated HCV-RNA and normalized serum transaminases after IFN therapy showed serum Mn-SOD levels similar to healthy controls (72.52 ± 18.48 vs. $76.19 \pm$

Fig. 4. Pre- and posttreatment values of Mn-SOD gene expression (as a ratio to β -actin) in PBMC from patients with chronic hepatitis C who showed sustained biochemical and virological response after interferon treatment.

17.38, NS). In viremic patients we found a close relationship between the levels of serum Mn-SOD protein and serum alanine aminotransferase (ALT) ($r = .75$, $p < .001$), probably reflecting the simultaneous release to plasma of Mn-SOD protein and ALT from damaged liver cells. No correlation was observed between Mn-SOD protein in serum and Mn-SOD mRNA values in PBMC.

No relationship was observed between Mn-SOD mRNA levels (in PBMC or in the liver) and serum transaminases. Similarly, we did not find any correlation between Mn-SOD mRNA values in the liver and the Knodell's score of the liver biopsy. When we analyzed the relationship between Mn-SOD expression and treatment outcome, we found no differences in pretreatment Mn-SOD mRNA values between those patients who subsequently showed sustained response to IFN and those who subsequently failed to respond to the therapy (1.16 ± 0.77 vs. 1.12 ± 0.66 , $p = .90$).

We found no correlation between viral load and values of Mn-SOD mRNA in PBMC ($r = .11$, $p = .53$). We also observed that the amount of Mn-SOD mRNA in PBMC was similar in the different HCV genotypes. Thus, Mn-SOD mRNA was 1.03 ± 0.34 , 1.19 ± 0.78 , 1.13 ± 0.86 , and 1.03 in HCV-RNA positive patients with genotypes 1a ($n = 6$), 1b ($n = 16$), 3a ($n = 12$), and 4 ($n = 1$), respectively.

Because Mn-SOD expression is believed to be stimulated by cytokines such as $\text{TNF}\alpha$, which in turn causes oxidative stress,⁸ we investigated the levels of $\text{TNF}\alpha$ mRNA in PBMC and in the liver from patients and controls where Mn-SOD mRNA had been previously evaluated. As shown in Table 1, $\text{TNF}\alpha$ mRNA levels in PBMC and in the liver were significantly elevated compared with controls in HCV-RNA-positive subjects, while the levels of $\text{TNF}\alpha$ mRNA in PBMC were within normal range in patients who cleared HCV-RNA after IFN therapy. Although the overall changes in the tran-

Table 1. Levels of $\text{TNF}\alpha$ mRNA (as a Ratio to β -Actin) in PBMC and in the Liver

	PBMC	Liver
Healthy controls	0.18 ± 0.11	0.86 ± 0.26
Hepatitis C Virus-RNA (+)	$0.39 \pm 0.31^*$	$1.49 \pm 0.89^*$
Hepatitis C Virus-RNA (-)	0.12 ± 0.06	

* $p < .05$ vs healthy controls and Hepatitis C Virus-RNA (-).

scriptional expression of $\text{TNF}\alpha$ paralleled those of Mn-SOD, no significant correlation was found between these two transcripts in PBMC or in the liver from patients with chronic hepatitis C.

DISCUSSION

The pathogenetic mechanisms through which HCV causes cell damage remain obscure, although it has been suggested that oxidative stress may play a pathogenetic role in this infection.^{2,3,25} Previous results from our own group have shown that antioxidants such as *N*-acetylcysteine, administered together with IFN, induced a significant decrease in serum transaminases (although not clearance of viremia) in patients previously resistant to IFN alone.²⁵

In this article we show that the transcriptional expression of Mn-SOD is increased in PBMC from patients with chronic hepatitis C. In this disease, both PBMC and the liver have been shown to be infected by HCV.^{1,26} The increase in Mn-SOD here reported appear to be related to active viral infection because Mn-SOD was found to significantly decrease in patients with sustained virological and biochemical response following IFN therapy. Because enhanced Mn-SOD expression appears to be an adaptive response to increased oxidative stress,^{15,16} our observations support the concept that disturbed intracellular redox state may be implicated in the pathogenesis of HCV infection. A significant elevation of Mn-SOD protein was also found in serum from patients with chronic HCV infection. Mn-SOD protein did not correlate with Mn-SOD mRNA values, but showed a close correlation with ALT values, indicating that increased serum levels of Mn-SOD may simply reflect enzyme leakage from damaged hepatocytes and, thus, the intensity of liver cell damage.

Increased oxidative stress has been demonstrated to occur in other viral diseases, including human immunodeficiency virus type 1 infection.^{27,28} In this condition, altered redox state has been ascribed to increased production of proinflammatory cytokines such as $\text{TNF}\alpha$.²⁷ The relationship between ROS and $\text{TNF}\alpha$ is bidirectional in that $\text{TNF}\alpha$ induces oxidative stress but also ROS activate latent nuclear factor κB ²⁹ and increase $\text{TNF}\alpha$

expression.³⁰ In addition to increasing the production of ROS, TNF α also induces the synthesis of Mn-SOD,^{31,32} and this latter effect can protect the cell against the cytotoxic effects of TNF α .^{31,32} As shown in the present report (which confirms our previous studies⁷), this cytokine is overexpressed in PBMC and in the liver in chronic hepatitis C. It thus seems possible that TNF α might participate in causing oxidative stress and Mn-SOD overexpression in PBMC in this condition.

We did not find any correlation between TNF α mRNA and Mn-SOD mRNA levels in PBMC or between the levels of the two transcripts in liver tissue. In fact, despite the presence of increased TNF α mRNA values, the levels of SOD message in the liver were not different from controls. This absence of correlations does not exclude the participation of TNF α in causing oxidative stress in patients with hepatitis C. Although the induction of Mn-SOD by TNF α occurs in most cells, there are some human tumors cells lines and HIV-infected T lymphocytes where Mn-SOD is not induced by TNF α .^{31,33} On the other hand, it should be considered that Mn-SOD expression is modulated by a diversity of cytokines and proinflammatory mediators in addition to TNF α .

Among these cytokines, interleukin-1 (IL-1) and IFN- γ have been shown to increase Mn-SOD synthesis, whereas transforming growth factor- β (TGF- β) markedly inhibits the expression of this enzyme.^{34,35} Interestingly TGF- β mRNA is considerably increased in the liver of patients with chronic hepatitis C,³⁶ while it is normal in PBMC from these patients (E. Larrea, unpublished data). This may contribute to explain why Mn-SOD mRNA is high in PBMC but not overexpressed in the liver in hepatitis C. If HCV infection causes similar oxidative stress in the liver as in PBMC, the lack of hepatic Mn-SOD induction in response to an altered redox state might deprive the liver of an efficient protective mechanism against the noxious effects of ROS,^{25,32,37} thus facilitating oxidative hepatocellular damage in this virus infection.

In summary, this article shows that in HCV infection, the adaptive response to oxygen free radicals, manifested by the induction of Mn-SOD, was present in PBMC but absent in the liver, suggesting that this organ may be less protected against oxidative damage. The involvement of oxidative stress in cell damage, viral persistence, and resistance to IFN requires further study.

Acknowledgements—This work was supported by grants 1853-95 from Gobierno de Navarra (Spain) and PM95-0205 from CICYT (Spain).

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ABBREVIATIONS

H_2O_2 —hydrogen peroxide
 HCV—hepatitis C virus
 IFN—interferon
 IL-1—interleukin-1
 $\text{O}_2^{\cdot-}$ —superoxide ion
 PBMC—peripheral blood mononuclear cells
 ROS—reactive oxygen species
 SOD—superoxide dismutase
 TGF- β —transforming growth factor- β
 TNF α —tumor necrosis factor- α