



# Oxidative damage, pro-inflammatory cytokines, TGF- $\alpha$ and c-myc in chronic HCV-related hepatitis and cirrhosis

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## Abstract

**AIM:** To assess whether a correlation exists between oxidative DNA damage occurring in chronic HCV-related hepatitis and expression levels of pro-inflammatory cytokines, TGF- $\alpha$  and c-myc.

**METHODS:** The series included 37 patients with chronic active HCV-related hepatitis and 11 with HCV-related compensated cirrhosis. Eight-hydroxydeoxyguanosine in liver biopsies was quantified using an electrochemical detector. The mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$  and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR.

**RESULTS:** TNF- $\alpha$  levels were significantly higher in hepatitis patients than in cirrhosis patients ( $P=0.05$ ). IL-1 $\beta$  was higher in cirrhosis patients ( $P=0.05$ ). A significant correlation was found between TNF- $\alpha$  and staging ( $P=0.05$ ) and between IL-1 $\beta$  levels and grading ( $P=0.04$ ). c-myc showed a significantly higher expression in cirrhosis patients ( $P=0.001$ ). Eight-hydroxydeoxyguanosine levels were significantly higher in cirrhosis patients ( $P=0.05$ ) and in HCV genotype 1 ( $P=0.03$ ). Considering all patients, 8-hydroxydeoxyguanosine levels were found to be correlated with genotype ( $P=0.04$ ) and grading ( $P=0.007$ ). Also multiple logistic regression analysis demonstrated a significant correlation among the number of DNA adducts, TNF- $\alpha$  expression and HCV genotype ( $P=0.02$ ).

**CONCLUSION:** In chronic HCV-related liver damage, oxidative DNA damage correlates with HCV genotype, grading and TNF- $\alpha$  levels. As HCV-related liver damage progresses, TNF- $\alpha$  levels drop while IL-1 $\beta$  and c-myc levels increase, which may be relevant to liver carcinogenesis.

## INTRODUCTION

Oxidative damage may affect a number of cell targets, including DNA<sup>[1-2]</sup>. Eight-hydroxydeoxyguanosine (8OHdG), a modified DNA base generated by genomic material interacting with reactive oxygen species, is a mutation that causes G-C to T-A transversion at DNA replication<sup>[3]</sup>. This adduct is a marker of oxidative DNA damage and one of the most widely-investigated lesions, since its consequences may well be linked to carcinogenic mechanisms<sup>[4-6]</sup>.

Oxidative damage in general and 8OHdG accumulation in particular, have been described in experimental and clinical HCV infection, with HCV-related oxidative damage playing a major part in the induction of liver diseases<sup>[7-9]</sup>. Although it is well known that reactive oxygen species induction lies at the center of a complex network of tissue and inflammatory responses involving the expression of cytokines, growth factors and oncogenes, this network has not been thoroughly investigated in HCV-related liver diseases.

Liver injury is reportedly associated with a chronic inflammatory response involving tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), etc. The former plays a central role in liver injury, triggering the production of other cytokines that in turn recruit inflammatory cells, promote fibrogenesis and further activate oxidative burst<sup>[10]</sup>. The initiation of a number of intracellular signal pathways involving apoptotic and/or anti-apoptotic signals should also be included amongst the effects of TNF- $\alpha$ <sup>[11]</sup> and HCV infection is indeed associated with an increase in TNF- $\alpha$  production, and the expression of viral proteins apparently results in more severe liver injury and hepatocyte death<sup>[12-14]</sup>.

On the other hand, IL-1 $\beta$  gives rise to the cascade of

the inflammatory response and recent reports have shown that its levels are higher in HCV-related liver diseases than in other forms of liver damage<sup>[15]</sup>. Its polymorphisms behind are related to the risk of progression to HCC<sup>[16-17]</sup>.

Our hypothesis is that oxidative DNA damage prompted by pro-inflammatory cytokines and/or by a specific effect of HCV core protein<sup>[18-19]</sup>, and associated with an imbalance between apoptosis and cytoproliferation<sup>[20]</sup>, is a fundamental event in HCV-related liver carcinogenesis.

Since a number of additional mediators are involved in liver carcinogenesis, including oncogenes such as c-myc that controls hepatocyte proliferation<sup>[21]</sup> and growth factors such as TGF- $\alpha$  involved in controlling liver regeneration and tumoral progression<sup>[22]</sup>, particularly when they are co-expressed<sup>[23]</sup>, the present study was to seek any correlations between oxidative DNA damage and the levels of pro-inflammatory cytokines like TGF- $\alpha$  and of c-myc in chronic HCV-related liver damage.

## MATERIALS AND METHODS

### Patients

Forty-eight patients consecutively recruited (34 M / 14 F, mean age  $42 \pm 12$  years) with liver disease characterized by abnormal serum transaminase levels for more than 6 months, were admitted to the Division of Gastroenterology for diagnostic liver biopsy. Informed consent was obtained from all patients. Patients taking medication or vitamins capable of interfering with oxidative balance or liver damage were excluded from the study. The study was approved by the Human Research Committee of the University of Padova. Thirty-seven patients (24 M / 13 F, mean age  $40.5 \pm 11$  years) were assigned to chronic active HCV-related hepatitis (CAH) group and 11 patients (7 M / 4 F, mean age  $50.5 \pm 14$  years) were assigned to HCV-related compensated (Child class A) cirrhosis (CIRR) group. Before biopsy, each patient was tested to measure HCV antibodies using a second-generation ELISA and all positive sera were confirmed by RIBA II assay. In all patients, anti-HCV seropositivity was confirmed by positive HCV-RNA levels using the Amplicor HCV test (Amplicor PCR Diagnostic, Hoffman-La Roche, Basel Switzerland). A standardized genotyping assay (Inno-Lipa HCV III, Inno-genetics, Gent, Belgium) was used. HCV genotypes were classified as genotype 1, subtypes 1a and 1b, genotypes 2, 3 and 4 and their subtypes. All the following studies were performed prior to any treatment.

### Morphological evaluation

Biopsies (one per patient) were taken using a 16-17 gauge modified Menghini needle under ultrasound guidance and local anesthesia. Only patients whose biopsy material was adequate (i.e. about 4 cm long) were included in the study to avoid taking a second biopsy. At least 2 cm of biopsy material was cut, fixed in 10% buffered formaldehyde and handed over to the pathologists. The tissue was embedded in paraffin, cut and routinely stained with H&E and PAS for routine evaluation. Together with the overall diagnosis, the pathologist (who was unaware of the clinical diagnosis) also gave a semi-quantitative score (0-3) for the presence and extent of macro- and micro-vesicular steatosis and the

Knodell index<sup>[24]</sup>, as modified by Ishak *et al*<sup>[25]</sup>, including both a grading and a staging of hepatic disease.

### Biochemical findings

Serum levels of ferritin, transaminases and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT) were determined as part of the routine clinical procedure. The tissue for biochemical determination was around 15 mg wet weight. Samples were processed immediately and stored at  $-80^{\circ}\text{C}$ .

### Quantification of 8OHdG from hepatic biopsies

Liver biopsy specimens obtained at endoscopy were stored at  $-80^{\circ}\text{C}$  for no longer than 3 wk. Preliminary experiments indicated that storage under these conditions could not affect the results of the assessment obtainable with unfrozen samples and the samples might remain stable for as long as 1 month (data not shown).

After thawing, the specimens were homogenized in separation buffer (75 mM NaCl, 10 mM Tris/Cl pH 7.5, 5 mM EDTA pH 6, 0.5% sodium dodecyl sulfate) and proteinase K at  $55^{\circ}\text{C}$  overnight. After treatment with ribonuclease A, the DNA was purified according to Fraga *et al*<sup>[26]</sup>. Following nuclease P1 and alkaline phosphatase hydrolysis, samples were filtered through  $0.22 \mu\text{m}$  nylon filter units (Scientific Resources, Inc., Alfattech, Genova - Italy), and approximately 20  $\mu\text{g}$  of DNA per sample was injected into the HPLC (Shimadzu, Kyoto, Japan). 8OHdG and normal deoxynucleosides were separated in a  $3 \mu\text{m}$  Supelcosil LC-18-DB analytical column ( $7.5 \text{ cm} \times 4.6 \text{ mm}$ , Supelco, Bellefonte, PA) equipped with a  $5 \mu\text{m}$  SupelguardTM LC-18-DB guard column cartridge. The solvent system consisted of an isocratic mixture of 90% 50 mmol/L potassium phosphate (pH 5.5) and 10% methanol at the 1 mL/min flow rate. 8OHdG was detected using an electrochemical detector (ECD; ESA Coulochem II 5200A, Bedford, MA) equipped with a high-sensitivity analytical cell model 5011 with the oxidation potentials of electrodes 1 and 2 adjusted to 0.15 V and 0.35 V, respectively. 8OHdG levels were referred to the amount of deoxyguanosine (dG) detected in the same sample by UV absorbency at 254 nm. The amount of DNA was determined according to a calibration curve versus known amounts of calf thymus DNA. 8OHdG levels were expressed as the number of 8OHdG adducts per  $10^5$  dG bases. An 8OHdG standard (Sigma) prepared immediately before determination, was injected before any set of samples. The coefficient of variation was  $<10\%$  and the amount of DNA required for the assay (expressed in  $\mu\text{g}$  of DNA) was 100  $\mu\text{g}$ . Samples with lower amounts of DNA were rejected, since the risk of methodological error was only acceptable above this cut-off.

### TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$ and c-myc determination

The mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$  and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR. Total RNA extracted from frozen liver tissue (stored at  $-80^{\circ}\text{C}$ ) by acid guanidium thiocyanate-phenol-chloroform according to the Chomczynski and Sacchi method<sup>[27]</sup>, was quantified spectrophotometrically. Integrity of the RNA sample was assessed by electrophoresis on 2% agarose gel (FMC Bio Product, Rockland,

**Table 1** Eight-hydroxydeoxyguanosine levels in chronic HCV-mediated liver damage (mean  $\pm$  SD)

	n° 8OHdG/10 <sup>5</sup> dG		n° 8OHdG/10 <sup>5</sup> dG
CAH	42.3 $\pm$ 25.3	HCV genotype 1	74 $\pm$ 36
CIRR	73.64 $\pm$ 28.2	Other genotypes	46.9 $\pm$ 23
P	0.05		0.03

MC, USA). One  $\mu$ g of RNA was reverse transcribed in cDNA in the presence of 1 $\times$  PCR buffer, 1 mM each of dNTPs, 1 U RNase inhibitor, 2.5  $\mu$ M random exomers and 2.5 U of murine leukemia virus. cDNA was amplified in a final volume of 50  $\mu$ L of PCR buffer, 2 U Amplitaq DNA polymerase, 0.056  $\mu$ mol/L of Taq Start antibody, 0.2 mM of each of the dNTPs, 0.4  $\mu$ mol/L of each primers for TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$ , c-myc and  $\beta$ -actin. PCR products underwent a vertical electrophoresis on polyacrilamide gel. Electrophoretic bands were stained with silver nitrate and scanned on a densitometer image analyzer system (Quantity-one Biorad, Hercules, CA, USA). The results were expressed as the optical density ratio of TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$  and c-myc to control  $\beta$ -actin.

### Statistical analysis

The data were examined statistically by one-way ANOVA and Student's *t*-test, Kruskal-Wallis and linear regression. Multiple logistic regression analysis was also used by including the following variables: 8OHdG levels, diagnosis, age, expression of TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$  and c-myc, genotype.

## RESULTS

### Patient characteristics

No difference in the patients' age or gender distribution was observed between the CAH and CIRR groups. ALT levels were significantly higher in patients with chronic hepatitis than in patients with cirrhosis (87.9 $\pm$ 49 *vs* 50.5 $\pm$ 14, *P* = 0.002 by *t*), while AST levels were significantly higher in CIRR group than in CAH group (178 $\pm$ 95 *vs* 52 $\pm$ 23, *P* = 0.002 by *t*). Serum ferritin and  $\gamma$ GT levels did not differ significantly between the two groups of patients.

According to the classification Ishak *et al*<sup>[25]</sup>, the stages of disease were, by definition, significantly higher in CIRR than in CAH patients (5.25 $\pm$ 0.4 *vs* 2.5 $\pm$ 0.8, *P* = 0.0001 by *t*), while grading was similar in the two groups. All patients were HCV-RNA positive. Type 1 (1a/1b) infection was the most prevalent (45%), followed by subtype 3a (29%), type 2 (17%) and finally type 4 (3%).

### Oxidative DNA damage, TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$ and c-myc expression

8OHdG levels were significantly higher in CIRR patients (*P* = 0.05 by *t*) and when oxidative damage was correlated with different HCV genotypes, 8OHdG levels were higher in HCV genotype 1 hepatitis than in the other genotypes (*P* = 0.03 by *t*). The results of 8OHdG are shown in Table 1. Considering all patients, 8OHdG levels correlated

**Table 2** TNF- $\alpha$ , IL-1 $\beta$ , TGF- $\alpha$  and c-myc expression in chronic HCV-mediated liver damage (mean  $\pm$  SD)

	TNF- $\alpha$ / $\beta$ -actin	IL-1 $\beta$ / $\beta$ -actin	TGF- $\alpha$ / $\beta$ -actin	c-myc/ $\beta$ -actin
CAH	0.7 $\pm$ 0.2	1.1 $\pm$ 0.3	0.41 $\pm$ 0.1	0.09 $\pm$ 0.05
CIRR	0.5 $\pm$ 0.2	1.4 $\pm$ 0.6	0.43 $\pm$ 0.2	0.46 $\pm$ 0.2
P	0.05	0.05	N.S.	0.001

significantly with genotypes (*P* = 0.04 Spearman's rank correlation) and grading (*P* = 0.007 Spearman's rank correlation). The results for pro-inflammatory cytokines, TGF- $\alpha$  and c-myc are shown in Table 2. TNF- $\alpha$  expression was significantly higher in CAH group than in CIRR group (0.7 $\pm$ 0.2 *vs* 0.5 $\pm$ 0.2, *P* = 0.05 by *t*), whereas IL-1 $\beta$  expression was significantly higher in CIRR group than in CAH group (1.4 $\pm$ 0.6 *vs* 1.1 $\pm$ 0.3, *P* = 0.05 by *t*). The previously mentioned higher oxidative DNA levels in genotype 1 HCV infection correlated with TNF- $\alpha$  (*P* = 0.04). A significant correlation was also found between IL-1 $\beta$  levels and grading (*P* = 0.04), and between TNF- $\alpha$  and staging (*P* = 0.05). No significant correlations were found between pro-inflammatory cytokine levels, steatosis score or genotype. TGF- $\alpha$  levels were similar in the two groups of patients (0.41 $\pm$ 0.18 *vs* 0.43 $\pm$ 0.21, *P* = NS by *t*), while c-myc expression was significantly higher in patients with cirrhosis (0.46 $\pm$ 0.2 *vs* 0.09 $\pm$ 0.05, *P* = 0.001 by *t*). No significant correlations were found between c-myc, steatosis score or genotype. Finally, multiple logistic regression analysis confirmed the previously reported significant correlation among the number of DNA adducts, TNF- $\alpha$  expression and genotype (*P* = 0.02).

## DISCUSSION

We have previously reported that oxidative DNA damage in the liver is, at least to some degree, a specific feature of HCV infection, in which it reaches its maximal levels<sup>[7]</sup>. Even though it occurs in the early stages too, 8OHdG accumulation parallels the progression of the disease and is more striking in subjects with HCV genotype 1b infection<sup>[28]</sup>.

This paper provides data on patients with HCV-related liver damage, partly describing the complex network of relationships between DNA oxidative damage, cytokine synthesis and release, c-myc and TGF- $\alpha$  expression that may both be strongly involved in liver cancerogenesis<sup>[29-31]</sup>. Numerous data link oxidative damage (and the parameters considered here) with the progression of liver disease and the onset of liver cancer. In primary murine hepatocyte cultures, TNF- $\alpha$  expression causes 8OHdG formation and an increase in cell cycle progression indicates a possible role of TNF- $\alpha$  in early malignant transformation of hepatocytes<sup>[32]</sup>.

The first set of our results was related to TNF- $\alpha$  and IL-1 $\beta$  which plays a direct role in causing growth arrest and a chronic role in inducing TNF- $\alpha$  expression<sup>[10-11]</sup>. This effect was not confirmed in our series, since a correlation between IL-1 $\beta$  and TNF- $\alpha$  was not detected. On the other hand, IL-1 $\beta$  expression was higher in the later stages of HCV-related liver disease, as previously dem-



onstrated by Gramantieri *et al*<sup>[33]</sup>, while the opposite was true of TNF- $\alpha$ , whose levels of expression were higher in CAH patients. We have previously reported that the balance between cytoproliferation and apoptosis is disrupted in HCV infection<sup>[20]</sup>. It is worth stressing that both TNF- $\alpha$  and IL-1 $\beta$  are involved in controlling the above described balance, thus taking part in determining the liver cell's fate and progression to liver cancer. In fact, the binding of TNF- $\alpha$  and IL-1 $\beta$  to their receptors leads to the activation of transcriptional factors, such as NF $\kappa$ B and AP-1, again involved in controlling cell proliferation<sup>[10]</sup>. What role does persistent oxidative stress play in this scenario? The overproduction of oxidative species, linked to over-expression of inflammatory cytokines (as shown by the positive correlation between TNF- $\alpha$  and 8OHdG levels in the liver), might be responsible for inhibiting the apoptotic process, most likely by activating the NF $\kappa$ B-dependent pathway<sup>[34]</sup>.

Last but not the least, oxidative damage may be related to the expression of proto-oncogenes, such as c-myc<sup>[35]</sup>. In our study, c-myc transcript levels were significantly higher in cirrhotic than in non-cirrhotic tissues, indicating that tissue damage progression from hepatitis to cirrhosis, with the related cell growth changes, may be mediated to some degree by c-myc, which indeed is considered one of the activators of cell proliferation<sup>[36]</sup>. In this series, we could detect no relationship between 8OHdG and c-myc, suggesting that they have different and independent proto-oncogene activation mechanisms.

It was reported that TGF- $\alpha$  /c-myc double transgenic mice exhibit enhanced cell proliferation and build up extensive oxidative DNA damage which possibly accounts for massive DNA damage and accelerated neoplastic development in the liver<sup>[37]</sup>. In the present study, all liver samples with or without cirrhosis, expressed low levels of TGF- $\alpha$  mRNA and revealed no correlation with any of the other parameters investigated. This may not be totally surprising, since a strong and prominent localization of TGF- $\alpha$  in ground-glass hepatocytes of HBV-related liver disease in association with HBV pre-S1 antigen has been reported and this may mean that TGF- $\alpha$  is more involved in HBV than in HCV liver disease<sup>[38]</sup>.

In our study DNA oxidative damage correlated with TNF- $\alpha$  over-expression in chronic HCV-mediated liver damage. Evolution to cirrhosis was characterized by an increased oxidative DNA damage, c-myc expression and IL-1 $\beta$  release. When disease activity was severe, it was paralleled by an increased expression of IL-1 $\beta$  and c-myc associated with genotype 1b infection and accumulation of 8OHdG. The above findings suggest that chronic HCV-mediated oxidative DNA damage in the liver may have an impact not only on hepatocyte proliferation rate through c-myc activation but also on cell proliferation and apoptosis through TNF- $\alpha$  activation.

In conclusion, HCV infection is associated with increasing cell proliferation unaccompanied with any substantial increase in apoptosis<sup>[20]</sup>, while TNF- $\alpha$  activation in this scenario has more to do with cell proliferation rather than with cell apoptosis.

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