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HCV-specific cellular immune responses in subjects exposed to but uninfected by HCV

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Gut

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Polymorphic Differences in SOD-2 May Influence HCV Viral Clearance

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Hepatitis C virus (HCV) is a pathogen causing chronic hepatitis, cirrhosis, and liver cancer occurring in about 3% of the world's population. Most individuals infected with HCV develop persistent viremia. Oxidative stress may play an important role in the pathogenesis of a number of diseases including HCV infection and diabetes mellitus. Polymorphisms in the antioxidant genes may determine cellular oxidative stress levels as a primary pathogenic role in HCV and/or in its complications. Patients with HCV and normal, healthy controls were investigated for a superoxide dismutase (SOD-2) polymorphism in the mitochondrial targeting sequence with Ala/Val (C-9T) substitution. Polymorphisms in antioxidant gene SOD-2 were carried out by PCR, restriction fragment length polymorphism assays and by polyacrylamide gel electrophoresis. For the SOD-2 polymorphism, the RNA positive group showed a higher percentage of "CT" genotype than the RNA negative group (89.3% vs. 66.1%, $P=0.001$, $\chi^2=11.9$). The RNA negative group had more TT genotypes than the RNA positive group (27.4% vs. 6.80%, $P=0.01$, $\chi^2=11.6$). The exposed uninfected group had an increased frequency of the "CT" genotype (86.2% vs. 66.1%, $P=0.02$, $\chi^2=5.5$). The RNA positives had a higher frequency of the "CT" from the normal controls (72.1% vs. 89.2%, $P=0.005$, $\chi^2=7.8$). **J. Med. Virol.** 86:941–947, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: SOD-2; antioxidant; gene polymorphism; hepatitis C; virus clearance

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis, and liver cancer occurring in up

to 3% of the world's population [Alter, 1999]. Many patients with HCV are asymptomatic, however 15% of the patients clear spontaneously HCV virus efficiently while 85% remain viremic and we are interested in genetic differences between these two groups [van der Poel, 1994]. Of those who remain RNA positive, the outcome appears extremely variable. Parenteral exposure to HCV usually results in infection and patients can be identified as infected by detection of HCV antibodies. Those who clear the viremia do not have any detectable virus RNA and are described as RNA negative but do have a positive HCV antibody test. An RNA positive result indicates that the virus is still present and those who remain viremic have a significant risk of developing chronic liver disease, cirrhosis and hepatocellular carcinoma.

The role of free radical reactions in protein oxidation, DNA damage and lipid peroxidation is strongly debated in relation to human disease and has been implicated in many disease states. It is not clear whether reactive oxygen species (ROS) are solely a major cause of tissue damage in disease or if they need to be accompanied by other factors as well as the tissue injury. It is clear that reactions with free radicals occur more readily than normal in tissues that are diseased or damaged and this may exacerbate disease states. Increased oxidizability of the damaged tissues can be due to the inactivation or leakage of antioxidants from cells. Proliferative cells that are exposed to sub-cytotoxic oxidative species (OS), such as hydrogen peroxide, ultraviolet light and ethanol, display mitochondrial DNA deletions, cell

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morphology, histochemistry changes, cell cycle regulation, and gene expression differences [Sozou and Kirkwood, 2001]. Inflammatory conditions that control pathways of cell death, gene transcription and stellate cell activation are under redox potential control and cells have developed homeostatic controls to overcome such environmental situations [Guimarães et al., 2006; Muriel, 2009; Circu and Aw, 2010]. Hepatic damage in a variety of liver disorders can progress as a result of oxidative stress as a pathogenic mechanism [Sen et al., 2002].

OS plays an important role in the pathogenesis of toxic liver diseases and of other hepatic alterations. Almost all experimental and clinical conditions of chronic liver disease that has been studied have detected OS [Parola and Robino, 2001]. Part of the cytopathic immunological response to viral hepatitis is the production of ROS and reactive aldehydes which can directly activate stellate cells transforming them into myofibroblasts and leading to hepatic fibrosis and cirrhosis [Svegliati Baroni et al., 1998; Galli et al., 2005]. Kupffer cells and other liver macrophages release the profibrotic cytokine TGF alpha as a result of redox sensitive reactions [Poli, 2000]. Higher levels of OS in the form of ROS have been detected in non-alcoholic steatosis and steatohepatitis [Pessayre et al., 2002].

Free oxygen radicals take part in the pathogenesis of chronic HCV in children by decreasing the barrier efficiency, diminishing catalase and superoxide dismutase (SOD) levels [Chrobot et al., 2000].

HCV infection has shown significantly increased manganese superoxide dismutase (SOD-2) in peripheral mononuclear cells (PBMCs) and patients with HCV infection display differential expression of SOD-2 in different tissue. HCV infection has been found to induce SOD-2 in PBMCs but its induction was not enhanced in the liver [Larrea et al., 1998]. Further to this, patients with positive viremia and chronic HCV infection showed a significantly increased SOD-2 expression in PBMCs compared to cases that had resolved the viremia [Larrea et al., 1998]. The lower SOD-2 response in the liver compared to the periphery and the lower SOD-2 producing genotype may combine and produce a higher ROS environment resulting in poor viral replication as HCV replication is inhibited by the presence of ROS.

Manganese superoxide dismutase (MnSOD) is involved in controlling dioxygen toxicity in the mitochondria, an organelle of extreme oxidative load. Over-expression of the antioxidant SOD-2 abolished the signal generated by ROS [Brownlee, 2001].

Decreased levels of SOD-2 may contribute to the development of certain diseases. Mice without the gene that encodes SOD-2 die 10 days after birth with cardiomyopathy and lipid accumulation in the liver and skeletal muscles [Li et al., 1995]. The SOD-2 gene may determine cellular oxidative stress levels in HCV infection. They may also determine the extent of liver damage resulting from HCV infection.

We also studied, as a comparison, a diabetic cohort in comparison and antioxidant responses to hyperglycemia have shown that SOD-2 responses did not change between patients with diabetic complications group or in normal controls [Hodgkinson et al., 2003].

OS, as reflected in blood and urine samples using a wide range of pro- and antioxidant markers, is a significant feature in HCV infection. The oxidative stress is more marked with the patients with cirrhosis but there is clear evidence of oxidant stress in non-cirrhotic patients. In fact oxidative stress has been detected in almost all experimental and clinical conditions of chronic liver disease [Jain et al., 2002].

The oxidative burst in HCV infection has been evaluated and hydrogen peroxide production was higher in chronically infected HCV cases than in controls and may be an additional factor in the development of liver lesions [Toro et al., 1998]. Lymphocyte function and the degree of chronicity of HCV infection is thought to be influenced by oxidative stress in peripheral blood mononuclear cells from HCV patients [Boya et al., 1999]. More specifically oxidative stress can inhibit the Th-cell and NK cell responses involved in HCV infection immune response [Thoren et al., 2004].

In hepatoma cells constitutively replicating HCV RNA to model the state of HCV infection, mitochondria generated increased levels of ROS, and showed mitochondrial injury and degeneration with increased lipid accumulation. This alteration of the cellular redox state changes significantly the level of autophagy, suggesting an association between oxidative stress and HCV-activated autophagy. There was also an upregulation of SOD-2 in HCV expressing cells with a subgenomic replicon [Chu et al., 2011].

Polymorphic genetic differences may change the antioxidant gene expression in a similar way to somatic mutations caused by OS. Disease susceptibility and resistance have been well investigated with relation to HLA polymorphisms in HCV patients [Cramp et al., 1998].

The SOD-2 targeting signal sequence polymorphism has been identified on chromosome 6q [Todd and Farrall, 1996]. This polymorphism in the mitochondrial targeting signal sequence could affect the transport of the enzyme through the mitochondrial membrane and a defect may alter the membrane receptor recognition site resulting in less of the enzyme protein entering the cell thus lowering the antioxidant response to oxidative stress. Other polymorphisms involved in host immune responses that play a role in the mechanisms that determine viral clearance or persistence in HCV infection with regard to the initial cytokine secretion of IL-12 on antigen recognition [Houldsworth et al., 2003, 2005; Hegazy et al., 2008].

IL28B rs.1297860-CC polymorphism is associated with a population of individuals that remain uninfected with HCV and are antibody negative, despite proved exposure to the virus and are a distinctly

different population from patients that spontaneously resolve infection after testing HCV antibody positive [Knapp et al., 2011].

MATERIALS AND METHODS

Patients

HCV cohort. European Caucasoid HCV antibody positive patients were included in the study. This is a retrospective study of DNA samples collected from patients visiting an HCV clinic after diagnosis, intravenous drug use (IVDU) individuals that had shared contaminated needles but remained HCV antibody positive and healthy controls not at risk of infection. The DNA samples were divided into three groups according to HCV RNA status, one group being HCV RNA negative, another HCV RNA positive and lastly a third group of individuals who were exposed to HCV but remained RNA negative. This latter group were screened and identified after completing questionnaires and had stated that they had used contaminated needles from patients who were diagnosed with HCV infection. The HCV RNA negative group all had detectable HCV antibodies as evidence of previous HCV infection but were repeatedly HCV RNA seronegative with persistently normal liver function tests over a period of at least 18 months, and none had clinical evidence of liver disease. The majority had risk factors for the acquisition of HCV infection and none had received antiviral treatment at any stage. All remained healthy many years after the likely time of infection and were considered to have cleared spontaneously virus and recovered from HCV infection.

The HCV RNA positive group were all both HCV antibody and HCV RNA positive. All 123 patients had abnormal liver function tests and/or biopsy evidence of HCV related liver disease. The RNA positive and RNA negative groups were comparable in terms of age, sex, and estimated duration of infection (Table I) and route of infection (Table II).

The exposed uninfected group were defined by the absence of any evidence of HCV infection (i.e., HCV antibodies and HCV negative) despite a clear history of risk from parenteral exposure to HCV. Cases were identified of recipients of pooled blood products, recipients of blood of HCV infected donors, and cases with a long history of IVDU use. Nineteen cases were recruited from London, of which four were hemophiliacs, three received HCV contaminated blood transfusions, three received other blood products, and nine were IVDU. Forty cases IVDU were recruited from day rehabilitation centers and prisons in the Plymouth area [Horne et al., 2004].

The European Union patients from London were identified from day rehabilitation centers in South London, from the blood transfusion centers and from the hemophiliac center at St Thomas' Hospital.

Controls. One-hundred five controls matched ethnically were studied. Control DNA was obtained from the cord blood of European Caucasoid subjects collected sequentially after normal obstetric delivery from the Obstetric Department, Derriford Hospital (Plymouth, UK).

Ethics committee approval and patient consent was obtained for all studies performed.

Virological testing. In both RNA positive and RNA negative groups, the presence of HCV antibodies was determined by second or third generation assay (Abbott IMx, Abbott Diagnostics, Maidenhead, UK). The presence or absence of HCV RNA was determined by a commercially available assay (Amplicor, Roche, Basel, Switzerland).

Genotyping

Genomic DNA was extracted conventionally from whole blood using a "salting out" method. The restriction site was found at -9 on the mitochondrial targeting sequence of the manganese superoxide dismutase, which is a C to T substitution resulting in an amino acid change of alanine to valine.

TABLE I. Percentage Genotype Frequency of Mitochondrial Targeting Sequence of SOD-2 C/T Polymorphism in South East HCV Cases

Genotype frequency	Exposed uninfected	RNA positive	RNA negative	Normal controls
Number of cases	n = 58; 48M:10F	n = 103; 63M:40F	N = 62; 40M:22F	n = 79; 40M:39F
Mean age/years ± SEM	33.2 ± 1.06	48.9 ± 0.83	46.7 ± 1.38	NA
Mean duration of disease	NA	18.7 ± 0.9	17.3 ± 1.1	NA
Genotyping C/C	3.5% (2)	3.9% (4)	6.5% (4)	11.4% (9)
C/T	86.2% (50) ^a	89.3% (92) ^a	66.1% (41) ^a	72.1% (57)
T/T	10.3% (6)	6.8% (7) ^b	27.4% (17) ^b	16.5% (13)
Allelic frequency				
Number of alleles or chromosomes	n = 116	n = 206	n = 124	n = 158
C	46.5% (54)	48.5% (100)	39.5% (49)	47.5% (75)
T	53.5% (62)	51.5% (106)	60.5% (75)	52.5% (83)

Hardy-Weinberg = 1.00 for controls and all patients.

^aEU (86.2%) versus RNA negative (66.1%) were significantly different for CT; $P = 0.02$, $\chi^2 = 5.54$, OR = 3.20 (1.19 < OR < 8.85), RNA positive versus RNA negative were also significantly different for CT (89.3% vs. 66.1%) $P = 0.001$, $\chi^2 = 11.87$, OR = 0.23 (0.09 < OR < 0.57).

^bRNA negative (27.4%) versus RNA positive (6.8%) were significantly different for TT; $P = 0.001$, $\chi^2 = 11.64$, OR = 5.18 (1.85 < OR < 14.96).

TABLE II. Sources of Infection for HCV Cases

	RNA positive	RNA negative
Number of cases, n	n = 103	n = 62
IVDU	42.7% (44)	61.3% (38)
Blood/blood products	34.0% (35)	17.7% (11)
Sexual/familial	1.0% (1)	4.8% (3)
No known risk factors	22.3% (23)	16.1% (10)

This table shows the different sources of HCV infection in the South East patient group SOD-2 data.

Two primers were used to amplify SOD-2 (ala, -9, val), the forward primer was 5'-AGC CCA GCC TGC GTA GAC-3 and the reverse primer 5'-TAC TTC TCC TCG GTG ACG-3'.

The restriction enzyme used to digest the amplified fragment was BsaW1 (*Bacillus stearothermophilus* W1718 Chen) (5'A^CCGG A3', 3'T GGCC^T 5'). The C1183T polymorphism in the mitochondrial sequence of MnSOD was digested by BsaW1 in a water bath at 6°C for 90 min produced two fragments of 82 and 164 bp. Samples where the cut site was abolished due to the presence of the "T" allele produced fragments of 246 bp. The migration of the DNA was visualized under ultra violet light and the bands were photographed using a transilluminator linked to specialist computer software. In order to genotype the samples, the position of the bands was determined relative to the DNA ladder fragments.

Statistical Analysis

The frequency of the alleles and genotypes in both patient subgroups and controls were compared for significance, using contingency tables and Chi-squared test with Yates correction where appropriate. *P* values of <0.05 were considered to be significant. Odds ratios with 95% confidence limits were also included where appropriate. The results are described using percentage frequency, *P* values, Chi-squared and odds ratio values. A Yates correction was applied to all *P* values.

A power calculation was performed by a university statistician who specializes in medical research projects and it was agreed that the sample size was adequate to give sufficient power to find significant statistical difference in this cohort, however it was understood that optimum sample size may not always be achievable with a this kind of retrospective study.

In order to ensure that differences between the patient groups were not found by chance, a Bonferroni calculation was applied to the data [Benjamini and Hochberg, 1995].

Hardy-Weinberg Distribution

In a non-selected population, the relative frequencies of different alleles tend to be constant and are described by a single equation. Several factors may

distort the gene frequencies in a population. The patients infected with HCV were recruited from the South East of England whereas the healthy control subjects were from the South East. A high frequency may indicate the possibility that evolutionary forces are in operation and applying selective pressure in favor of heterozygotes or homozygotes for mutant genes that cause the more common inherited disorders. We tested for Hardy-Weinberg distribution in the population of the genotypes that were studied using the calculation ($p^2 + 2pq + q^2 = 1$). The frequency of C = *p* and the frequency of T = *q*. This tested for genetic variation of a population in equilibrium [Emigh, 1980].

RESULTS

SOD-2 Genotype for HCV Patients

European Caucasoid HCV antibody positive patients were included for the SOD-2 polymorphism investigation and were recruited from The Institute of Liver Studies at King's College Hospital. Three groups of cases were studied; HCV antibody positive (n = 165, 103M:62F) but HCV RNA negative cases with spontaneous viral clearance and a mean age of 46.7 years ±1.38 SEM (n = 62, 40M:22F), HCV antibody and HCV RNA positive chronically infected cases with a mean age of 48.9 years ±0.83 SEM (n = 103, 63M:40F) and also cases with a long history of intravenous drug usage or exposed to blood products and at a high risk of HCV infection, termed HCV exposed but uninfected cases (EU) with an average age of 33.2 years ±1.06 SEM (n = 58, 48M:10F).

There was a significant difference between the RNA negative group (66.1%) and the RNA positive group (89.3%) for the "CT" heterozygous cases [*P* = 0.001, $\chi^2 = 11.87$, OR = 0.23 (0.09 < OR < 0.57)]. There were also more EU than RNA negative "CT" genotypes (86.2% vs. 66.1%, *P* = 0.02, $\chi^2 = 5.54$, OR = 3.20 (1.19 < OR < 8.85). The homozygous cases for "TT" were also significantly different between the RNA negative cases (27.4%) and the RNA positive cases (6.8%) with a *P*-value of *P* = 0.0006, $\chi^2 = 11.64$, (OR = 5.18 (1.85 < OR < 14.96; Table I).

DISCUSSION

Clearly impaired oxidative balance may have a prognostic significance on disease activity and determines the severity or the disease outcome in chronic HCV patients. It has also been observed that symptom free HCV carriers with impaired redox state are also at a higher risk of aminotransferase flare-up [Vendemiale et al., 2001]. Thus, understanding of these mechanisms led to the consideration of antioxidant therapy as a promising therapy for a number of liver diseases but this therapy has not yet shown to be significantly beneficial in HCV patients, although small reductions of serum alanine aminotransferase

have been detected with vitamin E treatment. Specific liver tissue delivery seems to be a limiting factor to its efficacy and the heterogeneous nature of the antioxidant treatments used made interpretation of results complicated [Singal et al., 2011].

The single nucleotide polymorphism results in a substitution of C to T and causes an amino acid change from alanine to valine. The variation in amino acid from alanine to valine in the SOD-2 leader signal affects the processing efficiency of the enzyme. The valine form may be less efficiently transported into the mitochondria than the alanine form of the enzyme. Studies have indicated that basal SOD-2 activity may be highest for Ala/Ala, followed by Ala/Val and then Val/Val [Shimoda-Matsubayashi et al., 1996]. No allelic differences were found in frequency between the HCV patients groups or the T1DM patient groups. There was, however, a lower frequency of heterozygous cases in the RNA negative group that spontaneously resolved their viremia and a higher frequency of "TT" homozygous cases than the RNA positives. The T allele relates to the valine amino acid, which is considered to have a lower basal level of SOD-2 activity [Chistyakov et al., 2001] but there is little experimental evidence to back this hypothesis yet, especially in HCV patients. SOD-2 levels have been measured in HCV infected patients but they have not been assessed according to genotype. It is interesting that a lower SOD-2 activity producing genotype should be associated with HCV viral clearance. It has, however, been reported that reactive oxygen species (ROS) suppress the replication of HCV in human hepatoma cells [Choi et al., 2004] thus the valine variant of SOD-2 may be more detrimental to the replication of HCV due to a higher level of ROS. Ala16val SOD-2 polymorphism was investigated in a group of French HCV cirrhosis patients and variants did not seem to modulate the progression of hepatocellular carcinoma in these patients [Nahon et al., 2012]. Further, the alanine SOD-2 genotype has been associated with an increased risk for HCC than in controls in a group of Moroccan patients [Ezzikouri et al., 2010]. This anomaly may be a factor of the delicate balance of HCV interactions with the host cells, the highly complicated host immune response to the virus and various other environmental factors. We do not know how the two different genotypes respond to HCV infection and whether there are differences in expression due to the presence of the virus. HCV does activate NF κ B, the transcription factor for SOD-2, and SOD-2 transcription is also enhanced by IFN- γ , a cytokine commonly expressed during HCV infection [Bigger et al., 2001]. The valine, beta sheet variant, may bind or respond differently in the presence of HCV than it does in normal circumstances. If the SOD-2 polymorphism has an influence on and is initiated by host inflammatory responses then it is interesting that the HCV data and the patients with diabetic nephropathy have opposing significance with

regard to disease progression. Enhanced SOD-2 expression enhances T cell and NK cell responses to HCV infection and early effective T cell responses are important in the spontaneous resolution of HCV infection. SOD-2 polymorphisms that enhance SOD-2 expression may support this process along with other as yet unknown factors. In acute infection, HCV-specific Th cell responses occur more frequently and are stronger in individuals who clear viremia [Diepolder et al., 1995]. However, once HCV infection is established, SOD-2 expression may contribute to chronic inflammation in the RNA positive group by facilitating less oxidative stress and an enhanced environment for viral replication in the liver (see Fig. 1). Phenotype differences in this area of research warrants much further investigation into differential SOD-2 translocation and its effects on mitochondrial function, nuclear DNA integrity and cellular survival in various tissues to further clarify this finding.

In the diabetic patient cohort our results agreed with a Russian researcher, Chistyakov where there was a lower frequency of "CC" homozygotes in the patients with nephropathy (DN) than those without. The findings in the present study differed with the Chistyakov group on the frequency of "TT" homozygotes. We observed similar levels of heterozygosity in the control group as Chistyakov's research group and agree that there is an increase in Ala/-9/Val dimorphism in DN patients.

Patients with HCV infection have shown significantly increased SOD-2 in peripheral mononuclear cells (PBMCs). It has been observed that HCV infected cases display differential expression of SOD-2 in different tissue and HCV infection induces SOD-2 in PBMCs but SOD-2 induction was not enhanced in the liver [Larrea et al., 1998]. Further to this, patients with positive viremia and chronic HCV infection showed a significantly increased SOD-2 expression in PBMCs compared to cases that had resolved the viremia [Larrea et al., 1998]. The lower SOD-2 response in the liver compared to the periphery and the lower SOD-2 producing genotype may combine and produce a higher ROS environment resulting in poor viral replication.

It is known that SOD-2 levels are reduced in some other chronic inflammatory conditions, but it is not known how HCV proteins affect SOD-2 expression or activity. HCV core protein does cause oxidative injury and may have a direct effect on the mitochondria [Okuda et al., 2002], which may affect the antioxidant response and SOD-2 production.

Many questions arise from this study and further studies are required to investigate other patients infected with HCV with different outcomes in order to extend the understanding of HCV infection. Also other single nucleotide polymorphisms linked with the SOD-2 gene or function would be interesting avenues to pursue for future research studies.

Figure 1 describes the findings of this study and the complicated affects of polymorphic variations in

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