

Original Paper

# Lack of *in vivo* blockade of Fas- and TNFR1-mediated hepatocyte apoptosis by the hepatitis C virus

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## Abstract

*In vitro* data have shown that the hepatitis C virus (HCV) core protein binds to protein members of the tumour necrosis factor receptor (TNFR) superfamily. Since this interaction could be relevant to HCV persistence and oncogenesis, this study assessed whether HCV may interfere with the apoptotic cascade *in vivo*. Apoptosis (by TUNEL) and Fas and TNFR1 expression (by immunohistochemistry) were scored in the liver of 60 chronic hepatitis C patients. Results were compared with the liver disease grading and staging scores and the HCV replication level in serum and liver. Apoptotic hepatocytes were stained in 29 cases. Fas was expressed in 35 cases and TNFR1 in 21, 15 patients (25%) being negative for both receptors. Overall, the numbers of TUNEL-, Fas- and TNFR-positive hepatocytes did not correlate with the extent of intrahepatic CD8+ T-lymphocyte infiltration, the grading and staging of liver disease, or the serum or liver HCV RNA levels. Furthermore, when patients expressing either Fas or TNFR1 were stratified according to serum HCV RNA levels, cases with detectable hepatocyte apoptosis had higher HCV viraemias. In conclusion, an HCV-mediated, *in vivo* blockade of hepatocyte apoptosis via the Fas- or TNFR1-dependent pathways seems unlikely. Copyright © 2002 John Wiley & Sons, Ltd.

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## Introduction

The molecular cloning of the hepatitis C virus (HCV) in 1989 marked the end of many years of searching for an elusive agent responsible for non-A, non-B hepatitis (NANB) [1]. A major clinical burden of HCV infection is represented by its marked tendency to persist after the acute phase [2]; also, a spontaneous recovery from chronic HCV infection is rare [3]. This situation reflects the inability of the host immune system to clear HCV at all stages of infection. Recent data have shown that an effective, specific cytotoxic T-lymphocyte (CTL) response is critical to the elimination of HCV [4]. Since such a response is invariably present in HCV-infected individuals [5], why is it mostly ineffective?

The modulation of apoptosis signal transduction is one of the several mechanisms proposed to account for the persistence of HCV infection [5]. CTL-mediated killing of hepatocytes occurs by triggering the apoptotic cascade via interaction of proteins on the lymphocyte membrane with specific receptors at the surface of the target cell [6]. Three major systems are known to be involved in this mechanism: the tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and its receptors (TNFR1 and TNFR2), the Fas-ligand and the Fas receptor, and the perforin-granzyme system [7]. Several HCV proteins have been reported, in a variety of transient or stable *in vitro* expression systems, to modulate apoptosis of the transfected cells upon different stimuli

[8–18]. Moreover, some experimental data show that the HCV nucleocapsid protein may interact non-covalently with a cytoplasmic domain (the ‘death domain’) of the lymphotoxin beta receptor and the TNFR1 [10,12,19]. However, the data obtained *in vitro* are contradictory, since in some cases the transfected cells are protected against the apoptotic stimulus [8,13,14,16], whereas in others they do not seem protected at all [15], and may even be sensitized [9–12]. The disparate effects of HCV proteins observed *in vitro* are unexplained. They may depend on the physiological status of the cell line, on genetic elements specific to single HCV isolates, or on clonal variation within the same tumour cell line, as well discussed in recent reviews [20,21]. Sensitization to apoptosis may be disadvantageous for HCV, since it would lead to an increased death rate of infected cells. In contrast, inhibition of the apoptosis cascade may be relevant for the persistence of HCV infection, as shown for other viruses [22], and may also be important in modulating the oncogenic properties of HCV [20]. Neither phenomenon has so far been documented *in vivo*.

To evaluate whether this interaction may occur in the liver of chronic hepatitis C patients, we studied the level of liver cell apoptosis, as evaluated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay, and the expression of some apoptosis mediators (Fas receptor and TNFR1). We then correlated the results with the

activity of liver disease, the extent of CD8+ infiltration, and the levels of HCV replication in serum and liver.

## Materials and methods

### Patient population

We studied 60 chronic hepatitis C patients. All had abnormal serum alanine aminotransferase activities; persistent infection with HCV, as documented by detection of HCV RNA in serum by a qualitative RT-PCR assay (Amplicor<sup>™</sup>, Roche, Switzerland; sensitivity limit 1000 copies/ml) for at least 6 months; and histologically-proven chronic hepatitis.

A first group of controls included 24 chronic hepatitis B patients. The two chronic hepatitis patient groups were comparable in terms of liver disease grading and staging scores (Table 1). A further control group included 24 histologically normal livers taken at autopsy from patients who had died of extrahepatic diseases.

This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

### Serum assays

Serum assays included detection of HCV RNA by the above-mentioned qualitative RT-PCR and by a quantitative signal amplification-based, branched DNA assay (Quantiplex<sup>™</sup> version 2, Chiron Corp., Emeryville, CA, USA). HCV RNA genotyping was performed by restriction fragment length polymorphism (RFLP) [23].

### Liver histology

Liver tissue was obtained from all patients by percutaneous biopsy performed for diagnostic purposes and from the normal livers at autopsy. All tissue specimens were fixed in 10% neutral buffered formalin and

Table 1. Baseline characteristics and liver cell apoptosis levels in 60 chronic hepatitis C and 24 chronic hepatitis B patients

|                                          | Chronic hepatitis C (n=60) | Chronic hepatitis B (n=24) | p      |
|------------------------------------------|----------------------------|----------------------------|--------|
| Liver disease grading                    |                            |                            |        |
| Lobular                                  | 1.78 ± 0.92                | 1.7 ± 1.06                 | NS     |
| Periportal                               | 1.78 ± 0.98                | 1.74 ± 1.32                | NS     |
| Portal                                   | 2.32 ± 0.77                | 2.12 ± 1.01                | NS     |
| Liver disease staging                    | 2.32 ± 2.11                | 3.09 ± 2.39                | NS     |
| Hepatocyte apoptosis (cells per HPF)     | 0.017 ± 0.024              | 0.026 ± 0.035              | NS     |
| Non-hepatocyte apoptosis (cells per HPF) | 0.016 ± 0.035              | 0.06 ± 0.076               | <0.001 |

paraffin-embedded. Five-micrometre sections were stained with haematoxylin and eosin (H&E), reticulin, Masson's trichrome, and Perl's stain. Histological diagnosis was based on internationally accepted criteria, which include a semi-quantitative assessment of liver disease grading and staging [24].

### Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL)

TUNEL (Roche Diagnostic AG, Rotkreuz, Switzerland) was performed for the histological detection of apoptosis of liver cells (hepatocytes and mononuclear cells). Liver tissue sections were deparaffinized, rehydrated, and digested with 3 µg/ml proteinase K for 15 min at room temperature. After washing, sections were incubated in 25 mM cobalt chloride, 0.01 mM biotin-16-dUTP, and 25 U/ml terminal deoxynucleotidyltransferase. After blocking endogenous peroxidase by immersion in distilled water containing 25% H<sub>2</sub>O<sub>2</sub> for 3 min, sections were washed, incubated with horse-radish peroxidase-conjugated streptavidin, developed in diaminobenzidine-H<sub>2</sub>O<sub>2</sub>, and counterstained with haematoxylin. Positive hepatocytes were identified by their location in hepatic cords and inflammatory cells were identified by their position along sinusoids. The positive nuclei were counted on high-power fields (HPFs) (400×) and the numbers of apoptotic hepatocytes and inflammatory cells were scored separately. Each HPF contained on average 250 hepatocytes. The apoptotic index was defined as the number of TUNEL-labelled cells per average HPF. It was calculated by dividing the total number of positive cells by the total number of HPFs scored on each specimen.

### Immunohistochemistry

Immunohistochemical staining was performed using primary antibodies to human Fas receptor (Dako Diagnostic AG, Zug, Switzerland), TNFR1 (Santa Cruz, Glaser AG, Basel, Switzerland), and CD8 (Dako). Briefly, 3–5 µm sections were mounted on silane-coated glass slides, deparaffinized, rehydrated, and pretreated with H<sub>2</sub>O<sub>2</sub>/methanol to block endogenous peroxidase activity. After incubation for 1 h at room temperature with the diluted primary antibodies, reactions were revealed with the avidin-biotin-peroxidase complex method (Vectastain<sup>™</sup>, Vector, Switzerland). Peroxidase activity was revealed with 30% 3,3'-diaminobenzidine as chromogen in PBS containing 0.015% H<sub>2</sub>O<sub>2</sub>. Sections were counterstained with Mayer's haematoxylin.

The degree of Fas receptor expression was semi-quantitatively scored as follows: 0, none; 1, less than 1/3 of positive hepatocytes; 2, involvement of 1/3 to 2/3 of hepatocytes; and 3, more than 2/3 of positive hepatocytes [25]. TNFR1 expression was evaluated qualitatively (detectable or undetectable) [26]. CD8+ lymphocytes were scored as follows: for portal infiltrates, as a percentage of CD8+ cells vs. total inflammatory cells, scored by H&E staining, with 0, none; 1, less than 30% of portal inflammatory cells; 2, involvement

of 30–60% of portal inflammatory cells; and 3, greater than 60% of portal inflammatory cells; for lobular infiltrates as 0, none; 1, less than 1/3 of positive lobules; 2, 1/3 to 2/3 positive lobules; and 3, more than 2/3 of positive lobules. The average, absolute number of CD8+ cells per portal tract or per lobule was also reported.

#### Strand-specific detection of genomic- and negative-strand HCV RNA in the liver

A specimen adjacent to the one being processed for routine histological studies was obtained in all 60 liver biopsies, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total liver RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform procedure [27]. A strand-specific RT-PCR assay was used to assess the presence and relative titres of either HCV RNA strand [28,29]. Semi-quantitation was achieved by performing a nested RT-PCR to the end point on two- to four-fold dilutions [in  $10\ \mu\text{g}/\text{ml}$  *Escherichia coli* tRNA (Sigma)] of an initial amount of 100 ng of total liver RNA. Titres were expressed as the last dilution giving a visible band of the appropriate size on a 1.6% agarose gel stained by ethidium bromide. Intrahepatic genomic- and minus-strand HCV RNA titres were normalized to an arbitrary  $\beta$ -actin mRNA titre of 1024, as measured on the same specimen [30]. The strand specificity, sensitivity, and reproducibility of the

RT-PCR detection of liver HCV RNA have been reported before [28]. In particular, this assay was shown to detect as few as 15 copies of each HCV RNA strand per assay, independently of genotype [29].

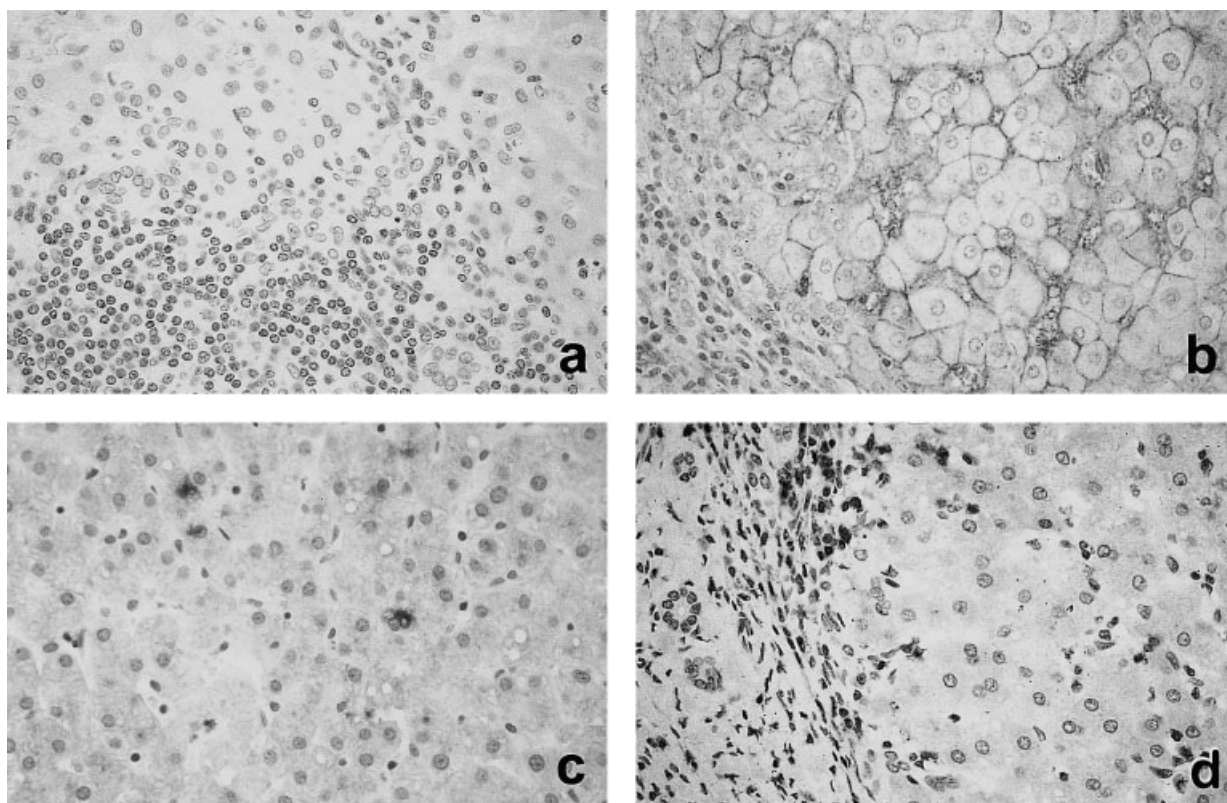
#### Statistical analysis

Apoptotic indices are reported throughout the Results section as average  $\pm$  SD. However, before statistical analysis, both apoptotic indices and serum HCV RNA levels were  $\log_{10}$ -transformed. Differences were evaluated by the Mann–Whitney *U*-test. The coefficients of correlation among non-parametric variables were evaluated by Spearman's rank correlation test. Tables of contingency were evaluated by the  $\chi^2$ -method or Fisher's exact test, when appropriate.

## Results

### Frequency and distribution of intrahepatic apoptosis

TUNEL-labelled nuclei were single and widely scattered within the liver (Figure 1a). Positive hepatocytes were seen both in the periportal and in the intralobular areas. Infiltrating mononuclear cells were stained in both portal tracts and lobules. In chronic hepatitis C patients, the hepatocellular apoptotic index ranged from 0 to 0.1 cells per HPF ( $0.017 \pm 0.024$ ). In 31/60



**Figure 1.** Light microscopy of a tissue section from a patient with chronic hepatitis C. (a) Two apoptotic hepatocytes located at the level of interface hepatitis show TUNEL-positive nuclei. (b) Immunohistochemical staining reveals strong and diffuse membranous expression of Fas receptor on hepatocytes. (c) TNFR1 is expressed in perinuclear cytoplasm of some hepatocytes. (d) Several CD8+ lymphocytes are concentrated at the level of interface hepatitis and are sparsely distributed in the lobules.  $\times 360$

cases (52%), hepatocellular apoptosis was undetectable. The infiltrating mononuclear cell apoptotic index ranged from 0 to 0.194 cells per HPF ( $0.016 \pm 0.035$ ). In 39/60 cases (65%), no mononuclear cells were stained for apoptosis.

In chronic hepatitis B patients, the hepatocellular apoptotic index ranged from 0 to 0.118 cells per HPF ( $0.026 \pm 0.035$ ). In 12/24 cases (50%), hepatocellular apoptosis was undetectable. The infiltrating mononuclear cell apoptotic index ranged from 0 to 0.3 cells per HPF ( $0.06 \pm 0.076$ ). Infiltrating cells could not be stained in eight cases (33%).

There was no statistically significant difference in the apoptotic index of hepatocytes between chronic hepatitis C and chronic hepatitis B patients ( $p=0.218$ ) (Table 1). In contrast, not only were the infiltrating mononuclear cells more frequently stained in chronic hepatitis B than in chronic hepatitis C patients ( $p=0.015$ ), but the apoptotic index was significantly higher in the former than in the latter ( $p<0.001$ ).

The histologically normal livers had a hepatocyte apoptotic index of  $0.004 \pm 0.006$  cells per HPF. This value was significantly lower than that observed in chronic hepatitis C ( $p=0.011$ ) and chronic hepatitis B ( $p=0.006$ ) patients.

#### Frequency and distribution of apoptosis mediators by immunohistochemistry

Among 60 liver specimens of patients with chronic hepatitis C, Fas receptor staining was present on the hepatocellular membrane in 35 cases (58.3%) (Figure 1b). The Fas receptor staining score was 0 in 25 cases (41.7%), 1 in 18 (30%), 2 in 11 (18.3%), and 3 in 6 (10%). TNFR1 in hepatocytes was detected in 21 cases (35%) (Figure 1c). In 15 patients, neither Fas nor TNFR1 could be detected by immunohistochemistry.

#### Expression of CD8 in infiltrating mononuclear cells

CD8+ cells were detected in all 32 cases where material was available for study (Figure 1d). In all cases, CD8+ cells were found in both the portal tracts and the lobules. The distribution of CD8+ cells in portal tracts was diffuse in 27 cases and predominantly at the interface in five cases.

The scores of CD8 portal staining varied from 1 in 18 cases (56.2%), 2 in 11 cases (34.4%) to 3 in three cases (9.4%). The scores of CD8 lobular staining varied from 1 in 18 cases (56.2%), 2 in eight cases (25.6%) to 3 in six cases (19.2%). In no liver specimen were the inflammatory infiltrates completely devoid of CD8+ cells. The absolute number of CD8+ cells was  $30.4 \pm 13.7$  cells per portal tract, and did not correlate with either the hepatocellular ( $r=0.15$ ) or the infiltrating mononuclear cell apoptotic index ( $r=0.13$ ). Similarly, the absolute number of CD8+ cells per lobule was  $19.9 \pm 11.2$  cells; again, this failed to correlate with either the hepatocellular ( $r=-0.33$ ) or the infiltrating mononuclear cell apoptotic index ( $r=-0.16$ ).

#### Correlation of apoptosis scores and expression levels of apoptosis mediators with liver histology

We could not establish any correlation between the apoptotic indices and the expression pattern/level of Fas or TNFR1 (data not shown). The hepatocellular apoptotic index did not correlate with the lobular ( $r=-0.17$ ), periportal ( $r=-0.15$ ) or portal ( $r=0.18$ ) inflammatory scores, or with the fibrosis score ( $r=-0.09$ ). Similarly, the infiltrating mononuclear cell apoptotic index was not correlated with the lobular ( $r=-0.22$ ), periportal ( $r=-0.05$ ) or portal ( $r=0.04$ ) inflammatory scores, nor with the fibrosis score ( $r=0.18$ ).

#### Expression of liver cell apoptosis/apoptosis mediator scores and HCV genotype and replication level

Among the 57 patients in whom the HCV genotype could be determined, 23 were infected with type 1, eight with type 2, 19 with type 3, and seven with type 4. In three patients, we could not assign the viral type, based on the above RFLP method. The highest hepatocellular apoptotic index was found in patients infected with HCV type 1 ( $0.021 \pm 0.027$  cells per HPF), whereas patients with types 2, 3, and 4 had  $0.005 \pm 0.013$ ,  $0.017 \pm 0.025$ , and  $0.013 \pm 0.017$  cells per HPF, respectively. Moreover, none of these differences was statistically significant. The highest infiltrating mononuclear cell apoptotic index was again that of patients with type 1, with a value of  $0.025 \pm 0.047$  cells per HPF. In patients with types 2, 3, and 4, the infiltrating mononuclear cell apoptotic index was  $0.007 \pm 0.013$ ,  $0.007 \pm 0.012$ , and  $0.021 \pm 0.045$  cells per HPF, respectively. Again, none of these differences reached statistical significance.

Overall, there was no correlation between the expression pattern/level of apoptosis mediators and apoptotic indices, on the one hand, and the serum HCV RNA levels or the intrahepatic genomic- and negative-strand HCV RNA titres on the other hand (data not shown).

We then divided patients into several groups, depending on the following parameters: (1) presence/absence of detectable apoptosis in hepatocytes or mononuclear cells; and (2) levels of HCV RNA in serum higher/lower than an arbitrary threshold level of 1 000 000 genome-equivalents/ml.

Table 2 shows the distribution of all patients,

Table 2. Presence of TUNEL-positive liver cells according to serum HCV RNA levels in 60 chronic hepatitis C patients

|                          | Hepatocytes |      |          | Non-hepatocytes |      |          |
|--------------------------|-------------|------|----------|-----------------|------|----------|
|                          | APO+        | APO- | <i>p</i> | APO+            | APO- | <i>p</i> |
| Serum HCV RNA level      |             |      |          |                 |      |          |
| > 1 000 000 genome-Eq/ml | 22          | 20   | NS       | 16              | 26   | NS       |
| < 1 000 000 genome-Eq/ml | 7           | 11   |          | 9               | 9    |          |

Table 3. Presence of TUNEL-positive liver cells according to serum HCV RNA levels in 35 chronic hepatitis C patients expressing Fas in the liver

|                          | Hepatocytes |      |          | Non-hepatocytes |      |          |
|--------------------------|-------------|------|----------|-----------------|------|----------|
|                          | APO+        | APO- | <i>p</i> | APO+            | APO- | <i>p</i> |
| Serum HCV RNA level      |             |      |          |                 |      |          |
| > 1 000 000 genome-Eq/ml | 17          | 8    | 0.01     | 11              | 14   | NS       |
| < 1 000 000 genome-Eq/ml | 2           | 8    |          | 2               | 8    |          |

irrespective of the expression of the apoptosis receptors in the liver. There was no association between the level of HCV replication and the presence of TUNEL-positive cells in the liver.

Table 3 shows a similar stratification, but limited to patients in whom the Fas receptor could be stained (irrespective of the staining score). Although the nuclear TUNEL staining of infiltrating cells was not associated with the level of HCV replication, the hepatocyte apoptotic index was significantly higher among the patients with higher serum HCV RNA levels ( $p=0.01$ ). Similarly, the hepatocytes (but not the infiltrating cells) were more likely to be apoptotic among patients expressing TNFR1 and having higher levels of HCV viraemia (Table 4) ( $p=0.02$ ).

Finally, and in contrast with the above, when neither Fas nor TNFR1 was detected, higher levels of HCV RNA in serum were significantly associated with the lack of detectable hepatocyte apoptosis (Table 5)

Table 4. Presence of TUNEL-positive liver cells according to serum HCV RNA levels in 21 chronic hepatitis C patients expressing TNFR1 in the liver

|                          | Hepatocytes |      |          | Non-hepatocytes |      |          |
|--------------------------|-------------|------|----------|-----------------|------|----------|
|                          | APO+        | APO- | <i>p</i> | APO+            | APO- | <i>p</i> |
| Serum HCV RNA level      |             |      |          |                 |      |          |
| > 1 000 000 genome-Eq/ml | 12          | 2    | 0.02     | 7               | 7    | NS       |
| < 1 000 000 genome-Eq/ml | 4           | 3    |          | 2               | 5    |          |

Table 5. Presence of TUNEL-positive liver cells according to serum HCV RNA levels in 15 chronic hepatitis C patients lacking expression of both Fas and TNFR1 in the liver

|                          | Hepatocytes |      |          | Non-hepatocytes |      |          |
|--------------------------|-------------|------|----------|-----------------|------|----------|
|                          | APO+        | APO- | <i>p</i> | APO+            | APO- | <i>p</i> |
| Serum HCV RNA level      |             |      |          |                 |      |          |
| > 1 000 000 genome-Eq/ml | 0           | 11   | 0.009    | 0               | 11   | 0.06     |
| < 1 000 000 genome-Eq/ml | 3           | 1    |          | 2               | 2    |          |

( $p=0.009$ ). For liver infiltrating cells, the association approached, but did not reach, statistical significance ( $p=0.06$ ).

## Discussion

The high tendency of HCV to establish a chronic infection is unexplained, although several mechanisms have been postulated [5]. The possible interaction with the apoptotic cascade is an attractive one, since it may also account for the oncogenic potential of HCV [17,20,21,31]. Several HCV proteins have been shown to alter the effect of apoptotic stimuli on *in vitro* transfected cell lines. The proteins studied include the core protein [8–15], the non-structural (NS) protein 3 [18], and NS5A [17]. In some cases, a direct, non-covalent interaction of the core protein with the death domain of the lymphotoxin beta receptor [10,19] and of TNFR1 [12] was reported. However, the effects of viral proteins in these systems vary markedly, as they may alternatively protect the transfected cells from apoptosis, or sensitize them. To add further to the confusion, the effects of the same proteins (mostly the core protein) on the activation of specific transcription factors involved in the apoptotic signalling pathways (such as NF $\kappa$ B) are far from being unequivocal [12,14,16,31,32]. The reasons for these discrepancies are unclear and they may depend on several factors [21]. Clearly, an inhibition of the apoptotic cascade would be advantageous for HCV, since it may enhance viral replication and contribute to viral persistence and oncogenesis. Inhibition of apoptosis has been shown in several viral infections [22]. Mechanisms vary from the direct encoding of anti-apoptotic molecules by the viral genome (such as homologues to cellular proteins involved in apoptosis regulation, e.g. caspase inhibitors, serpins, and cytokine receptors), to the up-regulation of cell anti-apoptotic genes (such as *Bcl2* and *A20*) or the down-regulation of pro-apoptotic factors (such as Bax), to the induction of Fas-ligand [22]. These effects converge towards the protection of infected cells by cell-autonomous apoptosis and/or towards the evasion of viral antigen-specific cytotoxic lymphocyte killing [22].

In the present study, we noted that the overall apoptotic index of hepatocytes was comparable to that of a control population infected with HBV. We also saw that all liver specimens had a CD8+ infiltrate and that most of them expressed Fas or TNFR1, or both. However, we still wanted to analyse whether HCV could interact with some members of the death receptor family (Fas and TNFR1). We assumed that the effect that may be likely to prevail *in vivo* (if any) is the inhibition of the apoptotic cascade; hence we analysed the correlation between level of apoptosis and HCV replication. We based this assumption on several clinical observations: first, the high tendency to persistence of acute HCV infection; and second, the fact that both autoimmune phenomena and malignancies of both the

liver and the haematopoietic lineage have been found to be associated with HCV infection, especially primary liver cancer and a subset of B-cell non-Hodgkin lymphoma [33]. Defects in the control of the immune system homeostasis by apoptosis are in fact relevant for the pathogenesis of both autoimmune and lymphoproliferative disorders [34] and another study suggests that even other extrahepatic malignancies may be more frequent in HCV-infected patients than in the general population [35].

At variance with our expectations, we found that when only patients expressing Fas or TNFR were considered (the two death receptors most studied *in vitro*), higher levels of HCV replication were found in patients in whom hepatocyte apoptosis was more easily detected. So, HCV replication not only does not seem to protect from apoptosis, when Fas or TNFR1 is expressed, but it may even sensitize infected cells to the apoptotic stimulus, in agreement with some *in vitro* data [9–12]. We must emphasize the fact that the inverse relationship between viral replication and levels of apoptosis was not evident when all the patients were considered and this may be due to the fact that the sensitizing effect seen in Fas- and/or TNFR-positive patients was counterbalanced by a 'protective' effect in patients lacking the expression of both death receptors. In this small subgroup, in fact, higher levels of HCV replication were found in patients in whom hepatocyte apoptosis was below the threshold of the TUNEL assay. We also have to assume that the simple intrahepatic expression of Fas or TNFR1 may not be sufficient to trigger hepatocyte apoptosis, since this may require a specific intracellular environment. For example, TNF engagement with TNFR1 triggers apoptosis only if protein synthesis is simultaneously inhibited, such as by the virally induced activation of PKR (reviewed in [ref. 21]). HCV has been shown to rescue the PKR-mediated inhibition of translation and cells expressing the viral NS5a protein alone become refractory to apoptosis [36]. So, if the inhibition of protein synthesis is a prerequisite of apoptosis activation via TNF- $\alpha$ , it probably results from mechanisms other than activation of PKR. The analysis of these interactions was beyond the scope of the present study and probably only a single-cell analysis by combined immunohistochemistry and/or *in situ* hybridization imaging techniques may appropriately address this issue in the future.

Finally, as far as the apoptotic indices of infiltrating mononuclear cells are concerned, we could establish no correlations with the HCV replicative levels or with any other parameter. The only exception may be represented by patients not expressing either Fas or TNFR1, in whom the association between higher viraemia levels and undetectable apoptosis of mononuclear cells approached statistical significance. This association is worth studying in a larger population of patients, also taking into consideration the HCV tropism for mononuclear cells [37–39]. Furthermore, the apoptotic index of mononuclear cells in the control group of patients

with chronic hepatitis B was significantly higher than that found in chronic hepatitis C patients. This difference is unexplained and may have interesting implications as to the persistence and pathogenesis of HBV infection.

Thus, we suggest that two populations of chronic hepatitis C patients seem to exist, in whom the interaction between HCV product(s) and the apoptotic pathway(s) may differ. When Fas or TNFR is expressed, the degree of apoptosis of infected cells (either spontaneous or CTL-mediated) seems to increase in parallel with viral replication. In other words, the Fas- and TNFR-dependent apoptotic pathways may not be blocked by HCV. Since all of these patients have a chronic hepatitis, HCV probably exploits alternative pathways to evade immune recognition, other than interfering with apoptosis. A second group of chronically HCV-infected patients may exist, however, in whom HCV may block hepatocyte apoptosis via pathways other than the Fas- or TNFR-dependent ones. Why the immune system fails to activate proper expression of Fas and TNFR in these patients is unknown. The anti-apoptotic strategy of HCV in these cases can only be a matter of speculation. Recently [40], the t(14;18) translocation and *Bcl2* overexpression in lymphoid cells were observed in chronic hepatitis C patients. Since HCV infects B cells [36–38], a direct interaction between one or more HCV product(s) and the anti-apoptotic BCL2 activity cannot be excluded, and may be worth investigating also in hepatocytes, which are the primary target of HCV replication [41,42].

In conclusion, if HCV exerts anti-apoptotic activity *in vivo*, this does not seem to be mediated by an interaction with the Fas- or the TNFR-dependent pathways. Our data, however, seem to suggest that an anti-apoptotic effect may be acting in a small proportion of chronic hepatitis C patients, via undetermined mechanisms, which warrant further investigation.

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