Hepatic apoptotic markers are not predictors for the virological response to interferon-based therapy in chronic hepatitis C patients

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Introduction Chronic hepatitis C virus (HCV) infection is a major health problem worldwide. The majority of cases involving HCV infection develop into chronic hepatitis because of a failure to develop an effective immune response. Apoptosis of the hepatocytes plays a significant role in the pathogenesis of HCV infection: the interaction between the Fas antigen on hepatocytes and the Fas ligand on T cells corresponds to the main mechanism for hepatocyte damage. Interferon (IFN)-α has antiviral, immunoregulatory, and antiproliferative properties, and apoptosis seems to be a critical event in the action mechanisms of both IFNs. In this study, we aimed to detect any relationship between apoptotic markers in the liver and the response to the treatment.
Materials and methods The study included 180 chronic HCV patients treated with IFN and ribavirin in four centers. Apoptotic markers (Fas, Fas ligand, Fas-associated death domain, caspases 3, 8, and 9, and in-situ apoptosis) were studied in the liver. The age, sex of the patients, response to therapy, ALT level, viral load, and genotype were recorded.
Results The results of the study showed that the histological activity index and fibrosis correlated with CD95 staining density, caspase-8 intensiveness, and portal and parenchymal Fas ligand scores. The apoptotic parameters of the responsive cases were not significantly different from those of the unresponsive cases.
Conclusion The apoptotic parameters studied in liver tissue are associated with inflammation and fibrosis; however, these parameters may not predict response to treatment. Eur J Gastroenterol Hepatol 27:1057–1062

Introduction

Chronic hepatitis C virus (HCV) infection is a major health problem worldwide, with an estimated prevalence of 2–3% [1]. The majority of cases involving HCV infection develop into chronic hepatitis, with failure to develop an effective immune response appearing to be a key factor in developing the chronic condition [2]. It is clear that apoptosis of the hepatocytes plays a significant role in the pathogenesis of HCV infection [3].

Insufficient apoptotic course causes virus persistence and hepatocellular carcinoma, whereas excess apoptotic course causes liver damage [4,5]. Apoptosis primarily develops from interaction between the Fas antigen on hepatocytes and the Fas ligand (FasL) on T cells, corresponding to the main mechanism for hepatocyte damage [6]. Normal hepatocytes do not express the Fas antigen, whereas hepatocytes infected by HCV express it at varying levels [7]. The Fas/Apo 1 molecule is also known as CD95, which is a member of the TNF receptor family and stimulates the death signal after binding to its ligand. It has been established that the level of Fas antigen in the peripheral blood of patients with HCV is increased [8]. Interaction between Fas and FasL also activates apoptosis by activating caspases. The binding of T lymphocytes to T-cell receptors results in the secretion of granzymes and perforin stored in granules [9]. The perforins form pores in the plasma membrane and granzymes cause caspase activation by penetrating into cells through these pores.

Interferon (IFN)-α has antiviral, immunoregulatory, and antiproliferative properties [10]. It stimulates transcriptional activation of several genes with antiviral properties. Pegylated IFN combined with ribavirin provides a sustained virological response (SVR) of ~50% in patients infected with genotype 1. In addition to the other mechanisms implicated, clearance of apoptosis seems to be a critical event in the mechanism of action of both IFN and ribavirin [11,12]. There may be a link between the pattern of response and apoptosis: a recent study reported that patients who achieved SVR had lower levels of post-treatment soluble Fas (sFas) concentrations compared with nonresponders and those who developed relapse [13].

Apoptosis is suggested to be associated with liver histology. In patients with chronic HCV infection, the sFas level has been correlated with liver fibrosis [14] and necroinflammatory activity [15,16]. Also, higher levels of

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FasL were detected in HCV patients with more severe necroinflammatory activity [16].

In this study, we aimed to detect any relationship between apoptotic markers [Fas, FasL, Fas-associated death domain (FADD), caspases 3, 8, and 9, and in-situ apoptosis] in liver tissue, biochemical, and virological parameters, as well as their response to treatment in patients with HCV infection.

Materials and methods

Two university hospitals and two training hospitals participated in this study. The study period was January 2007–December 2012. The study involved chronic HCV infection patients with biochemical [alanine aminotransferase (ALT) level normal to < 10-fold of upper limit of normal], serological (anti-HCV and HCV-RNA positive), and histopathological findings compatible with chronic hepatitis C. Patients coinfected with HIV, hepatitis B and D, patients with alcoholic hepatitis, hemodialysis patients, and intravenous drug users were excluded.

Patients treated with the pegylated IFN (α2a or 2b) and ribavirin combination were included. Patients who did not complete the planned treatment schedule, those with interrupted therapy, and those with insufficient data were not included. Patients who had been treated previously were also excluded.

Chronic HCV patients who have been treated for up to 1 year and have been observed for at least 6 months were divided into two groups: responders and nonresponders.

Responders

Virologic response is considered 'rapid' when HCV RNA is undetectable 4 weeks after starting the therapy.

It is considered 'early' when the HCV RNA level decreases by at least two logs (or becomes undetectable) 12 weeks after starting.

Undetectable virus at the end of therapy is referred to as an end-of-treatment response.

A SVR is defined as undetectable HCV RNA in the serum by a sensitive test at the end of treatment and 6 months later.

Nonresponders

Patients in whom HCV RNA levels fail to decrease by at least two logs in 24 weeks are considered nonresponders.

Patients in whom HCV RNA levels decrease, but never become undetectable are referred to as partial responders.

Relapse is defined as detectable HCV RNA in individuals who achieved an end-of-treatment response.

Demographic (age, sex) and clinical (ALT level, HCV-RNA level, genotype, treatment dose, treatment duration, presence or absence of virological response, end of treatment, viral and biochemical response) data were obtained from patient files.

Liver biopsy

Those who had undergone a liver biopsy within the 3 years before the start of treatment were included. Paraffin blocks and/or slides were obtained. Two pathologists who were not aware of the patients' data carried out all of the tissue studies.

Studies of liver tissue

Liver histology

Although every patient had histology before treatment, inflammation (histologic activity index) and fibrosis were restudied in a blinded manner.

Histology was evaluated according to the Knodell score in each case [17]. The sections were stained with hematoxylin and eosin.

Apoptotic markers in the liver tissue

Serial 7 sections in 5 μm were obtained from each block. Then, the deparaffinization and rehydration processes were applied. The sections were prepared in a 750 W microwave oven, at pH 6.0, in a buffered citrate solution (10 mmol/l) for 15 min without interruption. Bearing in mind the manufacturer's recommendations of time periods, the biotin–streptavidin–alkaline phosphatase method of improved primary antibodies was applied using enhanced antibodies for CD95, caspase-3, caspase-8, caspase-9, Fas, and FasL. Fast red substrate levamisole was used as the chromogen. Contrast dye was performed using Mayer's hematoxylin. The same processes were performed without using the primary antibodies as a negative control. Immunoreactivity was scored semiquantitatively in four different areas that were the most intensely stained.

A terminal transferase reaction, which is an in-situ labeling method determining nucleosomal DNA fragmentation, was used in the detection of apoptosis. As recommended by the manufacturer, a commercial kit (ApopTag; Oncor Inc., Gaithersburg, Maryland, USA) was applied to the sections. Briefly, after deparaffinization and rehydration, the sections were incubated in the proteinase K (20 μg/ml) for 10 min at room temperature. After the blockage of endogenous peroxidase activity, the sections were washed twice, for 5 min each time, with a PBS. Then, two drops of balancing buffer were applied for 10–15 s on the sections at room temperature. It was incubated for 1 h in a moist chamber at 37°C and was buffered with TdT enzyme with a 2/1 ratio. The sections were held in the wash buffer solution (1 ml wash buffer + 34 ml distilled water) at room temperature for 10 min and then washed three times, again for 5 min each time, with PBS. After dripping two drips of antidigoxigenin peroxidase onto each section, they were washed with PBS for 30 min in a moist chamber at room temperature. The reaction was visualized by diaminobenzidine/hydrogen peroxide. The negative control was obtained by applying a similar method to remove the TdT enzyme from one of the serial sections. Expression of ApopTag at ×400 magnification in light microscopy was evaluated in four separate areas with higher immune expression, preferably counting a total of 1000 cells (≥500 cells).

The apoptotic index was calculated by dividing the number of cells showing nuclear staining by the total number of cells.

Immunohistochemical studies

Immunohistochemical studies were evaluated semiquantitatively on the basis of the previously described methods [18–20].

CD95

Membranous staining and/or cytoplasmic staining were searched in the hepatocytes. For membranous staining, both staining density and the extent of staining were evaluated semiquantitatively. For staining density, an absence of density of staining was scored as 0; if staining was visible at ×200 magnification, it was scored as 1; if staining was visible at ×100 magnification, it was scored as 2; and if staining was visible at ×40 magnification, it was scored as 3.

For staining extensity, no extensity was scored as 0; focal staining in small numbers of hepatocytes was scored as 1; if hepatocytes were stained in wide foci, it was scored as 2; and if there was widespread membranous staining, it was scored as 3.

Fas-associated death domain

For the cytoplasmic staining of hepatocytes, both staining density and extent of staining were evaluated semiquantitatively. To evaluate the staining density, an absence of density of staining was scored as 0; if staining was visible at ×200 magnification, it was scored as 1; if staining was visible at ×100 magnification, it was scored as 2; and if staining was visible at ×40 magnification, it was scored as 3.

For staining extensity, no identifiable extensity was scored as 0; focal staining in small numbers of hepatocytes at the periportal and/or pericentral area was scored as 1; if hepatocytes were stained in wide foci, it was scored as 2; and if there was widespread staining, it was scored as 3.

Caspase-3

Diffuse, fine granular, and/or rough granular staining was screened in hepatocyte cytoplasm. The staining density and extent of staining were evaluated semiquantitatively with the same scoring as described for FADD.

Caspase-8

Diffuse and rough granular staining was searched in the cytoplasm of hepatocytes. Immunohistochemistry expression was scored semiquantitatively between 0 and 3: if no staining was observed, it was scored as 0; if three or less hepatocyte lines at periportal and/or pericentral area were stained, it was scored as 1; if more than 3 hepatocyte lines at periportal and/or pericentral area were stained and/or staining in zone 2 were detected, it was scored as 2; and widespread staining in the liver parenchyma was scored as 3.

Caspase-9

Diffuse, fine granular, and/or rough granular staining was observed in hepatocyte cytoplasm. The density and extent of staining were evaluated semiquantitatively with the same scores described for FADD and for caspase-8, respectively.

Fas ligand

Evaluation was performed considering the number of T lymphocytes showing immunoexpression at the portal areas and parenchyma. At least four portal and lobular areas were evaluated. At the portal area, if the T lymphocytes showing immunoexpression were less than 5, it was scored as 0; if it was between 5 and 10, it was scored as 2, if it was between 11 and 15, it was scored as 2; and if it was more than 16, it was scored as 3. At the parenchyma, if the number of lymphocytes showing immunoexpression per lobule was less than 5, it was scored as 1; if it was higher than 2, it was scored as 2.

Apoptotic index

Expression of ApopTag at ×400 magnification in light microscopy was evaluated in four separate areas with higher immune expression, preferably counting a total of 1000 cells (≥500 cells). The apoptotic index was calculated by dividing the number of cells showing nuclear staining by the total number of cells.

Ethics

For the study, a retrospective consent from the patients was not obtained. The study was approved by The Committee of Ethics of Cerrahpasa Medical School.

Statistical evaluation

Evaluation was carried out using the statistical package for social sciences software (SPSS Inc. Version 17.0. Chicago, Illinois, USA). Primary assessment was performed using Pearson's correlation analysis. On the basis of these results, the factors affecting the response to treatment were determined by regression analysis. Comparison of continuous variables between the groups was performed using Student's *t*-test. The χ^2-test was used to compare categorical variables. *P* less than 0.05 was considered significant.

Results

There were 180 patients with complete clinical and laboratory data and liver biopsy samples. Demographic characteristics, genotypes, ALT and HCV-RNA levels, and histology of 86 (48%) responder patients and 94 (52%) nonresponder patients are presented in Table 1.

Apoptotic studies yielded results, except density of caspase-8; most of the samples were stained in high density [Figs 1a,b,c, 2a and b, 3a and b, Supporting Fig. 1A (Supplemental digital content 1, *http://links.lww.com/EJGH/A30*), 1B (Supplemental digital content 2, *http://links.lww.com/EJGH/A31*), 2A (Supplemental digital content 3, *http://links.lww.com/EJGH/A32*), 2B (Supplemental digital content 4, *http://links.lww.com/EJGH/A33*), 3 (Supplemental digital content 5, *http://links.lww.com/EJGH/A34*), 4 (Supplemental digital content 6, *http://links.lww.com/EJGH/A35*)]. Apoptotic parameters studied in the liver tissue were comparable in responders and nonresponders (Table 2).

The histology correlated with some apoptotic parameters

The histologic activity index correlated positively with the density of CD95 ($P = 0.02$, $r = 0.24$), extensity of caspase-8

Table 1. Demographic and clinical characteristics of responder and nonresponder patients

	Responder patients ($n=86$)	Nonresponder patients ($n=94$)	P
Age (mean ± SD) (years)	47.7 ± 10	51.9 ± 8	< 0.05
Sex (male/female)	35/51	37/57	> 0.05
BMI (kg/m^2)	26.1	27.8	< 0.05 (0.048)
ALT (mean) (IU/l)	96.1	93.2	< 0.05
HCV-RNA (mean) (IU/l)	1 959 982	2 677 326	< 0.05
Genotype (1b/1a/others)	78/4/4	82/8/4	> 0.05
Histologic activity index (mean)	7.27	7.12	< 0.05
Fibrosis score (mean)	1.78	1.66	< 0.05

ALT, alanine aminotransferase; HCV, hepatitis C virus.

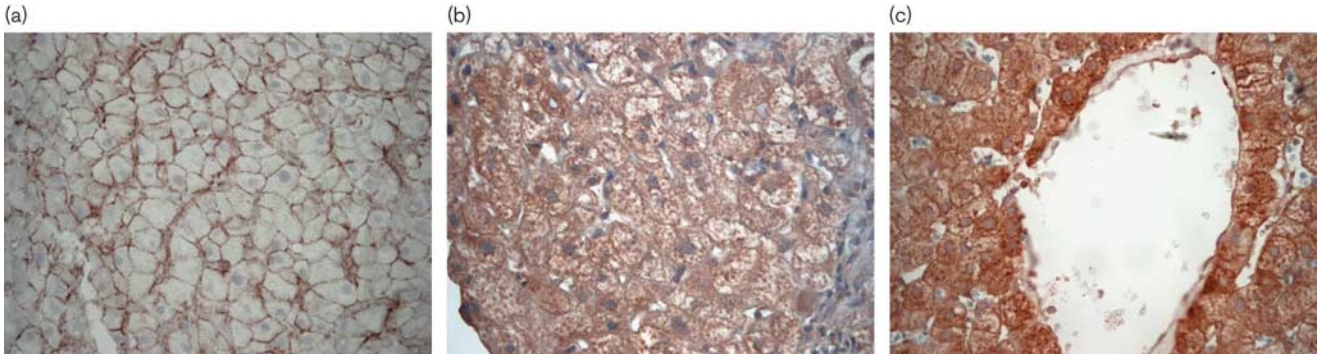

Fig. 1. (a) CD95, ×400, widespread membranous staining. (b) CD95, ×400, granular cytoplasmic staining. (c) CD95, ×400, membranous and granular cytoplasmic staining.

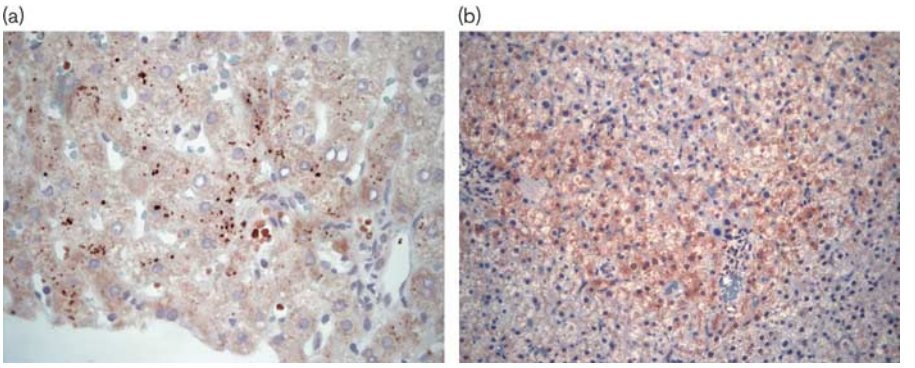

Fig. 2. (a) Caspase-3, ×400, rough granular staining in the cytoplasm of hepatocytes. (b) Caspase-3, ×200, in most hepatocytes, fine granular diffusely staining and a few examples of rough granular staining.

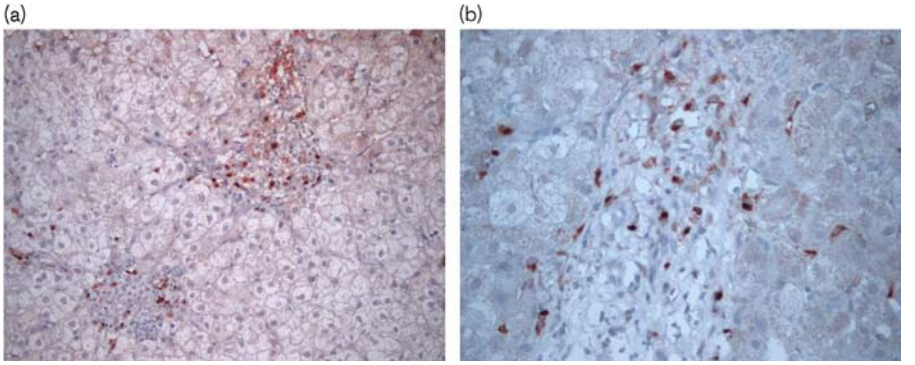

Fig. 3. (a) Ligand of Fas, ×200, large number of positive-stained lymphocytes at the two portal area. (b) Ligand of Fas, ×400, positive-stained lymphocytes in the portal area and surrounding sinusoids.

Table 2. Apoptotic studies in the liver tissue in responder and nonresponder patients

Score	Responder patients ($n=86$)	Nonresponder patients ($n=94$)	P
Density of CD95	1.80±0.8	1.89±0.8	>0.05
Extensity of CD95	1.76±0.9	1.80±0.8	>0.05
Density of caspase-3	1.63±0.9	1.60±0.9	>0.05
Extensity caspase-3	1.51±0.9	1.47±0.9	>0.05
Density of caspase-8[a]	3.0±0	3.0±0	>0.05
Extensity of caspase-8	1.65±0.8	1.62±0.9	>0.05
Density of caspase-9	2.05±0.9	2.00±0.9	>0.05
Extensity of caspase-9	1.30±0.7	1.30±0.7	>0.05
Density of FADD	0.61±0.8	1.69±0.8	>0.05
Extensity of FADD	0.58±0.8	0.64±0.8	>0.05
FasL, portal	1.44±0.9	1.54±0.9	>0.05
FasL, parenchymal	0.81±0.8	0.82±0.7	>0.05
Apoptotic index	3.38±2.9	3.67±3.5	>0.05

[a]Most of the samples were stained with caspase-8 at high density.
FADD, Fas-associated death domain; FasL, Fas ligand.

($P=0.02$, $r=0.21$), portal ($P<0.0001$, $r=0.34$), and parenchymal ($P=0.012$, $r=0.23$) FasL scores.

Fibrosis also correlated positively with the density of CD95 ($P=0.02$, $r=0.24$), extensity of caspase-8 ($P=0.02$, $r=0.21$), portal ($P<0.0001$, $r=0.34$), and parenchymal ($P=0.012$, $r=0.23$) FasL scores.

The histologic activity index correlated positively with fibrosis ($P<0.0001$, $r=0.59$). The relationship between HCV-RNA level and histologic and apoptotic parameters is not determined.

Discussion

The apoptotic mechanism is an important process in the elimination of infected cells. It is known that this process is also involved in HCV infection [21], whereas clearance of infected hepatocytes by apoptosis is suggested to be a critical event in HCV treatment [11]. On the surface of hepatocytes, Fas (CD95) is expressed and the binding of FasL (an effector molecule of cytotoxic T cells) to Fas triggers the extrinsic pathway of apoptosis. This binding subsequently activates the caspases [22]. We assessed several steps involved in the apoptotic process and investigated any relationship between these parameters and treatment response as well as other known parameters shown to predict the response.

Apoptosis of hepatocytes and HCV interaction has not been clearly explored. Some studies have suggested that HCV core protein suppresses apoptosis [23,24], whereas others suggest that HCV core protein sensitizes Fas and is a positive regulator of apoptosis [25].

Clinical studies have attempted to discover an indirect explanation for HCV–apoptosis interaction.

In 72 patients treated with IFN (10 MU/day for the first week three times per week for the next 22 weeks), the response was identified. Fas antigens (immunohistochemistry, anti-Fas monoclonal antibody) were studied in paraffin-embedded liver tissues [26–28]. Fas expression was reported to predict the short-term and long-term response to treatment. In another study of 67 HCV patients, a worse response to treatment was reported in patients with high levels of Fas antigen [29].

sFas is produced either by the splicing of transcripts or by the shedding of the extracellular portion of Fas [30]. sFas can reduce Fas/FasL-mediated apoptosis [31]. The sFas level was reported to correlate with liver fibrosis and inflammation in chronic HCV infection [14,15]. Higher levels of sFas were reported in more severe inflammation in HCV patients [16].

sFasL is produced by proteolytic cleavage of FasL and shows proapoptotic properties [32]. Higher levels of sFasL were reported in HCV patients with more severe inflammatory activities [16]. In a recent study, Schiavon *et al.* [13] found higher baseline sFasL levels in nonresponders compared with responders. In our study, we could not test sFas or sFasL levels in the blood. We found no correlation between histology and Fas or FasL in liver tissue. A probable explanation for this may be the fact that HCV-specific T cells migrate to the liver, become activated, and then express FasL that tranduces the apoptotic cell death signal to the infected hepatocytes. Therefore, Fas expression and the degree of liver inflammation may not correlate with intrahepatic viral load [22,33].

Confirming many other studies, being younger and leaner, with higher ALT, lower HCV-RNA, and a higher necroinflammatory score are found to be favorable factors for predicting the HCV treatment response. However, apoptotic parameters are found to be comparable in responders and nonresponders. Although some apoptotic parameters do correlate with inflammation and fibrosis, they cannot predict the treatment responses.

The use of paraffin-embedded liver tissue samples for studying apoptotic parameters seems to be a limitation of this study. However, other studies encourage using paraffin-embedded liver tissue samples for apoptotic studies: rat liver [34,35], rat and mouse liver [36], human and rat liver [37], and human liver studies [26,28,37–41] supported archived tissue studies. Fukuzawa *et al.* [26] compared fresh-frozen samples and paraffin-embedded tissue samples in their study of the apoptotic index and in-situ hybridization in liver tissue. They showed that both samples yielded valid and reproducible results, although fixing through fresh freezing may interrupt the evaluation of some intrahepatic structures.

Furthermore, Gown and Willingham [27] reported that the archived paraffin-embedded tissue samples show the activity of caspases with high sensitivity and specificity. The apoptotic index was successfully studied in archived paraffin-embedded samples of breast tissue from women with breast cancer [42].

This study has shown that inflammation and fibrosis, the basic histopathological features of the liver, are associated with some apoptotic indicators. However, apoptotic parameters may not be used in isolation to predict the response to the treatment.

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Conflicts of interest

There are no conflicts of interest.

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