

# Effects of interferon plus ribavirin treatment on NF- $\kappa$ B, TGF- $\beta$ 1, and metalloproteinase activity in chronic hepatitis C

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Little is known about the cellular and molecular mechanisms underlying the effects of anti-viral therapy on the regression of liver inflammation and fibrosis in chronic hepatitis C. The aim of this study was to evaluate the effects of interferon alpha and ribavirin in combination therapy on the tissue expression of nuclear-factor  $\kappa$ B (NF- $\kappa$ B) (a transcription factor coordinating the expression of stress genes involved in immune response and inflammation), of the polypeptide transforming growth factor beta-1 (TGF- $\beta$ 1) and matrix metalloproteinases 1 (MMP-1) (both of which play an important part in the pathological process of liver fibrogenesis), and on the serum levels of soluble TGF- $\beta$ 1, tissue inhibitors of metalloproteinases (TIMP)-1, and active endogenous MMP-2 and MMP-9 in paired (pre- and post-treatment) liver biopsy and serum samples of subjects with chronic hepatitis C. Serum levels of TGF- $\beta$ 1, TIMP-1, MMP-2, and MMP-9 were evaluated by enzyme-linked immunosorbent assay. Liver expression of muscle-specific  $\alpha$ -actin, NF- $\kappa$ B, TGF- $\beta$ 1, and MMP-1 was studied immunohistochemically using commercially available mono- and polyclonal antisera in an avidin–biotin complex method. Combination therapy induced a reduction in the liver expression of TGF- $\beta$  and NF- $\kappa$ B and an increased expression of MMP-1, regardless of the virological response to the treatment. The greater expression of MMP-1 and lesser expression of NF- $\kappa$ B were both associated with an improvement in fibrosis score. These effects paralleled the significant increase in soluble MMP-9/TIMP-1 ratio in post-therapy sera. Combination therapy with interferon and ribavirin affects the tissue expression of TGF- $\beta$ 1 and NF- $\kappa$ B and favors metalloproteinase activity, and may thereby modulate hepatic fibrogenetic events.

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Chronic infection with hepatitis C virus (HCV) is estimated to affect 100–200 million individuals worldwide.<sup>1</sup> The natural history of chronic hepatitis C may involve a gradual progression of hepatic fibrosis, eventually leading to cirrhosis, liver failure, and hepatocellular carcinoma in a significant pro-

portion of patients,<sup>2,3</sup> so the main therapeutic goal in patients with chronic hepatitis C is to eradicate the virus and prevent or contain the progression of liver fibrosis, thus avoiding severe long-term clinical complications. The currently most effective initial therapy for patients with chronic hepatitis C is a combination of interferon alpha (IFN- $\alpha$ ) or pegylated IFN- $\alpha$  (peg-IFN- $\alpha$ ) with ribavirin (RBV), which achieves a sustained viral response (SVR) in more than 50% of patients.<sup>4–6</sup> Histopathological analysis of paired liver biopsy specimens has shown that treatment with IFN alone<sup>7,8</sup> and, more recently, with Peg-IFN alone,<sup>9</sup> or with a combination of IFN or Peg-IFN- $\alpha$  and ribavirin,<sup>10,11</sup> is associated with an improvement in necro-inflammatory activity and

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hepatic fibrosis in patients who achieve a SVR. Little is known about the cellular and molecular mechanisms underlying the effects of IFN- $\alpha$  on the regression of liver inflammation and fibrosis in chronic hepatitis C.

HCV-related liver damage and progression of hepatic fibrosis are associated with the release of injury-related cytokines, the activation of various stress response genes and hepatic stellate cells (HSC), and abnormalities in the functional cross-talk between extracellular matrix protein production and degradation.<sup>12–16</sup>

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor coordinating the expression of genes involved in immune response and inflammation.<sup>17</sup> Five subunits of the NF- $\kappa$ B protein family have been identified in humans and it has been reported that NF- $\kappa$ B activates a variety of target genes involved in acute and chronic stress response pathways.<sup>18</sup> The few studies evaluating the expression of NF- $\kappa$ B in HCV-related liver damage report conflicting results, suggesting both apoptotic and antiapoptotic effects and a protective role against the progression of fibrosis.<sup>19,20</sup>

Experimental evidence suggests that the polypeptide transforming growth factor beta-1 (TGF- $\beta$ 1) greatly contributes to liver fibrogenesis.<sup>21,22</sup> High serum levels of TGF- $\beta$ 1 have been reported in patients with chronic HCV hepatitis and it has been claimed that TGF- $\beta$ 1 expression perpetuates HSC activation, thus sustaining fibrogenesis.<sup>23,24</sup>

Matrix metalloproteinases (MMPs) are endopeptidases synthesized by HSCs, classifiable in four groups depending on their substrate.<sup>25</sup> MMPs play an important part in tissue remodeling and repair in both physiological and pathological conditions.<sup>26</sup> MMPs activity depends on interaction with specific tissue inhibitors of metalloproteinases (TIMPs), so the balance between MMPs and TIMPs is an important determinant of fibrosis progression. TGF- $\beta$  induces a downregulation of the gene encoding for MMP-1, associated with an upregulation of the gene for TIMP.<sup>25,27</sup> Studies in patients with HCV hepatitis have reported that abnormal serum levels of MMPs and TIMPs may be correlated with liver fibrosis and cirrhosis.<sup>28–31</sup>

In the present study on patients with chronic hepatitis C, we evaluated the effects of IFN- $\alpha$  and ribavirin combination therapy on the tissue expression of TGF- $\beta$ 1, MMP-1, and NF- $\kappa$ B in paired (pre- and post-treatment) liver biopsies and the corresponding soluble TGF- $\beta$ 1, TIMP-1, and active MMP-2 and MMP-9 in sera collected at the same time as the liver biopsies.

## Materials and methods

### Patients

We studied 27 consecutive subjects with chronic hepatitis C who had received antiviral treatment,

retrospectively selected on the basis of the availability of paired, pre- and post-treatment, liver biopsies and serum samples collected on the same day. Sera from 17 blood donors served as normal controls.

The study group included eight female and 19 male subjects with a mean age of  $41 \pm 8.5$  years (range 27–67). All patients had high serum alanine aminotransferase (ALT) levels before therapy (at least 1.5 times the upper limit of normal) and were negative for other viral infections (including hepatitis B virus and human immunodeficiency virus) and hepatotoxic factors. All patients reported being total abstainers from alcohol. A total of 17 were naive patients enrolled in a trial of combination therapy with consensus IFN- $\alpha$  and ribavirin<sup>32</sup> and 10 were patients who had relapsed or failed to respond after IFN monotherapy, enrolled in trials for retreatment with IFN- $\alpha$  plus ribavirin.<sup>33,34</sup> SVR was defined as HCV-RNA in the serum below the detection limit (100 copies per milliliter) 24 weeks after completing the treatment. Relapse was defined as the reappearance of HCV-RNA 24 weeks after the completed therapy had produced a virological response. Patients who tested positive for HCV-RNA after 24 weeks of combination therapy were defined as nonresponders. For the purposes of the present study, relapsers and nonresponders were collapsed for analysis into a single group and defined as nonresponders (NRs) throughout the manuscript. The cases in the study included 18 SVRs and 9 NRs. The baseline characteristics of the cases studied are shown in Table 1.

### Serological Study

#### Virus

Anti-HCV was assessed using third-generation enzyme-linked immunosorbent assay (ELISA-3). Serum HCV-RNA was measured using an 'in-house' nested reverse transcription-polymerase chain reaction assay with a lower limit of detection of 100 copies per milliliter at a single laboratory.<sup>33</sup> The patients' HCV genotype was identified at the baseline using commercially available probe-specific hybridization assay (Innolipa assay, Innogenetics, Gent, Belgium).

#### ELISA

All sera were collected on the same day as the liver biopsies (see below) and immediately stored at  $-80^\circ\text{C}$  until they were used. Serum TGF- $\beta$ 1 and TIMP-1 levels were evaluated using a commercially available human TGF- $\beta$ 1 and TIMP-1 ELISA kit according to the manufacturer's instructions (TGF- $\beta$ 1: R&D System, Abingdon, UK; TIMP-1: Amersham, Bucks, UK). Serum MMP-2 and MMP-9 levels were measured with a commercially available human MMP-2 and MMP-9 ELISA kit (Amersham, Bucks, UK) according to the manufacturer's instruc-

**Table 1** Baseline characteristics of chronic hepatitis C patients

	All patients (n = 27)	SVRs (n = 18)	Nonresponders (n = 9)
Mean age, year ( $\pm$ s.d., range)	41 ( $\pm$ 8.5, 27–67)	41 ( $\pm$ 7.8, 27–55)	41 ( $\pm$ 10.3, 33–67)
Sex, n (%)			
Male	19 (70)	12 (67)	7 (78)
Female	8 (30)	6 (33)	2 (22)
Genotype, n (%)			
1	8 (30)	5 (28)	3 (33)
2	5 (18)	4 (22)	1 (11)
3	12 (45)	9 (50)	3 (34)
4	2 (7)	—	2 (22)
HCV treatment status, n (%)			
Naïve	17 (63)	10 (56)	7 (78)
Experienced	10 (37)	8 (44)	2 (22)
Mean duration between biopsy, months ( $\pm$ s.d., range)	31 ( $\pm$ 12, 14–57)	33 ( $\pm$ 13, 14–57)	26 ( $\pm$ 8, 14–36)

SVRs, sustained virological responders.

tions. The TIMP-1 ELISA kit recognizes total human TIMP-1. The MMP-2 and MMP-9 ELISA kit recognizes pro and active forms of MMP-2 and MMP-9.

### Histological Study

Liver biopsies were obtained using a percutaneous procedure 1–24 months before starting the treatment and then 6–25 months after it was stopped. The mean time elapsing between the first and second biopsies and serum samples was 31 months (Table 1). All liver samples were formalin-fixed and paraffin-embedded and routinely stained with hematoxylin and eosin, PAS after diastase digestion, Perls' method for iron and Van Gieson's method for evaluating fibrosis. All biopsies were reviewed by the same pathologist (MG), who was unaware of any clinical and serological information, or of the chronological sequence of the biopsies. The number of complete portal tracts included in each specimen was recorded to meet recent criteria for adequacy.<sup>35</sup> Grade (HAI) and stage were evaluated according to Ishak's scoring system.<sup>36</sup> Three grading categories were considered: low-grade activity = HAI 1–6; moderate activity = HAI >6  $\leq$  12, and high-grade activity = HAI > 12. Fibrosis was defined as mild (scores < 3) or clinically relevant (ie presence of complete septal fibrosis or cirrhosis = score  $\geq$  3). Steatosis was also evaluated and graded as the percentage of steatotic hepatocytes: less than 5% of steatotic hepatocytes was considered as minimal steatosis, up to 30% was classed as mild steatosis, and more than 30% was defined as moderate/severe steatosis.

### Immunohistochemical Study

- Paraffin sections 4 mm thick were stained with:
- monoclonal anti- $\alpha$ -smooth muscle actin (Dako, Carpinteria, CA, USA) (1:50 dilution, 1 h room temperature).

- polyclonal anti-NF- $\kappa$ B antibody (p50; Santa Cruz Biotechnology, Inc.) (1:400 dilution overnight; antigen retrieval with citrate buffer, 98°C for 40 min.)
- polyclonal anti-TGF- $\beta$ -1 antibody (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) at 1:20 dilution 1 h at room temperature (antigen retrieval with citrate buffer in microwave).
- monoclonal anti-MMP-1 antibody (3B6; Santa Cruz Biotechnology, Inc.) (1:10 dilution for 1 h room temperature; antigen retrieval with citrate buffer in microwave).

All sections were stained according to the avidin–biotin peroxidase complex method using a commercially available kit, then counterstained with hematoxylin. All the antibodies involved have been used extensively and their specificity has been documented.

Immunoreactivity was assessed throughout the liver specimens, using an arbitrary scale of 0–3, where 0 = no stained cells, 1 = focal expression by a few cells (less than 10%), only detectable under a high-power field; 2 = more stained cells (10–30%), easily detectable under a medium-power field, and 3 = numerous stained cells (>30%), easily detectable even under a low-power field.

### Statistical Analysis

The  $\chi^2$  test or Fisher's exact test was used for statistical analysis of the comparisons between group frequencies. Serum and histological features were compared with Student's *t*-test when appropriate. Spearman's rank correlation coefficients were calculated to assess the relationship between histological and serum findings.

## Results

### Serological Study

Table 2 shows the results of serum analysis. By comparison with the normal controls, the subjects

with chronic hepatitis C showed a significant increase in serum TGF- $\beta$ 1, active endogenous MMP-2 and TIMP-1 levels, while the serum levels of active endogenous MMP-9 were significantly lower. In addition, both MMP-2/TIMP-1 and MMP-9/TIMP-1 ratios were significantly lower in patients with chronic hepatitis C than in controls. After the treatment, serum levels of active endogenous MMP-9 increased significantly, while soluble TIMP-1 decreased significantly. These changes were reflected by changes in the MMP-9/TIMP1 ratio, which increased significantly after INF- $\alpha$  plus ribavirin treatment (Table 2). No significant changes were observed in soluble TGF- $\beta$ 1 and MMP-2 levels after the therapy and serum levels of TGF- $\beta$ 1 did not correlate with liver TGF- $\beta$ 1 expression: this may be due either to preformed sources of TGF- $\beta$ 1 or to the method used to determine serum TGF- $\beta$ 1 levels failing to discriminate between latent and active forms of TGF- $\beta$ 1.

## Histological Study

### Before therapy

A number of portal tracts  $\geq 11$  was detected in 16 cases. There were 6–10 portal tracts in six cases and fewer than six in five cases. The mean activity grade was  $6.96 \pm 2.69$  (range 2–11). The final label was mild activity in 11 (41%) cases and moderate in 16 (59%). HAI showed no significant correlation with the serum levels of TGF- $\beta$ 1, MMP-2, MMP-9, and TIMP-1.

The stage of fibrosis was mild (score  $< 3$ ) in 17 cases (63%) and clinically relevant in 10 (37%), including three cases of fully developed cirrhosis. Subjects with mild fibrosis had a significantly higher ( $P = 0.004$ ) MMP2/TIMP1 ratio than those with more severe fibrosis (score  $\geq 3$ ):  $0.3 \pm 0.09$  vs  $0.2 \pm 0.08$ .

Steatosis was observed in 18/27 cases (66.6%); it was minimal in three cases, mild in 10, and moderate/severe in five.

### After therapy

A number of portal tracts  $\geq 11$  was detected in 19 cases, 6–10 in five and less than six in three. The mean HAI was  $4.1 \pm 2.24$  (range 1–9). The difference

in activity grade before and after therapy was significant ( $P < 0.001$ ) in both SVR and NR. To be specific, a decrease in HAI  $\geq 2$  scores was observed in 15/18 SVR and in 5/9 NR.

The stage of fibrosis was mild in 18 cases and clinically relevant in nine. Although the differences between before and after therapy were not statistically significant, the fibrosis score dropped 2 points in three cases and 1 point in another three in the SVR group. One case progressed from stage 2 to cirrhosis. The samples were considered adequate in all these cases.<sup>35</sup> In the NR group, three cases had a 1 point reduction in fibrosis score after therapy. In both groups, SVR and NR, the cirrhosis diagnosed before the treatment was confirmed afterwards.

After therapy, steatosis was lower in 11 cases and disappeared in three. The difference between biopsies taken before and after therapy was highly significant ( $P = 0.01$ ).

## Immunohistochemical Study

Table 3 summarizes the outcome of the immunohistochemical analysis on the liver biopsies obtained before and after therapy.

### Prior to therapy

$\alpha$ -SMA immunohistochemical expression by sinusoidal cells (Figure 1) was observed in all cases and the immunoreaction was not topographically related to inflammation. TGF- $\beta$ 1 immunostaining was seen in 70% of biopsies, involving inflammatory cells (Figure 2) and, in a few cases, bile duct epithelium. Hepatocytes were negative in all cases. No association was found between tissue and serum expression of TGF- $\beta$ 1. MMP1 immunostaining was detected in inflammatory and sinusoidal cells (Figure 3) in 62.9% of biopsies. Expression by sinusoidal cells was topographically unrelated to the necro-inflammatory lobular lesions. Epithelial cells did not express MMP1. A total of 19 cases stained with NF- $\kappa$ B, detected in both the cytoplasm and the nucleus of inflammatory cells (Figure 4), in peri-sinusoidal cell cytoplasm and, occasionally, in bile duct epithelial cells (nuclear and cytoplasm positivity). No hepatocyte expression was observed.

**Table 2** Serum levels of TGF- $\beta$ 1, TIMP-1, active endogenous MMP-2, MMP-9, and MMP-2/TIMP-1 and MMP-9/TIMP-1 ratio in healthy controls and CHC patients before and after therapy

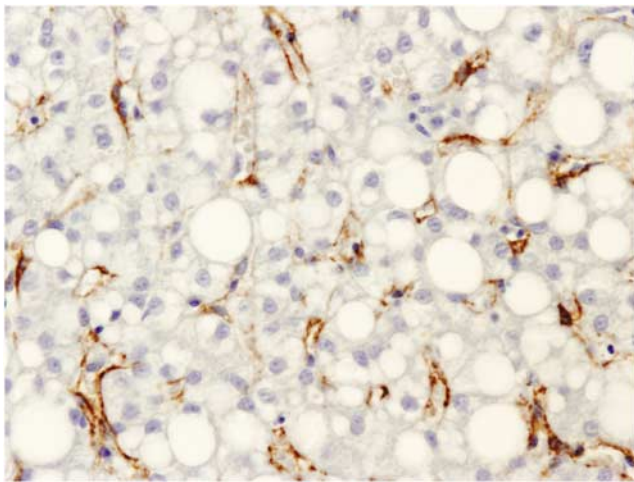
	Controls (n = 17)	CHC patients pre-therapy (n = 27)	CHC patients post-therapy (n = 27)	P-value controls vs CHC patients	P-value CHC patients pre- vs post-therapy
TGF- $\beta$ 1 (pg/ml)	$32.5 \pm 3.3$	$47.1 \pm 12.2$	$44.06 \pm 12.8$	0.0000	NS
TIMP-1 (ng/ml)	$646 \pm 63.9$	$992 \pm 254.8$	$872.7 \pm 161.2$	0.0000	0.05
MMP-2 (ng/ml)	$222.8 \pm 9.9$	$283 \pm 68$	$297.7 \pm 61.4$	0.0004	NS
MMP-9 (ng/ml)	$30.1 \pm 2.6$	$13 \pm 4.1$	$17.9 \pm 9.3$	0.0000	0.05
MMP-2/TIMP-1	$0.34 \pm 0.03$	$0.3 \pm 0.09$	$0.713 \pm 1.987$	0.01	NS
MMP-9/TIMP-1	$0.046 \pm 0.005$	$0.0139 \pm 0.003$	$0.0183 \pm 0.01$	0.0000	0.03

Data are presented as mean  $\pm$  s.d.; CHC = chronic hepatitis C.

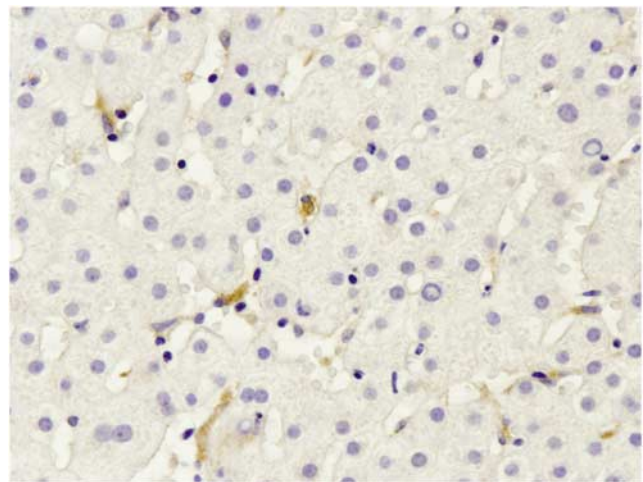
**Table 3** Immunohistochemical expression of  $\alpha$ -SMA, TGF- $\beta$ 1, MMP1, and NF- $\kappa$ B in liver biopsies obtained before and after therapy (27 cases)

	Before therapy					After therapy			
	Score 0	Score 1	Score 2	Score 3		Score 0	Score 1	Score 2	Score 3
$\alpha$ -SMA (sinusoidal cells)	0	15	8	4	$P=NS$	0	15	9	3
TGF- $\beta$ 1 (inflammatory cells)	8	6	8	5	$P=0.03$	15	4	6	2
MMP1 (sinusoidal cells)	8	10	5	4	$P=0.07$ (NS)	6	4	9	8
NF- $\kappa$ B (inflammatory cells)	8	5	9	5	$P=NS$	8	3	10	6
NF- $\kappa$ B (sinusoidal cells)	10	9	7	1	$P=NS$	6	4	9	8

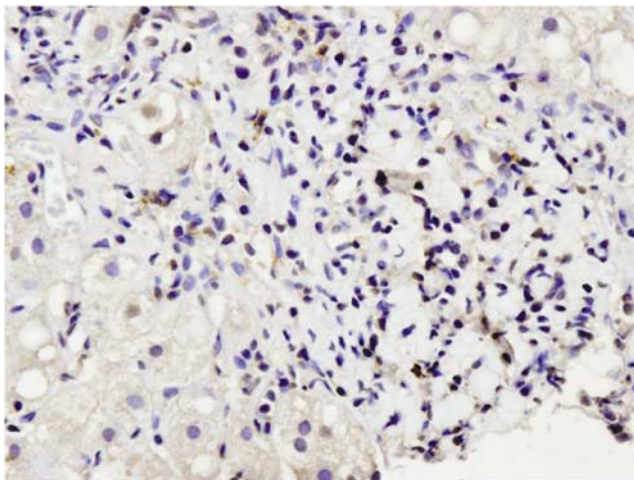
Score 1 = few cell stained (less than 10%); score 2 = more stained cells (10–30%); score 3 = >30% stained cells (see also the text).



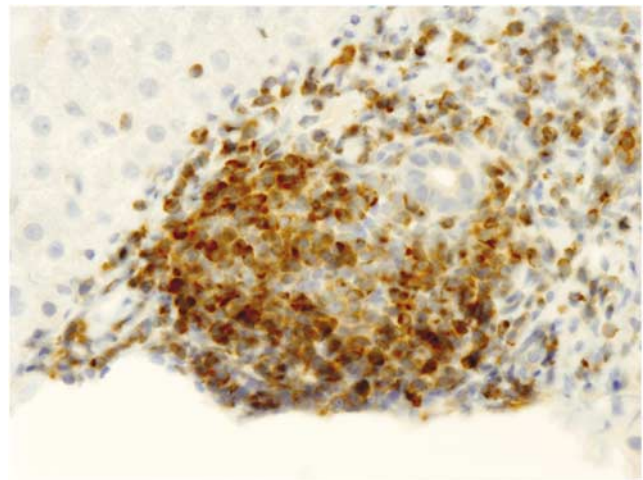
**Figure 1** Diffuse  $\alpha$ -SMA expression by hepatic stellate cells in chronic hepatitis C (avidin–biotin peroxidase complex method, original magnification  $\times 80$ ).



**Figure 3** Expression of MMP-1 in sinusoidal cells (avidin–biotin peroxidase complex method; original magnification  $\times 80$ ).



**Figure 2** TGF- $\beta$  expression by portal inflammatory cells in chronic hepatitis C (avidin–biotin peroxidase complex method, original magnification  $\times 80$ ).



**Figure 4** NF- $\kappa$ B expression in intra-portal inflammatory cells (avidin–biotin peroxidase complex method; original magnification  $\times 80$ ).

*After therapy*

- $\alpha$ -SMA expression decreased in eight of 27 cases after therapy, although the difference was not statistically significant.

- TGF- $\beta$ 1 expression declined significantly after therapy ( $P=0.03$ ), in terms of both the prevalence of positive cases and the score, which dropped in 14/19 (74%) cases. The decline in the tissue expression of TGF- $\beta$ 1 did not correlate with changes in grade and stage.

- MMP-1 immunostaining in sinusoidal cells increased after therapy in 13 cases, including 6/8 (75%) previously negative cases and 7/17 cases already positive prior to treatment. The difference between before and after therapy was only marginally significant ( $P=0.07$ ). Eight of these 13 cases (61%) had a lower fibrosis score after treatment. MMP-1 expression by inflammatory cells showed the same tendency to increase after the therapy (data not shown).
- NF- $\kappa$ B expression in inflammatory cells decreased after therapy in nine cases, increased in 10, and remained the same in eight. In six of the nine cases in which it decreased, a lower fibrosis score was also recorded, while only one of the 10 cases in which the NF- $\kappa$ B increased had a reduction in fibrosis; this difference reached statistical significance ( $P=0.01$ ). The prevalence of cases with NF- $\kappa$ B-expressing sinusoidal cells was higher (21/27; 78%) and the score increased after the treatment in 16 cases. The difference between before and after therapy was statistically significant ( $P=0.01$ ). The increase in NF- $\kappa$ B sinusoidal expression after therapy was greater in subjects whose fibrosis persisted or increased than in those whose scores decreased: 72% (13/18) vs 33% (3/9). This difference was not statistically significant, however ( $P=NS$ ).

None of the changes observed after therapy showed any significant association with virological response.

## Discussion

This study used paired (pre and post-therapy) tissue and serum samples to evaluate the effects of a combination IFN- $\alpha$  and ribavirin treatment for chronic hepatitis C on factors involved in necro-inflammatory lesions and liver fibrogenetic events.

The main result of this study is that IFN- $\alpha$  plus ribavirin treatment reduces the liver expression of TGF- $\beta$ 1 and NF- $\kappa$ B by inflammatory cells and increases the expression of MMP-1 by sinusoidal cells. These effects coincided with a significant increase in the soluble MMP-9/TIMP-1 ratio in post-therapy sera, suggesting that the treatment favored a collagenolytic activity. Although previous studies reported serum levels of gelatinase-type MMPs being associated with more severe fibrosis,<sup>28,30,31,37</sup> the role of these MMPs is far from being fully elucidated. In fact, an *in vivo* study recently reported different results from those observed *in vitro*, suggesting that MMP-2 may have several functions.<sup>38</sup> Since the action of MMPs is regulated by TIMPs, we propose the MMPs/TIMPs ratio as an indicator for evaluating collagenolytic enzyme activity. The reasons for no significant improvement in fibrosis score being observed after therapy probably relate to the fact that the post-therapy liver biopsies were obtained before any reversion of histological

fibrosis could take place, since this takes longer than changes in the products of signaling pathways.

Lower plasma TGF- $\beta$ 1 levels in chronic hepatitis C after IFN- $\alpha$  therapy have already been associated with a regression of hepatic fibrosis.<sup>39</sup> Our data on intrahepatic TGF- $\beta$ 1 expression after the treatment are also consistent with previous studies on a limited number of cases,<sup>40</sup> and we confirmed the absence of any correlation between TGF- $\beta$  expression and the degree of liver damage.<sup>41</sup>

The functional interactions between TGF- $\beta$ 1 and NF- $\kappa$ B in chronic hepatitis C, and how they may be affected by antiviral therapy, are not known. Studies using different models<sup>42</sup> have suggested a dynamic cross-talk between TGF- $\beta$ 1 and NF- $\kappa$ B in the pro-inflammatory cascade and data from the present *in vivo* study seem to support this hypothesis. Here, we found that NF- $\kappa$ B expression by sinusoidal cells increased after therapy, particularly in subjects whose fibrosis score increased or remained unchanged. Although this expression was detected only in the cytoplasm (and therefore in an apparently nonactivated state), it nonetheless seems to support the hypothesis that higher levels of NF- $\kappa$ B expression in chronic hepatitis C are associated with a higher likelihood of liver fibrosis progression. In contrast, Boya *et al*<sup>19</sup> suggested a protective role for NF- $\kappa$ B against fibrosis progression in hepatitis C, while Tai *et al*<sup>43</sup> found that NF- $\kappa$ B activation promoted a chronic inflammatory response. Concerning Boya's data, we evaluated a different NF- $\kappa$ B subunit and this may account for the different patterns of immunohistochemical expression observed, suggesting that different subunits of the NF- $\kappa$ B protein family have different effects in humans with chronic hepatitis C.<sup>44</sup> In addition, evidence from different models suggests a possible dual role for NF- $\kappa$ B in either proinflammatory or anti-inflammatory pathways.<sup>45</sup> We can thus hypothesize that different levels of NF- $\kappa$ B activation may result in either the regression or the persistence of the inflammatory response leading to fibrosis.<sup>45,46</sup>

As we ourselves<sup>47</sup> and others<sup>48,49</sup> have already shown, HSC activation decreases after therapy in some cases. We found no significant relationship between HSC activation and liver expression of TGF- $\beta$ , MMP-1, and NF- $\kappa$ B, but this is probably due to the small number of cases studied.

The small number of cases involved may also explain the absence of any statistical association between changes in immunohistochemical expression of  $\alpha$ -SMA, NF- $\kappa$ B, TGF- $\beta$ 1, and MMP-1 and viral response to therapy. Data from the literature suggest that an improvement in activity grade and lower rate of fibrosis progression are observable even in patients failing to respond to the therapy, albeit to a lesser extent than in SVR cases, but no reasons are given for this situation.<sup>7,8</sup>

In conclusion, combination therapy with interferon and ribavirin may favor collagenolytic activity by modulating hepatic expression of TGF- $\beta$ -1,

NF- $\kappa$ B, and MMP1 and serum levels of TIMP-1 and MMPs. These effects might account for the beneficial consequences on necro-inflammatory activity and fibrosis observed after combination therapy in hepatitis C.

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## References

- 1 WHO Technical Consultation on Hepatitis C. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hep* 1999;6:35–47.
- 2 Seef LB. Natural history of chronic hepatitis C. *Hepatology* 2002;36:S35–S46.
- 3 Fattovich G, Giustina G, Degos F, *et al*. Morbidity and mortality in compensated cirrhosis C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997;112:463–472.
- 4 Manns MP, McHutchison JG, Gordon SC, *et al*. Peginterferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001;358:958–965.
- 5 Hadziyannis SJ, Sette Jr H, Morgan TR, *et al*. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004;140:346–355.
- 6 Strader DB, Wright T, Thomas DL, *et al*. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004;39:1147–1171.
- 7 Sobesky R, Mathurin P, Charlotte F, *et al*. Modeling the impact of interferon alpha treatment on liver fibrosis progression in chronic hepatitis C: a dynamic view. *Gastroenterology* 1999;116:378–386.
- 8 Shiratori Y, Imazeki F, Moriyama M, *et al*. Improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000;132:517–524.
- 9 Cammá C, Di Bona D, Schepis F, *et al*. Effect of Peginterferon alpha-2a on liver histology in chronic hepatitis C: a meta-analysis of individual patients data. *Hepatology* 2004;39:333–342.
- 10 Poynard T, McHutchison J, Davis GL, *et al*. Impact of interferon alpha-2b and ribavirin on progression of liver fibrosis in patients with chronic hepatitis C. *Hepatology* 2000;32:1131–1137.
- 11 Poynard T, McHutchison J, Manns M, *et al*. Impact of pegylated interferon alpha-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. *Gastroenterology* 2002;122:1303–1313.
- 12 Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000;275:2247–2250.
- 13 Schuppan D, Krebs A, Bauer M, *et al*. Hepatitis C and liver fibrosis. *Cell Death Differ* 2003;S1:59–67.
- 14 Rockey DC. Hepatic fibrogenesis and hepatitis C. *Semin Gastrointest Dis* 2000;11:69–83.
- 15 Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. *Semin Liver Dis* 2001;21:397–416.
- 16 Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209–218.
- 17 Baldwin AS. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001;107:3–6.
- 18 Schmid RM, Adler G. NF-kappaB/rel/IkappaB: implications in gastrointestinal diseases. *Gastroenterology* 2000;118:1208–1228.
- 19 Boya P, Larrea E, Sola I, *et al*. Nuclear factor-kappa B in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. *Hepatology* 2001;34:1041–1048.
- 20 Gaweco AS, Wiesner RH, Porayko M, *et al*. Intra-graft localization of activated nuclear factor kappaB in recurrent hepatitis C virus disease following liver transplantation. *Hepatology* 2000;31:1183–1191.
- 21 Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med* 2000;342:1350–1358.
- 22 Gressner AM, Weiskirchen R, Breitkopf K, *et al*. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002;7:793–807.
- 23 Nelson DR, Gonzalez-Peralta RP, Qian K, *et al*. Transforming growth factor-beta 1 in chronic hepatitis C. *J Viral Hepat* 1997;4:29–35.
- 24 Nelson DR, Gonzalez-Peralta RP, Qian K, *et al*. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-beta. *J Viral Hepat* 2001;8:430–437.
- 25 Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003;200:448–464.
- 26 Røijkind M. Role of metalloproteinases in liver fibrosis. *Alcohol Clin Exp Res* 1999;23:934–939.
- 27 Pinzani M, Rombouts K. Liver fibrosis: from the bench to clinical targets. *Dig Liver Dis* 2004;36:231–242.
- 28 Lichtinghagen R, Michels D, Haberkorn CI, *et al*. Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C. *J Hepatol* 2001;34:239–247.
- 29 Koulentaki M, Valatas V, Xidakis K, *et al*. Matrix metalloproteinases and their inhibitors in acute viral hepatitis. *J Viral Hepat* 2002;9:189–193.
- 30 Leroy V, Monier F, Bottari S, *et al*. Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am J Gastroenterol* 2004;99:271–279.
- 31 Nunez O, Fernandez-Martinez A, Majano PL, *et al*. Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut* 2004;53:1665–1672.
- 32 Fattovich G, Zagni I, Minola E, *et al*. A randomized trial of consensus interferon in combination with

- ribavirin as initial treatment for chronic hepatitis C. *J Hepatol* 2003;39:843–849.
- 33 Fattovich G, Zagni I, Fornaciari G, *et al*. Efficacy of prolonged 5 million units of interferon in combination with ribavirin for relapser patients with chronic hepatitis C. *J Viral Hepatitis* 2003;10:111–117.
- 34 Fattovich G, Zagni I, Ribero ML, *et al*. A randomized trial of prolonged high dose of interferon plus ribavirin for hepatitis C patients non-responders to interferon alone. *J Viral Hepatitis* 2004;11:543–551.
- 35 Colloredo G, Guido M, Sonzogni A, *et al*. Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. *J Hepatol* 2003;39:239–244.
- 36 Ishak K, Baptista A, Bianchi L, *et al*. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995;22:696–699.
- 37 Benyon RC, Iredale JP, Goddard S, *et al*. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. *Gastroenterology* 1996;110:821–831.
- 38 Radbill B, Gupta R, Alvarez C. Divergent role of matrix metalloproteinase-2 in hepatic stellate cell activation and liver fibrosis. *Hepatology* 2004;40(S1):546A.
- 39 Tsushima H, Kawata S, Tamura S, *et al*. Reduced plasma transforming growth factor-beta1 levels in patients with chronic hepatitis C after interferon-alpha therapy: association with regression of hepatic fibrosis. *J Hepatol* 1999;30:1–7.
- 40 Kinnman N, Andersson U, Hultcrantz R. *In situ* expression of transforming growth factor-beta1-3, latent transforming growth factor-beta binding protein and tumor necrosis factor-alpha in liver tissue from patients with chronic hepatitis C. *Scand J Gastroenterol* 2000;35:1294–1300.
- 41 Roulot D, Durand H, Coste T, *et al*. Quantitative analysis of transforming growth factor beta 1 messenger RNA in the liver of patients with chronic hepatitis C: absence of correlation between high levels and severity of disease. *Hepatology* 1995;21:298–304.
- 42 Lu L, Chen SS, Zhang JQ, *et al*. Activation of nuclear factor-kappaB and its proinflammatory mediator cascade in the infarcted rat heart. *Biochem Biophys Res Commun* 2004;321:879–885.
- 43 Tai DI, Tsai SL, Chen YM, *et al*. Activation of nuclear factor kB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 2000;31:656–664.
- 44 Yen TS. Nuclear factor kappaB and hepatitis C—is there a connection? *Hepatology* 2000;31:785–787.
- 45 Lawrence T, Gilroy DW, Colville-Nash PR, *et al*. Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 2001;7:1291–1297.
- 46 Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? *J Clin Invest* 2001;107:255–264.
- 47 Guido M, Rugge M, Chemello L, *et al*. Liver stellate cells in chronic viral hepatitis: the effect of interferon therapy. *J Hepatol* 1996;24:301–307.
- 48 Khan MA, Poulos JE, Brunt EM, *et al*. Hepatic alpha-smooth muscle actin expression in hepatitis C patients before and after interferon therapy. *Hepato-gastroenterol* 2001;48:212–215.
- 49 Sakaida I, Nagatomi A, Hironaka K, *et al*. Quantitative analysis of liver fibrosis and stellate cell changes in patients with chronic hepatitis C after interferon therapy. *Am J Gastroenterol* 1999;94:489–496.