Hepatitis C virus F protein: A double-edged sword in the potential contribution of chronic inflammation to carcinogenesis

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Received October 2, 2008; Accepted February 3, 2009

DOI: 10.3892/mmr_00000122

Abstract. Persistent hepatitis C virus (HCV) infection can lead to chronic inflammation and even carcinogenesis. This may in part be due to various viral proteins, which affect apoptosis and promote liver cell growth. Recently, researchers reported a novel F protein, an alternative reading frame protein expressed from the HCV core coding sequence. The functional properties of this protein are presently unclear. Thus, we investigated whether F protein participates in the process of chronic HCV inflammation leading to malignant transformation. Serum or peripheral blood from 42 chronic HCV patients or 38 HCV-associated hepatocellular carcinoma (HCC) patients was analyzed for the presence of T lymphocytes and antibodies specific to F protein. Then, the correlation between indicators of F-specific immune response and viral load or serum IL-6 levels was examined. We identified the presence of both T- and B-cell-mediated immune responses specific to such an antigen in subjects with chronic HCV and in HCC patients. Of note, the presence of anti-F-specific antibodies appeared to be negatively correlated with HCV RNA viral load level. Furthermore, circulating IL-6 levels were significantly higher in patients with HCC than in those with chronic HCV. These increased serum IL-6 levels were, in some HCC patients, positively correlated with the presence of anti-F-specific antibodies. Taken together, these results suggest that F protein may participate in viral clearance and have anti-tumor effects, but may also contribute to the persistence of HCV infection in patients with a low viral load. This latter effect may cause hepatic damage leading to carcinogenesis. Elucidation of these F protein-associated contradictory functional activities is required.

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Key words: hepatitis C virus, F protein, chronic inflammation, hepatocellular carcinoma, viral load, IL-6

Introduction

Hepatitis C virus (HCV) is a major etiologic agent of hepatitis, with the number of infected individuals worldwide estimated to be more than 170 million (1,2). The most important feature of persistent HCV infection is the development, in up to 80% of infected individuals, of chronic hepatitis. Approximately 20% of patients with chronic hepatitis C develop severe cirrhosis, leading to hepatocellular carcinoma (HCC) within two to three decades of infection. Little is known regarding the factors leading to persistent infection and malignant transformation, although the currently held belief is that events during the late stages of chronic viral infection as well as host immunity are influential in determining outcome.

HCV is a member of the *Flaviviridae* family, with a positive-sense and single-strand RNA genome of approximately 9.6 kb in length (3). The HCV genome exhibits a considerable degree of sequence variation. On the basis of these variations, HCV is now classified into at least 6 genotypes and more than 60 subtypes (4,5). HCV subtype 1b is the most prevalent in Asia, including China, and is more frequently associated with severe liver injury, the development of HCC and poor interferon treatment response than any other HCV subtype (6,7). The HCV genome encodes a polyprotein that is post-translationally cleaved by a combination of host cell signal peptidases and by two viral proteinases, resulting in three structural proteins, termed core, envelope 1 (E1), and E2, in a protein named p7, and in six non-structural (NS) proteins, termed NS2, NS3, NS4A, NS4B, NS5A and NS5B. Besides its apparent involvement in viral assembly, the core protein has many intriguing independent activities, and thus plays a pivotal role in viral pathogenesis. According to recent reports, the core protein renders cells more resistant to apoptosis (8,9) and promots *ras* oncogene-mediated transformation in cultured rodent cells (10,11). Moreover, transgenic mice expressing the HCV core protein in the liver were demonstrated to be at an increased risk of developing HCC (12).

In addition to the above 10 gene products, a new HCV protein has been reported by various investigators (13,14). Termed F protein (also known as ARFP or core +1 ORF), it is encoded from an alternative +1 reading frame overlapping the core genomic region. F protein is a short-lived endoplasmic reticulum-associated protein and does not appear to play a major role in HCV replication. It has been demonstrated

that HCV genotype 1a-derived F protein could be produced as a result of a +1 ribosomal frameshift at or near codon 11, with the protein ending at codon 162 (15). In contrast, in HCV genotype 1b, F protein seems to be located at codon 42. In certain patients, this could be followed by a ribosomal frame shift at the stop codon 144 (16). The recent discovery of this novel F protein certainly challenges whether core protein is responsible for the various activities attributed to it.

Basu *et al* (17) showed that the F protein, unlike the HCV core protein, does not significantly modulate c-myc, hTERT and p53 promoter activities. However, like the HCV core genomic region, which reportedly repressesed p21 promoter activity in a p53-independent manner (18), the F protein was shown to repress p21 expression. Meanwhile, other studies have indicated that F protein, unlike core protein, does not inhibit tumor necrosis factor α-mediated apoptosis of HepG2 cells or promote rat embryo fibroblast growth. These results suggest that F protein does not share the major functional properties previously attributed to HCV core protein and is not implicated in cell growth regulation, other than regulating p21 expression. Fiorucci *et al* (19) reported that cell types which have been shown to support HCV infection, including the human hepatoma Huh7 cell line, the THP-1 monocytic cell line, monocytes, dendritic cells and macrophages, secrete pro-inflammatory cytokines (IL-6, IL-8, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1) when infected with adenoviral constructs encoding wild-type core protein sequences. Moreover, this secretion probably involves F protein expression, though production of these cytokines was reduced or abolished. The associated molecular events likely contributed to viral persistence. Increased levels of these circulating cytokines may contribute to the pathogenesis of chronic HCV, as the cytokines have multiple biological effects on immune and inflammatory responses, including fibrogenesis and carcinogenesis.

In conclusion, the role of F protein in the development of persistent infection and malignant transformation remains unclear. In order to clarify the issues outlined above, we analyzed the functional activities of F protein from patients with chronic HCV and HCV-associated HCC, and identified the humoral and cellular immune responses specific to such an antigen. We conclude that the detection of an anti-F protein inflammatory response may be negatively correlated with the level of HCV replication. Moreover, we found that the serum levels of IL-6 (also known as hepatic stimulating factor) and the presence of anti-F-specific antibodies was positively correlated in most HCC patients.

Materials and methods

Blood samples. Sera and peripheral blood mononuclear cell (PBMC) samples were isolated from 80 anti-HCV antibody positive HBsAg-negative patients with and without HCC according to a protocol approved by the Institute of Medicine of Jiangshu Province, P.R. China. Based on physical indicators, symptoms, enhanced CT scans, magnetic resonance imaging and pathological assessment at time of surgery, 42 patients (24 male and 18 female, mean age 41.2 years) were diagnosed as having chronic hepatitis C and 38 patients (29 male and 9 female, mean age 57.3 years) as having HCV-related HCC. At the time of blood sample collection, 26 of the 42 HCV chronic patients were undergoing interferon therapy. The clinical characteristics of the patients are summarized in Table I. Samples from 10 healthy HCV-negative volunteers (6 male and 4 female, mean age 35.6 years) along with 10 HBs-positive patients (6 male and 4 female, mean age 48.5 years) were collected as controls. Informed consent was obtained from all patients and donors. PBMCs were isolated from donors by Ficoll-Hypaque density gradient centrifugation.

Expression and purification of HCV novel F protein. HCV RNA was extracted from 50 μl of serum with an RNeasy Mini kit (Qiagen, Valencia, CA). To amplify the entire core protein-coding region of the HCV genome, reverse transcription (RT) and PCR were successively performed using the primers sense 5'-GAGCACGAATCCTAAACCTCAAAG-3' and antisense 5'-CTTCATAAGCGGAAGCTGGG-3'. The reaction was initially carried out at 45˚C for 30 min and at 94˚C for 2 min for RT, followed by PCR over 30 cycles at 94˚C for 45 sec, 52˚C for 60 sec and 72˚C for 60 sec. Anti-HCV-negative sera served as a negative control in the RT-PCR analysis to monitor for possible cross-contamination between the samples. The F gene was amplified using, in the first round of PCR, the primers sense 5'-GAGCACGAATCCTAAACCTCAAAG-3' and antisense 5'-ACACCCAACCTGGGCCCT-3' and, in the second round, sense 5'-CAGGGGCCCAGGTTGGGTGT-3' and antisense 5'-TTAGAGGGGCGCCGACGAG-3'. PCR was performed over 36 cycles, with each cycle consisting of 1 min at 94, 56 and 72˚C, respectively. This gene construct contained 41 aa from the core sequence, followed by the +1 reading frame. The ribosomal frame shifting is illustrated in Fig. 1.

The expression and purification of F protein were carried out as described elsewhere (16). Briefly, the amplified F fragments were purified with a QIAquick PCR Purification kit (Qiagen) and directly sequenced. The purified F PCR products were cloned into the expression vector pET32a (Novagen, Madison, WI) upstream of a hexahistidine tail, allowing over-expression in *E. coli* and purification on a Ni-nitrolotriacetic acid agarose column.

Detection of anti-F antibodies in the sera of patients. The presence of antibodies specific to the novel F protein was evaluated in the serum of patients with an indirect enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated with 100 μl of purified F protein (1 μg/ml) in 50 mM sodium carbonate buffer (pH 9.6) by overnight incubation at 4˚C. After washing and saturation, a 1:100 dilution of each sample of serum was added and incubated at 37˚C for 2 h. After washing, the wells were incubated at 37˚C for 1 h with peroxidase-conjugated AffiniPure goat anti-human immunoglobulin G whole Ab (Sigma, St. Louis, MO) diluted 1:10,000. Finally, the color reaction, started by the addition of O-phenylenediamine dihydrochloride-H_2O_2 buffer, was stopped after 10 min by the addition of 2 N H_2SO_4. Absorbance was measured at 490 nm in a microplate reader. For each experiment, the cutoff was determined as the mean plus three standard deviations of the results from 3 HCV-negative serum samples. An OD value ≥2.1 times that of the negative control was considered positive.

Table I. Clinical characteristics of the chronic hepatitis C virus patients with or without hepatocellular carcinoma and of the controls at presentation.

Characteristic	Chronic HCV	HCC	HBs(+) control	Healthy control
No. of patients	42	38	10	10
Median age (years)	41.2	57.3	48.5	35.6
Gender (male:female)	24:18	29:9	6:4	6:4
No. (%) of patients under interferon treatment	18 (42.9)	-	-	-
HCV genotype				
1a	16	14	-	-
1b	24	21	-	-
2	1	2	-	-
3	0	0	-	-
4	0	0	-	-
Not determined	1	1	-	-
ALT (IU/l)	81±52	113±65	65±21	0

HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ALT, alanine transaminase.

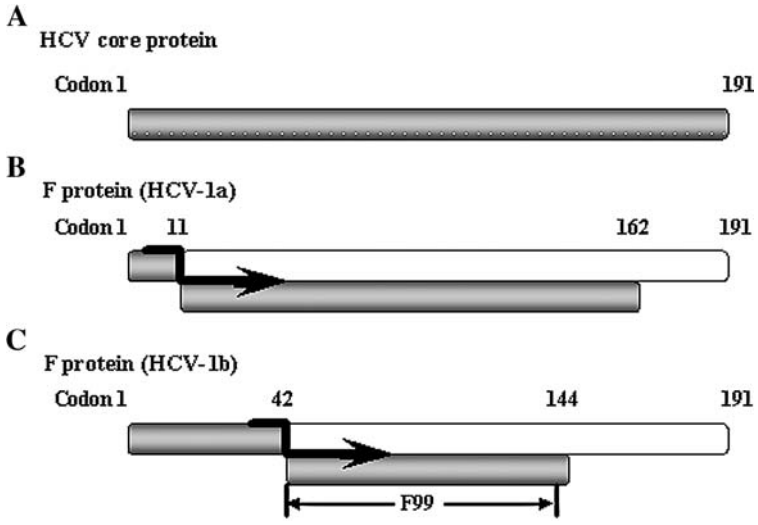

Figure 1. F protein encoded from an alternative +1 reading frame overlapping with the core region of HCV genotypes 1a and 1b. The HCV core sequence is illustrated in (A). HCV genotype 1a-derived F protein (B) could be produced as a result of a +1 ribosomal frameshift at or near codon 11, with the protein ending at codon 162. In HCV genotype 1b (C), F protein is located at codon 42. This could be followed by a ribosomal frame shift at the stop codon 144. F99 synthetic peptide (C) is 99 amino acids in length and is located between aa 42 and 141 of the F protein sequence.

ELISPOT assay. For the evaluation of T-cell immune response, the F99 synthetic peptide of F protein (99 amino acids in length and located between aa 42 and 141 of the F protein sequence; Fig. 1) was synthesized in place of the whole F sequence. Since F protein of the HCV 1b subtype construct contains 41 aa from the core sequence followed by the +1 reading frame, synthesizing the F99 peptide minimizes the antigenicity of the core protein. PBMCs were isolated and cultured as previously described (20). Briefly, PBMCs were cultured in RPMI-1640 medium containing 10% FCS for 2 h at 37˚C. Adherent cells were cultured for 6 days in RPMI-1640 medium containing 10% FCS, 1000 U/ml human GM-CSF and 500 U/ml human IL-4. On day 7 of the culture, 10 ng/ml tumor necrosis factor-α was added for the maturation of the dendritic cells. IL-10- and IFN-γ-producing cells were quantified using ELISPOT kits from Mabtech (Nacha, Sweden) according to the manufacturer's instructions. PBMCs from donors were stimulated with purified F99 peptide or RPMI-1640 complete medium as a negative control. The colored spots, representing cytokine-producing cells, were counted under a dissecting microscope. The number of spots observed in the negative control wells was subtracted from the number of spots observed in the experimental wells.

Analysis of PBMC antigen-specific proliferative response. PBMCs (2 to $3x10^5$ cells/well) were incubated in the presence of 5 μg/ml F99 peptide in U-bottomed 96-well culture plates. Antigen-specific T-cell responses were measured by [^{3}H]

thymidine incorporation during the last 16 h of incubation. After labeling, the cells were washed with phosphate-buffered saline, fixed in cold 10% trichloroacetic acid, and washed in 95% ethanol. Incorporated [^{3}H] thymidine was extracted in 0.2 M NaOH and measured in a liquid scintillation counter. Values were expressed as the means from 6 wells from triplicate experiments.

HCV RNA real-time RT-PCR quantification. HCV RNA was quantified from serum samples by real-time PCR after the reverse transcription of the 5' non-coding region of the genome using SYBR Green I detection, rather than labeled probes, based on the SYBR Green I dye and LightCycler fluorimeter (Roche, Basel, Switzerland). Briefly, total RNA was extracted from 200 μl of each serum sample using the Qiagen QIAamp Viral RNA Mini kit according to the manufacturer's protocol. For quantification of the serum samples, RNA was reverse transcribed as previously described (21). For limited-cycle nested PCR quantification, RT-PCR was performed in a one-step reaction with 15 cycles of PCR (the limited-cycle step), followed by real-time RT-PCR. HCV loads were measured by real-time PCR with outer primers: sense 5'-GCCATG GCGTTAGTAYGAGT-3' and antisense 5'-TTTCGCRAC CCAACRCTACT-3' for single-round reactions, or with outer primers plus inner primers: sense 5'-AGTGTCRTRCAGCCT CCAGG-3' and antisense 5'-ACCCAACRCTACTMGGCT AG-3' for limited-cycle nested reactions. Fluorescence was monitored at 530 nm.

Serum IL-6 measurement. Serum IL-6 levels were measured using a sensitive sandwich ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. All measurements were made in duplicate, and the average values were used in the statistical analyses.

Statistical analysis. Data are presented as the mean standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test and significant P-values ($P<0.05$) compared with the controls.

Results

Antibodies specific to F protein are detected in chronic HCV patients with or without HCC. If the novel F protein is expressed during chronic HCV inflammation, leading to malignant transformation, it is likely that patients will have antibodies recognizing epitopes of the F protein in addition to other anti-HCV antibodies. To test this possibility, serum samples from chronic HCV patients with or without HCC were evaluated for their reactivity against recombinant F protein expressed in *E. coli*. For this purpose, a cDNA fragment containing the core coding of the HCV-1b was treated as a template and amplified with two pairs of primers. The F PCR products were cloned into pET-32a and expressed the 16-kDa protein in *E. coli*. We then examined the sera using indirect ELISA to identify the presence of anti-F-specific antibodies. As expected, none of the healthy or HBs-positive individuals exhibited detectable anti-F antibodies in the sera samples. In contrast, as shown in Fig. 2A, we detected the presence of anti-F-specific antibodies ($P<0.05$) in 17 of the 42 chronic HCV patients (40.5%) and in 23 of the 38 patients with HCV-associated HCC (60.5%). Detection of the antibody was not restricted to patients with genotype 1b viruses. Six of the 18 chronic HCV patients and 9 of the 17 HCC patients infected with various genotypes (1a and 2a) were also found to be positive. Importantly, 10 of the 18 anti-F-positive HCV chronic patients were undergoing interferon therapy at the time of blood sample collection. The difference was significant in contrast to the non-interferon therapy patients (7/24) ($P<0.05$).

PBMCs from chronic HCV patients with or without HCC secrete IFN-γ or IL-10 in response to the F99 peptide derived from F protein. To document the existence of cellular immune responses against the F99 peptide derived from F protein, we used the ELISPOT kit to quantify the IL-10- and IFN-γ-producing cells. PBMCs were preincubated with antigen and later transferred to the ELISPOT plates. The numbers of IL-10- and IFN-γ-producing cells in response to the F99 peptide for the tested patients are shown in Fig. 2B. IL-10- or IFN-γ-producing cells were detected in 4 of the 42 chronic HCV patients and 3 of the 38 HCV-associated HCC patients. Two of the 4 chronic HCV patients and 2 of the 3 HCC patients also showed detectable anti-F antibodies in their serum. In 2 of the 4 chronic HCV patients, F99-specific T-cells produced IL-10 without IFN-γ. In 1 of the 4 chronic HCV patients, only IFN-γ-producing cells were detected. Finally, in 1 of the 4 chronic HCV patients, both IFN-γ- and IL-10-secreting cells were detected. Interestingly, only one type of cytokine-producing cell was found in 3 HCV-associated HCC patients; 2 patients had IFN-γ and 1 had IL-10 cytokine-producing cells.

This indicates that there was no obvious link between the detection of specific T-cells and clinical status in HCV patients with or without HCC. F99-specific T-cells were detected in both groups ($P>0.05$). Furthermore, the T-cell response was not associated with HCV subtype. IFN-γ- or IL-10-producing cells were also detected in HCV genotype 2a rather than 1b patients.

Proliferation of chronic HCV but not HCC PBMCs in response to the F99 peptide. After a 6-day culture, poor proliferation was induced by the preparations in a few of the donors. [^{3}H]-thymidine incorporation was detected upon stimulation of PBMCs with the F99 peptide. PBMCs from 2 chronic HCV patients, but from no HCC patients, exhibited a low degree of proliferative response to the F99 peptide. Fig. 2B shows the representative results obtained using cells from weak responders. No stimulation of proliferation was detected when PBMCs from HCC patients were incubated with the F99 peptide. Furthermore, although the F99 peptide had been derived from the HCV 1b genotype, proliferative responses were detected in chronic HCV patients with varying subtypes (2a). In 2 patients, only IL-10-producing cells were detected. Moreover, the proliferation and cytokine secretion of PBMCs were not correlated.

Chronic HCV patients with or without HCC display different patterns of cellular and humoral immune response specific to F protein. Overall (Fig. 2), there was an obvious correlation between the presence of humoral immune responses and the

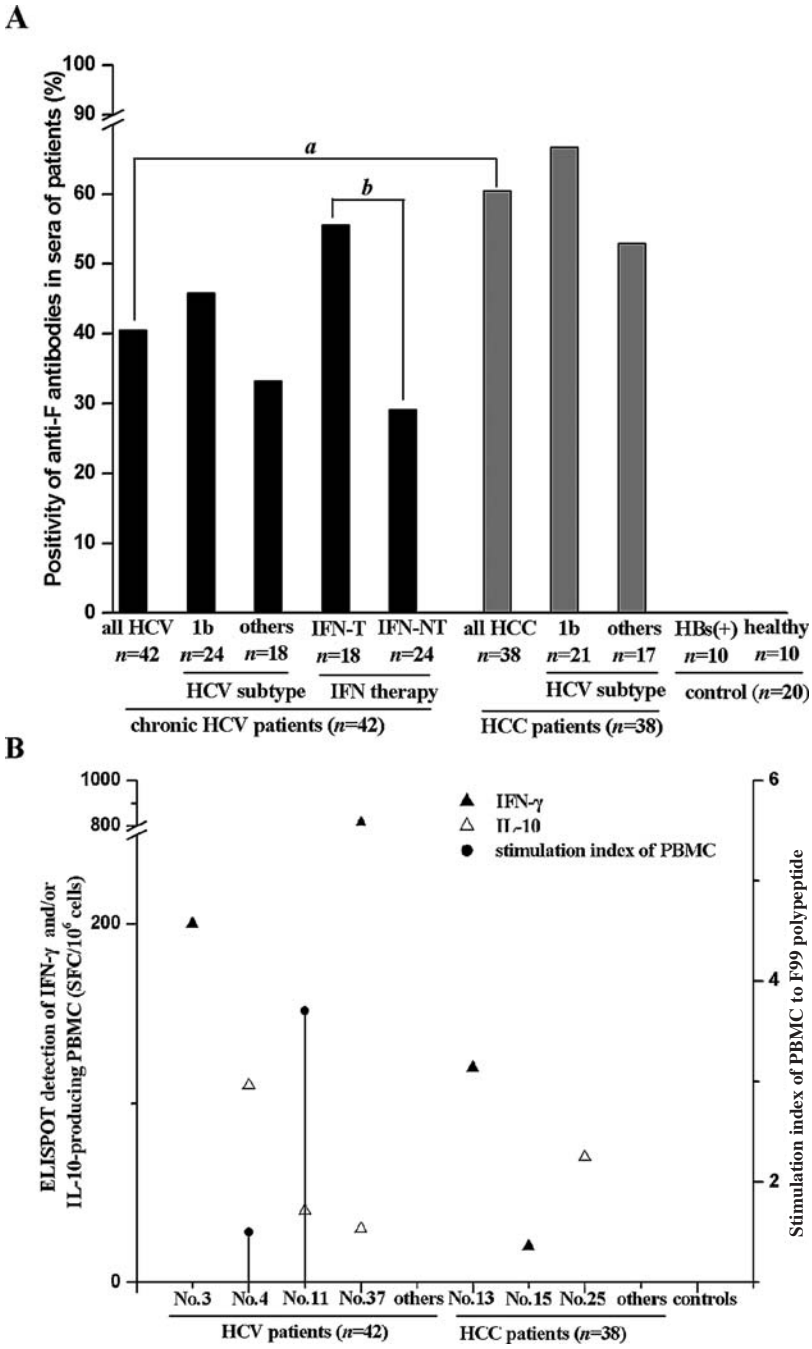

Figure 2. (A) Positivity of anti-F antibodies in sera samples from chronic HCV patients with or without HCC. The presence of anti-F antibodies in sera was determined using indirect ELISA as described in Materials and methods. Chronic HCV patients (n=42) were divided into the HCV-1b subtype (1b) (n=24) and HCV-other subtype (other) (n=18) groups, or into the interferon therapy (IFN-T) (n=18) and non-interferon therapy (IFN-NT) (n=24) groups. Similarly, HCC cohorts included the HCV-1b subtype (1b) (n=21) and HCV-other (other) (n=17) groups. Healthy HCV-negative (healthy) volunteers (n=10) and HBs-positive [HBs(+)] patients (n=10) were enrolled controls. [a]P<0.05, patients with chronic HCV vs. patients with HCC. [b]P<0.05, chronic HCV patients with IFN therapy vs. HCV patients without IFN therapy. (B) Detection of cellular immune responses specific to F protein. F-specific cytokine profiles and T-cell proliferation were analyzed in the different cohorts of patients. The number of IFN-γ (▲)- and IL-10 (△)-producing cells per 10^6 PBMCs following *in vitro* stimulation by F protein in chronic HCV and HCC patients were evaluated by ELISPOT (left Y axis). Four chronically infected HCV patients (nos. 3, 4, 11 and 37) and 3 HCC patients (nos. 13, 15 and 25) displayed F99-specific T-cell responses. Proliferative responses of PBMCs from different cohorts of patients to the F99 peptide derived from F protein were evaluated by [^{3}H]-thymidine incorporation. The stimulation index of PBMCs to the F99 peptide is shown (right Y axis). PBMCs from 2 chronic HCV patients (nos. 4 and 11), but from no HCC patients, showed proliferative responses to the F99 peptide.

malignant transformation status of patients. The positive ratio of F protein was greater in HCC patients (60.5%) than in chronic HCV patients (40.5%) (P<0.05). There were no significant differences between the two groups regarding F99-specific T-cell responses. No relationship between cytokine production and the proliferative response of PBMCs was observed. Seven of the 80 HCV patients with or without HCC displayed IFN-γ- and/or IL-10-secreting cells; only 2 of these 7 exhibited weak proliferation in response to the F99 peptide. Of these 7 patients with F99-specific T-cell responses, 4 (2 HCV patients with HCC and 2 without HCC) also had detectable anti-F antibodies in their sera.

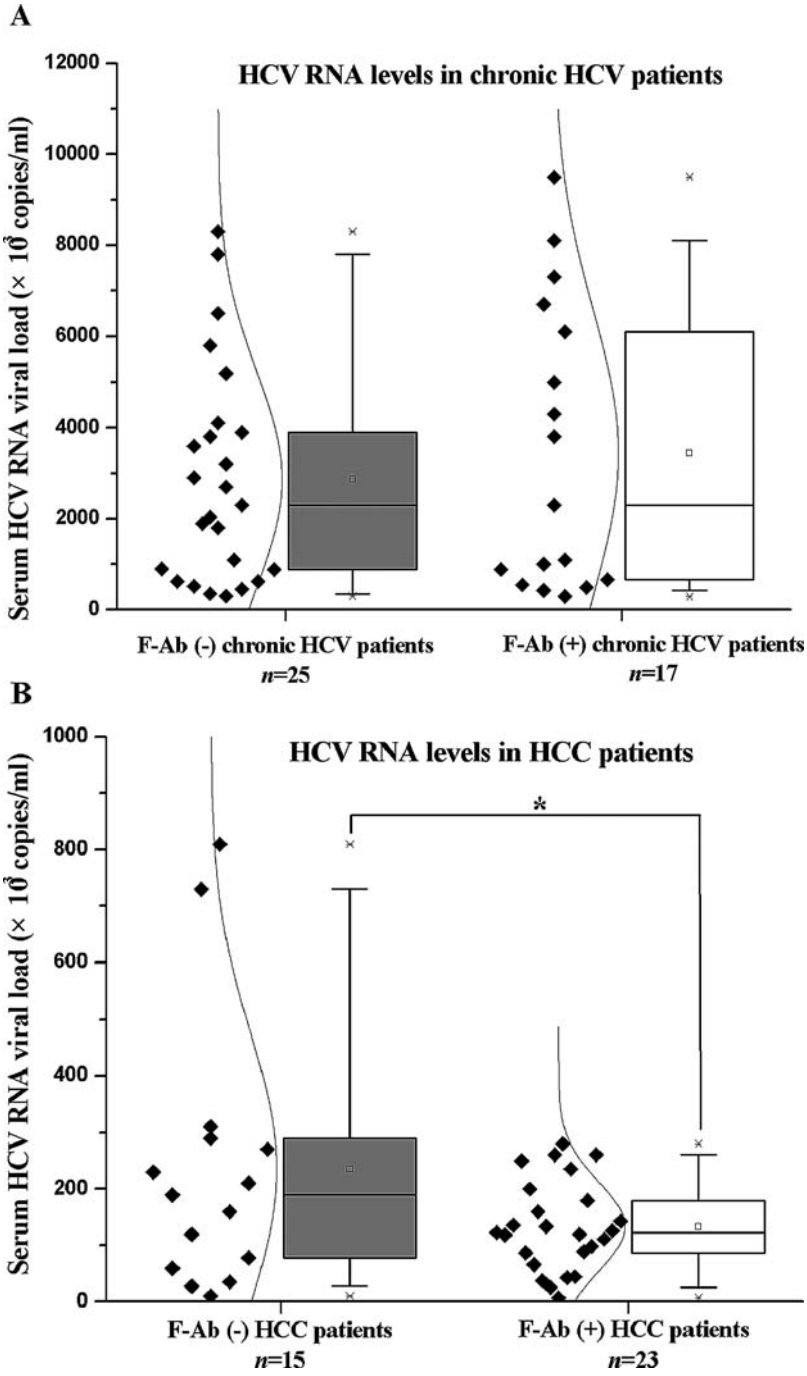

Figure 3. Comparison of serum HCV RNA viral load in F-antibody-positive or -negative chronic HCV patients with or without HCC. (A) HCV RNA serum levels in chronic HCV patients. (B) HCV RNA load levels in HCC cohorts. *P<0.05, F-antibody-positive HCC patients vs. F-antibody-negative HCC patients.

In short, no correlations were observed between the cellular and humoral immune responses specific to F protein in either HCV patients or HCC cohorts. These T- and B-cell responses were not restricted to HCV genotype 1b patients. Patients with the 1a and 2a genotypes also demonstrated immune responses.

Low serum HCV viral loads of chronic HCV and HCC patients. With synthetic HCV RNA as the template, the sensitivity of the real-time RT-PCR assay was ~10^3 HCV copies/ml. A wide linear relationship was observed (up to 10^7 copies/ml) between the number of PCR cycles needed to detect a fluorescent signal and the number of HCV copies. We measured the sera viral loads of all the patients with chronic HCV infection with or without HCC. As shown in Fig. 3A, 42 chronic HCV patients had viral loads ranging from $2.9x10^5$ to $9.7x10^6$ copies/ml. The viral loads of most HCC serum samples ranged from $7.1x10^3$ to $3.1x10^5$ copies/ml (Fig. 3B). In general, serum HCV RNA was ~10- to 100-fold less abundant in HCC than in chronic HCV cases. In only 2 HCC cases were the viral loads ($7.3x10^5$ and $8.1x10^5$ copies/ml) similar to those of the chronic HCV cases. As illustrated in Fig. 3B, the F-specific B-cell response in HCC patients was negatively correlated with the serum level of HCV RNA replication.

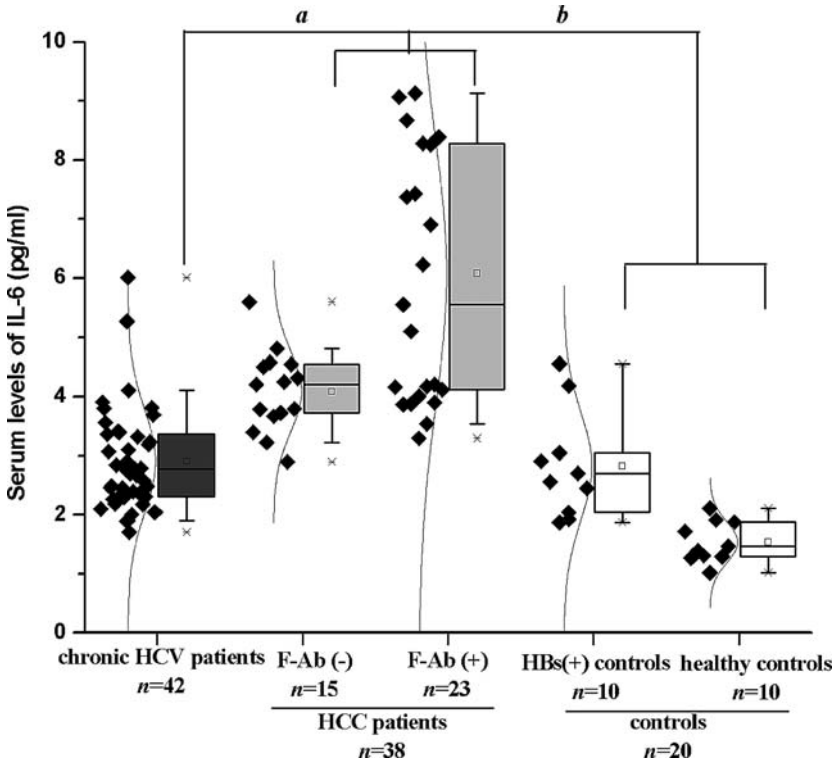

Figure 4. Serum IL-6 levels in patients with chronic HCV with or without HCC and in controls. [a]P<0.05, patients with HCC vs. patients with chronic HCV. [b]P<0.05, patients with HCC vs. controls.

IL-6 production positively correlates with the malignant status of HCV patients. In order to investigate the role of IL-6 in malignant transformation, we measured its serum levels using solid-phase Quantikine Immunoassays. Serum levels of IL-6 were measured in patients with HCV chronic infections and were significantly higher in these patients than in healthy subjects (Fig. 4). Furthermore, the analysis revealed that circulating IL-6 levels were significantly higher in patients with HCC than in patients with chronic HCV. As illustrated in Fig. 4, the median serum IL-6 level in patients with HCC was ~5.0 pg/ml. Among the 23 patients with HCC displaying anti-F antibodies, 11 patients (9 males, 2 females) had a serum IL-6 level higher than that of 15 anti-F sero-negative HCC patients. The IL-6 serum level of these 11 HCC patients ranged from ~6.0 to 10.0 pg/ml. However, the median IL-6 serum level of the other 15 anti-F seronegative HCC patients was ~3.0 pg/ml.

Discussion

Researchers have reported the synthesis of F protein encoded from alternative open reading frames (ORFs) from the HCV core genomic region. F protein is a short-lived endoplasmic reticulum-associated protein, and its stability can be maintained by the use of the proteasome inhibitor MG132 (22). The functional significance of F protein remains unclear. Although this protein is probably very unstable and/or expressed at very low levels, observations indicate that it has both conservative and diverse biological properties. F protein is highly conserved among different HCV isolates and is expressed during natural HCV infection. Walewski *et al* (23) observed mutation profiles in the genotype 1b core sequences and thus concluded that F protein is as conserved as conventional HCV proteins. Of the 125 positions of full-length F protein, 116 (85%) are invariant, while 185 of 191 positions (97%) of full-length core are invariant and 167 of 214 positions (78%) in full-length NS2 sequences are invariant. According to GenBank data, F protein can be expressed through a ribosomal frameshift within the core-coding sequence of all HCV genotypes. However, the primary structure of F protein shows high sequence diversity, and there is low homology between different genotypes isolated from patients. The high conservation of F protein indicates that it may play an important role in the HCV lifecycle. Meanwhile, the variability of its covalent structure may influence viral replication, pathogenicity or, alternatively, may regulate the curative effect of IFN.

Based on the above findings, in the present study we aimed to evaluate the correlation between F protein and the transformation from chronic inflammation to malignancy. We then observed differences in the pathological phenomena of F protein exhibited by chronic HCV patients and patients with HCV-associated HCC. Our data clearly indicate that: i) the presence of both T- and B-cell-mediated immune responses specific for such an antigen in subjects with chronic HCV and in HCC patients was identified; ii) in HCC patients, the detection of anti-F-specific antibodies and a low level of HCV RNA viral load was correlated, and there were significant differences in HCV viral loads between F-antibody-positive and F-antibody-negative HCC patients (P<0.05); iii) circulating IL-6 levels were significantly higher in patients with HCC than in those with chronic HCV. These increased serum IL-6 levels were positively correlated with the presence of anti-F-specific antibodies in some HCC patients.

Chronic inflammatory response can cause hepatic damage leading to carcinogenesis (24), and HCC represents a classic case of inflammation-linked cancer (25). In this study, we evaluated the existence of immune responses specific to F protein in cases of chronic HCV infection with or without HCC. The positive ratio of anti-F antibodies detected was higher in HCV patients with HCC than in chronic HCV patients ($P<0.05$). Of the 38 HCV patients with HCC, 23 (60.5%) presented anti-F-specific antibodies, while 17 of the 42 chronic HCV patients (40.5%) presented anti-F-specific antibodies. Furthermore, T lymphocytes specific to a 99-mer peptide derived from F protein were detected in 7.8% of HCC patients and 9.5% of chronic HCV patients. These cells were mainly characterized by their capacity to proliferate and produce IFN-γ and/or IL-10 cytokines. Of the 7 patients in the 2 cohorts presenting F-specific T-cell responses, 4 (2 HCV patients with HCC and 2 without HCC) also had detectable anti-F antibodies in their serum. These T-cell and B-cell responses were not restricted to HCV genotype 1b patients. Other genotypes, i.e., 1a and 2a patients, were also found to have immune responses. These results indicate considerable heterogeneity in detectable F-specific inflammatory responses.

The F protein does not appear to play a major role in viral RNA replication, as its absence did not abolish the replication of an HCV RNA replicon in Huh7 hepatoma cells (26,27). However, this does not mean that F protein has no correlation with HCV replication. It was reported that HCV replication complex-mediated RNA synthesis requires intact microtubule and actin polymerization (28). Therefore, interruption of normal microtubule formation likely results in decreased HCV replication. Tsao *et al* (29) found that F protein interacts with a cellular protein named prefoldin 2. Functional prefoldin delivers nascent actin and tubulin proteins to the eukaryotic cytosolic chaperonin for facilitated folding. The interaction of F protein with prefoldin 2 perturbs the normal function of the prefoldin complex, resulting in aberrant organization of the tubulin cytoskeleton and decreased HCV replication. Additionally, another research group reported *in vivo* results suggesting that mutations in the F region have a possible deleterious effect on HCV replication, and that F region stop mutant phenotypes were severely impaired for replication in Huh-7.5 cells (30). Our results also suggest that the detection of anti-F-specific antibodies and a relatively low level of HCV replication may be correlated in HCC patients. The protective T-cell-mediated immune response may play the same role in virus clearance *in vivo* as interferon therapy in chronic HCV patients. The majority of chronic HCV patients undergoing interferon therapy were found to have anti-F antibodies, indicating that interferon may stimulate HCV-infected cells to generate F protein, and that F protein might be a virulence factor that helps HCV survive adverse conditions, as previously reported (31). The molecular events involved likely contribute to viral persistence at a low level of replication, and this viral persistence may cause hepatic damage leading to carcinogenesis.

IL-6 is a proinflammatory cytokine that plays an important role in the hepatic response to infections or systemic inflammation. Serum IL-6 levels are considerably increased in situations of chronic liver inflammation, which may lead to the development of HCC (32). Studies with animal models have shown that transgenic mice expressing high levels of IL-6 develop hepatic nodular hyperplasia, suggesting that IL-6 could provide the primary stimulus for cell proliferation and is involved in the development of HCC (33). Another notable finding of this study is that increased serum IL-6 levels were positively correlated with the presence of anti-F-specific antibodies in some HCC patients. Serum levels of IL-6 were significantly elevated in HCC patients compared with chronic HCV patients, and high-risk male HCC patients had elevated IL-6 serum levels as compared with female patients (data not shown). This phenomenon may be explained by a recent study. To understand the mechanisms underlying gender disparity in HCC, Naugler *et al* (34) examined diethylnitrosamine (DEN)-induced hepatocarcinogenesis in male and female IL-6 knockout ($IL\text{-}6^{-/-}$) mice and wild-type (WT) controls. All male WT mice developed HCC, while only 13% of the WT females did. DEN administration caused greater increases in serum IL-6 concentrations in WT males than it did in WT females, while the ablation of IL-6 abolished gender differences in the hepatocarcinogenesis in mice, with less than 10% of the $IL\text{-}6^{-/-}$ males or females developing HCC.

In summary, we identified F protein, a core frameshifting protein, which may act as a double-edged sword in the pathogenesis of chronic hepatic inflammation leading to carcinogenesis. The protective T-cell-mediated immune response specific to F protein may participate in viral clearance and have anti-tumor effects. On the other hand, F protein may be a virulence factor that helps the HCV virus survive adverse conditions, with molecular events that likely contribute to viral persistence at a low level of replication. This may in turn cause hepatic damage leading to carcinogenesis. Further elucidation of the contradictory functional activities associated with F protein is required.

Acknowledgements

This study was supported in part by grants from the Natural Science Foundation of Jiangsu Province, P.R. China (BK2005004), and from the Tenth Five-Year Scientific Project of Jiangsu Province, P.R. China (BK2004011).

References

1. Poynard T, Yuen MF, Ratziu V and Lai CL: Viral hepatitis C. Lancet 362: 2095-2100, 2003.
2. World Health Organization: Hepatitis C: Global prevalence. Weekly Epidemiol Rec 72: 341-344, 1997.
3. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW and Houghton M: Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244: 359-362, 1989.
4. Doi H, Apichartpiyakul C, Ohba KI, Mizokami M and Hotta H: Hepatitis C virus (HCV) subtype prevalence in Chiang Mai, Thailand, and identification of novel subtypes of HCV major type 6. J Clin Microbiol 34: 569-574, 1996.
5. Mellor J, Holmes EC, Jarvis LM, Yap PL and Simmonds P: Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. J Gen Virol 76: 2493-2507, 1995.
6. Bruno S, Silini E, Crosignani A, *et al*: Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. Hepatology 25: 754-758, 1997.
7. Nousbaum JB, Pol S, Nalpas B, Landais P, Berthelot P and Bréchot C: Hepatitis C virus type 1b (II) infection in France and Italy. Ann Intern Med 122: 161-168, 1995.

8. Marusawa H, Hijikata M, Chiba T and Shimotohno K: Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J Virol 73: 4713-4720, 1999.
9. Ray RB, Meyer K and Ray R: Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 226: 176-182, 1996.
10. Chang J, Yang SH, Cho YG, Hwang SB, Hahn YS and Sung YC: Hepatitis C virus core from two different genotypes has an oncogenic potential but is not sufficient for transforming primary rat embryo fibroblasts in cooperation with the H-ras oncogene. J Virol 72: 3060-3065, 1998.
11. Ray RB, Lagging LM, Meyer K and Ray R: Hepatitis C virus core protein cooperates with ras and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J Virol 70: 4438-4443, 1996.
12. Moriya K, Fujie H, Shintani Y, *et al*: The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 4: 1065-1067, 1998.
13. Varaklioti A, Vassilaki N, Georgopoulou U and Mavromara P: Alternate translation occurs within the core coding region of the hepatitis C viral genome. J Biol Chem 277: 17713-17721, 2002.
14. Walewski JL, Keller TR, Stump DD and Branch AD: Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. RNA 7: 710-721, 2001.
15. Xu Z, Choi J, Yen TS, *et al*: Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. EMBO J 20: 3840-3848, 2001.
16. Boulant S, Becchi M, Penin F and Lavergne JP: Unusual multiple recoding events leading to alternative forms of hepatitis C virus core protein from genotype 1b. J Biol Chem 278: 45785-45792, 2003.
17. Basu A, Steele R, Ray R and Ray RB: Functional properties of a 16 kDa protein translated from an alternative open reading frame of the core-encoding genomic region of hepatitis C virus. J Gen Virol 85: 2299-2306, 2004.
18. Ray RB, Steele R, Meyer K and Ray R: Hepatitis C virus core protein represses p21WAF1/Cip1/Sid1 promoter activity. Gene 208: 331-336, 1998.
19. Fiorucci M, Boulant S, Fournillier A, *et al*: Expression of the alternative reading frame protein of hepatitis C virus induces cytokines involved in hepatic injuries. J Gen Virol 88: 1149-1162, 2007.
20. Kong J, Diao Z, Deng X, Zhong H, Yao W and Hu X: Anti-tumor effects of immunotherapeutic peptide on the treatment of hepatocellular carcinoma with HBc carrier. Oncol Rep 18: 279-285, 2007.
21. Hu KQ, Yu CH and Vierling JM: One-step RNA polymerase chain reaction for detection of hepatitis C virus RNA. Hepatology 18: 270-274, 1993.
22. Roussel J, Pillez A, Montpellier C, Duverlie G, Cahour A, Dubuisson J and Wychowski C: Characterization of the expression of the hepatitis C virus F protein. J Gen Virol 84: 1751-1759, 2003.
23. Walewski JL, Gutierrez JA, Branch-Elliman W, *et al*: Mutation master: profiles of substitutions in hepatitis C virus RNA of the core, alternate reading frame, and NS2 coding regions. RNA 8: 557-571, 2002.
24. Balkwill F and Mantovani A: Inflammation and cancer: back to Virchow? Lancet 357: 539-545, 2001.
25. Karin M and Greten FR: NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5: 749-759, 2005.
26. Lohmann V, Körner F, Koch J, Herian U, Theilmann L and Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285: 110-113, 1999.
27. Blight KJ, Kolykhalov AA and Rice CM: Efficient initiation of HCV RNA replication in cell culture. Science 290: 1972-1974, 2000.
28. Bost AG, Venable D, Liu L and Heinz BA: Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. J Virol 77: 4401-4408, 2003.
29. Tsao ML, Chao CH and Yeh CT: Interaction of hepatitis C virus F protein with prefoldin 2 perturbs tubulin cytoskeleton organization. Biochem Biophys Res Commun 348: 271-277, 2006.
30. McMullan LK, Grakoui A, Evans MJ, *et al*: Evidence for a functional RNA element in the hepatitis C virus core gene. Proc Natl Acad Sci USA 104: 2879-2884, 2007.
31. Branch, AD, Walewski, JL, Gutierrez, JA, *et al*: HCV alternate reading frame proteins (ARFPs) may be virulence factors that help the virus survive adverse conditions. Hepatology 38: 468A-469A, 2003.
32. Abiru S, Migita K, Maeda Y, *et al*: Serum cytokine and soluble cytokine receptor levels in patients with non-alcoholic steatohepatitis. Liver Int 26: 39-45, 2006.
33. Maione D, Di Carlo E, Li W, *et al*: Coexpression of IL-6 and soluble IL-6R causes nodular regenerative hyperplasia and adenomas of the liver. EMBO J 17: 5588-5597, 1998.
34. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM and Karin M: Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 317: 121-124, 2007.