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The novel immunoregulatory molecule FGL2: A potential biomarker for severity of chronic hepatitis C virus infection

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Background & Aims: This report describes the use of a novel sensitive and specific ELISA for the measurement of human fibrinogen-like protein 2 (FGL2/fibroleukin), a novel effector of natural regulatory T (Treg) cells, to predict the course of chronic hepatitis C viral infection (HCV).

Methods: Plasma levels of FGL2 were measured in HCV patients and compared to healthy controls and to patients with alcoholic liver disease.

Results: FGL2 levels were significantly higher in HCV patients (84.3 ± 89.1 ng/ml, $n = 80$) compared to healthy controls (36.4 ± 21.9 ng/ml, $n = 30$, $p < 0.001$), to a subset of patients who cleared HCV following anti-viral treatment (16.6 ± 19.7 ng/ml, $n = 32$, $p < 0.001$), and to patients with inactive alcoholic liver disease (18.8 ± 17.4 ng/ml, $n = 24$, $p < 0.001$). Among HCV patients, plasma levels of FGL2 correlated significantly with the stage of fibrosis ($p = 0.001$) and were significantly higher in patients with cirrhosis (164.1 + 121.8 ng/ml, $n = 60$) compared to non-cirrhotics (57.7 ± 52.8 ng/ml, $n = 20$, $p = 0.001$). Genotype 1 patients had significantly higher levels of FGL2 (98.1 ± 100.3 ng/ml, $n = 60$) compared to patients with genotype 2/3 (41.5 ± 38.6 ng/ml, $n = 20$, $p = 0.0008$). Patients with genotype 2/3 had FGL2 levels similar to healthy controls (41.5 ± 38.6 vs. 36.41 ± 21.9 ng/ml, p = ns). Infiltrating lymphocytes in liver biopsies of HCV patients were positive for either FGL2 or FoxP3 (a marker of Treg cells) or expressed both markers.

Conclusions: This report documents the development of a sensitive ELISA for measurement of plasma levels of FGL2 an effector Treg cells, which correlates with the severity of HCV infection.

Keywords: FGL2; HCV; Treg cells.

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Abbreviations: DC, dendritic cells; FGL2, fibrinogen-like protein 2; HBV, hepatitis B virus; HCV, hepatitis C virus; MHV-3, murine hepatitis virus strain 3; SVR, sustained virological response; Treg, $CD4^+CD25^+$ regulatory T cells.

Introduction

Fibrinogen-like protein 2 FGL2 is a member of the fibrinogen-like family of proteins, which includes tenascin and angiopoietin [1]. Our laboratory has extensively studied the role of FGL2 in the pathogenesis of murine viral hepatitis (MHV-3), an experimental model of fulminant viral hepatitis [2,3]. Susceptible mice (BALB/cJ) develop a fatal hepatitis within 3–5 days post-infection, whereas a resistant strain of mice (A/J) survives and clears the virus within 10–14 days of infection [3]. We previously reported that in mice infected with MHV-3, plasma levels of FGL2 pre- and post-MHV-3 infection were predictive of susceptibility and severity of disease [2,3]. Treatment with antibody to FGL2, fully protected susceptible BALB/cJ mice from the lethality of MHV-3. It has also been shown that FGL2 accounts for the fibrin deposition seen in both acute and chronic hepatitis in humans [4,5].

We and others reported that FGL2 is an effector molecule of $CD4^+CD25^+$ $FoxP3^+$ regulatory T cells (Treg), and has immunoregulatory activity [6–11]. Shevach reported that FGL2 is an important Treg product that primarily down-regulates dendritic cell (DC) function [12]. Treg have been implicated in suppressing T cell immune responses to viral infections including hepatitis B virus (HBV) [13,14] and hepatitis C virus (HCV) [15,16]. Patients with chronic HBV and HCV infection have increased numbers of Treg both in the blood and within the liver, which have been reported to impair immune responsiveness to these viruses [13,14,17]. In support for a role of Treg in the pathogenesis of HBV is the observation that depletion of Treg in a mouse model of chronic HBV infection results in enhanced anti-viral $CD8^+$ T cell responses [18].

Examining the role of Treg production of FGL2 in the pathogenesis of MHV-3 hepatitis, we showed that adoptive transfer of wild-type Treg cells into resistant *fgl2*$^{-/-}$ mice increased the mortality following MHV-3 infection [3]. The MHV-3 study demonstrates that FGL2 is an important effector cytokine of Treg that contributes to susceptibility to MHV-3-induced fulminant hepatitis.

Based on the mouse data, FGL2 may well play a role in chronic viral hepatitis in human. The further exploration of this role would be greatly facilitated by the availability of a sensitive assay for FGL2 in human samples. The present study was undertaken to

assess the potential usefulness of measurements of plasma levels of FGL2 using an enzyme-linked immunosorbent assay (ELISA) in monitoring outcomes in patients with HCV infection.

Materials and methods

Reagents

Monoclonal mouse anti-human FGL2 and polyclonal rabbit anti-human FGL2 were all produced in our laboratory as previously described [10,19].

Generation of recombinant FGL2

Recombinant human FGL2 was expressed in the mammalian Chinese Hamster Ovary (CHO) cell system as previously described [19]. Recombinant FGL2 was purified using a protein A resin column (PALL Corporation, Quebec, Canada). Purity of recombinant FGL2 was confirmed by SDS–PAGE and Western blot analysis as previously described [10,19].

Blood preparation

Whole blood was collected from normal healthy volunteers and patients with biopsy proven chronic hepatitis C or alcohol induced cirrhosis, and centrifuged at 1500*g* for 10 min at 21 °C in heparinized tubes. Plasma was subsequently collected and frozen at −80 °C until analysis.

Patient population

All human studies were conducted according to the Declaration of Helsinki principles and approved by the Human Ethics Committee of the University Health Network. Thirty healthy controls volunteers (16 females, 14 males, aged 18–56 years without any illness in the two weeks prior to enrolment) were recruited from the laboratory/hospital staff of the Multi Organ Transplant Program at the University Health Network. Eighty patients with chronic HCV infection (14 females, 66 males aged 25–65 years) were recruited from outpatient clinics of the Multi Organ Transplant Program, at the University Health Network. Inclusion criteria was HCV RNA positivity by PCR (Amplicor, Roche, Basel, Switzerland), regardless of the severity of the liver disease. Patients on anti-viral therapy, HCV infected patients with a current alcohol misuse, and co-infections with HBV or HIV, were excluded. Twenty-four patients (3 female, 21 male aged 45–69) with alcohol induced liver cirrhosis were also recruited from outpatient clinics of the Multi Organ Transplant Program, at the University Health Network as controls.

Sandwich ELISA for FGL2

A standard sandwich ELISA method was developed for measurement of plasma levels of FGL2 [20]. Briefly, 96-well microplates (Costar EIA/RIA, Corning Inc., Corning, NY) were coated at 4 °C overnight with 50 ng of monoclonal mouse anti-human FGL2 antibody. After washing in tris-buffer saline (TBS), the plates were incubated with 200 μl/well of Superblock solution (Pierce Biotechnology Inc., Rockford, IL) for one hour. Fifty μl of recombinant FGL2 or patient plasma (diluted 1/10 with 2.5% BSA) were added to each well and incubated for 1 h followed by incubation for 1 h with 50 μl of a polyclonal rabbit anti-human FGL2 antibody (1 μg/ml in 1% BSA in PBS). The plate was then washed and a goat anti-rabbit IgG HRP (horseradish peroxidase), (diluted 1/6000) was added to each well. Subsequently, 100 μl/well of tetramethyl benzidine (Sigma Aldrich, Oakville, ON) were added at room temperature for 5 min and the reaction was stopped by adding 100 μl of 2 M H_2SO_4. Absorbance was measured at an OD of 450 nm using an Appliskan multimode microplate reader (Thermo Fisher Scientific Inc., Waltham, MA).

Immunostaining for FGL2

Formalin-fixed, paraffin-embedded liver tissue from patients with chronic hepatitis C was sectioned and examined for expression of FGL2 and FoxP3 by immunohistochemistry [14]. Tissue sections were stained overnight at room temperature with a mouse monoclonal antibody against FGL2. FGL2 staining was detected by horse radish peroxidase (HRP) staining, whereas FoxP3 was detected by staining with tetrazolium blue.

Determination of the severity of HCV disease

Liver biopsies were available in 71 out of 80 patients with chronic HCV infection. All biopsies were reviewed by a pathologist who was blinded to both the patient's clinical outcome and levels of FGL2. Necro-inflammatory activity and fibrosis stage were scored according to Metavir [21].

Statistical analysis

Results are reported as mean and standard deviation (SD) unless otherwise specified. One-way or two-way ANOVA followed by the Bonferroni test for post hoc analysis were used for group comparison. Linear correlation or Spearman rank correlation was used as appropriate. A p value <0.05 was considered significant.

Results

Sensitivity, specificity and reproducibility of the ELISA for measurement of FGL2

To assess the sensitivity of the assay, serial dilutions of recombinant FGL2 were used to establish a standard curve. A representative standard dose–response curve of the ELISA is presented in Fig. 1 (R^2 = 0.98). FGL2 was detectable over a wide range of concentration (0.3–125 ng/ml). The specificity of the assay was confirmed by absence of cross-reactivity with fibrinogen. The reproducibility of the assay was assessed comparing values of undiluted plasma with the same plasma samples diluted by 1/10, 1/20, 1/50, and 1/100 in BSA. The dilution studies using plasma showed that for optimal results, a minimum dilution of 1/10 of the plasma sample was necessary as has been reported for other biologic assays using plasma. The reproducibility of the assay was further confirmed with serial measurements of standards and patient samples. The mean concentrations with standard deviation and coefficients of variation within the same assay (intra-assay) and between the assays (inter-assay) are shown in Table 1A (standard) and B (samples).

Plasma levels of FGL2 in healthy human controls

To determine levels of plasma FGL2 in humans, 10 ml of plasma from 30 healthy individuals who served as controls were col-

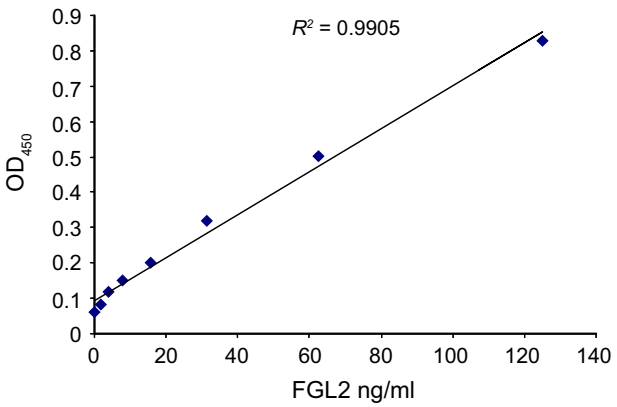

Fig. 1. FGL2 ELISA standard curve. The sensitivity of the ELISA was assessed by measuring various concentrations of recombinant FGL2. The assay shows a strong linear correlation between absorbance at 450 nm and concentration of FGL2 (R^2 = 0.99). The detection range was between 0.3 and 125 ng/ml.

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lected and analyzed. The baseline characteristics of these human controls are presented in Table 2. Plasma levels of FGL2 were measured by ELISA. Mean plasma levels of FGL2 were 36.41 ± 21.9 ng/ml. No significant difference in mean plasma levels of FGL2 was observed between males vs. females (38.8 ± 23.4 ng/ml vs. 34.2 ± 22.0 ng/ml, respectively) or Caucasians vs. Asians (37.9 ± 22.9 ng/ml vs. 35.3 ± 22.4 ng/ml, respectively) (Fig. 2A and B).

Plasma levels of FGL2 in patients with chronic HCV infection

The plasma levels of FGL2 were measured in 80 patients with biopsy proven chronic HCV infection of whom 20 had cirrhosis, and in 24 patients with alcohol induced cirrhosis. Baseline characteristics of these patients are presented in Table 2. Mean plasma levels of FGL2 were significantly higher in patients with chronic HCV infection (84.3 ± 89.1 ng/ml, $n = 80$) compared to healthy controls (36.41 ± 21.9 ng/ml, $n = 30$, $p < 0.001$) or to patients with alcoholic cirrhosis (18.8 ± 17.4 ng/ml, $n = 24$, $p < 0.001$), and HCV patients who had received anti-viral therapy and reached a sustained virological response (SVR) (16.6 ± 19.7 ng/ml, $n = 32$, $p < 0.001$), (Fig. 3A). Among HCV patients, mean plasma levels of FGL2 were significantly higher in patients with cirrhosis (164.1 ± 121.8 ng/ml, $n = 20$) compared to non-cirrhotics (57.7 ± 52.8 ng/ml, $n = 60$, $p = 0.001$) (Fig. 3B).

Correlation of FGL2 plasma levels with routine laboratory parameters in chronic HCV

We first examined the relationship of plasma levels of FGL2 with routine laboratory parameters associated with severity of the disease. Levels of FGL2 correlated positively and significantly with serum levels of aspartate transaminase (AST) ($Rs = 0.50$,

Table 1. Reproducibility of the ELISA assay. Several replicates of different (A) standard dilutions or (B) plasma samples were performed. The mean concentrations with standard deviation and coefficients of variation within the same assay (intra-assay) and between the assays (inter-assay) are shown below.

	n^a	FGL2 (ng/ml)		% CV
		Mean	STD	
(A)				
Intra-assay variation	4	60.6	0.89	1.4
	4	15.6	0.62	3.9
	4	3.6	0.26	7.2
Inter-assay variation	4	54.3	1.65	3
	4	6.7	0.29	4.3
	4	1.8	0.13	7.2
(B)				
Intra-assay variation	3	39.07	1.11	2
	3	18.2	0.75	4
	3	10.01	0.34	3
Inter-assay variation	2	37.9	1.65	4
	2	17.33	1.23	7
	2	10.02	0.1	1

[a] Number of samples measured in triplicate.

Table 2. Baseline characteristics of healthy volunteers and patients with chronic HCV infection.

	Healthy controls $n^a = 30$	HCV patients $n = 80$	Alcoholic patients $n = 24$
Age mean (range)	32 (18–56)	60 (25–65)	65 (45–69)
Female *n* (%)	14 (46)	14 (17)	6 (25)
Caucasian *n* (%)	18 (60)	58 (72.5)	23 (95)
Asian *n* (%)	12 (40)	20 (25)	1 (5)
African-American *n* (%)	NA	2 (2.5)	NA
Genotype 1 *n*	NA	60	NA
Genotype 2/3 *n*	NA	20	NA

[a] *n* = number of patients.

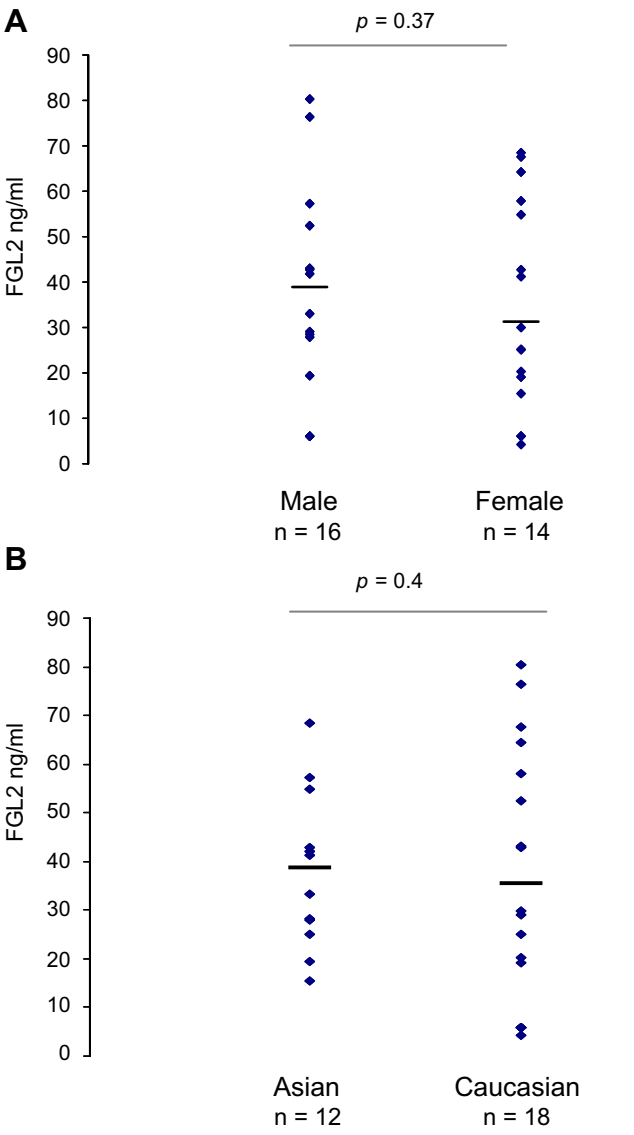

Fig. 2. Mean plasma levels of FGL2 in healthy controls according to gender and ethnicity. Ten ml of heparinized blood was collected from healthy controls ($n = 30$) and analyzed for presence of FGL2. Levels of FGL2 did not differ significantly according to gender (A) (male vs. female: 38.8 ± 23.4 ng/ml vs. 34.2 ± 22.0 ng/ml, $p = 0.5$), or ethnicity (B) (Caucasian vs. Asian: 37.9 ± 22.9 ng/ml vs. 35.3 ± 22.4 ng/ml, $p = 0.2$).

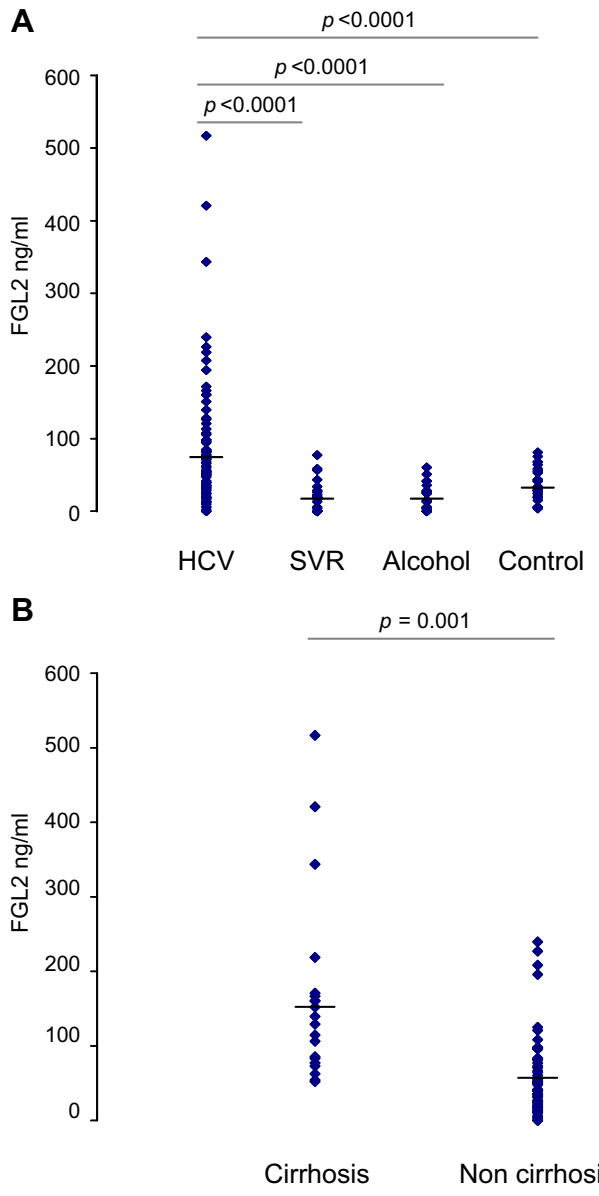

Fig. 3. Mean plasma levels of FGL2 in patients with chronic HCV infection. (A) Ten ml of heparinized blood was collected from 80 patients with chronic HCV infection, which had not received anti-viral therapy. Mean plasma levels of FGL2 in these patients were compared to 30 healthy controls, 24 patients with inactive alcoholic cirrhosis, and 32 patients with chronic HCV who cleared the virus following successful anti-viral therapy (sustained virological responders, SVR). Mean plasma levels of FGL2 were significantly higher in patients with chronic HCV infection (84.3 ± 89.1 ng/ml, $n = 80$) compared to healthy controls (36.41 ± 21.9 ng/ml, $n = 30$, $p < 0.001$), patients with alcoholic cirrhosis (18.8 ± 17.4 ng/ml, $n = 24$, $p < 0.001$), and patients with SVR (16.6 ± 19.7 ng/ml, $n = 32$, $p < 0.001$). (B) Among HCV patients, mean plasma levels of FGL2 were significantly higher in patients with cirrhosis (164.1 ± 121.8 ng/ml, $n = 20$) compared to non-cirrhotics (57.7 ± 52.8 ng/ml, $n = 60$, $p = 0.001$).

$p = 0.0001$) (Fig. 4A), prothrombin time (INR) ($Rs = 0.46$, $p = 0.0001$) (Fig. 4B), and bilirubin ($Rs = 0.41$ $p = 0.001$) (Fig. 4C) but only weakly with levels of alanine transaminase (ALT) ($Rs = 0.21$, p = NS) (Fig. 4A). There was a negative correlation of levels of FGL2 with serum albumin ($Rs = -0.32$, p = NS) (Fig. 4D).

Correlation of FGL2 plasma levels with histologic grade and stage of chronic HCV

Fibrosis stage is a well known marker for disease severity and indication for anti-viral treatment. Liver biopsies performed within 12 months of collecting blood for FGL2 determination were available for assessment of fibrosis stage (Metavir) in 71 out of the 80 patients with HCV infection. FGL2 plasma levels were significantly higher in patients with advanced fibrosis (stage 3–4, $n = 22$) compared to the patients with mild fibrosis (stage <1, $n = 35$) 148.3 ± 143.0 vs. 44.4 ± 52.4 ng/ml, $p = 0.001$ and moderate fibrosis (stage 2, $n = 19$) 148.3 ± 143.0 vs. 72.9 ± 65.9 ng/ml, $p = 0.01$ (Fig. 5A). Furthermore, HCV patients with stage 2 fibrosis had significantly higher levels of FGL2 compared to patients with stage <1 (72.9 ± 65.9 vs. 44.4 ± 52.4 ng/ml, $p = 0.05$). Similarly, analysis of plasma levels of FGL2 according to the activity grade (METAVIR) (Fig. 5B) showed a significant difference between patients with higher inflammation as assessed by activity grade (grade >2, $n = 26$), compared to those with lower grade of inflammation (grade <2) (74.4 ± 69.8 vs. 36.8 + 37.1 ng/ml, $n = 46$, $p = 0.01$).

Correlation of FGL2 plasma levels with virological parameters of HCV infection

Plasma levels of FGL2 correlated positively with viral titers (Fig. 6A). Analysis of the plasma levels of FGL2 according to the HCV genotype showed that genotype 1 patients, known to have lower response rates to anti-viral therapy, had significantly higher levels of plasma FGL2 compared to genotype 2/3 patients (98.0 ± 100.3 vs. 41.5 ± 38.6 ng/ml, $p = 0.0008$) (Fig. 6B). Furthermore, the mean plasma levels of FGL2 in a subset of HCV patients ($n = 32$), who had cleared the virus following treatment with pegylated interferon (alpha 2b 1.5 μg/kg body weight. s.c. once weekly or pegylated interferon alpha 2b 180 μg s.c. once weekly) and ribavirin (800 mg [body weight <75 kg] or 1000 mg [body weight ⩾75 kg] p.o. in two divided doses) and developed a sustained virological response (SVR) for more than 6 months post-therapy, were significantly lower compared to patients with active disease (16.6 ± 19.7 vs. 84.3 ± 89.1 ng/ml, $n = 80$, $p < 0.001$) (Fig. 3A).

FGL2 expression in the liver of patients with chronic HCV infection

To evaluate the expression of FGL2 in the liver, immunostaining for FGL2 protein and the transcription factor FoxP3 was performed as described above. Typical features of HCV histopathology were observed including a heavy infiltration of lymphocytes and other cells including macrophages in both the portal and periportal areas. Immunostaining for FGL2 and FoxP3 was observed within infiltrating cells in the liver of patients with chronic HCV (Fig. 7A and B). FoxP3/FGL2 co-staining showed that only a small proportion of lymphocytes stained for both FGL2 and FoxP3, while the majority of $FoxP3^+$ cells were FGL2 negative and many FGL2 positive cells were Fox P3 negative (Fig. 7C).

Discussion

In this study, we describe the development of a reproducible, sensitive, and specific ELISA for the measurement of plasma lev-

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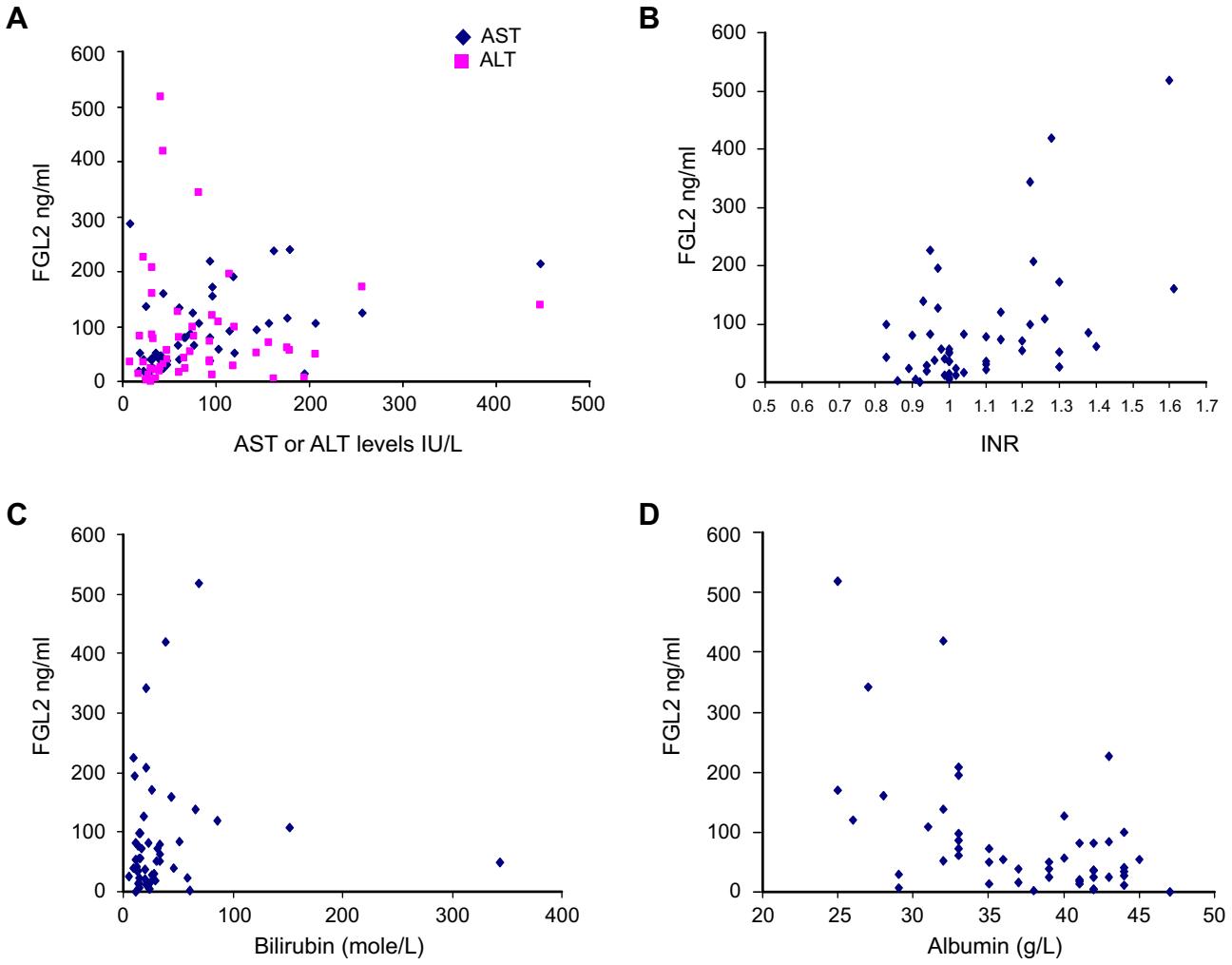

Fig. 4. Correlation between FGL2 levels and levels of aspartate transaminase (AST), alanine transaminase (ALT), coagulation (INR), and bilirubin. A positive correlation was found between plasma levels of FGL2 and AST, ALT (A), coagulation (INR) (B), and bilirubin (C) whereas a negative correlation was observed between levels of FGL2 and albumin (D).

els of FGL2, which correlates with the severity of HCV disease. The ELISA was capable of measuring plasma levels of FGL2 over a wide range, with excellent reproducibility. We demonstrated that patients with HCV infection had significantly higher plasma levels of FGL2 than healthy controls and patients with inactive end stage alcoholic cirrhosis. Furthermore, plasma levels of FGL2 significantly correlated with the stage of fibrosis and the degree of necro-inflammatory activity on liver biopsies. HCV patients with cirrhosis had significantly higher levels of FGL2 compared to patients without cirrhosis, and patients with inactive alcoholic cirrhosis, suggesting that the presence of cirrhosis or advanced fibrosis alone did not account for the high levels of FGL2 in the HCV patients but rather was related to the activity and severity of HCV infection. Patients with genotype 1, known to have a poorer response to treatment with anti-viral therapy, had significantly higher levels of FGL2 than patients with genotype 2 and 3. Furthermore, in HCV patients, who cleared the virus following anti-viral therapy, and developed a sustained virological response, levels of FGL2 was significantly lower than patients with active chronic HCV. Collectively, these results suggest that levels of FGL2 in plasma of HCV patients correlate with and may therefore serve as an easily accessible biomarker for the severity of the disease.

FoxP3$^+$ CD4$^+$CD25$^+$ natural Treg cells (Treg) are known to be actively engaged in the control of a variety of physiological and pathological immune responses [22]. Evidence that Treg may underlie the attenuated HCV specific T cell responses in chronically infected patients comes from the finding that Treg isolated from the peripheral blood of HCV patients suppress HCV specific responses *in vitro* [15,16]. Furthermore, Treg are elevated in the circulating blood of HCV patients and not in those patients who clear the virus [17]. A number of other studies have suggested that depletion or reduction of Treg leads to enhanced immune responses against various infectious pathogens including HBV and HCV [14,17,23].

We and others have recently demonstrated that FGL2 is highly expressed by Treg and contributes to their suppressive activity [6–11]. *In vitro* studies by Chan et al. from our laboratory provided the first evidence that FGL2 has immunomodulatory activity [10]. Recombinant FGL2, inhibited T cell proliferation in

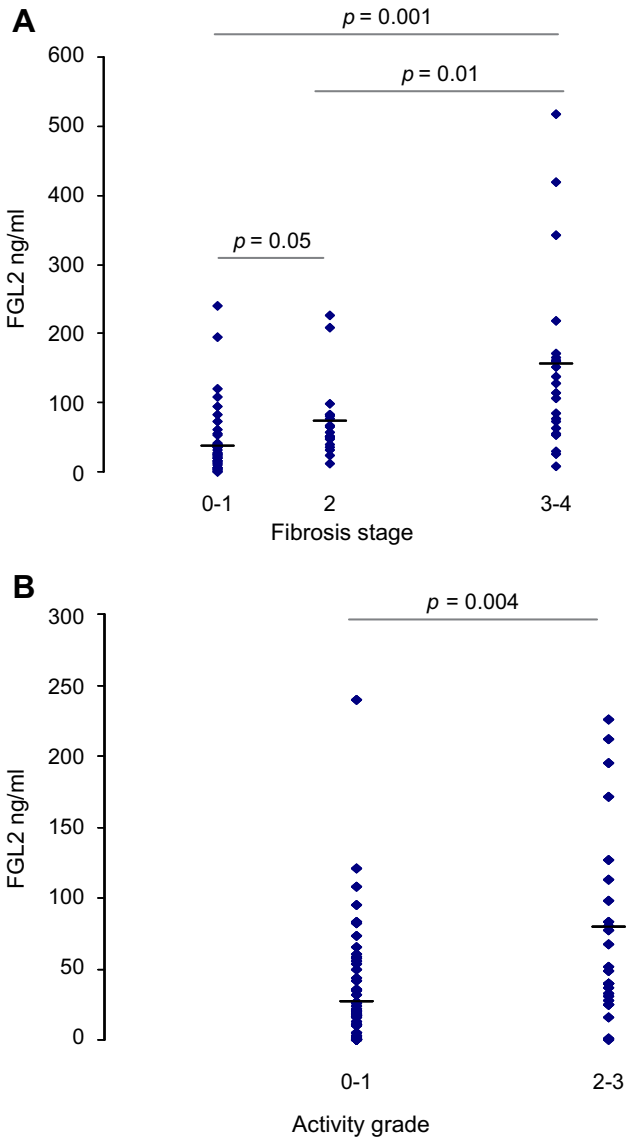

Fig. 5. Mean plasma levels of FGL2 in patients with chronic HCV infection according to the stage of fibrosis. (A) Plasma levels of FGL2 were significantly higher in HCV patients with advanced fibrosis (stage 3–4, $n = 26$) compared to the patients with lower stage of fibrosis (148.3 + 143.0 vs. 44.4 ± 52.4 ng/ml for stage <1, $n = 33$, $p = 0.001$) and (148.3 ± 143.0 vs. 72.9 ± 65.9 ng/ml for stage 2, $n = 12$, $p = 0.01$). (B) Plasma levels of FGL2 is also significantly higher in patients with higher activity grade (grade >2, $n = 26$), compared to those with lower activity grade (grade <2, $n = 46$), (74.4 ± 69.8 vs. 36.8 ± 37.1 ng/ml, $p = 0.01$).

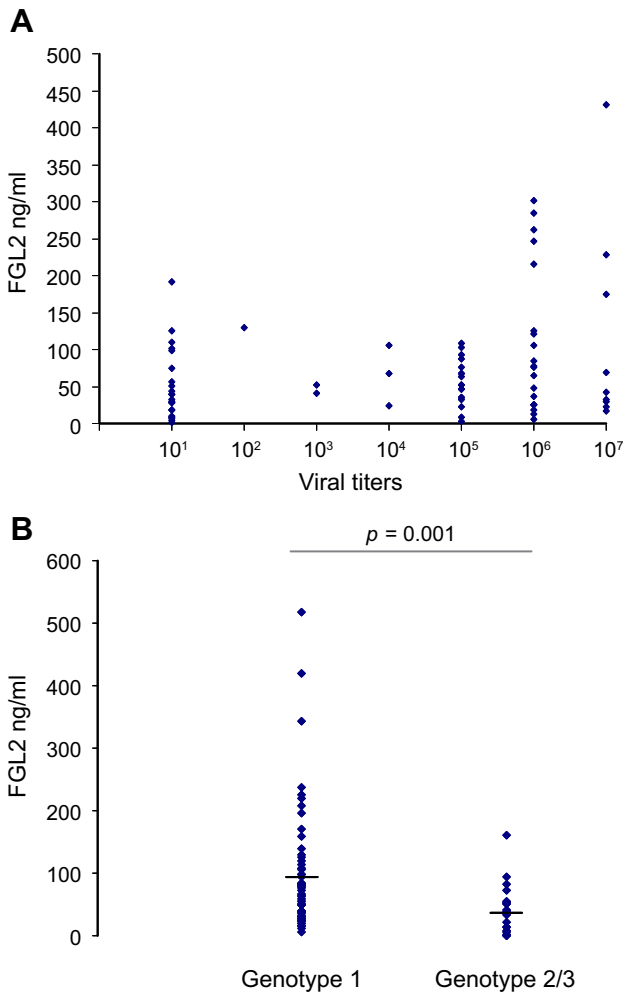

Fig. 6. Mean plasma levels of FGL2 in patients with chronic HCV infection according virological parameters. (A) A positive correlation was found between plasma levels of FGL2 and viral titers. (B) Plasma levels of FGL2 were significantly higher in patients with genotype 1 ($n = 60$) compared to genotype 2/3 ($n = 20$) infection (12.4 ± 12.3 vs. 5.5 ± 5.1 ng/ml, $p = 0.008$).

response to alloantigens, anti-CD3/anti-CD28 monoclonal antibody and Con A in a dose-dependent manner, whereas it had no direct inhibitory effect on CTL activity [10]. More recently, we reported that deletion of FGL2 resulted in impaired Treg activity, which was associated with increased immune reactivity and development of autoimmune kidney disease [11].

Our laboratory has extensively studied the role of FGL2 in a model of fulminant hepatitis caused by MHV-3 [3]. Several lines of evidence suggest that production of FGL2 contributes to the lethality of MHV-3 induced hepatitis. First, only in susceptible animals was there an induction of FGL2 by MHV-3 [3]. Second, serial measurements of FGL2 levels in mice, which are susceptible or resistant to MHV-3, both pre- and post-infection showed a significant difference between animals which are susceptible and resistant to viral infection [2,3]. The difference in the levels of FGL2 both pre- and post-infection correlated well with susceptibility and disease progression.

Levels of FGL2 in healthy human volunteers were not markedly influenced by ethnicity or gender. Similarly, no significant differences in levels of FGL2 were observed between different age groups or during a circadian cycle (data not shown). In contrast, our data demonstrate that FGL2 levels were significantly higher in patients with chronic HCV and correlated with disease severity as indicated by the stage of liver fibrosis. HCV patients with advanced fibrosis (stage 3–4) had a significantly higher levels of plasma FGL2 compared to HCV patients with mild (stage <1) and moderate fibrosis stages (stage 2). Similarly, plasma

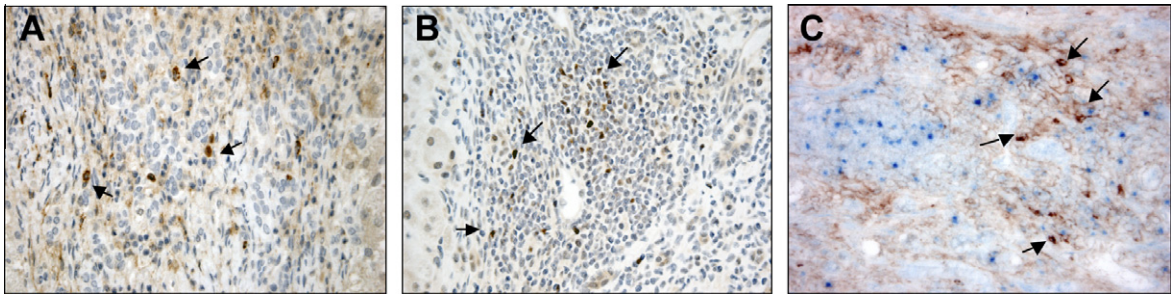

Fig. 7. FGL2 and FoxP3 expression in the liver of patients with chronic HCV infection. (A) Staining for FGL2. Heavy, predominantly lymphocytic infiltrates were seen within the portal and periportal areas of the liver. Note that many infiltrating cells stained positively for FGL2 (brown stain) which was expressed both within the cytoplasm and at the cell surface (arrows). (B) FoxP3 staining. Similar infiltrates as in (A), but showing nuclear FoxP3 staining (brown stain, arrows). (C) Co-localization of FoxP3 and FGL2 was seen in some (arrow) but not all cells staining with either FoxP3 (Tetrazolium blue, nuclear) or FGL2 (brown, cytoplasmic/membranous). Original magnification, (A–C) = 200×.

levels of FGL2 were significantly higher in patients with stage 2 fibrosis compared to the patients with none or mild fibrosis (stage <1). Furthermore, analysis of plasma levels of FGL2 according to the activity grade as a marker of necroinflammation showed a significant difference between patients with higher necroinflammation activity (grade >2) compared to those with lower necro-inflammatory activity (grade <2). Collectively, these results indicate that plasma levels of FGL2 may be a useful non-invasive biomarker for the assessment of disease severity in patients with chronic HCV infection.

Of interest, it was observed that plasma FGL2 levels in patients with chronic HCV genotype 1 infection were not uniform, but rather spanned a wide range of values. Thus, some genotype 1 patients had near normal plasma levels of FGL2 (<100 ng/ml), whereas others had markedly elevated levels (>150 ng/ml). Based on this and the observation of lower FGL2 plasma levels in genotype 2/3 patients, it is tempting to speculate that patients with near normal plasma levels of FGL2 may have a higher probability of responding to anti-viral therapy than those with highly elevated levels. This needs to be further explored in a prospective study that is currently underway.

Liver tissue from patients with chronic HCV showed increased numbers of FoxP3$^+$ T cells consistent with previous reports by others [17]. Not all FoxP3$^+$ cells stained for FGL2 and although the significance of this finding is not clear at this point, recent reports have suggested that FoxP3 staining in humans in contrast to mice is not a specific marker for Treg. It is known that effector T cells can transiently express FoxP3 [24]. Secondly, other Treg subsets including CD8$\alpha\alpha$, NKT cells, and double negative T cells, which are FoxP3 negative, have been shown to express high levels of FGL2 [25,26], and thus FGL2 positive cells may represent other subset of regulatory T cells. Alternatively, as it is known that FoxP3$^+$ Treg are heterogeneous, it is possible that not all FoxP3$^+$ Treg express FGL2 [27] similar to the observation that not all regulatory T cells express IL-10 [28].

In summary, we have established a sensitive and specific ELISA, which allows for measurement of plasma levels of FGL2 in humans. These results suggest that monitoring plasma levels of FGL2 in patients with chronic HCV infection might be a means of predicting disease severity and/or potentially a marker of response to anti-viral therapy. To confirm this, we have now initiated a prospective clinical trial in patients with chronic HCV infection.

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