A weak relationship between positivity of anti-HBc and the development of HCC was observed in the present study, although it was not statistically significant ($P = 0.097$). The role of resolved or occult HBV infection in promoting the development of HCC in patients with CHC is highly controversial. Some investigators have emphasized its role in hepatocarcinogenesis,[30–32] and others have reported evidence that does not support this.[33,34] Undoubtedly, the accumulation of CHC patients with a SVR who subsequently developed HCC is necessary to elucidate whether or not the presence of isolated anti-HBc is a risk factor for the development of HCC in CHC patients with a SVR.

In Japan, public health insurance has covered IFN α-2b plus ribavirin therapy[35] since January 2002 and peginterferon α-2a monotherapy[36] since January 2004. Furthermore, combination therapy of peginterferon α-2b and ribavirin[37] is now available. These alternative therapies were demonstrated to be more effective than IFN monotherapy in CHC patients with HCV genotype 1 infection, in those with high HCV viral load in the circulation, and in those with severe fibrosis of the liver.[35–37] However, such patients are also at high risk for developing HCC, and it is very likely that the number of patients who develop HCC even after clearance of serum HCV RNA following more effective IFN therapy administered with or without ribavirin, may increase in the future, indicating the necessity of careful follow-up of such patients.

In conclusion, CHC patients who respond to IFN monotherapy or combination therapy should be followed as closely as possible, even after eradication of HCV, paying special attention to those who had severe fibrosis (F3 or F4) in the liver, those who had taken moderate amounts of alcohol (≥ 27 g/day), and those who were ≥ 65 years at the start of IFN treatment, to detect small and controllable HCC.

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Factors regarding increase of platelet counts in chronic hepatitis C patients with sustained virological response to interferon—Relation to serum thrombopoietin levels

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Abstract

Thrombocytopenia is frequently found in patients with chronic liver disease, and associated with advanced fibrosis stage and with decreased liver function. Serum thrombopoietin (TPO) levels also decrease as the disease progresses from mild fibrosis to cirrhosis. On the other hand, platelet counts increase associated with improvement of fibrosis in chronic hepatitis C (CH-C) patients with sustained virological response (SVR) to interferon (IFN) therapy. Then, we studied if the increase of platelet counts in SVR associate with elevated TPO production or a reduction of spleen size. Liver fibrosis, spleen size, serum TPO levels, albumin, zinc turbidity test (ZTT), platelet counts were compared in fifteen CH-C patients with SVR before and after IFN therapy.

Results: Albumin increased from 4.2 ± 0.3 to 4.3 ± 0.3 g/dl ($p = 0.067$), ZTT decreased from 17.7 ± 5.9 to 8.9 ± 3.9 K-U ($p < 0.001$), platelet counts increased from $15.5 \pm 6.8 \times 10^4$ to $19.9 \pm 5.8 \times 10^4/\mu l$ ($p < 0.01$) and serum TPO levels increased from 1.65 ± 0.94 to 2.06 ± 1.22 fmol/ml ($p = 0.073$). Spleen size was measured by ultrasonography, and the spleen index was calculated by multiplication of the long and short axes from hilus, which decreased from 14.6 ± 5.0 to 10 ± 3.1 ($p < 0.001$) after IFN therapy.

In conclusion, increase of platelet counts in SVR may be related to the reduction of spleen size and increased serum TPO levels associated with improvement of fibrosis after IFN therapy.

Keywords: Thrombopoietin; Platelet counts; Sustained virological responder; Chronic hepatitis C; Interferon

1. Introduction

Chronic hepatitis C (CH-C) progresses to cirrhosis insidiously after hepatitis C virus (HCV) infection accompanying with the gradual decline in the platelet counts. A low platelet count is an accurate marker of hepatic fibrosis and an excellent predictive noninvasive marker of cirrhosis in the absence of clinical, biological, endoscopic or ultrasonographic signs of portal hypertension [1]. Saito et al. [2] reported that platelet counts significantly correlated with the fibrotic stage, that is, F1, 19.2; F2, 17.2; F3, 13.2; F4, 7.8 ($\times 10^4/\mu l$). The main cause of thrombocytopenia has been attributed to an increased sequestration and pooling of platelets by an enlarged spleen secondary to portal hypertension, especially in severe case such as liver cirrhosis [3,4]. This theory has long been controversial and studies of platelet-turnover have yielded conflicting results [5]. Other mechanisms, such as an autoimmune [6] or viral megakaryocyte infection [7], have also recently been postulated in patients with HCV infection.

Thrombopoietin (TPO) is the most potent and specific cytokine for the growth and maturation of megakaryocyte and platelet production [8–10]. TPO messenger RNA (mRNA) transcripts have been found predominantly in the liver with lesser amounts also detected in the kidneys, bone marrow and spleen [11]. Most TPO is bound to receptors, c-Mpl, on

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platelet and the serum level is low. When thrombocytopenia develops, binding receptors decrease and serum TPO level increases. Elevated TPO level stimulates megakaryopoiesis and results in increasing platelet production [12–15]. Kawasaki et al. [16] and Adinolfi et al. [17] reported that serum TPO levels were decreased, as the disease progressed from mild fibrosis to cirrhosis, in patients with chronic hepatitis and liver cirrhosis. Moreover, the former authors identified a correlation between serum TPO levels and prothrombin activity, thus suggesting that low TPO levels might be expression of decreased liver function even in patients with chronic hepatitis.

We previously reported that platelet counts increased associated with improvement of fibrosis in CH-C patients with sustained virological response (SVR) to interferon (IFN) [18]. The aim of this study was to look for possible factors regarding the increase of platelet counts, such as serum TPO levels, spleen size, liver histology and liver function test in CH-C patients with SVR to IFN therapy.

2. Patients and methods

2.1. Patients

We studied 15 CH-C patients with SVR (undetectable HCV RNA after IFN therapy) (14 male, 1 female; mean age 59.7 ± 6.9 years old) who were biopsied before and after IFN therapy. Time interval between two times of liver biopsy was mean 7.6 ± 2.0 years (range 3.4–10.7 years). Histological degree of liver fibrosis was graded according to the severity as follows; F1 stage is expansion of portal area, F2 stage is bridging fibrosis without lobular distortion, F3 stage is lobular distortion, F4 stage is cirrhosis. Fibrosis score of each patients before IFN therapy was as follows; 1 patient had F1, 4 patients had F2 and 10 patients had F3. Platelet counts, albumin, zinc turbidity test (ZTT), serum TPO levels and spleen size were compared before and after IFN therapy, respectively. A peripheral blood sample was obtained on the day of liver biopsy.

2.2. TPO assay

Samples were stored at −80 °C until analyzed. The serum TPO were measured by a commercially available sandwich enzyme-linked immunosorbant assay (ELISA) using a monoclonal antibody and a polyclonal antibody to recombinant human TPO as previously described. Normal range: male, 0.79 ± 0.35 fmol/ml; female, 0.70 ± 0.26 fmol/ml [8].

2.3. Spleen size

Spleen size was measured by ultrasonography, and the spleen index (SI) was calculated by multiplication of the long and short axes from hilus.

2.4. Statistical analysis

The data are expressed as mean ± S.D. Wilcoxon signed rank test were used for statistical analysis according to the data analyzed. For all tests, $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Changes of albumin, ZTT and platelet counts before and after IFN therapy

Mean albumin increased from 4.2 ± 0.3 to 4.3 ± 0.3 g/dl ($p = 0.067$), ZTT significantly decreased from 17.7 ± 5.9 K-U to 8.9 ± 3.9 K-U ($p < 0.001$) and platelet counts significantly increased from 15.5 ± 6.8 × 10^4 to 19.9 ± 5.8 × 10^4/μl ($p < 0.01$), respectively (Fig. 1).

3.2. Changes of serum TPO levels before and after IFN therapy

Mean serum TPO levels increased from 1.65 ± 0.94 to 2.06 ± 1.22 fmol/ml ($p = 0.073$) after IFN therapy (Fig. 2). Serum TPO levels increased after IFN therapy in 12 out of 15 patients.

3.3. Changes of fibrosis score before and after IFN therapy

Fibrosis score improved after IFN therapy as follows; from F1 to F1 in one patient, from F2 to F1 in four patients, from F3 to F1 in five patients, from F3 to F2 in three patients and from F3 to F3 in two patients in which the width of fibrous septum became narrower, respectively (Fig. 3).

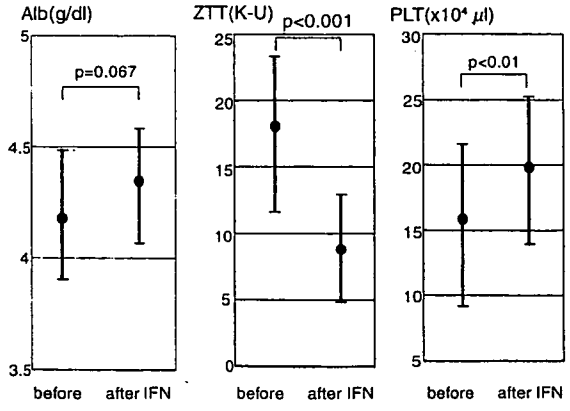

Fig. 1. Changes of albumin, ZTT and platelet counts before and after IFN therapy. Mean albumin increased from 4.2 ± 0.3 to 4.3 ± 0.3 g/dl ($p = 0.067$), ZTT significantly decreased from 17.7 ± 5.9 K-U to 8.9 ± 3.9 K-U ($p < 0.001$) and platelet counts significantly increased from 15.5 ± 6.8 × 10^4 to 19.9 ± 5.8 × 10^4/μl ($p < 0.01$) after 7.6 ± 2.0 years of IFN therapy, respectively.

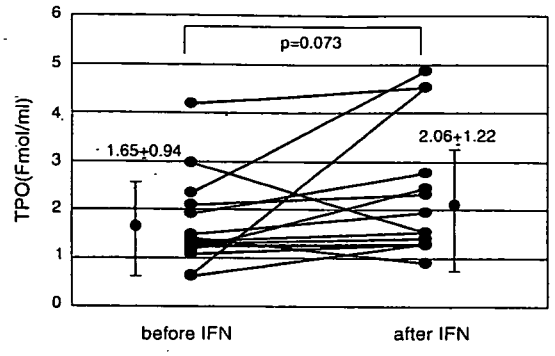

Fig. 2. Changes of serum TPO levels before and after IFN therapy. Serum TPO levels increased from 1.65 ± 0.94 to 2.06 ± 1.22 fmol/ml ($p = 0.073$) after 7.6 ± 2.0 years of IFN therapy.

3.4. Changes of spleen size before and after IFN therapy

Mean spleen index (SI) decreased from 14.6 ± 5.0 to 10 ± 3.1 ($p < 0.001$) after 7.6 ± 2.0 years of IFN therapy, which equaled to the rate of decrease of 0.7 ± 0.2 times (range 0.50–0.97 times) as compared to that before IFN therapy (Fig. 4).

3.5. Correlation between platelet counts and serum TPO levels or spleen index (SI) in the rate of change before and after IFN therapy

There was no significant correlation between platelet counts and serum TPO levels in the rate of increase nor between the rate of increase of platelet counts and the rate of decrease of SI; correlation coefficient = 0.143 ($p = 0.189$) and 0.214 ($p = 0.127$), respectively.

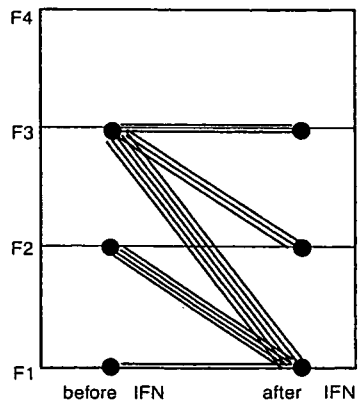

Fig. 3. Changes of fibrosis score before and after IFN therapy. Fibrosis score improved after IFN therapy as follows; from F1 to F1 in one patient, from F2 to F1 in four patients, from F3 to F1 in five patients, from F3 to F2 in three patients and from F3 to F3 in two patients in which the width of fibrous septum became narrower after 7.6 ± 2.0 years of IFN therapy, respectively.

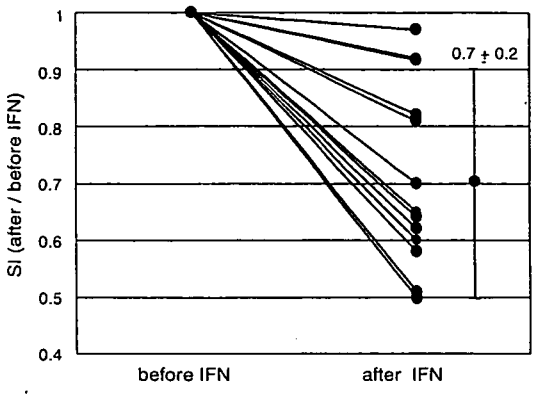

Fig. 4. Changes of spleen size before and after IFN therapy. Spleen index (SI) was calculated by multiplication of the long and short axes from hilus on ultrasonography, and decreased from 14.6 ± 5.0 to 10 ± 3.1 ($p < 0.001$), which equaled to the rate of decrease of 0.7 ± 0.2 times (range 0.50–0.97 times) as compared to that before IFN therapy.

4. Discussion

Thrombocytopenia is frequently found in patients with chronic liver disease, and associated with advanced fibrosis stage and with decreased liver function. Moreover, thrombocytopenia is included in one of risk factors for development of hepatocellular carcinoma (HCC) concomitant with male gender, age 55 years or older, prothrombin activity 75% or less and anti-HCV positivity [19], and alcohol, tobacco and obesity as synergistic risk factors [20]. Kubo et al. [21] reported that the proportion of patients with multicentric HCC was significantly higher among patients with low platelet counts (below $10^5/mm^3$) than patients with a higher count. These findings suggest that platelet counts are relevant to not only development of HCC but also prognosis of HCC. From this point of view, we studied on the significance of SVR in which platelet counts increased and incidence of HCC decreased as compared with those of NR [18]. Further, serum TPO levels decrease as the disease progresses from mild fibrosis to cirrhosis [16,17]. In the present study we studied if the increase of platelet counts in CH-C patients with SVR to IFN therapy associate with elevated TPO production or a reduction of spleen size. There have been no reports regarding the relationship between platelet counts and the change of spleen size after IFN therapy. Bizollon et al. [22] reported that biochemical and virological responder to combination therapy of ribavirin and IFN associated with marked histological improvement and Shiota et al. [23] reported that serum TPO levels increased following SVR to IFN therapy in patients with chronic hepatitis C. Itoh et al. [24] showed a significant decrease in fibrosis stage paralleled by an increase in platelet count and serum TPO levels in patients with SVR to IFN therapy after 4 years of follow-up. We also recently reported that the increase of platelet counts and serum albumin and decrease of ZTT associated with improvement of fibrosis in CH-C patients with SVR to IFN therapy [18]. In the present study, we demonstrated that a reduction of spleen size and the

increase of serum TPO levels were relevant factors regarding the increase of platelet counts in SVR. We investigated factors relating to the reduction rate of SI after IFN therapy, such as fibrosis stage, albumin, ZTT, platelet counts, TPO, age and SI before IFN therapy, and the ratio of the value after IFN therapy in those items to the value before IFN therapy; however, no significant difference was seen between two groups—one group with less than 10% ($n = 3$) and the other group over 30% ($n = 10$) of the reduction rate. Serum TPO levels did not increase in 3 out of 15 patients after IFN therapy regardless of the decrease of spleen size in all patients. These may show that the decrease of spleen size have a stronger influence on the increase of platelet counts than the increase of serum TPO levels. The increase of albumin and decrease of ZTT also seems to be the result from the regeneration and the amelioration of necroinflammatory change of hepatic cells, respectively.

Chen-Wei et al. [25] described that serum TPO levels elevation response to consensus interferon (CIFN) therapy is higher in SVR than in nonresponder, which means less hepatic fibrosis and better hepatic function reserve in SVR. Therefore, the serum TPO response to CIFN-induced thrombocytopenia may possibly serve as a marker for the severity of hepatic fibrosis. Koruk et al. [26] reported a positive correlation between serum TPO and albumin levels in patients with LC, revealing that serum TPO concentration may decrease with deterioration of protein producing ability of liver in LC. Kato et al. [27] showed a 30–40% reduction of total liver TPO mRNA content and thrombocytopenia in patients with cirrhosis compared to patients without cirrhosis. Giannini et al. [28] also demonstrated that serum TPO levels was correlated to liver functional impairment evaluated by means of [^{13}C]aminopyrine breath test and liver fibrosis.

From these findings, it is assumed that improvement of liver fibrosis induced by IFN therapy resulted in the increase of serum TPO levels and serum albumin, and further the decrease of spleen size leading to the increase of platelet counts. However, we did not evaluate other factors regarding thrombocytopenia such as anti-platelet-autoantibodies, platelet-turnover, measurement of portal hyperpressure. Therefore, these mechanisms could not be completely ruled out.

In conclusion, it appears that the increase of platelet counts in CH-C patients with SVR relates to both the reduction of spleen size and the increased TPO levels associated with improvement of liver histology after IFN therapy.

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今月の症例

ペグインターフェロンα-2a導入後早期にBasedow病を発症したC型慢性肝炎の1例

独立行政法人国立病院機構横浜医療センター臨床研究部
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概要　症例は23歳，女性，C型慢性肝炎の治療のため，pegylated interferon α-2a（PEG-IFNα-2a）を投与したところ，治療開始約1カ月後にBasedow病を発症した．IFN治療による甲状腺機能亢進症の多くは無痛性甲状腺炎であり，Basedow病は稀である．また本症例は，その発症時期が非常に早期であったことからも興味深い症例と考え報告する．〔日内会誌　94：2600～2602，2005〕

Key words：C型慢性肝炎，ペグインターフェロンα-2a，Basedow病

症　例

患者：23歳女性．主訴：全身倦怠感．既往歴・家族歴：特記事項なし．輸血歴：なし．生活歴：アルコール焼酎水割り3杯/日×5年間．タバコ15本/日．現病歴：2003年12月全身倦怠感があり，近医受診．肝機能障害を指摘され，12月初旬当科入院．入院時AST 267IU/*l*，ALT 507IU/*l*，T-bil 0.6mg/dl，HCV抗体陽性，HCV genotype2a，HCV-RNA 410KIU/ml，肝生検では慢性肝炎（F1/A1）の所見であった．本人の希望により一時退院後，2月下旬よりPEG-IFNα-2a180μg週1回の投与を開始した．IFN開始前の甲状腺ホルモン値は正常範囲，thyroid test，microsome testは400倍であった．AST，ALTは速やかに改善した．WBC減少のため6回目よりPEG-IFNα-2aは90μgに減量．3月下旬頃より全身倦怠感と眼痛が出現し，3月下旬精査加療目的で入院となった．入院時現症：血圧；100/60mmHg，脈拍；109bpm整，体温；37.2℃．眼瞼結膜貧血なし，眼球結膜黄染なし．頸部に弾性硬の甲状腺腫大を認めた．心音，呼吸音正常．腹部腸蠕動音正常，圧痛なし，肝，脾触知せず．四肢浮腫なし．手指の振戦を認めた．入院時検査所見：尿所見異常なし，Hb 13.0g/dl，WBC 1,700/μl（Neut 47%，Ly 14%，Mo 10%，Bas 0%，Eos 1%），Plt 14.4×10⁴/μl，T.Bil 0.6mg/dl，AST 94IU/*l*，ALT 172IU/*l*，ALP 271IU/*l*，γGTP 30IU/*l*，T-chol 107mg/dl，TG 35mg/dl，TSH 0.005μIU/*l*，fT_3 20.51pg/ml，fT_4 5.29ng/dl，TSH受容体抗体（TRAb）20IU/*l*，超音波検査では甲状腺のびまん性腫大と血流増加を認めた（図1）．

臨床経過（図2）

本症例はPEG-IFNα-2a投与開始5週後に甲状腺機能亢進症状と思われる全身倦怠感，眼痛を認め，TSHの低下，fT3，fT4の著明な高値，TRAb陽性，甲状腺超音波検査所見などよりBasedow病と診断した．後日保存血清により測定した結果では，治療開始後4週の時点でTSHの低下，

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Chronic hepatitis C with early complication of Grave's disease during the treatment of pegylated interferon α-2a.

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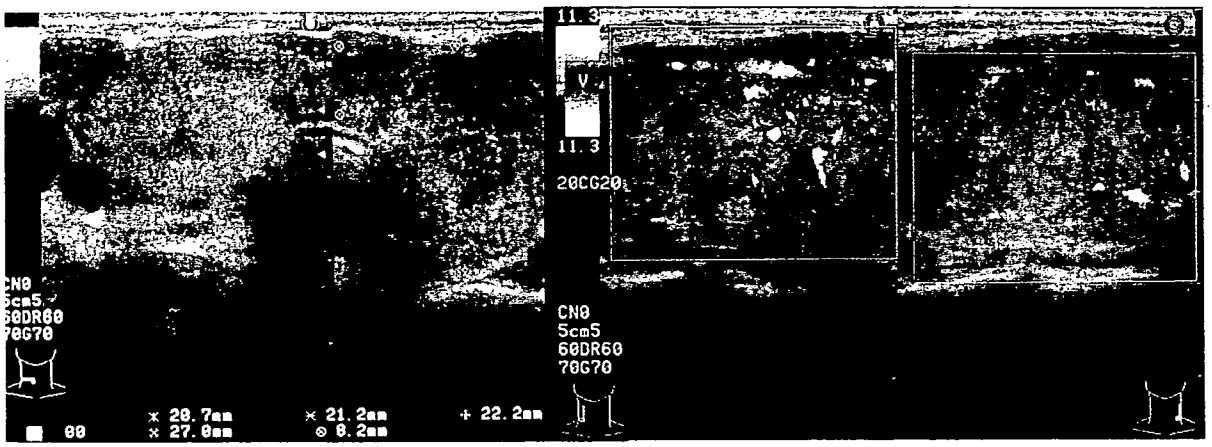

図1. 甲状腺エコーではRt；67.5×20.7×25.5mm，Lt；58.0×22.2×20.9mm，びまん性に腫大しており，表面はやや不整，内部不均一であった．カラードップラーエコーでは内部血流は増加（火焔状）していることよりBasedow病と診断．

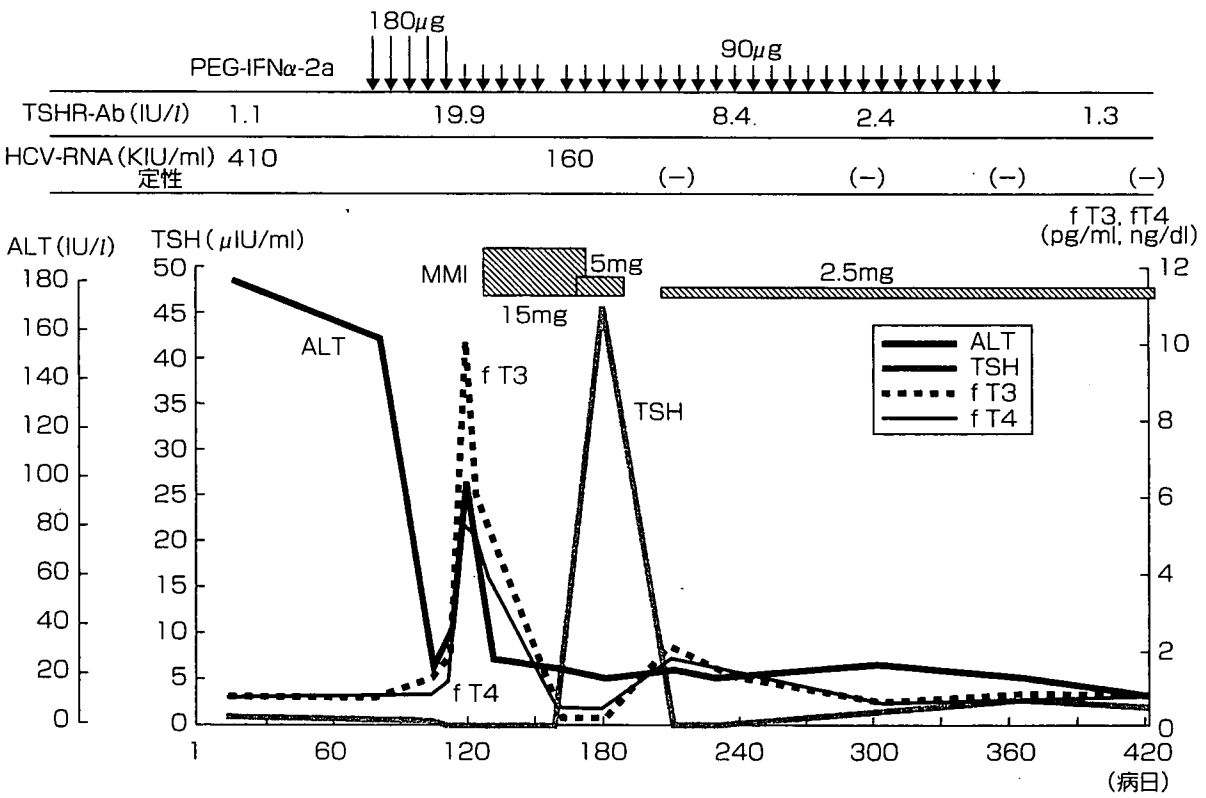

図2. 臨床経過

fT3，fT4の上昇がみられた．また治療開始前のTRAbは陰性（1.0IU/l未満）であった．チアマゾール（MMI）15mg/日から投与開始し，症状は改善．MMI漸減し，現在5mgの隔日投与で機能正常である．またPEG-IFNα-2aは予定通り48週間投与し，治療終了6カ月後までAST，ALT

は正常，HCV-RNAも陰性で経過している．

考　察

IFN治療による甲状腺機能異常の出現頻度は10%前後といわれている．女性，甲状腺疾患の家族歴，治療前の甲状腺自己抗体陽性例で，甲状腺機能異常が出現する可能性が高い[1,2]．本症例ではIFN投与前の甲状腺機能は正常であったが，thyroid test，microsome testが陽性であり，慎重にIFNを投与した．IFN治療誘発性の甲状腺機能異常は甲状腺機能低下症や無痛性甲状腺炎が多く，Basedow病は稀である[3,4]．甲状腺機能異常の病因としては，免疫修飾作用が主と考えられるが，その他IFNαによる直接の甲状腺機能抑制作用や，IL-6を介して甲状腺ホルモンの末梢代謝に及ぼす影響も報告されている[5]．今までの報告では，IFN投与開始からBasedow病の出現時期まで，ほとんどの症例が3カ月以上経過しており，その平均は1年との報告もある[4,6]．本症例は，IFN投与開始後約1カ月で甲状腺機能亢進症が出現しており，これまでの報告に比べ非常に早期である．このことはPEG-IFNα-2a製剤（あるいはPEG-IFN製剤）に特徴的なことなのか，本症例が特殊なケースなのか，興味深い点である．IFN長期（1年以上）投与症例の増加に伴い，今後Basedow病発症例も増加することが予想される．本例のように早期に診断し，適切に治療することによりIFN治療も継続可能であることから，自覚症状の有無にかかわらず甲状腺機能検査を定期的に施行することは重要であると思われる[7]．

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Immunomodulatory effects of selective leucocytapheresis as a new adjunct to interferon-α2b plus ribavirin combination therapy: a prospective study in patients with high plasma HCV viraemia

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SUMMARY. Efficacy of interferon-α2b (IFN) + ribavirin (IFN/RBV) combination in patients with high plasma hepatitis C virus (HCV) is very poor. Dysregulated CD4+ /CD8+ T cells is involved in both impaired cell-mediated immunity and resistance to IFN. Adsorptive granulocytes and monocytes apheresis (GMA) can remove infected leucocytes which are extrahepatic HCV reservoirs and also has been associated with intriguing immunomodulation and increases in CD4+ T cells. Our aim was to see if GMA enhances the efficacy of IFN/RBV. Twenty-four patients, 13 IFN resistant and 11 IFN naive were enrolled. Seventeen were genotype 1b and 7 were 2a or 2b. Mean plasma HCV-RNA was 612.9 (100–850) kIU/mL and alanine aminotransferase, 108 (41–373) U/L. GMA was performed with Adacolumn at one session/day for five consecutive days and IFN/RBV was started within 24 h after the last GMA session. Daily 6 million units of IFN, six times/week for 2 weeks and then three times/week for 22 weeks were given with RBV (600–800 mg/day/patient). Patients were followed for 6 months. GMA was associated with a significant increase in lymphocyte counts, complement activation fragment C3a and falls in tissue necrosis factor-alpha, and IL-8 produced by peripheral blood leucocytes. At week 24, 20 of 24 patients (83%) were HCV negative and by end of follow-up (week 49), the remission was sustained in 14 of 24 patients (58%) including 100% of patients with 2a or 2b. In conclusion, enhanced efficacy of IFN/RBV following GMA might be attributed to a more efficient immune function and a renewed IFN signaling towards HCV.

Keywords: chronic hepatitis C, complement activation fragments, granulocyte and monocyte adsorptive apheresis, interferon-α2b, lymphocytes, ribavirin.

INTRODUCTION

Hepatitis C virus (HCV) has an estimated worldwide prevalence of 170 million cases, including 2 million in Japan [1,2]. The clinicopathological features of the infected population include persistently elevated alanine aminotransferase (ALT) levels or normal liver function. The natural history of HCV shows that infection with relatively mild disease may progress to liver cirrhosis and hepatocellular carcinoma (HCC) during 20–30 years [1–4]; the infection becomes chronic in 50–85% of cases [5].

The treatment of HCV is currently [1,5,6] based on a combination of interferon-alpha (IFN) with ribavirin (RBV). With this regimen, a failure to eradicate HCV occurs in most patients infected by genotype 1b who present with a high viral load [5–7]. Factors associated with HCV resistance to IFN/RBV combination are not fully understood yet, but dysregulated functional T cells (CD4+/CD8+ T cells) is thought to be involved in both impaired cell-mediated immunity against HCV and resistance to anti-HCV drug therapy [8–11]. Thus, IFN resistance is thought to play a role at the early stages of infection, while a qualitative and

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GMA, granulocyte and monocyte apheresis; Hb, haemoglubin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon-alpha; IFN/RBV, IFN-α2b in combination with ribavirin; RT nested-PCR, reverse transcription nested polymerase chain reaction; SOCS, suppressor of cytokine signaling; SRL, special research laboratory; TNF-α, tissue necrosis factor-alpha; WBC, white blood cell counts.

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quantitative defect of both CD4+ and CD8+ immune response appears as the main determinant of viral persistence [5].

A seemingly unexpected strategy to enhance the immune function has emerged to be selective depletion of excess and activated granulocytes and monocytes [12,13]. The Adacolumn [12] was originally developed for selective depletion of excess and activated granulocytes and monocytes/macrophages in cancer patients who were found to have raised neutrophil counts, but low lymphocyte counts and impaired anti-tumour cytotoxic T-cell function [12,14]. The Adacolumn leucocytapheresis carriers adsorb granulocytes and monocytes/macrophages (FcγR and complement receptors bearing leucocytes [12]. The carriers typically adsorb 65% of granulocytes and 55% of monocytes from the blood in the column [12,15,16]. Studies in patients with rheumatoid arthritis revealed sustained increases in lymphocyte counts ($CD4^+$ T cells) and downregulation of inflammatory cytokines produced by leucocytes [12]. These effects suggested that it might enhance the efficacy of anti-HCV drug therapy. Further, granulocytes and monocytes/macrophages are extrahepatic replication sites and reservoirs for HCV dissemination [17–19] which can be eliminated. Additionally, the column carriers generate complement activation fragments including C3a, C5a, SC5b-9 complex and the opsonin C3bi [12,20] which theoretically should contribute to HCV clearance via opsonization of viral particles and enhanced cell-mediated immunity.

METHODS

Objectives

Adsorptive granulocyte and monocyte apheresis (GMA) was expected to benefit patients with HCV by depleting infected leucocytes and enhancing the immune function against the virus. Therefore, one major objective was to assess its effects on the efficacy of IFN/RBV combination in patients with high plasma HCV viraemia, mostly of genotype 1b. We were also interested to see the effects of GMA on peripheral blood leucocytes.

Patients selection

The demography of 24 patients of this study is shown in Table 1. Major inclusion criteria included: (i) a diagnosis of chronic hepatitis during the past 6 months as confirmed by liver biopsy; (ii) plasma HCV-RNA at least 100 kIU/mL by reverse transcription nested polymerase chain reaction (RT nested-PCR) within the past 3 months; (iii) abnormally high ALT level during 1 month prior to the initiation of the study; (iv) negative for hepatitis B virus and autoimmune hepatitis; (v) not receiving antiviral drugs or immunosuppressants within 3 months prior to the study; (vi) negative for HCC; (vii) neutrophil count >2000/μL, platelet count >80 000/μL, haemoglobin (Hb) >8.5 g/dL; (viii) absence of severe cardiovascular disease or renal failure; (ix) weight >35 kg; age above 12 and under 76 years; and (x) no history of adverse reaction to anticoagulants used during GMA (heparin).

Adsorptive GMA

GMA was performed with Adacolumn which is a new adsorptive carrier-based selective leucocytapheresis device [12]. The unit was provided by Japan Immunoresearch Laboratories (Takasaki, Japan). This medical device is CE marked (validated) by TUV (notified body). The column is filled with cellulose acetate beads of 2 mm in diameter as the column adsorptive carriers, bathed in sterile saline. The column was placed in an extracorporeal setting with a perfusion rate of 30 mL/min, duration 60 min similar to the settings used in patients with rheumatoid arthritis and ulcerative colitis [12,15,16]. Each patient received five GMA, one session/day over five consecutive days.

Drug therapy

IFN-α2b in combination with ribavirin (IFN/RBV) was started within 24 h after five GMA sessions. The dose of IFN was daily 6 million units six times/week for 2 weeks and then 6 million units three times/week for 22 weeks together with 600–800 mg ribavirin/patient/day (600 mg for patients weighing <60 kg and 800 mg for patients weighing 60 kg and higher). Patients were then followed for a further 24 weeks (total 49 weeks) without any anti-HCV medication during follow-up.

Ethics

The study protocol was reviewed and approved by the Institutional Review Board for ethics of clinical studies involving humans at each institution. Further, prior to GMA, informed consent was obtained from all patients verbally and in writing after they were informed of the purpose of the study and the nature of the procedures involved. Patients were advised that they were free to withdraw from the study at anytime without jeopardizing their subsequent care and treatment.

Assessment of efficacy and safety

To monitor treatment efficacy and safety, plasma HCV-RNA, serum ALT, aspartate aminotransferase (AST), total bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase, lactic dehydrogenase, total protein, creatinine, iron, ferritin, white blood cell counts (WBC), Hb and platelet counts were measured before first, third, fifth session, and once a month up to the end of the follow-up period or otherwise as shown in Figs 1–3.

Table 1 Patients' background and results of granulocyte and monocyte adsorptive apheresis (GMA) followed by IFN-α2b + ribavirin combination therapy. IFN-α2b + ribavirin was given after five GMA sessions over five consecutive days

Patient no.	Genotype	Liver biopsy	HCV-RNA (kU/mL)	Past IFN result	24 weeks IFN + ribavirin therapy								24 weeks observation					
					1	2	4	8	12	16	20	24	28	32	36	40	44	48
1	1b	A1F2-3	220	Relapsed	NT	–	→	→	→	+	+	+	+	+	+	+	+	+
2	1b	A2F2	>850	Relapsed	NT	–	NT	0.9	–	→	→	→	+	+	+	+	+	+
3	1b	A2F2	590	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
4	1b	A2F3-4	260	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
5	1b	A1F3	790	Naive	NT	–	→	→	→	→	→	→	→	+	+	+	+	+
6*	1b	A2F2	430	Relapsed	+	–	–	–	–	+	NT	NT	NT	NT	NT	NT	NT	NT
7	1b	A2F3	490	Relapsed	+	+	+	NT	+	+	+	+	+	+	+	+	+	+
8	1b	–	>850	Naïve	1.5	–	→	→	→	→	→	→	→	+	+	+	+	+
9	1b	A2F1	700	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
10	1b	A1F1	600	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
11	1b	A2F1	370	Naïve	340	62	84	3.8	–	→	→	→	+	+	+	+	+	+
12†	1b	A2F2	>850	Naïve	+	+	+	+	–	→	→	→	→	→	→	→	→	→
13	1b	A1F1	>850	Relapsed	NT	NT	0.7	–	→	→	→	→	→	+	+	+	+	+
14	1b	A1F0	>850	Naïve	NT	NT	–	→	→	→	→	→	→	+	+	+	+	+
15	1b	A1F1	160	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
16	1b	A2F3	>850	Relapsed	NT	1.8	NT	+	+	–	→	→	→	→	→	→	→	→
17	1b	A1F3	>850	Relapsed	340	NT	440	630	850	850	850	850	850	850	850	850	850	850
18	2a	A2F3	>850	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
19	2a	A2F1	110	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
20	2a	A2F2	540	Relapsed	NT	–	→	→	→	→	→	→	→	→	→	→	→	→
21	2a	A1F1	>850	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
22	2b	A2F2	100	Naïve	–	→	→	→	NT	→	NT	NT	→	→	→	→	→	→
23	2b	A2F2	>850	Relapsed	NT	NT	–	→	→	→	→	→	→	→	→	→	→	→
24	2b	A2F1	>850	Relapsed	NT	NT	–	→	→	→	→	→	→	→	→	→	→	→

*Withdrew at week 12 because of a broken leg.
†Received IFN after week 24.
NT, not tested; –, absent; → , negative HCV-RNA was maintained; A1–4, slight, mild, moderate and extensive inflammatory cell infiltration respectively; F1–4, slight, mild, moderate and extensive liver fibrosis respectively.

Assays of HCV-RNA, ALT, AST and C3a

Plasma HCV-RNA was measured by RT nested-PCR [21]. ALT, AST and complement activation fragment C3a (by radioimmunoassay) were measured in serum samples. All assays were performed at a special research laboratory (SRL) using the most sensitive assay kits available (R&D Systems, Minneapolis, MN, USA) according to the package insert.

Measurement of TNF-α and IL-8

Tissue necrosis factor-alpha (TNF-α), a major inflammatory cytokine produced by peripheral blood mononuclear leucocytes and the chemokine, interleukin-8 (IL-8) produced by peripheral blood leucocytes were measured to test the effects of GMA on inflammatory factors. TNF-α assay was according to Chofflon et al. [22] while IL-8 was according to DeForge et al. [23]. Blood samples were taken from the Adacolumn inflow (peripheral blood) at the start of GMA and from the outflow towards the end of the 60-min GMA session. Tests at about 5×10^5/mL leucocytes were stimulated with 1.5 μg/mL lipopolysaccharide and the supernatants were sent to an SRL for assay of TNF-α and IL-8 by ELISA as indicated above.

Statistical analysis

The data are presented as the mean ± SD values and ranges unless stated otherwise. Comparisons between sets of data were carried out with the Mann–Whitney *U*-test on raw data by using Stat View Software. A significance level of 0.05 was used for all statistical tests, and two-tailed tests were applied when appropriate.

RESULTS

Changes in HCV-RNA during therapy

Table 1 shows entry HCV-RNA levels, liver biopsy outcomes, previous response to IFN and HCV-RNA profiles during the

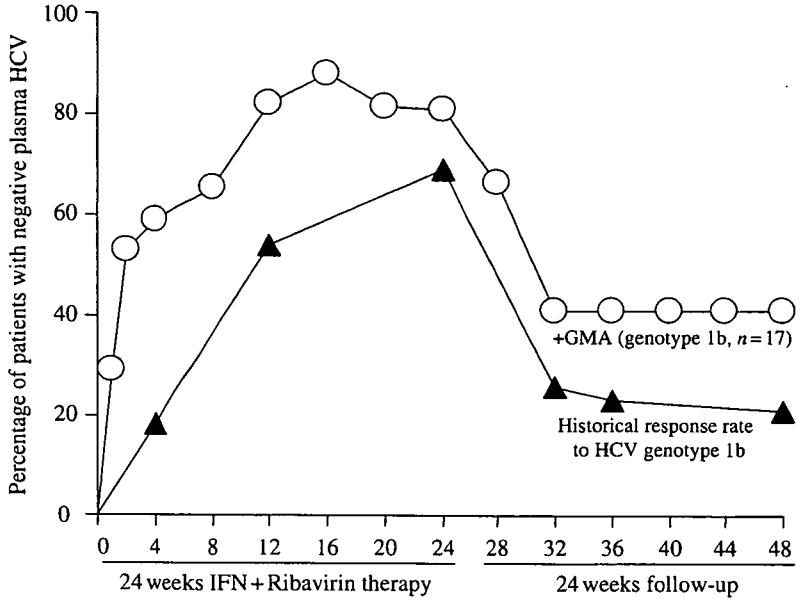

Fig. 1 The percentage of patients who became HCV (HCV-RNA) negative with time during IFN-α + ribavirin therapy and the follow-up for two groups of patients. The solid triangles represent the historical response to IFN-α2b + ribavirin in patients with HCV genotype 1b while the open circles represent the response to the same regimen in 17 patients with HCV genotype 1b who received five GMA sessions prior to initiation of IFN + ribavirin therapy. For clarity, SD values are not shown. It can be seen that in the group receiving GMA, the response was more rapid with a greater fraction of patients in remission throughout the study time. The historical data are according to Iino S. Future outlook for treatment of chronic hepatitis C: IFN + ribavirin combination therapy (Rinsho Byori, 2001; 49: 747–54 with permission of the publisher).

therapy and the follow-up period. Eleven of 24 patients were IFN naïve and 13 had relapsed following a short-period response to IFN. Plasma HCV-RNA was 612.9 ± 312.9 kU/mL (range 100–850 kIU/mL). As shown, at the end of the 24-week IFN/RBV therapy, 20 of 24 patients (83%) were HCV negative, and by the end of the follow-up period, 14 of 24 patients (58%) had maintained their remission. With respect to HCV genotypes, all seven patients with genotype 2a or 2b maintained their remission throughout the follow-up period (100% sustained response). To say that this small group of seven patients with 2a or 2b responded to the therapy more rapidly and fully compared with patients who harboured HCV genotype 1b. Additionally, in Fig. 1, the historical response to IFN/RBV in patients with HCV genotype 1b is presented for comparison with the results of this study on 17 patients who received GMA prior to IFN/RBV. In the GMA group, the response is more rapid with a greater fraction of patients achieving sustained remission.

Changes in serum ALT and AST levels during the study

At entry, the group ALT was 108.1 ± 80.4 IU/L (range 41–373 IU/L). The corresponding values for AST were 73.42 ± 45.09 IU/L and 31–226 IU/L. The mean ALT fell following the start of IFN/RBV therapy and was within normal range after 12 weeks ($P < 0.001$) and remained at this level during the 24-week follow-up period ($P < 0.0001$). The changes in serum AST were very similar to ALT (results not presented).

Changes in peripheral blood leucocyte counts

Figure 2 shows total peripheral blood WBC and granulocyte (neutrophil) counts *vs* time during GMA, IFN/RBV therapy and follow-up. There was no marked fall in either WBC or granulocyte counts during the week in which patients received five GMA sessions. At entry, WBC count ($\times 10^3/\mu L$) was 4.826 ± 0.745 (range 3.92–6.61). The corresponding values after five GMA sessions were 4.602 ± 0.908 (range 6.40–3.11). Similarly, for neutrophils (%), entry values were 55.65 ± 8.64 (range 35–70.9). The values after five GMA sessions were 50.73 ± 12.04 (range 34–68). However, both WBC and granulocyte counts fell significantly during IFN/RBV therapy which were reversed during the follow-up.

Effects of the treatment on peripheral blood lymphocyte counts

Figure 3 shows lymphocyte counts at entry, at the end of the last GMA session and up to the end of the observation period. At entry, lymphocyte count (%) was 39.96 ± 8.87 (range 21.9–52.6). Just prior to the start of IFN/RBV therapy, it had

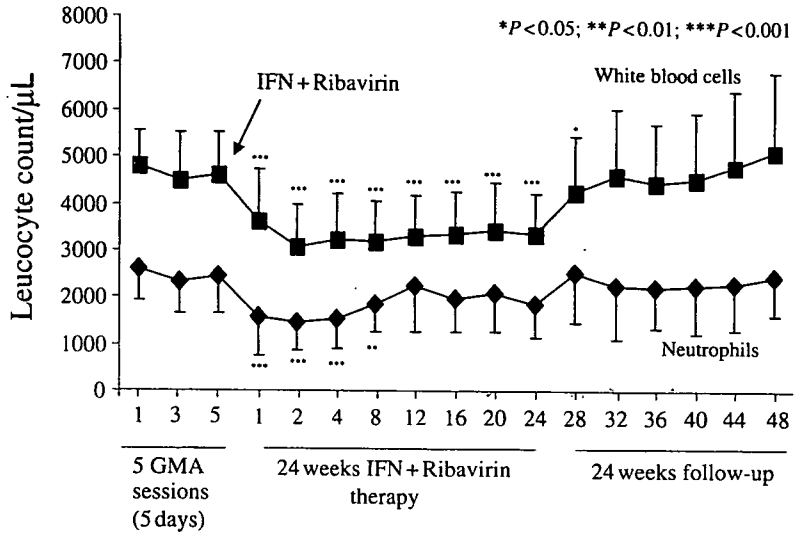

Fig. 2 Total white blood cell (WBC) and granulocyte counts with time during 5 days of GMA and IFN + ribavirin therapy together with the follow-up time. As shown, no marked fall in peripheral blood leucocytes was seen during GMA, but both WBC and neutrophil counts fell during IFN + ribavirin therapy with recovery during the follow-up. For additional comments see Discussion. The *P*-values for the time points shown are relative to the entry levels.

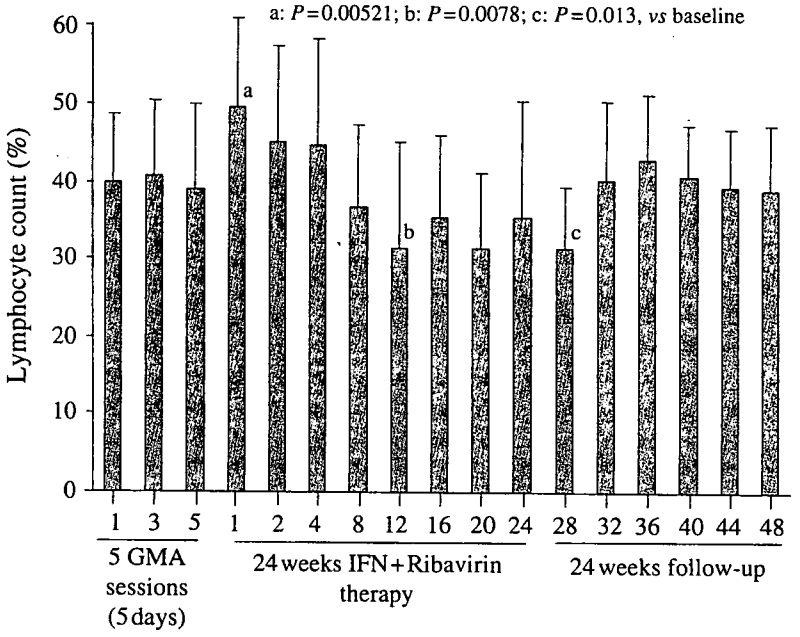

Fig. 3 Changes in lymphocyte counts at entry, at the end of the last GMA session (start of IFN/ribavirin therapy) and up to the end of the observation period. At entry, the lymphocyte count (%) was 39.96 ± 8.87 (range 21.9–52.6). At the start of IFN/ribavirin therapy, it had increased to 49.51 ± 11.55 (range 28–69) (P = 0.0052). Similar to WBC and granulocyte counts (Fig. 4), lymphocyte counts began to fall during IFN/ribavirin therapy and by week 12 were 31.47 ± 13.74 (range 9.0–54.4) (P < 0.0078). After cessation of IFN/ribavirin therapy (fallow-up) blood leucocytes began to rise again.

increased to 49.51 ± 11.55 (range 28–69) (P = 0.0052). Obviously, the upper limit is higher than normal laboratory values. The anomaly might be associated with HCV infection and/or IFN/RBV therapy. However, it is not attributable to removal of granulocytes by GMA because earlier work has shown that at the time of the rise in lymphocyte count, the peripheral blood granulocyte count returns to baseline level, within a few hours [12]; the lost granulocytes are replaced by CD10 negative (naive) neutrophils [13]. Indeed, Fig. 2 does not show any marked fall in WBC or neutrophils during the 5-day GMA course. Similar to WBC and granulocyte counts, lymphocyte counts began to fall during IFN/RBV therapy and by week 12 was 31.47 ± 13.74 (range 9.0–54.4) (P < 0.0078). After cessation of IFN/RBV therapy, blood lymphocyte counts began to rise again (Fig. 3).

Generation of the complement C3a during GMA

Figure 4 shows generation of complement activation fragment, C3a in the peripheral blood (Adacolumn inflow) and in the blood that emerged from the column (blood return to patients). The data show a dramatic increase in this major complement activation product at the column outflow, by more than 10-fold higher relative to inflow.

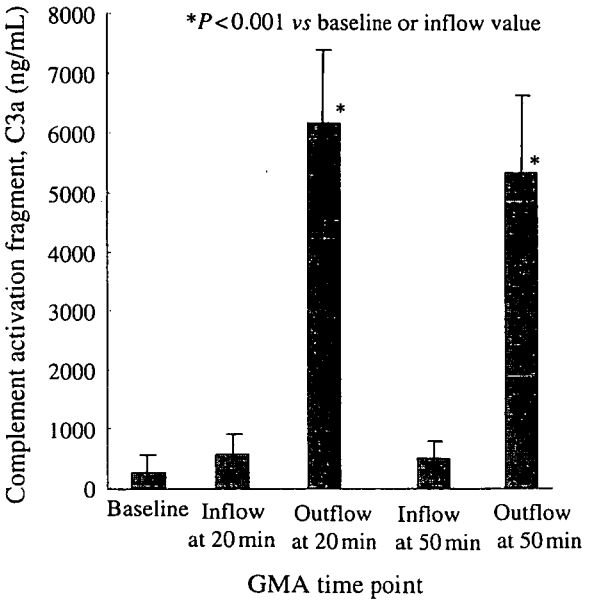

Fig. 4 Generation of complement activation fragment, C3a in the peripheral blood (Adacolumn inflow) and in the blood that emerged from the column (blood return to patients). The data show a dramatic increase in blood levels of the major active complement, C3a at the column outflow, by more than 10-fold higher relative to the column inflow.

Effects of GMA on TNF-α and IL-8

Figure 5 shows the effects of GMA on TNF-α and IL-8 produced by leucocytes. As indicated, blood samples were taken from the Adacolumn inflow at the start of GMA (peripheral blood) and outflow at 50 min. This was performed on two separate occasions, the first GMA session and the fifth (last) session. The amounts of TNF-α and IL-8 produced by leucocytes were significantly reduced not only by leucocytes that had passed through the column, but also by peripheral blood leucocytes after five sessions.

Treatment safety and patient compliance

All 24 patients completed their five GMA sessions according to the protocol and no severe adverse events related to GMA were seen. Further, during GMA, no marked fall in haematological or biochemical parameters were seen in spite of the fact that column removes up to 65% of neutrophils and 55% of monocytes from the blood in the column [12]. Only two incidences of transient mild headache were reported during GMA. In both cases, the symptoms receded within 3 h without medication. Two patients did not complete the follow-up; one had accidental broken leg at week 12 and the other received intermittent IFN/RBV during the follow-up according to the physician's discretion (see Table 1). As shown in Figs 2 and 3, during IFN/RBV therapy, both WBC, neutrophil and lymphocyte counts fell significantly and were reversed during the follow-up. Similarly, Hb (g/dL) fell from an entry value of 14.16 ± 1.19 (range 11.7–16.3) to 11.73 ± 1.67 (range 7.6–15.2) at week 12. The fall in Hb reversed during the follow-up.

DISCUSSION

The only novelty in this study is addition of GMA to the widely adopted [5,6] IFN/RBV therapy. GMA with Adacolumn in patients with rheumatoid arthritis and ulcerative colitis reported unexpected suppression of TNF-α, IL-1β, IL-6 and IL-8 produced by leucocytes [12,13,24–26], an increase in CD4+/CD8+ T cells [12], a rise in CD10 negative (naive) neutrophils [13] and generation of active complement including SC5b-9 complex and the opsonin C3bi by the column carriers [20]. In view of the evidence that granulocytes, monocytes/macrophages are extrahepatic replication sites for HCV [17–19] and an increase in CD4+/CD8+ T cells subsequent to a GMA course [12], we had the impression that GMA might enhance the efficacy of IFN/RBV. Earlier in a patient with ulcerative colitis–HCV co-morbidity, genotype 1b who was intolerant to IFN, we achieved sustained remission after GMA [27]. Following this case, we undertook a preliminary study in six patients with high plasma HCV-RNA. GMA was performed at one session/week for 5 weeks. Despite a fall of over 50% in HCV-RNA during the GMA procedure, HCV-RNA increased again during the waiting time for the next session [28]. It appeared that one session/week for five consecutive weeks was an inappropriate GMA frequency. Accordingly, in this study, we tried one GMA/day.

In Japan, the standard treatment for HCV is 6–10 million units of IFN for 24 weeks (daily for 2–4 weeks, then three times a week for 20–22 weeks) plus RBV; this was followed in this study. This regimen normalizes plasma AST and ALT, but plasma HCV-RNA becomes negative in <50% of patients after the treatment [29]. Patients who do not respond to this regimen are considered to harbour IFN-resistant HCV. However, the majority of patients included in this study had high plasma viraemia and were harbouring HCV genotype 1b which very poorly responds to IFN/RBV [5–7,30]. In spite of this, 20 of 24 patients (83%) became HCV negative and of these, 14 (58%) achieved sustained remission (40% genotype 1b and 100% of genotype 2a or 2b). An 83% initial response rate in patients with high plasma HCV load and a 58% sustained remission rate are the highest reported in the literature for genotype 1b [5–7,30]. In our experience, patients who maintain their remission up to 24 weeks following the end of treatment are unlikely to have a viral relapse. Our impression that an increase in circulating lymphocyte level was one factor for good initial response was overshadowed by the fall in lymphocytes during IFN/RBV therapy. Hence, it is likely that one or more course of GMA during drug therapy and follow-up can increase the number

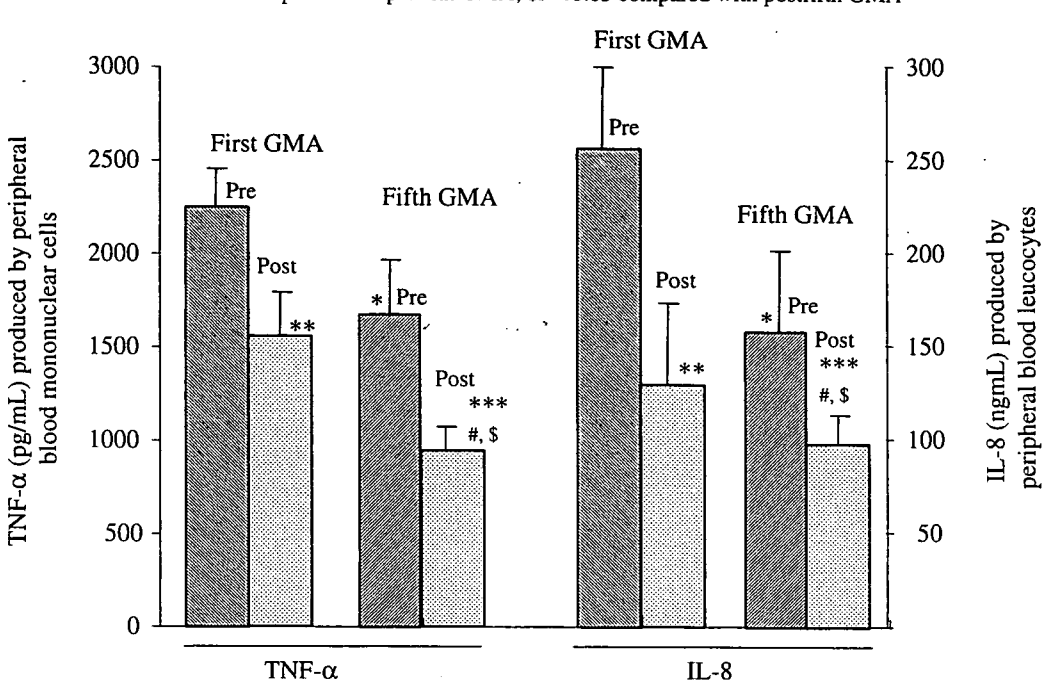

Fig. 5 Effects of GMA on TNF-α and IL-8 produced by lipopolysaccharide stimulated blood leucocytes. As indicated, blood samples were taken from the Adacolumn inflow at the start of GMA (peripheral blood) and outflow at 50 min. This was performed on two separate occasions, the first GMA session and the fifth (last) session. The amount of cytokine produced by leukocytes was significantly reduced not only by passing through the column but also by peripheral blood after five sessions. The results show that GMA is associated with reduced cytokine production by blood leucocytes.

patients with sustained remission. We also observed GMA-related suppression of TNF-α and IL-8 produced by blood leucocytes, a significant rise in lymphocyte counts and generation of the active complement, C3a.

Poor HCV response to IFN is associated with defective CD4+/CD8+ T-cell function [8–11]. Hence, a decrease in lymphocytes during IFN/RBV therapy is likely to be a negative factor in HCV response to INF/RBV. The mechanism(s) by which IFN/RBV causes a fall in blood leucocytes is not clear to us. A study by Zella et al. [31] reports that IFN-α2b increases the expression of apoptosis receptor CD95 and chemokine receptors CCR1 and CCR3 in monocytoid cells. Similarly, Kaser et al. [32] report that IFN-α promotes activation-induced T-cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression, while a study by Manna et al. [33] reports that IFN-α potentiates TNF-induced apoptosis.

For GMA-related enhanced efficacy of IFN/RBV, the following speculations are in line with the observed actions of GMA. First, as reported above, GMA generates complement activation products, C3a together with SC5b-9 complex and the opsonin C3bi [12,20]. Complement is an integral component of body's defence [34] and generation of C3a, SC5b-9 and C3bi theoretically should contribute to HCV killing both in the plasma and within the liver via opsonization of the viral particles and enhanced cell-mediated immunity. Secondly, GMA has been associated with a sustained fall in TNF-α, IL-1β, IL-6 and IL-8 released by peripheral blood leucocytes [12,13,24–26]. It is known [35–37] that down-regulation of pro-inflammatory cytokines compromises the activities of the so-called 'suppressor of cytokine signalling' (SOCS). These are several intracellular proteins induced by inflammatory cytokines [35–37]. SOCS are well known to have a strong inhibitory role on the activities of interferons [38–40] including the anti-HCV action of IFN-α [40]. This may lead us to assume that an enhanced anti-HCV efficacy of IFN/ribavirin following GMA reflects absence of a strong inhibitory effect on IFN-α by SOCS. Thirdly, GMA depletes infected leucocytes and increases lymphocytes which should provide a healthier immune function at the start of IFN/RBV therapy. Certainly, these speculations need to be further supported in future controlled studies and if true, they can serve as the basis for GMA to become an adjunct to IFN/RBV, a very safe, feasible and natural procedure. Additional issues which might affect the efficacy of GMA and need to be evaluated in future controlled trials include: (i) frequency of

GMA (once a day, three times a week etceteras); (ii) duration of one GMA session; and (iii) duration of one treatment course (1 week, 2 weeks or longer?).

In conclusion, this study has shown that adsorptive depletion of infected granulocytes and monocytes/macrophages enhances the anti-HCV efficacy of IFN/RBV therapy. Generation of active complement opsonins during GMA is thought to contribute to HCV killing, and additional mechanism(s) including increased lymphocytes and suppression of specific cytokines are likely to be involved. Clearly further controlled studies are necessary to determine the full efficacy of GMA in the treatment of HCV.

ACKNOWLEDGEMENTS

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Short Communication

Full-Length Sequence of Hepatitis B Virus Belonging to Genotype H Identified in a Japanese Patient with Chronic Hepatitis

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SUMMARY: We have isolated and cloned the full-length nucleotide sequence of the hepatitis B virus (HBV) genome (denoted HBV-IM806-2) recovered from a Japanese patient with chronic hepatitis. This patient had a history of travel to Bangkok, Thailand, and then suffered the onset of acute hepatitis B 3 months after his return to Japan. The HBV-IM806-2 isolate was composed of 3,215 nucleotides and showed the highest similarity to genotype H of HBV. Interestingly, 24 amino acid residues specific for genotype H were identified throughout the full genome sequence. Furthermore, phylogenetic analysis based on the full genome sequence confirmed that IM806-2 belonged to genotype H and was more closely related to the prototype of the Los Angeles strain than to the Nicaragua strain.

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) and are at risk of dying as a result of the occurrence of hepatocellular carcinoma accompanying HBV infection. By characterization of the viral genome, HBV has been classified into genotypes A through G with an inter-genotypic diversity of at least 8% in the full genome sequence (1,2). In addition to this classification, a newly described genotype H has been found in Nicaragua and the U. S. (3). It seems that the distribution of genotype H is restricted to the northern part of Latin America, however, the exact distribution of this genotype remains unclear due to the lack of a rapid and simple method of identification such as genotyping by PCR or PCR-RFLP. Among the sequence records deposited in the database, only 8 isolates of genotype H have been sequenced in the full genome to date. Here we report the identification and entire nucleotide sequence of the HBV belonging to genotype H isolated from a Japanese patient with chronic hepatitis.

A 61-year-old man underwent a medical examination at the International Medical Center of Japan, Tokyo and was diagnosed with chronic hepatitis. Serological findings were positive for HBsAg, HBeAg and anti-HBc, but negative for anti-HCV and HCV RNA. The patient had visited Bangkok, Thailand, 30 years previously and had had sexual contact with a woman there. Three months after his return to Japan, he suffered the onset of acute hepatitis with HBV infection which developed into chronic hepatitis B. We conducted a sequencing analysis of the HBV obtained from this patient. To obtain a full-length sequence, we amplified HBV DNA by PCR using a primer combination of HBV4 (sense; 5'-CCG GAA AGC TTA TGC TCT TCT TTT TCA CCT CTG CCT AAT CAT C-3'; the HindIII site is underlined) and HBV4R (antisense; 5'-CCG GAG AGC TCA TGC TCT TCA AAA AGT TGC ATG GTG CTG GTG-3'; the SacI site is underlined) as reported previously (4). Viral DNA was extracted from 100 μl of serum using a DNA/RNA extraction Kit (SepaGene RV-R, Sanko Junyaku Co., Ltd., Tokyo, Japan). The resulting pellet was resuspended in 50 μl of RNase-free water and maintained at −20°C until use. The PCR conditions included pre-incubation at 94°C, 2-min activation of Blend Taq-Plus DNA polymerase (Toyobo Co., Ltd., Tokyo, Japan) followed by 40 cycles of PCR (94°C for 15 sec, 55°C for 45 sec and 72°C for 3 min 20 sec with a final extension for 7 min at 72°C). The PCR products were separated by 1% agarose gel electrophoresis and purified using a QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, Calif., USA) in preparation for sequence analysis. Purified PCR products were cloned into the HindIII/SacI sites of pUC19 vector. Cloned HBV DNA was subjected to sequencing using an ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA). Sequences of cloned HBV DNA were determined using the automated DNA sequencer, ABI 3100-Avant Genetic Analyzer (Applied Biosystems).

The sequence reported in this paper have been deposited in the DDBJ/GenBank/EMBL under the accession number AB205010.

The HBV genome recovered in this study was compared with the 35 isolates of HBV with a full-length sequence in the database. Nucleotide sequences were multiple aligned using GENETYX for Windows version 7 software (Genetyx, Tokyo, Japan) and were calculated using the Kimura two-parameter method; phylogenetic trees were constructed by the neighbor-joining method (5). To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1,000 times.

The full genome sequence of the HBV was obtained from our Japanese patient with chronic hepatitis and was named HBV-IM806-2. IM806-2 was composed of 3,215 bases. When compared with other previously reported HBV isolates with a full genome sequence, IM806-2 showed high overall identity (98.9%) with the prototype of the Los Angeles strain (AY090460) and 97.4% identity with the Nicaragua

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