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Association of Serum Cytokine Levels With Treatment Response to Pegylated Interferon and Ribavirin Therapy in Genotype 1 Chronic Hepatitis C Patients

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Background. We sought to clarify the associations among serum cytokines, amino acid substitutions in the interferon sensitivity–determining region (ISDR) and core region, and treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients.

Methods. We quantified a total of 8 serum cytokines before, during, and after treatment in 79 genotype 1 chronic HCV patients. Viral ISDR and core region variants were determined by direct sequencing.

Results. High levels of interleukin (IL)-12 and IL-18 and more than 2 mutations in the ISDR were associated with a sustained virological response (SVR). Conversely, high baseline IL-10 levels and glutamine at amino acid 70 of the HCV core protein (Gln70) were significantly associated with a nonresponse to treatment, and patients with Gln70 had significantly higher IL-10 levels. In multivariate analysis, low IL-10, high IL-12, and high IL-18 levels were independently associated with an SVR. These 3 cytokine levels were decreased from baseline levels 4 weeks into treatment and remained low in patients with an SVR.

Conclusion. Serum IL-10, IL-12, and IL-18 levels are predictive of the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR and core region.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infections develop chronic hepatitis, which leads to liver cirrhosis or hepatocellular carcinoma (HCC) in at least 20% of cases [1, 2]. HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan [3, 4]. Because approximately 70%–80% of Japanese HCC patients are infected with HCV, viral eradication is important to decrease the incidence of HCC. Interferon-based therapy can reduce HCV to undetectable levels and improve prognosis. The primary aim of antiviral therapy in HCV patients is a sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after completion of therapy. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by antiviral therapy [5, 6].

Cytokines play an important role in the pathogenesis, progression, and treatment outcome of HCV infection. Because the control of cytokine production is highly complex and the effects of cytokines are widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers could best clarify the immunopathogenesis of the disease and predict responses to antiviral therapy. However, such analysis is difficult using enzyme-linked immunosorbent assay, which requires each biomarker be tested individually. In

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this study, we used a new broad-spectrum bead-based multiplex immunoassay to simultaneously test multiple factors in the sera of patients with chronic hepatitis C. Wan et al recently reported that some cytokines are elevated in non-SVR HCV patients using this bead system, but only 17 patients with genotype 1 were evaluated [7]. Thus, the association between multiple cytokines and treatment outcome are largely unknown.

The objective of this study was to determine which cytokines in patients with genotype 1 chronic hepatitis C relate to the clinical and virologic characteristics of hepatitis and how they affect the HCV response to pegylated interferon (PEG-IFN) and ribavirin therapy.

PATIENTS AND METHODS

Participants

We included 79 consecutive patients with genotype 1 chronic hepatitis C in this study. We based diagnosis of chronic hepatitis C on the following criteria, as reported previously [8]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen; and 3) exclusion of other causes of chronic liver disease. No patient had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of patients are shown in Table 1. We used a group of 26 healthy individuals with normal transaminase levels and negative serologic results for hepatitis B and hepatitis C as the control. All participants were negative for the antibody to the human immunodeficiency virus. The protocol of this study was approved by the ethics committee of the Shinshu University School of Medicine, and all patients provided written informed consent.

Laboratory Testing

We measured antibodies to HCV in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories). We determined serum levels of HCV RNA using the COBAS AMPLICOR assays (Roche Diagnostic Systems), which amplify HCV RNA by reverse transcriptase–polymerase chain reaction. The lower limit of the assay was 50 IU/mL. We determined HCV genotypes using INNO-LiPA HCV II (Innogenetics). We found that all patients in our test cohort were infected with genotype 1b. We performed alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests using standard methods [9].

Antiviral Therapy

All patients received body weight–adjusted PEG-IFNα-2b (PegIntron, Schering-Plough K.K.; ≤45 kg, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; ≥ 91 kg, 150 μg/dose), and ribavirin (Rebetol, Schering-Plough K.K.; ≤60 kg, 600 mg/day; 61 kg–80 kg, 800 mg/day; ≥81 kg, 1000 mg/day) for 48 weeks, as reported previously [10].

Definition of Viral Kinetic Response and Treatment Outcome

An early virological response (EVR) was defined as undetectable serum HCV RNA by 12 weeks of therapy. An SVR was classified as serum HCV RNA that was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of Amino Acid Substitutions in the Core and NS5A Regions

We determined the sequence of 1–191 amino acids (aa) in the core protein of genotype 1b HCV, and we evaluated substitutions at aa70 of arginine (Arg70) or glutamine (Gln70) [11] with the use of HCV-J as a reference [12]. We also determined the sequence of 2209–2248 aa in the NS5A region of genotype 1b HCV containing the interferon sensitivity–determining region (ISDR), and the number of aa substitutions in the ISDR was defined as wild-type (0), intermediate-type (1), or mutant-type

Table 1. Demographic and Clinical Characteristics of Patients with Hepatitis C Virus Infection

Characteristics	All (n = 79)	SVR (n = 31)	Non-SVR (n = 48)	P
Median age, y (range)	60 (17–74)	56 (28–72)	61 (17–74)	0.08
Male, n (%)	40 (51)	23 (74)	17 (35)	0.001
Median values (range)				
ALT, IU/L (range)	54 (22–389)	53 (24–172)	61 (22–389)	0.25
AST, IU/L (range)	44 (20–288)	36 (21–133)	48 (20–288)	0.012
HCV RNA, 10^5 IU/mL(range)	17 (1.1–51)	15 (1.1–50)	19 (2.2–51)	0.13
Substitutions				
Core aa 70(Arg70/Gln70)	47/28	22/6	25/22	0.028
ISDR of NS5A(wild/intermediate/mutant)	46/17/13	13/7/9	33/10/4	0.026

NOTE. HCV, hepatitis C virus; SVR, sustained virological response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; aa, amino acid; ISDR, interferon sensitivity–determining region.

(≥2) [13]. We determined all aa substitutions in the core region and ISDR by direct sequencing.

Detection of Cytokines

We quantified 8 cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-18, and vascular endothelial growth factor [VEGF]) using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit) for serum samples obtained before the start of treatment, 4 weeks after the start of treatment, and 24 weeks after treatment completion. All collected samples were immediately stored at −70°C and remained in storage until testing.

Statistical Analysis

We used the Mann–Whitney *U* test and Kruskal–Wallis test to analyze continuous variables where appropriate. We used the Friedman test to evaluate changes in serum cytokine levels over time. We used the Spearman rank correlations to evaluate the relationship between pairs of markers. We used the χ^2 test with the Yates correction for the analysis of categorical data. In cases where the number of participants was <5, we used the Fisher exact test. We considered a *P* value of ≤.05 statistically significant. To predict treatment outcome, cutoff points for continuous variables were decided by receiver-operating characteristic (ROC) curve analysis. Multivariate analysis was performed using a stepwise logistic regression model. Statistical analyses were performed using SPSS software version 18.0J.

RESULTS

Detection and Quantification of Serum Markers in Patients with Chronic Hepatitis C and Controls

Of the 79 patients receiving PEG-IFN and ribavirin therapy, 31 (39%) were sustained responders with accompanying normalization of ALT levels. Of the 48 patients without an SVR, 23 had a relapse and 25 did not respond to treatment. Patients with an SVR had a higher male ratio compared with patients without ($P = .001$) (Table 1). Before treatment, the median AST level in the SVR group was significantly lower than that in the non-SVR group (36 vs 48 IU/L; $P = .012$). Substitutions of aa 70 in the core region ($P = .028$) and in the ISDR ($P = .026$) were both significantly associated with treatment outcome.

Serum samples obtained prior to antiviral therapy were examined for the presence of 8 cytokines by multiplex assays. Of these, 6 could be reliably quantified in a large majority of samples. As shown in Figure 1, the median baseline serum concentrations of 4 cytokines [IL-10 (4.8 vs 4.3 pg/mL; $P = .032$), IL-12p40 (20.4 vs 8.5 pg/mL; $P < .001$), IL-12p70 (12.8 vs 1.0 pg/mL; $P < .001$), and IL-18 (21.9 vs 14.5 pg/mL; $P = .008$)] were significantly higher in patients with HCV infection than in healthy controls. Conversely, serum levels of IL-4 (7.3 vs 7.9 pg/mL; $P = .011$) and VEGF (57.5 vs 78.0 pg/mL; $P = .025$) were significantly lower in patients with HCV infection compared with those in controls.

Effects of Antiviral Therapy on Serum Cytokine Levels

The median baseline serum levels of 4 cytokines (IL-12p40 [24.1 vs 17.2 pg/mL; $P = .003$], IL-12p70 [15.9 vs 12.6 pg/mL; $P < .001$], IL-18 [27.9 vs 17.7 pg/mL; $P = .001$], and VEGF [93.0 vs 39.7 pg/mL; $P < .001$]) were significantly higher in patients who achieved an SVR than in those who did not (Figure 2). In contrast, SVR patients showed significantly lower baseline IL-10 concentrations (4.1 pg/mL) than non-SVR patients (7.3 pg/mL; $P = .002$).

Significantly higher baseline levels of 3 cytokines (IL-4 [7.8 vs 7.0 pg/mL; $P = .001$], IL-12p40 [24.1 vs 14.6 pg/mL; $P < .001$], and VEGF [65.5 vs 43.0 pg/mL; $P = .025$]) were observed in patients with a virological response compared with levels in those without. Conversely, IL-10 levels (4.3 vs 7.9 pg/mL; $P < .001$) were significantly lower in virological responders compared with that in nonresponders.

Several demographic (age and sex) and clinical (ALT level, AST level, and viral load) findings were examined for their correlation with serum cytokines in patients with HCV infection, but no significant associations were observed. However, serum IL-12p40 levels were significantly correlated with serum IL-18 ($P = .004$, $r = 0.325$) (Figure 3A) and VEGF ($P = .024$, $r = 0.253$) (Figure 3B). There was also a significant correlation between IL-18 and VEGF ($P < .001$, $r = 0.394$) (Figure 3C).

Prediction of Treatment Outcome in Patients with Chronic Hepatitis C

We performed ROC curve analyses to determine the optimal cutoff values for serum cytokines in predicting treatment outcome for genotype 1 HCV–infected patients. We obtained the ROC curve for serum IL-10 via calculations using the values obtained from 25 nonresponders and 54 patients with a virological response. The ROC curves for serum IL-12p40, IL-18, and VEGF were obtained from 31 patients who achieved an SVR and 48 non-SVR patients. We selected optimal cutoff point values based on the cytokine level at which accuracy was maximal. The optimal cutoff value, sensitivity, specificity, positive predictive value, negative predictive value, and calculated area under the curve (AUC) for the 4 cytokines are listed in Table 2. The AUC values were consistently high and ranged between.70 (IL-12p40) and.86 (IL-10).

In addition, ROC curves for serum IL-10, IL-12p40, IL-18, and VEGF at 4 weeks after the start of treatment were obtained (Table 2). The AUCs for these 4 cytokines (.62–.86) were also high, but lower than those at baseline.

Correlation Between Core Region and Interferon Sensitivity–Determining Region Amino Acid Substitutions and Cytokine Production.

Because core region and ISDR substitutions have been associated with treatment outcome both in this study and elsewhere, we analyzed whether substitutions in these regions were correlated with baseline serum cytokine concentrations as

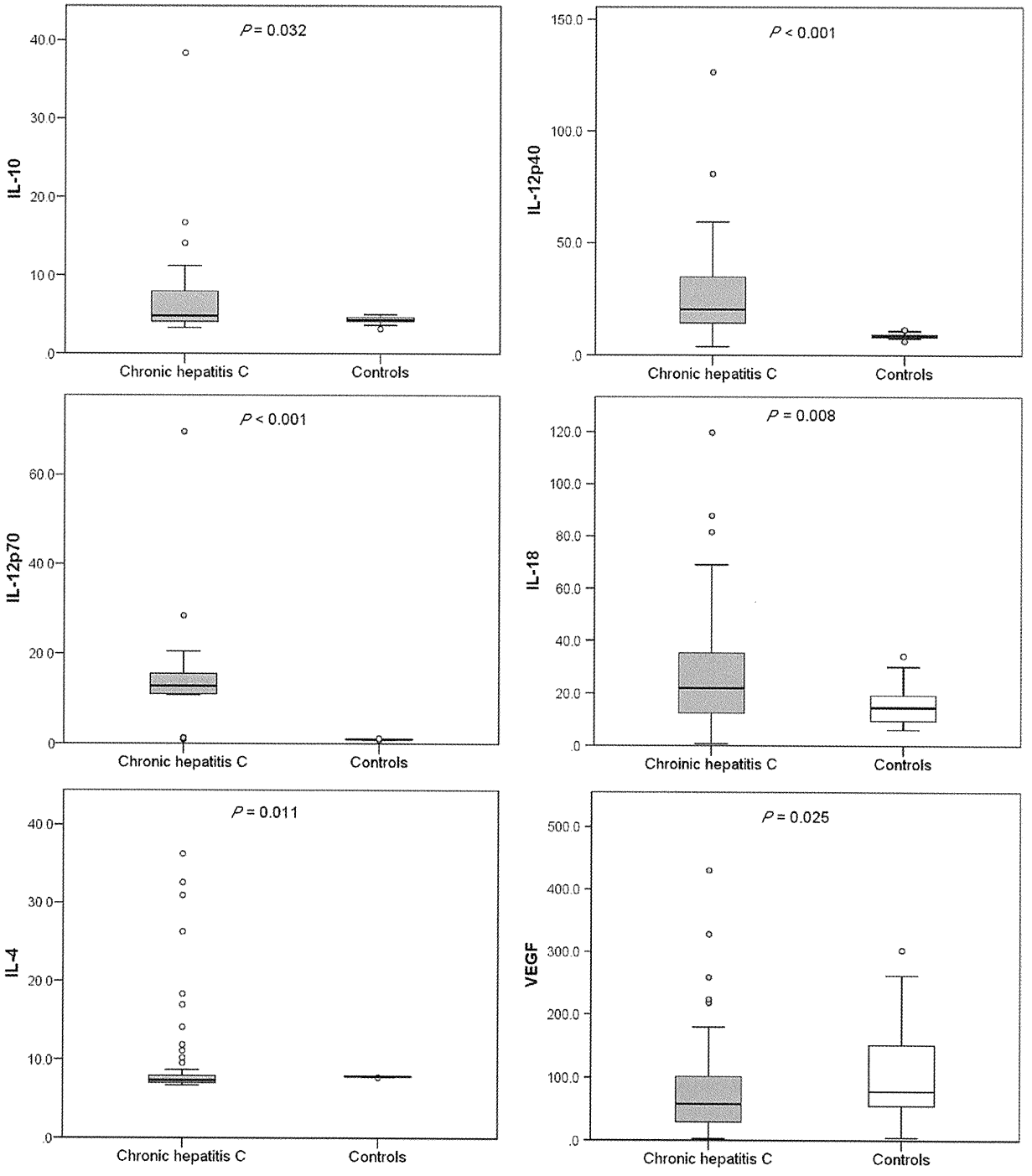

Figure 1. Detection of Serum Cytokines in Patients with HCV Infection and Healthy Subjects. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. Serum IL-10, IL-12p40, IL-12p70, IL-18, IL-4, and VEGF levels were detected in 79 patients with HCV infection and 26 controls. **NOTE.** HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

well. Before treatment, median IL-10 levels in patients with Gln70 (7.5 pg/mL) were significantly higher than those in patients with Arg70 (4.3 pg/mL; $P = .045$). The prevalence of higher serum IL-10 (≥5.0 pg/mL at baseline) was significantly greater in the nonresponse group than in the response group (25 of 25 patients [100%] vs 11 of 50 [22%]; $P < .001$). The frequencies of the combination of higher IL-10 and HCV with and without core Gln70 were 14 of 25 patients (56%) and 3 of 50

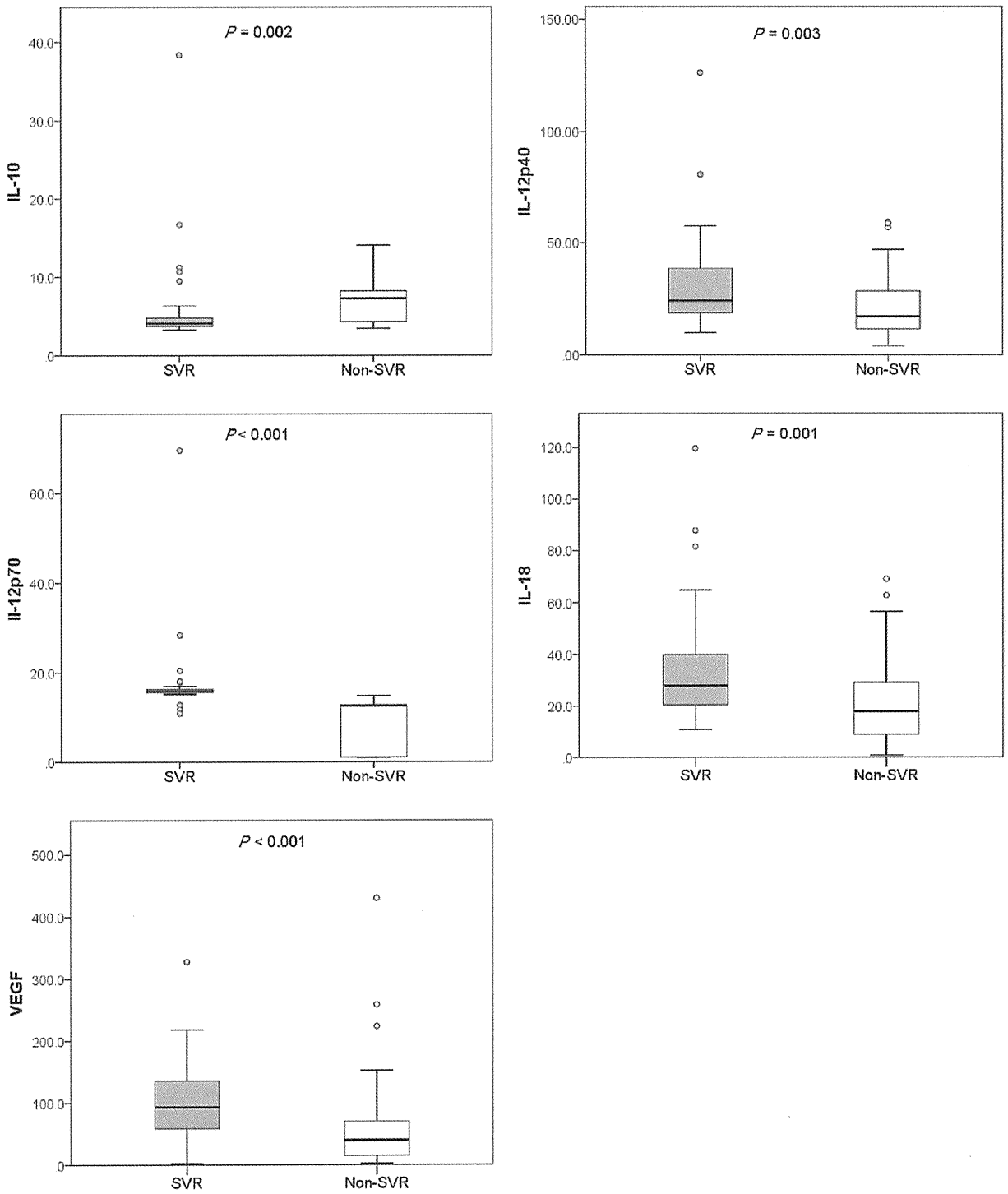

Figure 2. Serum Cytokines Related to Antiviral Therapy Outcome. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) Serum IL-10, IL-12p40, IL-12p70, IL-18, and VEGF were detected in 31 patients who achieved a sustained virological response and 48 patients who did not. **NOTE.** SVR, sustained virological response; IL, interleukin; VEGF, vascular endothelial growth factor.

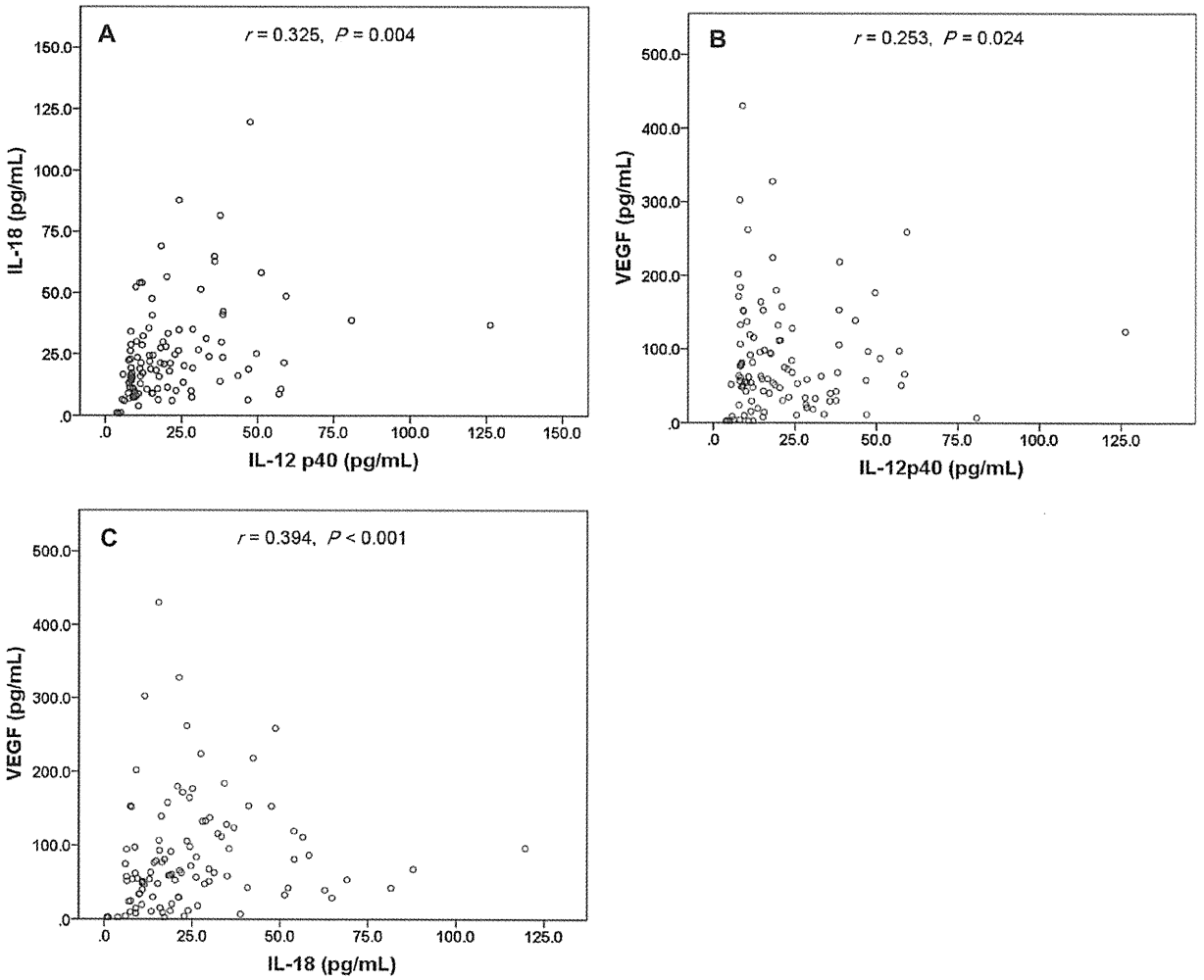

Figure 3. Correlation Between Serum Cytokines in 79 Patients with HCV Infection. (A–B) Serum IL-12p40 was significantly correlated with the level of (A) IL-18 ($r = .325$; $P = .004$) and (B) VEGF ($r = .253$; $P = .024$). (C) Serum IL-18 was correlated with the level of VEGF ($r = .394$; $P < .001$).
NOTE. HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

Table 2. Optimal Cutoff Value, Sensitivity, Specificity, Area Under The Curve, and Predictive Values of Serum IL-10, IL-12p40, IL-18, and VEGF at Baseline and After 4 Weeks of Treatment in 79 Patients with Chronic Hepatitis C

Cytokine	Collection Time	Cutoff Value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-10	baseline	5.0	100 (86–100)	80 (67–89)	.86 (.84–.98)	69	100
	4 wk	6.8	82 (69–91)	100 (86–100)	.86 (.78–.95)	100	71
IL-12p40	baseline	17.4	81 (63–93)	52 (37–67)	.70 (.59–.82)	52	81
	4 wk	21.3	81 (63–93)	60 (45–74)	.69 (.57–.81)	57	83
IL-18	baseline	15.4	97 (83–100)	46 (31–61)	.72 (.61–.83)	54	96
	4 wk	24.6	87 (70–96)	42 (28–57)	.62 (.50–.75)	49	83
VEGF	baseline	57.6	77 (59–90)	69 (54–81)	.74 (.63–.86)	62	83
	4 wk	62.6	74 (55–88)	67 (52–80)	.70 (.58–.82)	59	80

NOTE. CI, confidence interval; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; IL, interleukin

All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .01$ for all comparisons). Cutoff values were determined by constructing receiver operating characteristic curves and are expressed as pg/mL. IL-10 is predictive of a nonresponse. IL-12p40, IL-18, and VEGF are predictive of a sustained virological response.

Table 3. Multivariate Analysis of Factors Independently Associated with a Sustained Virological Response to Pegylated Interferon and Ribavirin Therapy in Patients Infected with Hepatitis C Virus Genotype 1

Factors	OR	95% CI	*P*
Gender: male	10.932	2.178–54.780	.004
AST ⩾ 40 IU/L	.946	.906–.989	.013
IL-10 ⩾ 5.0 pg/mL	.823	.704–.962	.014
IL-12p40 ⩾ 17.4 pg/mL	1.071	1.009–1.137	.024
IL-18 ⩾ 15.4 pg/mL	1.085	1.024–1.150	.006

NOTE. OR, odds ratio; CI, confidence interval; AST, aspartate aminotransferase;IL, interleukin.

Only variables that achieved statistical significance ($P < .05$) in multivariate logistic regression analysis are shown.

patients (6%), respectively, which was statistically significant ($P < .001$).

Serum levels of IL-12p70 were significantly correlated with the number of substitutions in the ISDR (Kruskal–Wallis; $P = .027$). In addition, median baseline serum IL-12p70 levels were significantly higher in patients with mutant-type ISDR than in those with wild or intermediate types (15.6 vs 12.7 pg/mL; $P = .009$).

Factors Independently Associated with a Sustained Virological Response

We evaluated several factors found in association with an SVR from PEG-IFN and ribavirin therapy for their independence by multivariate analysis (Table 3). Male (odds ratio 10.93 [95% confidence interval 2.18–54.87], $P = .004$), AST ⩾40 IL/L (.95 [.91–.99], $P = .013$), IL-10 ⩾ 5.0 pg/mL (.82 [.70–.96], $P = .014$), IL-12p40 ⩾ 17.4 pg/mL (1.07 [1.01–1.14], $P = .024$), and IL-18 ⩾ 15.4 pg/mL (1.09 [1.02–1.15], $P = .006$) were independent risk factors related to an SVR. Conversely, core region or ISDR substitutions were not significant independent associations in this study.

Serum Cytokine Changes During and After Treatment

We next measured cytokine levels 4 weeks after the initiation of therapy and 6 months after its completion (Table 4). The levels of IL-10 ($P < .001$, Friedman test), IL-12p40 ($P = .008$), and IL-18 ($P < .001$) were significantly decreased in samples collected from patients who achieved an SVR. The reduction in serum cytokine levels from baseline to 4 weeks of treatment was determined and compared between SVR and non-SVR groups, and showed that the ratio of IL-10 had a significant negative association with both an EVR ($P = .024$) and an SVR ($P = .001$).

DISCUSSION

In this study, we measured the levels of 8 cytokines in patients with genotype 1 chronic hepatitis C and analyzed their association with the outcome of PEG-IFN and ribavirin therapy using a newly developed bead-array multiplex system. Serum IL-10, IL-12p40, IL-12p70, and IL-18 were higher in patients with HCV infection than in healthy participants. In addition, cytokines IL-10, IL-12p40, and IL-18 all decreased during treatment and remained low in patients with an SVR. These findings suggest that cytokines may in fact compromise host immune responses to the virus.

A strong association between high baseline serum IL-10 and a nonresponse to PEG - IFN and ribavirin therapy was found in our cohort, which is consistent with previous studies [7, 14, 15]. We found achievement of an EVR or SVR to be diminished in patients who had a lower IL-10 ratio between baseline and 4 weeks of treatment. In addition, using ROC curve analysis, we found sensitivity, specificity, and AUC were all high for IL-10, suggesting that serum IL-10 values at baseline and 4 weeks of treatment are predictive markers for treatment nonresponse (Table 2). Although humoral immunity is said to play a minor role in recovery from HCV infection and B-cell immunity is strongest in those with persistent infection [8, 16], a strong natural killer cell–mediated and Th1 cell–mediated immune response seems to be a key factor in protection from HCV infection. IL-10 was originally described as a cytokine synthesis inhibitory factor [17, 18], but recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathologic effects of Th17 [19, 20]. Furthermore, there is strong evidence of a substantial genetic component to IL-10 production [21, 22]; the –1082 G/G genotype is known to be related to increased IL-

Table 4. Serum Cytokine Levels Changes During and After Treatment of Pegylated Interferon Plus Ribavirin

Cytokines	Treatment Outcome	Baseline	Week 4	Week 72	*P*
IL-10	SVR	4.1 (3.3–25.4)	3.7 (3.1–19.9)	3.5 (2.9–9.0)	< .001
	Non-SVR	7.3 (3.7–10.8)	7.5 (3.9–8.8)	7.4 (3.9–10.9)	0.962
IL-12p40	SVR	24.1 (11.3–99.0)	22.1 (11.6–75.2)	18.4 (7.8–76.5)	0.008
	Non-SVR	17.2 (4.6–57.9)	19.2 (8.1–50.1)	21.6 (5.8–77.0)	0.281
IL-18	SVR	27.9 (13.8–100.6)	25.1 (13.2–95.2)	23.3 (6.6–48.5)	< .001
	Non-SVR	17.7 (1.1–59.9)	31.3 (10.3–90.6)	17.4 (5.4–52.0)	< .001

NOTE. Data are median (5th–95th percentile) values. IL, interleukin; SVR, sustained virological response.

10 production and is associated with a high risk of inefficient HCV clearance [23, 24] and resistance to IFN treatment [25–28].

In agreement with our findings, recent studies have indicated that Gln70 substitutions in the HCV core region are associated with treatment failure [11, 29–32]. Additionally, patients with Gln70 had higher IL-10 levels compared with those with Arg70. Among the 28 HCV patients who had Gln70, all 14 nonresponders had higher IL-10 (≥5.0 pg/mL), whereas 11 of 14 responders had lower IL-10 levels ($P < .001$). This association between Gln70 and elevated IL-10 levels is intriguing. Dolganiuc et al reported that HCV core and NS3 proteins in monocytes and dendritic cells induce IL-10 [33], so further studies are needed to clarify the relationship between IL-10 and core region amino acid substitutions.

This report demonstrates the beneficial role of IL-12 in achieving an SVR during PEG - IFN and ribavirin therapy. IL-12 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function, and amplifies the cytotoxicity of cytotoxic T lymphocytes and natural killer cells [34]. Thus, production of IL-12 is directed toward the elimination of intracellular pathogens and viruses. Elevated serum IL-12 has been noted in patients with chronic HBV or HCV infection, and is even more prominent among responders to IFN-α treatment [35, 36]. In our study, we noted significantly higher serum IL-12p70 in participants carrying mutant-type ISDR than in those with intermediate- or wild-type ISDR. This correlation between IL-12 and ISDR substitutions is striking and requires further study to verify its favorable effect during PEG-IFN and ribavirin therapy.

It is believed that the dynamics of the Th1/Th2 response determine the outcome of antiviral therapy to chronic hepatitis C [10] and that IL-18 is an important mediator of the Th1/Th2 balance. IL-18 plays a critical role in host defense against infection by intracellular microbes but also induces autoimmune diseases and propagates inflammation [37]. IL-18 is significantly upregulated in patients with chronic HCV infection and is correlated with hepatic injury [38, 39], indicating a key role in disease pathogenesis. However, the effect of IL-18 on antiviral therapy for chronic hepatitis C is still unclear. We found that IL-18 levels were significantly higher in patients with chronic HCV infection compared with healthy controls, but they were also higher at baseline in patients who achieved an SVR than in those who did not. In addition, there was a significant correlation between IL-18 and IL-12; in the presence of IL-12, IL-18 stimulates *IFNG* expression, thus promoting the Th1-mediated immune response. Without IL-12, IL-18 stimulates Th2 responses [37]. In this study, because serum IFN-γ levels were below detection thresholds, we could not assess the association of such cytokines.

Lastly, we observed that pretreatment serum VEGF levels were associated with an SVR. A previous study showed no association between baseline VEGF and treatment outcome, but only 36 patients, including 19 with genotype 1, were studied [40]. Hence, it is still unclear if this angiogenesis marker plays a critical role in response to antiviral therapy in chronic HCV infection. Furthermore, we correlated VEGF with IL-12 and IL-18 in our study. In particular, IL-18 enhances the production of VEGF in rheumatoid arthritis synovial fibroblasts, suggesting that IL-18 could be an angiogenic mediator with triggering effects on VEGF production [41]. Although the preoperative serum VEGF level was found to be a significant predictor of tumor recurrence and overall survival in patients with HCC [42], there have been no reports regarding treatment response in patients with chronic hepatitis C during antiviral therapy.

In multivariate analysis of our cohort, low IL-10, high IL-12p40, and high IL-18 were independent factors related to an SVR in patients treated with PEG-IFN and ribavirin. Our results indicate that such 3-cytokine profiling may offer clinicians another tool in predicting treatment outcome of HCV infection. Further investigation must be done in vitro and using many samples to validate the significance of our findings.

In conclusion, several cytokines were seen to be elevated in patients with chronic hepatitis C using the multiplex bead assay. Serum IL-10 levels and amino acid substitutions at the 70 aa core region of HCV are useful for predicting a nonresponse to PEG-IFN and ribavirin therapy in patients with chronic hepatitis C genotype 1. A higher level of serum IL-12 is considered to be favorable for response to antiviral therapy, and is correlated with substitutions in the ISDR. Lastly, IL-18 is notably high in patients with chronic HCV infection, and is correlated with IL-12.

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Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture

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ABSTRACT

Adipose tissue-derived mesenchymal stem cells (ASCs) have been reported to be multipotent and to differentiate into various cell types, including osteocytes, adipocytes, chondrocytes, and neural cells. Recently, many authors have reported that ASCs are also able to differentiate into vascular endothelial cells (VECs) in vitro. However, these reports included the use of medium containing fetal bovine serum for endothelial differentiation. In the present study, we have developed a novel method for differentiating mouse ASCs into VECs under serum-free conditions. After the differentiation culture, over 80% of the cells expressed vascular endothelial-specific marker proteins and could take up low-density lipoprotein in vitro. This protocol should be helpful in clarifying the mechanisms of ASC differentiation into the VSC lineage.

1. Introduction

Recently, adipose tissue is an important source of adult stem cells [1]. Adipose-derived mesenchymal stem cells (ASCs) can be obtained in high yield with minimal discomfort under local anesthesia [2,3]. After the reports of Zuk et al. [4,5], many studies have examined the plasticity, induction ability, and individual characteristics of ASCs. Derived from the embryonic mesoderm, adipose tissue is a heterogeneous cell population that includes smooth muscle cells, fibroblasts, adipocytes, mast cells, and endothelial cells [6–8]. ASCs are an adherent cell population in vitro and maintain their mesenchymal phenotype and plasticity towards the mesenchymal lineage even after they propagate in culture for several passages. These cells can differentiate into several cell types in vitro, including adipocytes, chondrocytes, osteoblasts, cardiomyocytes, and endothelial cells [5,9–13]. Moreover, ASCs are reported to have positive effects on patients who received bone marrow transplantation and suffered from GVHD (graft versus host disease), suggesting that they have an immuno-modulatory function [14].

In the present study, we focused on whether ASCs are able to differentiate into vascular endothelial cells (VECs) in a chemically defined medium after expanding them. Although mouse [15], rat [16], and human [13,17–19] ASCs have already been reported to differentiate into VECs, all of the differentiation methods have utilized fetal bovine serum (FBS). When considering the clinical applications for regenerative medicine in the future, possible contamination by animal serum is a negative factor for safety. Unknown factors in FBS also prevent researchers from accurate analysis of the differentiation mechanism. Therefore, we attempted to develop a new method for differentiating ASCs into functional VECs without serum.

2. Materials and methods

2.1. Isolation of ASCs from mice

Inguinal adipose tissue was isolated from 12- to 14-week-old adult female and GFP-transgenic C57BL/6J mice. The tissue was minced into 2–3 mm pieces in DMEM (Gibco) containing 10% FBS, and incubated at 37 °C in 5% CO_2 incubator for 1 h. Then the suspension was centrifuged at 1300 rpm for 6 min at room temperature. To dissociate the cells, they were treated with 0.12% collagenase type I solution and incubated at 37 °C for 30 min and then centrifuged at 1300 rpm for 6 min at room temperature. The cells were cultured in DMEM containing 5% FBS, 10 units penicillin,

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and 10 μg/ml streptomycin (GIBCO) in a CO_2 incubator at 37 °C. After continuous culture for five passages, they were used for the differentiation experiments. To eliminate any intact VECs, CD31 positive cells were removed using anti-CD31 antibody-conjugated beads (MACS) after 2 h incubation of the preparation. The CD31 negative cells were cultured and serially passaged. Animal experiments were approved by the Animal Care and Use Committee of National Center for Global Health and Medicine.

2.2. Examination of ASCs differentiation capacity into adipogenic and osteogenic differentiation

To confirm the multipotency of our cultured ASCs, we tested whether they could differentiate into adipocytes and osteoblasts, as reported previously. Passage 5 cells were cultured in adipogenic medium for 2 weeks; hMSC Adipogenic Induction SingleQuots (Cambrex) supplemented with indometacin, IBMX, insulin, dexamethasone, NCGS, and L-glutamine. The cells were fixed in 10% formalin for 10 min and stained with Oil red-O solution (Merck) to detect lipid droplets. To confirm osteogenic differentiation, the cells were cultured in osteogenic medium: hMSC Osteogenic SingleQuots (Cambrex) supplemented with ascorbate, MCGS, β-glycerophosphate, and L-glutamine. After 2 weeks, the alkaline phosphatase activity of the cells was measured by Alkaline Phosphatase Kit (Takara Bio) and the expression of an osteogenic protein marker, osteopontin, was examined by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.3. Differentiation into vascular endothelial cells

To initially determine whether the ASCs could develop the characteristics of VECs or not, they were cultured in a commercially available vascular cell maintaining medium, EBM-2 (CAMBREX) containing 2% FBS and EGM-2 BulletKit (mixture of FGF2, VEGF, heparin, IGF-I, EGF, hydrocortisone, and ascorbic acid, CAMBREX) on collagen type IV coated dish, for 12 days; then their gene expression was verified. Next, we surveyed supplements that are able to replace FBS. We tested 2% KSR, B27, N2, G5, or ITS (Invitrogen); each candidate supplement was added in EBM-2 medium instead of FBS. And we also examined the other culture medium such as DMEM, IMDM, and DMEM/F12 instead of EBM-2. Finally, to determine the optimal concentration of FGF2 or VGEF, different concentrations (0, 5, 10, and 20 ng/ml) was tested for induction of endothelial cells. When the optimal culture medium for the VEC induction from ASCs was determined to be DMEM/F12 medium containing 10 ng/ml FGF2, 2% ITS, and EGM-2 BulletKit (without FGF2), further experiments for functional assay and transplantation employed this medium.

2.4. RT-PCR and real time PCR

Marker gene expression of VECs was determined by RT-PCR. After ASCs were cultured for 12 days in endothelial differentiation medium on a collagen type IV dish, total RNA was extracted by the use of Isogen (Nippon gene) as described by the manufacturer, and was treated with Superscript III (Invitrogen) to generate cDNA using oligo(dT) adaptor primer (Sigma). Then PCR amplification was performed for mouse *flk1, flt1, VE-cadherin*, and *CD31*. PCR cycles were as follows: 95 °C for 5 min, 95 °C for 30 s, annealing temperature for 30 s, 72 °C for 1 min (25–30 cycles), and 72 °C for 3 min. The RT-PCR products ware analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide. Primers for PCR were follows: *flk1* (5′-GCC AAT GAAGGG GAACTGAAGAC-3′, 5′-TCTGGCT GCTGGTGATGCTGTC-3′), *flt1* (5′-TGTGGAGAAACTTGGTGACCT-3′, 5′-TGGAGAACAGCAGGACTCCTT-3′), *ve-cadherin* (5′-TTGCCCAGCCC TACGAACCTAAAG-3′, 5′-ACCACCGCCCTCCTCATCGTAAGT-3′), *CD31* (5′-GGTGACACTGGACAAAAAGG-3′, 5′-CAGCTTCACTGCTTTGCTT G-3′), *gapdh* (5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, 5′-CATG TAGGCCATGAGGTCCACCAC-3′). For the real-time PCR, primers are follows: *tie2* (5′-GTGAAGATCAAGAATGCTACC-3′, 5′-GTGAAGATC AAGAATGCTACC-3′), *CD31* (5′-GTTTGTCAAGCGAAGGATAGATA A-3′, 5′-TCCTGCACGGTGACGTATTCACT-3′), *von Willebrand factor* (*vWF*, 5′-AACGGAAGTCCATGGTTCTG-3′, 5′-CCCCATTGAAGGCAT ACTCC-3′). Reactions were performed using SYBER Premix ExTaq (Takara Bio) and a MyiQ thermal cycler (BIORAD).

2.5. Immunocytochemistry

Differentiated endothelial-like cells from ASCs were fixed with 4% paraformaldehyde for 30 min at room temperature and then treated successively with 0.3% Triton X-100 (Wako Chemical) in PBS (Sigma) for 15 min followed by 3% bovine serum albumin (Sigma) for 30 min to reduce nonspecific reactions. The cells were reacted overnight with each of the following anti-endothelial marker antibodies at a 1:300 dilution at 4 °C; anti-flk1 (Becton Dickinson), anti-CD34 (Becton Dickinson), and anti-tie2 (Santa Cruz Biotechnology) antibodies. Then the cells were stained by Alexa Fluor 488 or 594 conjugated antibody (Molecular Probes) as the secondary antibody for 1 h at room temperature. Their nuclei were stained with DAPI for 10 min. The photographs were taken with a DP70 digital camera (Olympus) and analyzed by MetaMorph software (Molecular Devices).

2.6. Examination of cell function in vivo and in vitro

For the examination of tubular formation, the cells were seeded on Matrigel (Becton Dickinson) at 5×10^4 cells/35 mm dish. After 24 h, the morphology of the cells was examined, and phase-contrast images were photographed (Olympus IX70). For in vivo examination, the femoral muscle of a mouse was injured by liquid nitrogen and injected with the differentiated vascular endothelial-like cells (1×10^6 cells) from ASCs of GFP-transgenic mice. Two weeks after cell injection, we investigated whether the donor cells had formed vessel-like structures.

LDL uptake was assessed by incubating cells for 4 h at 37 °C with 2.5 μg/ml Alexa Fluor 488 conjugated acetyl-LDL (Molecular Probes). Cells were analyzed by fluorescence microscopy and a flow cytometer (EPICS XL, Beckman Coulter).

3. Results

3.1. In vitro differentiation of ASCs

To prove that the cultured cells from adipose tissue had retained their multipotent differentiation potential, we first confirmed that they differentiated into adipogenic and osteogenic lineages. When ASCs (Fig. 1A) were cultured in adipogenic medium for 2 weeks, more than 40% of the cells became lipid-retaining cells that stained by Oil-red O (Fig. 1B). In osteogenic medium, more than 50% of the cells were induced into an osteogenic lineage confirmed by alkaline phosphatase staining (Fig. 1C). The gene expression of *osteopontin* was also detected (Fig. 1D).

3.2. ASCs cultured in growth factor mix changed their gene expression pattern to closely resemble that of vascular endothelial cells

It was reported that the early passages of ASCs can contain small amounts of VECs and express VEC marker proteins [20]. Therefore, we used anti-CD31 antibody to remove any CD31 positive cells during the preparation of ASCs. To examine whether the ASCs could differentiate into VECs in "vascular endothelial maintaining

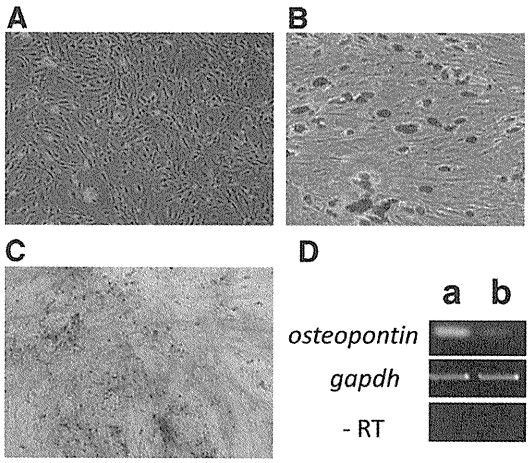

Fig. 1. Examination of mouse adipose-derived mesenchymal stem cell (ASCs) differentiation capacity into adipogenic and osteogenic lineages. (A) Mouse ASCs after five passages. (B) ASCs were cultured in adipogenic medium for 2 weeks. About 40% of the cells were stained by Oil-red O, indicating they differentiated into mature adipocytes. (C) More than 50% of the cells were positive for alkaline phosphatase staining after 2 weeks cultured in osteogenic medium, and they also express an osteopontin gene. (D) a: cultured in the osteogenic medium, b: cultured in normal ASCs medium.

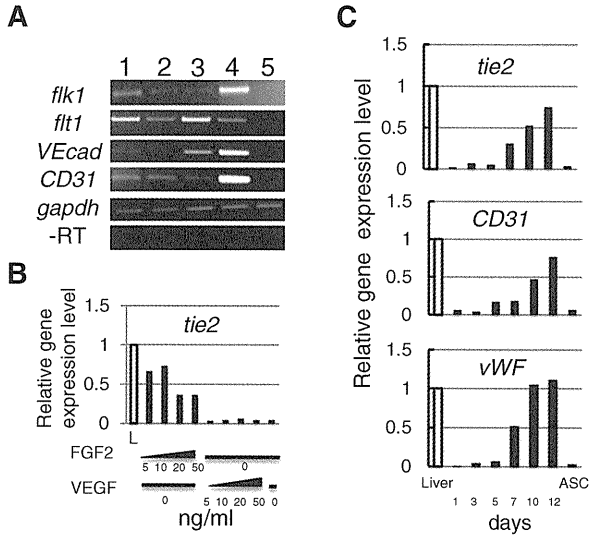

Fig. 2. Vascular endothelial-specific gene expression analyzes in different culture media. (A) RT-PCR analysis of ASCs cultured in EBM-2 medium containing EGM-2 BulletKit in the presence of FBS for 12 days (lane 1). ASCs cultured in EBM-2 containing EGM-2 BulletKit and ITS (lane 2). ASCs cultured in DMEM/F12 medium containing EGM-2 BulletKit and ITS (lane 3). Compared to the "vascular endothelial maintained medium" containing FBS (lane 1), *ve-cadherin* expression was observed almost the same level when ASCs cultured in serum-free DMEM/F12 medium (lane 3). For comparison, 8-week-old mouse liver cells (lane 4) and normal ASCs (lane 5) are shown. (B) Effects of FGF2 and VEGF in different concentrations on the differentiation of ASCs Expression of *tie2*, one of the vascular endothelial markers, was highest when 10 ng/ml FGF2 containing medium was used. *Tie2* gene expression did not elevate in any concentrations of VEGF in the absence of FGF2 and serum L (liver used as positive control). (C) Time course expression of *tie2*, *CD31*, and *vWF* genes. All of genes were upregulated 7–10 days after the beginning of differentiation culture. ASCs were differentiated in DMEM/F12 medium containing EGM-2 BulletKit (without FGF2), ITS, and 10 ng/ml FGF-2.

medium", we cultured ASCs in EBM-2 medium containing 2% FBS and EGM-2 BulletKit on a collagen type IV coated dish. After 12 days of culture, they expressed the vascular endothelial marker genes, *flk1, flt1, ve-cadherin,* and *CD31* (Fig. 2A, lane 1). Normal ASCs did not express these genes at all (Fig. 2A, lane 5). These results indicate that the ASCs have capacity to differentiate into VECs. The "vascular endothelial maintaining medium" contains 2% FBS, which may contain variable amounts of unknown factors including growth factors. To establish a stable method for differentiating VECs from ASCs, we tried to establish a serum-free culture method. We tested 2% KSR, B27, N2, G5, and ITS as replacements for FBS and found that the ITS supplement (insulin, transferrin, and selenium) had almost the same effects as FBS on endothelial differentiation. The cells expressed *flk1, flt1* and *CD31* but did not express *ve-cadherin* (Fig. 2A, lane 2). Others supplements were not so much upregulated the gene expressions except for ITS (data not shown). Therefore, we tested other types of basal medium, DMEM, IMDM, and DMEM/F12, as replacements for EBM-2. When we changed the medium from EBM-2 to DMEM/F12, the gene expression of *ve-cadherin* was proven (Fig. 2A, lane 3). There was no effect was observed when the medium was used DMEM or IMDM (data not shown).

Next, we attempted to determine the optimal concentrations of FGF2 and VEGF, because both factors are considered to be important for the differentiation of VECs [21,22]. The ASCs were seeded in DMEM/F12 medium containing ITS and EGM-2 Bulletkit (without FGF2 and VEGF). Then we added various concentrations of FGF2 or VEGF to the culture medium. After 12 days, we analyzed for expression of the early vascular endothelial marker gene, *tie2*, by real-time PCR. Without FGF2, expressions of vascular endothelial marker genes did not increase (Fig. 2B) even when the concentration of VEGF was elevated. On the other hand, in the presence of FGF2, *tie2* expression level increased, and 10 ng/ml FGF2 was the most efficient concentration (Fig. 2B). These results indicate that, when the ASCs differentiate into VECs in this serum-free medium, FGF2 plays a more important role than VEGF.

When we examined the time course expression of vascular endothelium-specific genes of ASCs cultured in this differentiation medium, *tie2* and *CD31* showed almost the same pattern of expression, beginning to express after 5–7 days culture and increasing gradually up to 12 days. Gene expression of *vWF* increased after 7 days culture and reached almost its highest level after 10 days culture (Fig. 2C).

It must be mentioned that we used two lines of ASCs prepared differently during primary culture; one was prepared directly without any selection and the other was the cell population from which CD31 positive cells were removed to avoid contamination by intact endothelial cells. After five passages, both lines were examined for vascular endothelial differentiation, no differences between the two groups with respect to the differentiation efficiency were detected.

3.3. Differentiated ASCs express vascular endothelial-specific protein

To examine whether the differentiated ASCs actually expressed vascular endothelial-specific marker proteins, we stained those cells with anti-flk1, anti-tie2, and anti-CD34 antibodies. Most of the cells positively expressed flk1, tie2, and CD34 (Fig. 3A–C). The average of percentages of positive cells calculated by MetaMorph software were as follows: flk1 (82%), tie2 (78%), and CD34 (87%), respectively. These results indicate that approximately 80–90% of the ASCs differentiated into vascular endothelial-like cells.

3.4. VECs differentiated from ASCs showed similar morphological and physiological functions in vitro and in vivo

After ASCs were cultured in DMEM/F12 medium with EGM-2 BulletKit (without FGF2), ITS and 10 ng/ml FGF2 on collagen type IV coated dishes, they were capable of forming tubular-like vascular structures when they were seeded in Matrigel dishes (Fig. 4C). In contrast, ASCs cultured in DMEM containing 5% FBS

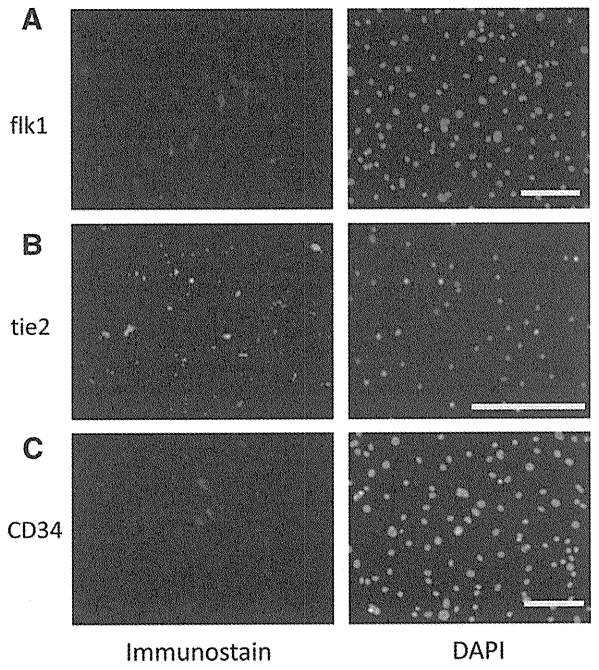

Fig. 3. Immunohistochemical staining of differentiated ASCs with anti-vascular endothelial marker proteins antibodies. All of three marker proteins (flk1, tie2, and CD34) were positively stained against the differentiated ASCs cultured for 12 days in the medium. Calculated with Metamorph software, average of flk1 (A), tie2 (B), and CD34 (C) positive cells were about 82%, 78%, and 87%, respectively ($n = 6$). Nuclei were stained with DAPI. Scale bar, 200 μm.

did not form such structures (Fig. 4A and B). We next examined whether the differentiated cells formed vessel-like structures in vivo. The femoral muscle of a C57BL/6J mouse was injured by liquid nitrogen. Then we injected vascular endothelial-like cells that had differentiated from ASCs derived from GFP mice into the muscle. Two weeks after the injection, we analyzed tissue samples to determine whether the cells histologically contribute to vessel-like structures. We found that GFP positive cells were incorporated into vessel-like structures, indicating that the VECs which were differentiated from ASCs successfully formed these structures (Fig. 4D) but control cells which cultured in DMEM medium containing 5% FBS did not (data not shown). These results suggest that the induced ASCs were able to form tubular structures almost as well as intact endothelial cells in vitro and in vivo.

Finally, we examined the efficiency of differentiation of ASCs into VECs by measuring LDL uptake. Alexa Flour 488-conjugated acetyl-LDL was added to the medium, and fluorescence positive cells were counted by a flow cytometer and observed under a fluorescence microscope. The differentiated ASCs showed very high acetyl-LDL uptake; over 80% of the cells were fluorescence positive (Fig. 4E and F). Undifferentiated ASCs did not uptake acetyl-LDL at all (Fig. 4F).

4. Discussion

In the present study, we showed that mouse ASCs can differentiate into VECs in serum-free medium in vitro. They formed tubular structures in Matrigel culture, and contributed to vessel-like structures in vivo. Analysis of the vascular endothelial function revealed that over 80% of cells incorporated acetyl-LDL during 6 h of culture, indicating most of the cells differentiated into the functional VECs. These results indicate our differentiation method can be useful for the efficient differentiation of VECs from ASCs.

It is well known that early passage ASCs contain VECs. However, these VECs are also reported to disappear when ASCs are continuously cultured until passage 5 [20], indicating that the VECs in adipose tissue do not propagate in the culture medium for ASCs. To rule out the possibility that VECs were contaminating our ASCs, (i) we removed the VECs using anti-CD31 antibody-conjugated beads before the primary culture and (ii) we used ASCs cultured for more than five passages. Both of the cell population almost equally differentiated into VECs; and there was no detectable difference. These results suggest that most of the intact VECs that may exist in a large population of ASCs at first disappear during continuous culture and propagation of ASCs.

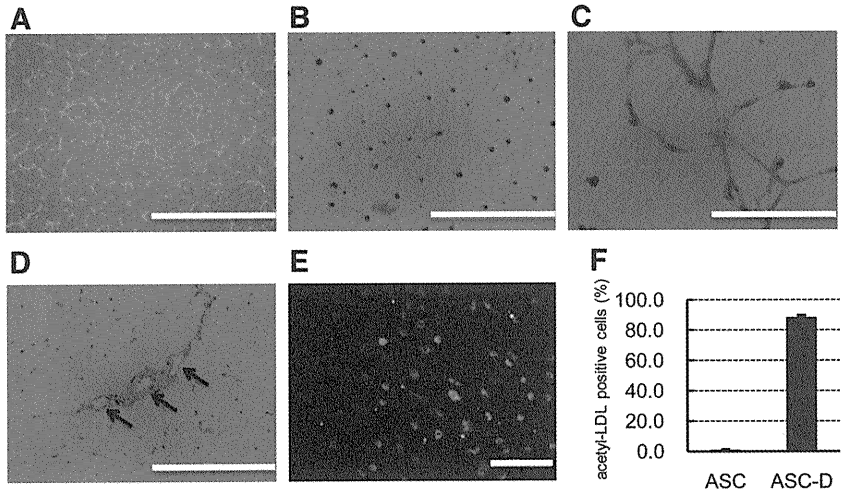

Fig. 4. Morphological and physiological functions of differentiated ASCs. Morphological changes in vascular endothelial-like cells differentiated from ASCs were examined. (A) Normal ASCs were cultured in matrigel for 6 h (A) and 24 h (B). Tubular formation was observed when vascular endothelial-like cells differentiated from ASCs were cultured in matrigel for 24 h (C). Vessel-like structures were formed in the mouse muscle and injected EGFP positive donor cells (brown and arrows) participated in the vessels (D). Acetyl-LDL conjugated fluorescence uptake of differentiated ASCs was examined. Most of the differentiated ASCs were green fluorescence positive when they were cultured in DMEM/F12 medium containing EGM-2 Bulletkit (without FGF2), ITS, and 10 ng/ml FGF2 for 12 days (E). (F) A flow cytometry analysis showed that more than 85% of the differentiated cells (ASC-D) incorporated acetyl-LDL. Normal ASCs did not acetyl-LDL uptake at all (ASC). Scale bar, 2 mm (A–C) and 200 μm (D and E).

FGF2 is a critical growth factor for the induction of VECs. It is reported to play an important role on angiogenesis and vasculogenesis [23–25]. VEGF is also reported to be a key growth factor during embryonic development and differentiation of vascular system [22,26]. We attempted to determine how important and effective these factors are for the induction of VECs in our serum-free condition. We examined the optimal concentrations of these growth factors and demonstrated that the presence of FGF2 upregulated the gene expression of vascular endothelial markers. Therefore, in serum-free conditions, FGF2 is considered an essential growth factor for ASC differentiation into VSC. It is reported that the biologic activity of FGF2 is dependent on the presence of heparin. Small heparin oligosaccharides of defined sizes can activate the mitogenic potential of FGF2 on appropriate target cells and are active in the binding of FGF2 to a soluble FGF2 receptor [27,28]. In the present study, the culture medium contains heparin and it probably upregulates the activity of FGF2. It is possible that heparin plays a critical role in the differentiation from ASCs into VECs in the absence of serum. One the other hand, VEGF did not influence the *tie2* gene expression at any concentration (Fig. 2B) when the culture medium did not contain FGF2. VEGF is reported to be secreted by both mouse [29] and human [30] cultured ASCs, and VEGF expression of VECs is induced by FGF2 [31]. Therefore, additional supplementation with VEGF was not effective for differentiation of VECs in the absence of FGF2 in our experiments.

ASCs were reported to differentiate into VECs when they were cultured in a medium containing FBS [15–18]. FBS contains various unknown factors in varying amounts and may prevent further analysis of the differentiation mechanisms. It is also a negative when cells are prepared for clinical use, because the cells could possibly incorporate proteins or carbohydrates derived from this [32]. The present study intended to establish a medium for differentiation from ASCs to VECs without the usage of FBS. Our method is useful for further analysis of the mechanisms of differentiation from ASCs to VECs and will shed new light on stem cell research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.029.

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