TABLE VII. Factors Associated With Non-Virological Response to Combination Therapy of Interferon Plus Ribavirin Identified by Multivariate Analysis in Patients With Genotype 1b

Factor	Category	Odds ratio (95% CI)	P
Amino acid substitutions in the core region[a]	0: No double-mutant	1	0.028
	1: Double-mutant	7.000 (1.238–39.566)	

Only the variable that achieved statistical significance ($P < 0.05$) on multivariate logistic regression is shown.
[a]The mutant aa 70 and 91 pattern was evaluated as double-mutant, and other patterns as non-double-mutant.

RBV combination therapy, a different dose of IFN was used in the present study to test whether a larger dosage of IFN improves the outcome of IFN therapy.

In this study, the larger dose did not increase sustained virological response nor decrease non-virological response. Instead, the dose reduction of IFN and/or RBV was significantly higher in the higher dose group (Table III). Furthermore, the incidence of depression was significantly higher in the high-dose group (Table III). These results suggest that a high dose of IFN is not beneficial to patients who receive IFN and RBV combination therapy, and probably who will receive the PEG-IFN and RBV combination therapy.

The predictive factors for sustained virological response and non-virological response to the combination therapy for patients with genotype 1b were analyzed. Logistic regression analyses identified pretreatment substitutions at both aa 70 and 91 in the core region (double-mutant) as a singular predictive factor for non-virological response (Table VII). Furthermore, the existence of aa substitution in the ISDR was significantly more frequent in virological responders compared to non-virological responders (Table VI), in agreement with previous reports [Puig-Basagoiti et al., 2001; Pascu et al., 2004]. It has been reported that the numbers of aa substitutions in the ISDR correlate with serum HCV RNA levels [Enomoto et al., 1996]. However, no apparent correlation was observed in this study. As shown in Figures 3 and 4, patients who had substitutions of aa 70 and/or 91 in the core region or no aa substitutions in ISDR had poor initial reduction in the HCV core antigen. These results are consistent with recent studies that have shown the importance of a rapid initial decline of the viral load in obtaining a better response rate [Fried et al., 2002; Davis et al., 2003]. These results suggest that aa substitution analysis should provide important information on treatment of patients with genotype 1b.

The core protein of the HCV has been reported to disturb the IFN signaling by interacting with STAT1 SH2 domain [Lin et al., 2006] or repressing IRF1 [Ciccaglione et al., 2007]. These studies did not analyze the effect of aa substitutions in the core region. Further study is necessary to clarify the effect of aa substitutions in the core region and to identify a molecular target to improve the therapy.

Although aa substitution in the core region was identified as an important predictor in patients with genotype 1b in this study, aa substitutions of the core region and ISDR in patients with genotype 2a/b infection were not analyzed. Although the sustained virological response rate in patients who completed the therapy was high (26/28 [93%], per protocol analysis), few patients were unable to achieve sustained virological response. Furthermore, a significant number of patients could not complete the treatment course because of adverse effects. A more effective and easy to complete therapy should be developed to treat such patients. The predictive factors in such patients should also be clarified.

The recent development of a new type of drug targeting NS3/4 protease may improve the outcome of treatment in patients with chronic hepatitis C [Reesink et al., 2006; Forestier et al., 2007; Kieffer et al., 2007; Sarrazin et al., 2007a,b]. However, drug resistant mutants might emerge against such a small molecule therapy targeting viral enzyme(s). The functions of virus proteins that resist IFN including core, ISDR and PePHD should be clarified further to develop a better therapy that can achieve a higher sustained virological response rate with fewer and milder side-effects.

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HEPATOLOGY

Dose comparison study of pegylated interferon- -2b plus ribavirin in naïve Japanese patients with hepatitis C virus genotype 2: A randomized clinical trial

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Key words
hepatitis C virus genotype 2, low-dose pegylated interferon, ribavirin, side-effect, sustained virological response.

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Abstract

Background and Aim: To compare the efficacy and safety of pegylated interferon (PEG-I) at 1 and 1.5 g/kg, and in combination with ribavirin (RBV) for 24 weeks in naïve Japanese patients infected with hepatitis C virus genotype 2.

Methods: The present study was an open-label, randomized trial of 55 patients receiving PEG-I (1 or 1.5 g/kg body weight [BW], subcutaneously, once a week) and RBV for 24 weeks. The patients were followed up for 24 weeks without treatment.

Results: The intention-to-treat analyses showed that the proportion of patients with a sustained virological response (SVR) in the 1- g/kg PEG-I–RBV group (38.5%, 10/26) was lower than that of the 1.5- g/kg PEG-I–RBV group (74.1%, 20/27; P = 0.013). The PEG-I dose was reduced in two of the 26 patients of the 1- g/kg PEG-I–RBV group (one because of thrombocytopenia at 2 weeks, and one because of generalized fatigue at 20 weeks), and four of the 27 patients of the 1.5- g/kg PEG-I–RBV group (one because of neutropenia at 20 weeks, and three because of generalized fatigue at 1, 5, and 8 weeks). The multivariate analysis identified age (< 60 years) and dose of PEG-I (1.5 g/kg) as significant determinants of SVR.

Conclusion: The dose of PEG-I to be used at the start of therapy should be 1.5- g/kg BW in naïve Japanese patients infected with hepatitis C virus genotype 2.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, with an estimated 170 million chronic carriers worldwide.[1] Chronic HCV infection is causally associated with liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[2–6] In Japan, 60–70% of patients with HCC or LC are HCV carriers.[7]

Pegylated interferon (PEG-I)- plus ribavirin (RBV), the current standard treatment for chronic HCV infection, can increase the sustained virological response (SVR) rate.[8–15] In this regard, a small scale, non-randomized study by the Hepatitis C Intervention Therapy Group on the use of PEG-I- 2b plus RBV reported that the SVR rate of patients infected with HCV genotype 2 treated for 24 weeks at a dose of 1.4 g/kg once per week (83%) was equivalent to that of patients (100%) treated with 0.7 g/kg once per week.[16] Moreover, Lindsay et al.[12] reported that the SVR rate of patients infected with HCV genotype 2 on 48-week PEG-I- 2b monotherapy (dose: 1.5 g/kg once per week, 41%) was similar to that of patients (42%) treated with 1 g/kg once per week. Meyer-Wyss et al.[17] reported that SVR rates in patients infected with HCV genotype 2 or 3 were similar to those if patients treated with 1 or 1.5 g/kg PEG-I body weight [BW], that is, SVR rates were achieved in 39 of 55 (71%) and 29 of 36 (81%) patients, respectively (P = ns). Mangia et al.[18] reported that the SVR rate of patients infected with HCV genotypes treated with PEG-I at 1 g/kg BW was 80%.

However, there are no reports on whether 1 and 1.5 g/kg doses of PEG-I plus RBV for 24 weeks have similar efficacies and safety in Japanese patients infected with HCV genotype 2. It is important to study the response to such low-dose interferon because some Japanese patients who receive treatment are older than 60 years. In addition, it is possible that the SVR rate to interferon is better in patients infected with HCV genotype 2.

The aim of the present study was to determine whether 1 and 1.5 g/kg doses of PEG-I plus RBV for 24 weeks have similar efficacies and safety in naïve Japanese patients infected with HCV genotype 2. For this purpose, we conducted a randomized clinical trial to evaluate the efficacy and safety of 1 g/kg versus 1.5 g/kg

PEG-I combined with RBV for 24 weeks in naïve-infected patients with HCV genotype 2.

Methods

Patients and study design

This study was an open-label, randomized clinical trial conducted in six centers across Japan. Enrolment spanned from February 2006 to October 2007. The inclusion criteria were male and female patients with chronic hepatitis C who were than 20 years. Naïve cases were infected with HCV genotype 2.

The exclusion criteria were as follows: (i) patients treated with Shosaiko-to, a Japanese herbal medicine considered to improve liver function; (ii) patients with autoimmune hepatitis; (iii) patients with a history of hypersensitivity to PEG-I- -2a or other interferons; (iv) patients with a history of hypersensitivity to biological products, such as vaccines; (v) patients with decompensated liver cirrhosis (LC); (vi) patients with hepatocellular carcinoma (HCC) or malignant tumors in other tissues; (vii) patients with or without a history of severe psychosis, such as being severely depressed and/or suicidal; (viii) women who were pregnant or lactating or who were suspected of being pregnant; and (ix) patients judged by the investigator not to be appropriate for inclusion in this study.

The patients were randomly allocated (1:1, groups of four, central randomization) to one of the following two parallel treatment groups: the 1- g/kg PEG-I–RBV group and the 1.5- g/kg PEG-I–RBV group. The patients of the former group received 1 g/kg BW PEG-I subcutaneously once a week. The RBV dose was adjusted according to BW: 600 mg for ≤ 60 kg BW, 800 mg for > 60 kg BW, but ≤ 80 kg BW and 1000 mg for > 80 kg BW, based on the drug information for RBV supplied by the manufacturer. These durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare.

A lower dose of RBV was selected by the Japanese Ministry of Health, Labor and Welfare. Patients of the latter group were treated with 1.5 g/kg BW PEG-I subcutaneously once a week. The RBV dose was also adjusted according to BW as described earlier. The daily dose of RBV was reduced by 200 mg when hemoglobin (Hb) fell below 10 g/dL, there was an acute decrease followed by the stabilization of Hb concentrations at more than 3 g/dL from baseline, or the appearance of clinical symptoms of anemia (e.g. palpitation, dyspnea on efforts, and fatigue) associated with a decrease in Hb of > 2 g/dL from baseline. Once the RBV dose was reduced, it was maintained at that level throughout the rest of study when patients complained of anemia-related symptoms of fatigue or pallor. However, RBV was discontinued when Hb fell below 8.5 g/dL or when patients manifested more severe anemia, including orthostatic hypotension. After the end of the 24-week active treatment, the patients were followed up for a further 24 weeks without treatment.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committees of all of the participating centers. Written informed consent was obtained from all participating patients.

Data collection

Visits were scheduled at baseline, after 1, 2, 3, 4, and 8 weeks of treatment, at 4-week intervals until the end of treatment, and finally, 4 and 24 weeks after the completion of treatment. At each visit, blood samples were analyzed for hematology and blood chemistry at the local hospital laboratory using standard methodology. Serum HCV-RNA was determined at baseline, after 4, 8, 12, 16, and 20 weeks of treatment, at the end of treatment, and at the end of the 24-week, drug-free follow-up period. HCV-RNA was centrally assessed by qualitative reverse transcription–polymerase chain reaction. The histopathological stage was conducted before treatment and determined based on the histological scoring system of Desmet et al.[19]

At each visit, information on possible side-effects was obtained by questioning the patients in a structured manner about specific, commonly observed, and expected side-effects of the study medication, such as flu-like symptoms, fatigue, nausea, vomiting, diarrhea, dizziness, depression, and hair loss.

Data management and statistical analysis

The primary objectives of the study were to show the efficacy and safety of PEG-I at 1 g/kg versus 1.5 g/kg. The primary study end-point was SVR, defined as HCV-RNA below the detection limit at the end of the follow-up period, that is, 24 weeks after the completion of treatment. The secondary end-points were initial and end-of-treatment virological responses at weeks 4 and 24, and virological breakthrough and relapse, that is, the reappearance of HCV-RNA during therapy and follow up, respectively. Safety and tolerability, as reflected by clinical and laboratory side-effects, were analyzed descriptively.

Non-parametric tests were used to compare variables between groups (Mann–Whitney U-test, two-tailed test, and Fisher's exact probability test). Missing HCV-RNA values were treated on a worst-case basis, that is, they were treated as if they would have remained above the detection limit. Thus, patients with missing values and those who abandoned the study prematurely were classified as treatment failures at the time points following withdrawal, regardless of the reason for discontinuation. The intention-to-treat (ITT) analyses for efficacy and safety were performed based on the patients who received at least one dose of the study medication.

Univariate and multivariate logistic regression analyses were used to determine the predictors of SVR. We also calculated the odds ratios and 95% confidence intervals (95%CI). All P-values less than 0.05 by two-tailed tests were considered significant. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) upon the univariate analysis were entered into the multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR included the following variables: sex, age, body mass index, genotype (2a or 2b), aspartate aminotransferase, alanine aminotransferase, platelet count, serum iron, serum ferritin, hyaluronic acid, viremia level, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL-C), pathological staging, dose of PEG-I, RBV dose/BW, > 80% of RBV total dose, and reaching undetectable levels by week 4. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

A total of 55 patients were enrolled. Of these, two patients were excluded from randomization because of decompensated cirrhosis.

A total of 53 patients were randomized. Thus, 53 patients (n = 26, for the 1- g/kg PEG-I–RBV group, and n = 27 for the 1.5- g/kg PEG-I–RBV group) received at least one course of treatment (Fig. 1). Table 1 summarizes the baseline characteristics of the 53 patients. These were similar in the two treatment groups; the majority of patients were males, with a median age of ≥ 50 years and median BW of > 50 kg.

Efficacy

ITT analysis

The proportion of patients in the 1- g/kg PEG-I–RBV group who exhibited a rapid decrease in HCV-RNA to undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 4 (57.7%, 15/26) was not

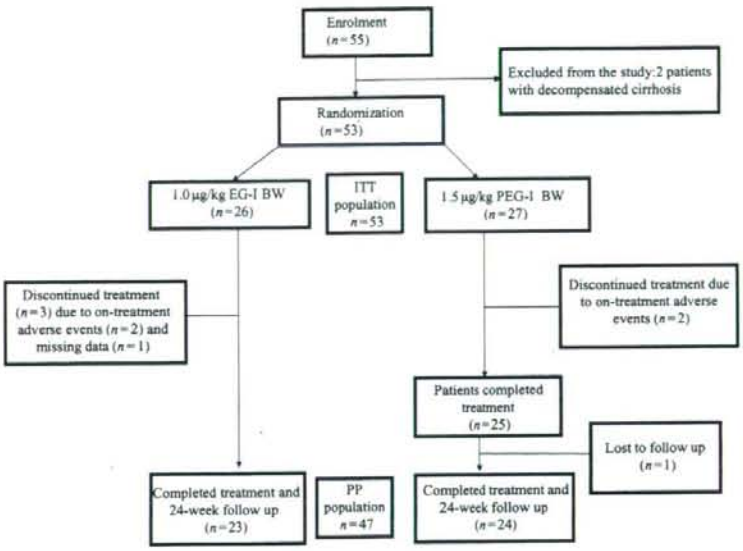

Figure 1 Flow diagram showing the number of patients who enrolled in the study and those who withdrew from the study. BW, body weight; ITT, intention to treat; PP, per protocol.

Table 1 Baseline characteristics

	1 g/kg pegylated interferon + ribavirin (*n* = 26)	1.5 g/kg pegylated interferon + ribavirin (*n* = 27)	*P*-value
Age (years)*	57 (31–77)	55 (41–75)	NS
Sex (male/female)	9/17	15/12	NS
Body weight (kg)*	53 (40–85)	61 (38–83)	NS
Body mass index (kg/m^2)*	22.1 (16.9–34.0)	23.9 (16.1–28.9)	NS
Genotype (2a/2b)	13/13	13/14	NS
White blood cell (10^3/ L)*	5.8 (4.0–7.2)	5.2 (3.9–6.4)	NS
Neutrophil cell (10^3/ L)*	3.0 (2.0–4.0)	2.8 (1.8–3.5)	NS
Hemoglobin (g/dL)*	13.3 (9.8–16.4)	13.7 (11.3–17.6)	NS
Platelet count (10^4/mm^3)*	20.9 (15.5–27.9)	20.1 (16.2–26.9)	NS
Serum aspartate aminotransferase (IU/L)*	32 (18–164)	37 (18–203)	NS
Alanine aminotransferase (IU/L)*	31 (16–164)	34 (17–180)	NS
Serum iron (g/dL)*	129 (12–246)	107 (60–275)	NS
Serum ferritin (g/dL)*	75.3 (4.9–389.3)	92 (4.9–671)	NS
Total cholesterol (mg/dL)*	198 (134–249)	175 (117–279)	NS
High-density lipoprotein cholesterol (mg/dL)*	55 (25–98)	55 (32–78)	NS
Low-density lipoprotein cholesterol (mg/dL)*	124 (63.8–176.4)	104.6 (44.2–188.2)	NS
Triglycerides (mg/dL)*	93 (12.8–210)	95 (46–210)	NS
Hyaluronic acid*	51 (9–411)	47 (10.3–411)	NS
Hepatitis C virus viremia (KIU/mL)*	1100 (200–> 5000)	1700 (300–> ,000)	NS
Histological stage† (F0/F1/F2/F3)	1/14/8/3	0/13/9/5	NS

*Values are median (range); †as assessed by the local pathologist. NS, not significant.

Table 2 Adherence to therapy

Treatment	1 g/kg pegylated interferon + ribavirin (n = 26)	1.5 g/kg pegylated interferon + ribavirin (n = 27)	P-value
> 80% of pegylated interferon dose/ < 80% of pegylated interferon dose†	22/1	24/1	NS
Premature withdrawal of pegylated interferon	3	2	NS
Ribavirin dose (mg/kg)*	11.5 (9.4–15.0)	11.6 (10.0–16.0)	NS
> 80% of ribavirin dose/ < 80% of ribavirin dose†	21/ 2	22 / 3	NS
Premature withdrawal of ribavirin	3	2	NS

*Values are median (range); †actual dose was > 80% of prescribed pegylated interferon and ribavirin dose. Patients who received full-length treatment, but required dose reductions (< 80% of the originally assigned dose). NS, not significant.

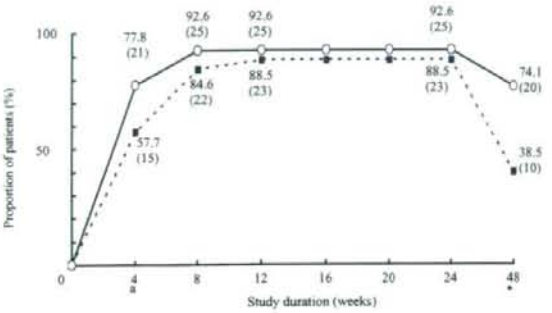

Figure 2 Results of the intention-to-treat analyses. Numbers are percentage and (number) of patients of each treatment group. Data represent the proportion of responders at the end of the indicated week of therapy (0–24 weeks) and those who achieved a sustained virological response (at 48 weeks). With regard to the virological response to combination therapy, patients of both groups exhibited a rapid decrease in HCV-RNA, reaching undetectable levels (HCV-RNA ≤ 100 copies/mL) each week. †P = 0.13; *P = 0.013. →, 1 g (n = 26); ◇, 1.5 g (n = 27).

significantly different from that of the 1.5- g/kg PEG-I–RBV group (77.8%, 21/27; P = 0.13). Further analysis showed that 10 of 15 (66.7%) patients of the 1- g/kg PEG-I–RBV group who exhibited a rapid decrease in HCV-RNA to undetectable levels by week 4 achieved SVR. Twenty of 21 (95.2%) patients of the 1.5- g/kg PEG-I–RBV who exhibited a rapid decrease in HCV-RNA to undetectable levels by week 4 achieved SVR (Fig. 2).

The proportion of end-of-therapy responders of the 1- g/kg PEG-I–RBV group (88.5%, 23/26) was similar to that of the 1.5- g/kg PEG-I–RBV group (92.6%, 25/27) (Fig. 2). The proportion of patients of the 1- g/kg PEG-I–RBV group who showed SVR (38.5%, 10/26) was significantly lower than that of the 1.5- g/kg PEG-I–RBV group (74.1%, 20/27; P = 0.013). Furthermore, the proportion of patients of the 1- g/kg PEG-I–RBV group who developed viral relapse after the end of treatment (50%, 13/26) was significantly higher than that of the 1.5- g/kg PEG-I–RBV group (18.5%, 5/27; P = 0.047; Fig. 2).

Tolerance of therapy and adverse events

There was no difference in the proportion of drop-out patients from the 1- g/kg PEG-I–RBV group (7.7%, 2/26, one for depression, and one for generalized fatigue) and that of the 1.5- g/kg PEG-I–RBV group (7.4%, 2/27, one for excitability, and one for generalized fatigue).

The dose of PEG-I was reduced in two of the 26 (7.7%) patients of the 1- g/kg PEG-I–RBV group (one patient for thrombocytopenia at 2 weeks, and one patient for generalized fatigue at 20 weeks), and four of the 27 (14.8%) patients of the 1.5- g/kg PEG-I–RBV group (one patient for neutropenia at 20 weeks, and three patients for generalized fatigue [one patient at 3 weeks, one patient at 5 weeks, and one patient at 8 weeks]). There was no significant difference in the proportion of patients who required a PEG-I dose reduction (P = 1.0).

The changes in leukocyte and platelet counts during the 24-week treatment period were similar between the two groups. However, the neutrophil cell count was significantly different between the two groups at 1 and 24 weeks. The dose of RBV was reduced due to anemia in 15 of the 26 (57.7%) patients of the 1- g/kg PEG-I–RBV group, and for the same reason in 10 of the 27 (37%) patients of the 1.5- g/kg PEG-I–RBV group. In addition, 21 (80.7%) patients of the 1- g/kg PEG-I–RBV group administered > 80% of the prescribed RBV dose, while the > 80% dose was administered by 22 (81.5%) of the 1.5- g/kg PEG-I–RBV group (P = not significant). There was no significant difference between the two groups with regard to the number of patients who required a RBV dose reduction (Table 2).

Predictors of SVR

The univariate analysis identified nine parameters that influenced SVR: age (< 60 years; P = 0.001), Hb (> 13 g/dL; P = 0.021), serum iron (< 120 g/dL; P = 0.044), triglycerides (≥ 100 mg/dL; P = 0.034), LDL-C (< 120 mg/dL; P = 0.061), dose of PEG-I (1.5 g/kg; P = 0.009), total RBV dose (> 80%; P = 0.003), and reaching undetectable levels of HCV-RNA (HCV-RNA ≤ 100 copies/mL) by week 4 (≤ 100 copies/mL; P = 0.028). The multivariate analysis identified two parameters that independently influenced the SVR: age (< 60 years; odds ratio 11.93, 95%CI 1.75 81.19; P = 0.011), and the dose of PEG-I (1.5 g/kg; odds ratio 5.502, 95%CI 1.248–24.26; P = 0.024; Table 3). These results indicated that age and the dose of PEG-I are significant and independent predictors of SVR.

Discussion

Although the number of patients in this clinical trial was relatively small, our results showed a significantly lower SVR in patients of

Table 3 Multivariate analysis of factors associated with sustained virological response to pegylated interferon–ribavirin combination therapy in patients infected with hepatitis C virus

Factors	Category	Odds ratio (95% confidence interval)	*P*-value
Age (years)	1 ≥ 60	1	
	2 < 60	11.93 (1.75–81.19)	0.011
Dose of pegylated interferon	1 g/kg	1	
	1.5 g/kg	5.502 (1.248–24.26)	0.024

the 1- g/kg PEG-I–RBV group than that of the 1.5- g/kg PEG-I–RBV group in the ITT analysis. Furthermore, the frequency of viral relapse at the end of treatment was higher in the lower PEG-I dose group than in the higher dose group. The cause of the high relapse rate was probably a result of the slower viral response of HCV-RNA in the 1- g/kg PEG-I–RBV group. Although Meyer-Wyss et al.[17] reported that the virological response rates towards the commencement of treatment at week 8 and at the end of treatment at week 48 were not significantly different between the two treatment groups, in the present study, the proportion of patients of the 1- g/kg PEG-I–RBV group who showed viral response (57.7%, 15/26) at 4 weeks tended to be lower than that of the 1.5- g/kg PEG-I–RBV group (77.8%, 21/27; P = 0.13). This in agreement with the results of Rumi et al.,[20] who reported that failure of PEG-I therapy could be predicted by the lack of a rapid virological response in patients infected with HCV genotype 2. Moreover, patients with an early virological response seemed to have a high rate of SVR.[21–23] Accordingly, the time of viral response in the 1- g/kg PEG-I–RBV group will be achieved later than that of the 1.5- g/kg PEG-I–RBV group. This suggests that if an early virological response is not evident, treatment with PEG-I should probably be extended to 48 weeks in order to increase viral clearance and improve the SVR rate. In this regard, treatment with PEG-I–RBV for 16 weeks in patients infected with HCV genotype 2 or 3 is reported to achieve a lower overall SVR rate than the standard 24-week regimen.[24]

The SVR rate of the 1- g/kg PEG-I–RBV group was lower than that reported in previous studies.[17,18,25–27] It is possible that these differences are related to differences in race, age of studied patients, and the dose of RBV. The mean age of our patients was 50 years, but has been reported to be 30–40 years in previous studies.[7,18,25,26] In addition, while previous studies evaluated patients infected with HCV genotypes 2 and 3, the number of patients infected with genotype 2 was small.[17] Although Meyer-Wyss et al.[17] reported that although SVR rates in patients infected with HCV genotypes 2 and 3 were similar between patients treated with 1 or 1.5 g/kg PEG-I BW, were differences between the virological response (85%) at the end of treatment and the virological response (71%) at the end of follow up, in patients treated with 1- g/kg PEG-I. This means that a high proportion of patients of the 1- g/kg PEG-I–RBV group developed viral relapse after the end of treatment.

The dose of RBV used in the present study was lower than that used in previous studies.[17,18,25–27] The above durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. A lower dose was selected by the Japanese Ministry of Health, Labor and Welfare. In this regard, 21 (80.7%) patients of the 1- g/kg PEG-I–RBV group were administered > 80% of the prescribed RBV dose, while the > 80% dose was used by 22 (81.5%) patients of the 1.5- g/kg PEG-I–RBV group (P = not significant). There was no significant difference between the two groups with regard to the number of patients who required RBV dose reduction. In addition, there was no significant difference between the two groups with regard to the concentration of RBV at 8 weeks (data not shown).

In our study, the proportion of patients of the 1.5- g/kg PEG-I–RBV group who showed SVR was significantly higher than that of the 1- g/kg PEG-I–RBV group, despite the lack of a significant difference in the exposure to RBV. The proportion of end-of-therapy responders of the 1- g/kg PEG-I–RBV group was similar to that of the 1.5- g/kg PEG-I–RBV group. Although the proportion of patients who exhibited a rapid decrease in HCV-RNA to undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 4 was similar in the two treatment groups, the proportion of patients of the 1- g/kg PEG-I–RBV group who showed viral response (57.7%, 15/26) at 4 weeks tended to be lower than that of the 1.5- g/kg PEG-I–RBV group (77.8%, 21/27; P = 0.13). This finding suggests the clinical importance of the time period during which HCV-RNA is at undetectable levels (≤ 100 copies/mL).

In the IDEAL (Individualized Dosing Efficacy Versus Flat Dosing To Assess Optimal Pegylated Interferon Therapy) study, the proportion of 1- g/kg PEG-I–RBV patients who developed SVR was similar to that of the 1.5- g/kg PEG-I–RBV group.[27] We believe that there was no significant difference in the exposure to PEG-I between the IDEAL study and the present one with respect to SVR. However, no information was provided in the IDEAL study on the rapid virological response and end-of-therapy response. Therefore, we could not compare our rapid virological and end-of-therapy responses with those of that study.

Based on the above results, we believe that the time period during which HCV-RNA is at undetectable levels (≤ 100 copies/mL) is more important than the dosage of PEG-I in 24-week treatment regimens, that is, we prefer to use the 1.5- g/kg PEG-I–RBV regimen since it is more likely to achieve a rapid virological response than the 1- g/kg PEG-I–RBV regimen.

Our study showed no significant difference between the two treatment groups in the tolerance of therapy and adverse events. At the start of the study, we believed that the number of patients of the 1.5- g/kg PEG-I–RBV group who would require a PEG-I dose reduction or termination of such therapy would be greater than that of the 1- g/kg PEG-I–RBV group. However, the data analysis showed no significant difference in the proportions of such patients between the two groups. Furthermore, there were no significant differences in the rate of change in leukocyte and platelet counts over 24 weeks of therapy between the two groups, and neutrophil cell counts of the 1.5- g/kg PEG-I BW group at 1 and 24 weeks were significantly lower than those of the 1- g/kg PEG-I BW group. These results indicated that 1.5 g/kg PEG-I BW is a safe regimen.

The multivariate analysis showed that SVR was dependent on the age of the patient and the dose of PEG-I. Other studies reported a higher SVR rate for young patients than older patients.[28–30] Interestingly, the dose of PEG-I was a significant and independent predictor of SVR. Furthermore, other studies reported that no or mild hepatocyte steatosis was a significant factor associated with SVR.[31] However, we did not investigate hepatocyte steatosis because of the small sample used in this study.

In conclusion, the dose of PEG-I to be used at start of therapy of naïve Japanese patients infected with HCV genotype 2 should be 1.5 g/kg BW.

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Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia

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Abstract

Nonalcoholic steatohepatitis (NASH) is the hepatic manifestation of the metabolic syndrome. Currently, there is no established therapy for NASH. The aim of the present study was to evaluate the efficacy of atorvastatin in the treatment of NASH associated with hyperlipidemia. This prospective study included 31 patients with biopsy-proven NASH with hyperlipidemia. Body mass index, serum lipids, liver function tests, fibrosis markers, and adipocytokines (adiponectin, leptin, tumor necrosis factor–α) were measured periodically during an open-label study of atorvastatin (10 mg daily) for 24 months. Standard weight-loss counseling was continued during the treatment period. Oral glucose tolerance test and liver density assessed by computerized tomography were performed before and after treatment. Follow-up liver biopsy was performed in 17 patients. All 31 patients had high cholesterol levels at baseline, and 20 also presented high triglyceride levels. The body mass index and serum glucose levels did not change during the treatment. After treatment, 23 patients (74.2%) presented normal transaminase levels. Adiponectin levels were significantly increased, and the levels of tumor necrosis factor–α were significantly decreased. However, leptin levels were not changed significantly. The concentration of long-chain fatty acids was decreased; and significant decreases were observed in C18:2,n-6 (linoleic acid, −21%) and C20:4,n-6 (arachidonic acid, −22%). Liver steatosis and nonalcoholic fatty liver disease activity score were significantly improved, whereas 4 patients had increased fibrosis stage. The NASH-related metabolic parameters improved with therapy, including fibrosis in some patients. However, 4 of 17 patients had progression of fibrosis over the 2-year period, with 3 of them progressing to stage 3. It is unclear whether this divergent response represents sampling error, heterogeneity in the population, or untreated postprandial hyperglyceridemia. Controlled trials are needed to further investigate and resolve this.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Whereas simple steatosis seems to be a benign and nonprogressive condition, NASH is recognized as a potentially progressive disease that can lead to cirrhosis, liver failure, and hepatocellular carcinoma [1-4]. In Western countries, the prevalence in the general population of NASH ranges from 1% to 5%; and that of NAFLD ranges from 15% to 39% [5,6]. In Japan, a quarter of Japanese adults have become overweight, approximately 20% of Japanese adults have NAFLD, and about 1% of those are estimated to be have NASH as well [7,8]. Thus, the prevalence of NAFLD and NASH is increasing and becoming a major target disease not only in Western countries but also in Japan.

Nonalcoholic steatohepatitis is considered the hepatic manifestation of the metabolic syndrome and is particularly associated with insulin resistance (IR), obesity, hypertension, and abnormalities in glucose and lipid metabolism [9-12]. Currently, there are no proven effective therapies available for the treatment of NASH; and strategies have mainly led to treat underlying risk factors [13,14]. Promising treatments for NASH include antioxidants, hepatoprotective agents, antidiabetic agents, insulin sensitizers, lipid-lowering agents, and angiotensin II receptor antagonist [14,15]. Approximately 70% of patients with NASH have dyslipidemia

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[5,16]. Controlling dyslipidemia with diet, exercise, and lipid-lowering agents may help stabilize or reverse NAFLD. Atorvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl–coenzyme A reductase, has been reported to be effective in patients with NAFLD with dyslipidemia [17-20]. These reports have demonstrated that therapy with atorvastatin in NAFLD patients with dyslipidemia was effective for the reduction of serum aminotransferases and lipid levels. Kiyici et al [17] have demonstrated that the use of atorvastatin in NASH patients with hyperlipidemia was effective on the improvement of serum transaminases and liver density analyzed by computed tomography (CT). They have also stated that serum aminotransferases were normalized in approximately 60% of patients by the treatment of atorvastatin for 6 months, whereas those were normalized in approximately 20% of patients treated by ursodeoxycholic acid [17]. However, the efficacy of atorvastatin treatment for the histologic changes was not available. These observations let us to prove the effectiveness of atorvastatin in the treatment of NASH.

Therefore, in the present study, to evaluate the efficacy of long-term treatment of atorvastatin for NASH patients with hypercholesterolemia, we administered atorvastatin for 24 months to those who had failed to respond adequately to diet and exercise therapy; and we compared the resulting changes in clinical parameters, as well as the histologic changes.

2. Materials and methods

2.1. Patients

The prospective study included 31 patients with biopsy-proven NASH with dyslipidemia. Informed consent was obtained from each enrolled patient, and the study was conducted in conformity to the ethical guidelines of the 1975 Declaration of Helsinki [21] and was approved by the ethics and research committees of our hospital. In all patients, current and past daily alcohol intake was less than 20 g/wk; details regarding alcohol consumption were obtained independently by at least 2 physicians and confirmed by close family members. None of the patients had received any medication that could cause NASH [22]. In all of these patients, positive tests for the following disorders were excluded: secondary causes of steatohepatitis and drug-induced liver disease (eg, amiodarone [23] and tamoxifen [24]), alcoholic liver disease, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, α_1-antitrypsin deficiency, hemochromatosis, Wilson disease, and biliary obstruction [22,25].

All patients received atorvastatin (10 mg/d) for 24 months. In addition, all patients were given standard weight-loss counseling and encouraged to follow a low-fat and low-carbohydrate diet before and during the treatment.

2.2. Clinical and laboratory evaluation

A complete physical examination was performed on each patient before and after treatment. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. *Obesity* was defined as a BMI greater than 25 kg/m^2, according to the criteria of the Japan Society for the Study of Obesity [26]. A CT scan was used to determine areas of visceral fat at the level of the umbilicus [27]. Hyperlipidemia was diagnosed for patients with cholesterol levels greater than 220 mg/dL and/or triglyceride level greater than 150 mg/dL. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure of at least 130/85 mm Hg on at least 2 occasions.

Venous blood samples were taken in the morning after a 12-hour overnight fast. The laboratory evaluation in all patients included a blood cell count; and the levels of aspartate aminotransferase, alanine aminotransferase (ALT), γ–glutamyl transpeptidase, total cholesterol, triglyceride, fasting plasma glucose, hemoglobin A_{1c} (HbA_{1c}), free fatty acid (FFA), hyaluronic acids, ferritin, and high-sensitivity C-reactive protein (CRP) were measured using the standard techniques of clinical chemistry laboratories before and after treatment. Adiponectin, leptin, tumor necrosis factor (TNF)–α, insulin, malondialdehyde (MDA), type IV collagen, and procollagen type III propeptide levels were measured before and after treatment, as previously reported [22].

A standard 75-g oral glucose tolerance test (OGTT) was performed on all patients before and after treatment. After a 12-hour fast, patients were given 75 g oral glucose solution. Plasma glucose and immunoreactive insulin were measured at 0, 30, 60, 120, and 180 minutes after the oral glucose load. Impaired glucose tolerance (IGT) was ascertained when at least 1 value was either greater than 110 mg/dL at 0 minute or greater than 140 mg/dL at 120 minutes, and diabetes was diagnosed at a 120-minute value of greater than 200 mg/dL according to the recently published recommendations of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [28]. Insulin resistance was calculated by the homeostasis model (HOMA-IR) using the following formula: HOMA-IR = fasting insulin (in microunits per milliliter) × plasma glucose (in milligrams per deciliter)/405 [29].

Long-chain fatty acids in total plasma lipids were determined by using gas chromatography. In brief, total lipids were extracted from 0.5 mL of plasma using the method by Folch et al [30]; and isolated lipid fractions were prepared by transesterification under N_2 with 14% boron trifluoride in methanol at 100°C for 20 minutes. Fatty acid methyl esters were analyzed by gas chromatography using a Shimadzu gas chromatograph (GC-17A; Shimadzu, Kyoto, Japan) with flame ionization detector, a 0.25-mm inner diameter, and a 30-m capillary column containing Omega-wax stationary phase (Supelco, Bellefonte, PA) as reported previously [31,32]. Peaks of fatty acid methyl esters were identified by comparing fatty acid retention times with standard mixtures of fatty acid methyl esters (Supelco). Fatty acids were quantified using heneicosanoic acid methyl ester (21:0) as internal standard. Sample measurements were carried out in triplicates. Twenty-four fatty acid species were

determined in this study (C12:0, C14:0, C14:1ω5, C16:0, C16:1ω7, C18:0, C18:1ω9, C18:2ω6, C18:3ω6, C18:3ω3, C20:0, C20:1ω9, C20:2ω6, C20:3ω9, C20:3ω6, C20:4ω6, C20:5ω3, C22:0, C22:1ω9, C22:4ω6, C22:5ω3, C24:0, C22:6ω3, and C24:1ω9). Based upon these results, total fatty acids (TFA), saturated fatty acids (SFA), total unsaturated fatty acids (TUFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were calculated.

2.3. Pathology

Patients enrolled in this study underwent a percutaneous liver biopsy under ultrasonic guidance using a 16-gauge SOLO CUT aspiration needle (Create Medic, Yokohama, Japan) when the informed consent was obtained. The mean length of the liver biopsy specimens was 30 ± 0.4 mm. Formalin-fixed, paraffin-embedded liver sections were stained routinely with hematoxylin-eosin, silver reticulin, Masson trichrome, Perls Prussian blue, and diastase-resistant periodic acid–Schiff. All the specimens were examined by an experienced pathologist who was unaware of the clinical and biochemical data of the patients. All cases of NASH were scored using the method of Brunt et al [33] as previously reported [22]. Steatosis was graded as follows: grade 1 (≥5% and <33% of hepatocytes affected), grade 2 (33%-66% of hepatocytes affected), or grade 3 (>66% of hepatocytes affected). Necroinflammation was graded 0 (absent) to 3 (1, occasional ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intraacinar inflammation and portal inflammation). Fibrosis was graded 0 (absent) to 4 (1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis). Ballooning was graded 0 (none) to 2 (1, few balloon cells; 2, many cells/prominent ballooning). The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2) as reported by Kleiner et al [34].

2.4. Statistical analyses

Results are presented as the medians and ranges for quantitative data or as numbers with percentages in parentheses for qualitative data. Statistical differences in quantitative data were determined using the Mann-Whitney U test and the Kruskal-Wallis test, when applicable. Fisher exact probability test was used for qualitative data. Correlation coefficients were calculated by Spearman rank correlation analysis. Differences were considered statistically significant at all P values less than .05.

3. Results

3.1. Patients enrolled

Thirty-one patients (20 male) of NASH with dyslipidemia, with a mean age of 52.5 ± 12.4 years, were enrolled in the study. The histologic findings before treatment are shown in Table 1. No patient had cirrhosis, and 26% had stage 3 fibrosis. Clinical and laboratory characteristics of enrolled patients are shown in Table 2. Body mass index ranged from 21.1 to 33.6 kg/m^2 and averaged 27.1 kg/m^2; 80.6% of enrolled patients had obesity according to the criteria of the Japan Society for the Study of Obesity [26]. Serum ALT levels ranged from 29 to 203 U/L and averaged 89.4 U/L. All patients had hypercholesterolemia, and 61.3% had concomitant hypertriglyceridemia. Six patients had fasting hyperglycemia, and 5 patients were diagnosed as diabetic by 75-g OGTT. A 75-g OGTT was performed in all enrolled patients. Twenty-nine percent of the patients showed normal glucose tolerance (NGT); 42%, IGT; and 29%, diabetes mellitus (DM).

Table 1
Histologic findings of patients with NASH

	NASH (N = 31)
Steatosis grade	
1	21 (68%)
2	8 (26%)
3	2 (6%)
Necroinflammatory grade	
1	22 (71%)
2	8 (26%)
3	1 (3%)
Fibrosis stage	
1	10 (32%)
2	13 (42%)
3	8 (26%)
4	0 (0%)
Ballooning score	
0	0 (0%)
1	23 (74%)
2	8 (26%)

Values are number (%).

3.2. Biochemical and metabolic responses

After 24 months of treatment, all patients showed a significant reduction of liver transaminase and γ–glutamyl transpeptidase levels (Table 3). Both AST and ALT levels were in the reference range in 23 patients (74.2%) after treatment. Serum ALT levels fell from an average of 89.4 U/L at baseline to 35.9 U/L at 24 months (individual changes of ALT levels are shown in Fig. 1). Mean BMI was 27.1 ± 2.7 kg/m^2 at baseline and 26.7 ± 2.9 kg/m^2 at 24 months ($P > .39$). Significant improvement of serum lipid profile is shown in Table 3. Serum total cholesterol levels decreased from 237 ± 39 mg/dL at baseline to 163 ± 32 mg/dL after treatment. Serum triglyceride levels decreased from 199 ± 90 to 132 ± 44 mg/dL. Serum high-density lipoprotein cholesterol levels increased from 50 ± 12 to 55 ± 12 mg/dL. Serum low-density lipoprotein cholesterol levels decreased from 147 ± 31 to 81 ± 27 mg/dL. Serum fasting glucose and HbA_{1c} levels did not change significantly before and after treatment.

Table 2
Clinical and laboratory characteristics of patients with NASH before treatment

Characteristic	NASH (N = 31)
Sex (male/female)	20/11
Age (y)	52.5 (27-68)
BMI (kg/m^2)	27.1 (21.1-33.6)
<25	6 (19.4%)
25-29	20 (64.5%)
≥30	5 (16.1%)
Obesity (%)	25 (80.6%)
Hypercholesterolemia	31 (100%)
Hypertriglyceridemia	19 (61.3%)
Hypertension	15 (48.4%)
Fasting glucose (>110 mg/dL)	6 (19.4%)
75-g OGTT	
NGT	9 (29.0%)
IGT	13 (42.0%)
DM	9 (29.0%)

Results are presented as numbers with percentages in parentheses for qualitative data and as medians and ranges for quantitative data.

Adipocytokines and serologic parameters before and after treatment are shown in Table 4. Plasma adiponectin levels were significantly increased by 25% and plasma TNF-α levels were significantly decreased by 43% at the end of the treatment. The leptin levels were not changed significantly both in male and female subjects. Free fatty acid levels did not change, and MDA levels were significantly decreased by 20%. The liver fibrosis markers type IV collagen and hyaluronic acid levels were decreased significantly. Ferritin levels decreased significantly. High-sensitivity CRP, a marker for inflammation, decreased significantly. Plasma glucose and insulin levels during glucose tolerance test showed high

Table 3
Clinical and laboratory characteristics of the patients with NASH before and after treatment

	Before treatment (N = 31)	After treatment (N = 31)
BMI (kg/m^2)	27.1 ± 2.7	26.7 ± 2.9
AST (U/L)	51.1 ± 23.3	25.8 ± 7.3**
ALT (U/L)	89.4 ± 46.3	35.9 ± 13.5**
γ-Glutamyl transferase (U/L)	87 ± 74	51 ± 16**
Bilirubin, total (mg/dL)	1.0 ± 0.6	1.0 ± 0.5
Bilirubin, direct (mg/dL)	0.2 ± 0.1	0.2 ± 0.1
Albumin (g/L)	4.6 ± 0.3	4.6 ± 0.3
Total cholesterol (mg/dL)	237 ± 39	163 ± 32**
Triglyceride (mg/dL)	199 ± 90	132 ± 44**
HDL cholesterol (mg/dL)	50 ± 12	55 ± 12*
LDL cholesterol (mg/dL)	147 ± 31	81 ± 27**
Fasting Glucose (mg/dL)	107 ± 17	107 ± 16
HbA_{1c}	5.6 ± 0.7	5.7 ± 0.7

Results are expressed as means ± SD. *P* values for qualitative data were calculated using Fisher exact probability test, and *P* values for quantitative data were calculated using Mann-Whitney *U* test. AST indicates aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* $P < .05$, compared with the values before treatment.

** $P < .001$, compared with the values before treatment.

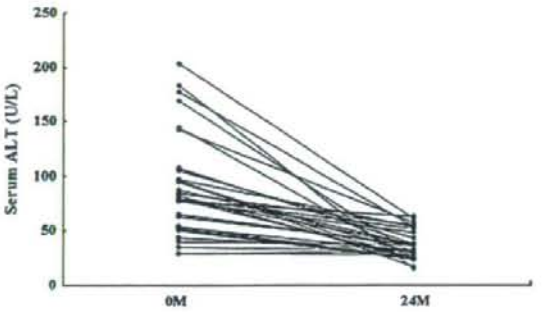

Fig. 1. Changes of serum ALT levels at baseline and after treatment with atorvastatin. (N = 31).

levels of postprandial glucose, insulin hypersecretion, and delayed peak of insulin secretion. These trends were not significantly changed by the treatment (at baseline and after treatment, 29% and 32%, 42% and 39%, and 29% and 29% of the patients showed NGT, IGT, and DM, respectively). Insulin resistance as determined by HOMA-IR tended to decrease, but statistical significance was not obtained.

Table 4
Adipocytokines, serologic parameters, and radiological analyses of patients with NASH

	Before treatment (N = 31)	After treatment (N = 31)
Adiponectin (μg/mL)	5.3 ± 2.1	6.6 ± 2.4**
Leptin (ng/mL)	12.1 ± 10.8	9.8 ± 6.0
Male	7.3 ± 3.8	7.2 ± 2.6
Female	20.7 ± 13.9	15.0 ± 7.4
TNF-α (pg/mL)	17.2 ± 4.9	9.8 ± 5.3**
FFA (mEq/L)	0.5 ± 0.2	0.5 ± 0.2
MDA (nmol/mL)	0.5 ± 0.3	0.4 ± 0.2*
Type IV collagen (ng/mL)	4.4 ± 1.1	3.9 ± 0.8*
P-III-P (U/mL)	0.7 ± 0.3	0.6 ± 0.2
Hyaluronic acid (ng/mL)	41 ± 39	30 ± 25*
Ferritin (ng/mL)	247 ± 197	149 ± 111*
High-sensitivity CRP	0.15 ± 0.1	0.06 ± 0.05*
Visceral fat area (cm^2)	154 ± 20	139 ± 77
CT liver-spleen ratio	0.54 ± 0.25	0.97 ± 0.26**
HOMA-IR	3.46 ± 2.23	3.01 ± 1.62

75-g OGTT	Glucose (mg/dL)	IRI (mU/mL)	Glucose (mg/dL)	IRI (mU/mL)
0 min	100 ± 15	14 ± 7	107 ± 19	13 ± 5
30 min	186 ± 41	84 ± 48	185 ± 49	98 ± 98
60 min	212 ± 66	116 ± 63	211 ± 79	116 ± 72
120 min	178 ± 61	128 ± 80	183 ± 62	104 ± 43
180 min	115 ± 52	61 ± 44	135 ± 75	53 ± 41
ΣBSorΣIRI	757 ± 245	386 ± 189	820 ± 256	383 ± 151

Results are expressed as means ± SD. *P* values for qualitative data were calculated using Fisher exact probability test, and *P* values for quantitative data were calculated using Mann-Whitney *U* test. P-III-P indicates procollagen III N-terminal propeptide; IRI, immunoreactive insulin.

* $P < .05$, compared with the values before treatment.

** $P < .001$, compared with the values before treatment.

Long-chain fatty acids in total plasma lipids are shown in Table 5A. The concentrations of TFA, SFA, TUFA, MUFA, PUFA, n3-PUFA, and n6-PUFA were all elevated and decreased significantly after treatment. However, the ratios of SFA, TUFA, MUFA, PUFA, n3-PUFA, and n6-PUFA were not changed before and after treatment. The n-6/n-3 PUFA ratios were not significantly changed before and after treatment. When the fatty acid composition of serum total lipids was determined, a significant decrease was observed in C18:2,n-6 (linoleic acid, −21%) and C20:4,n-6 (arachidonic acid, −22%) (Table 5B).

Table 5
Fatty acid composition and concentration of plasma total lipids in patients with NASH before and after treatment

A. Fatty acid composition of plasma total lipids

	Before treatment, μg/mL (%)	After treatment, μg/mL (%)
TFA	3568 ± 635	2731 ± 293*
SFA	1266 ± 247	985 ± 155*
	(35.4% ± 1.7%)	(36.0% ± 2.7%)
TUFA	2303 ± 552	1746 ± 312*
	(25.8% ± 3.5%)	(25.8% ± 2.7%)
MUFA	924 ± 232	709 ± 138*
	(25.8% ± 3.5%)	(25.8% ± 2.7%)
PUFA	1378 ± 321	1037 ± 174*
	(38.8% ± 6.6%)	(38.2% ± 7.8%)
n-3 PUFA	313 ± 86	214 ± 96*
	(8.9% ± 2.7%)	(7.7% ± 2.6%)
n-6 PUFA	1064 ± 234	821 ± 78*
	(29.8% ± 3.9%)	(30.4% ± 5.2%)
n-6/n-3 PUFA ratio	3.39 ± 2.73	3.83 ± 0.82
Saturated-unsaturated FA ratio	0.55 ± 0.02	0.56 ± 0.03

B. Fatty acid concentration of plasma lipids

	Before treatment (μg/mL)	After treatment (μg/mL)
C12:0	1.7 ± 0.6	1.8 ± 0.4
C14:0	35.6 ± 12.1	31.5 ± 7.3
C14:1	0.1 ± 0.0	0.1 ± 0.0
C16:0	917.7 ± 177.9	698.1 ± 117.1
C16:1	92.8 ± 13.9	66.6 ± 12.8
C18:0	266.6 ± 45.0	217.6 ± 26.1
C18:1	784.0 ± 208.9	604.3 ± 120.6
C18:2	871.8 ± 189.3	666.0 ± 50.1*
C18:3	9.8 ± 3.5	11.4 ± 6.2
C18:3	30.4 ± 7.8	23.5 ± 8.5
C20:0	9.0 ± 1.8	7.8 ± 1.1
C20:1	6.7 ± 1.7	5.8 ± 1.8
C20:2	6.1 ± 1.3	5.1 ± 1.3
C20:3	1.4 ± 0.4	1.5 ± 2.6
C20:3	34.7 ± 8.3	30.0 ± 2.6
C20:4	137.8 ± 30.7	105.3 ± 17.1*
C20:5	100.7 ± 54.0	72.4 ± 54.9
C22:0	19.3 ± 4.5	15.2 ± 2.0
C22:1	3.1 ± 0.6	3.1 ± 0.5
C22:4	3.9 ± 1.4	3.1 ± 0.7
C22:5	24.7 ± 4.8	20.5 ± 10.9
C24:0	15.9 ± 5.1	12.8 ± 1.3
C22:6	157.1 ± 19.2	97.8 ± 21.5
C24:1	37.6 ± 6.7	29.4 ± 2.6

* $P < .05$, compared with the values before treatment.

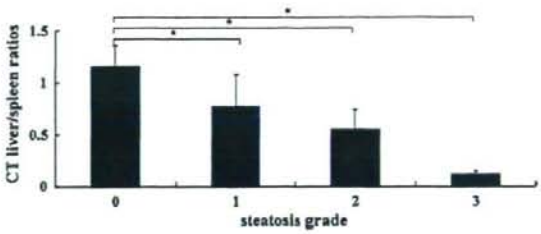

Fig. 2. Relationship between steatosis grade and liver density as measured by CT liver-spleen ratios. Results are expressed as means ± SD. * $P < .05$.

3.3. Liver density and plasma adipocytokines

Liver density was assessed by liver to spleen ratios as measured by means of abdominal CT scanning. Liver to spleen ratios were significantly increased from 0.54 ± 0.25 at baseline to 0.97 ± 0.26 at the end of treatment. Visceral fat area decreased from an average of 154 to 139 cm^2; however, statistical significance was not obtained. Liver density levels were inversely correlated with liver steatosis score (Fig. 2).

Table 6
Histologic changes before and after treatment

A. Histologic findings of patients with NASH

	Before treatment (n = 17)	After treatment (n = 17)
Steatosis grade	1.6 ± 0.1	0.8 ± 0.1**
Necroinflammatory grade	1.2 ± 0.1	1.0 ± 0.1
Fibrosis stage	1.8 ± 0.2	1.9 ± 0.2
Ballooning score	1.2 ± 0.1	1.0 ± 0.1
NAS	4.1 ± 0.3	2.9 ± 0.2**

B. Changes of distribution in grades and stages in patients with NASH

	Before treatment (n = 17)	After treatment (n = 17)
Steatosis grade		
0	–	4 (24%)
1	8 (47%)	12 (70%)
2	8 (47%)	1 (6%)
3	1 (6%)	–
Necroinflammatory grade		
1	13 (76%)	16 (94%)
2	4 (24%)	1 (6%)
3	–	–
Fibrosis stage		
1	6 (35%)	5 (29%)
2	8 (47%)	8 (47%)
3	3 (18%)	4 (24%)
4	–	–
Ballooning score		
0	1 (6%)	1 (6%)
1	11 (65%)	15 (88%)
2	5 (29%)	1 (6%)

Values are expressed as means ± SD (in micrograms per milliliter, n = 5). A. Values are expressed as means ± SD. B. Values are number (%).
** $P < .001$, compared with the values before treatment.

Changes of adiponectin levels were inversely correlated with those of steatosis grade ($P < .001$) and NAS ($P < .001$). Changes of TNF-α levels were positively correlated with those of steatosis grade ($P < .001$) and NAS ($P < .0001$). However, changes of leptin levels were not correlated with both steatosis grade and NAS.

3.4. Histologic responses

Follow-up liver biopsies were available on 17 patients. Table 6 shows the histologic changes before and after treatment. After treatment, macrovesicular steatosis, Mallory bodies, lipogranulomas, and NAS were improved significantly. Perisinusoidal, portal, and bridging fibroses were not changed. In brief, 13 patients (76%) had improvement and 4 had no change in NAS. Bridging fibrosis was found in 3 patients at baseline, and it vanished in 2 patients after treatment. Fibrosis stage increased in 4 patients (24%; 1 patient: from stage 1 to 2; 1 patient: from stage 1 to 3; 2 patients: from stage 2 to 3; before and after treatment) and did not change in 11 patients (65%).

4. Discussion

In this study, we assessed whether long-term treatment of atorvastatin would improve biochemical and histologic features of disease activity in NASH patients with dyslipidemia. All patients who received treatment with atorvastatin for 24 months showed an improvement or normalization of their serum lipid profiles. All 31 patients enrolled had improvements in serum aminotransferase levels. Alanine aminotransferase levels became normal in 74.2%. Imbalance of adipocytokines (reduced plasma adiponectin and increased plasma TNF-α levels), lipid peroxidation products (MDA levels), fibrosis markers (type IV collagen and hyaluronic acid levels), ferritin levels, and high-sensitivity CRP levels were significantly improved. Liver density was significantly improved or normalized without significant changes of visceral fat area. Plasma glucose levels, insulin levels, and total secretion amounts of glucose and insulin during OGTT were not affected by the atorvastatin treatment. Long-chain fatty acids in total plasma lipids were reduced significantly; reduction was specially evident in the n-6 series (C18:2,n-6 and C20:4,n-6). The histologic features of steatohepatitis (indicated by reduced score of NAS) were reduced. Whereas overall changes of fibrosis stage were not significantly changed, those were improved or not deteriorated in 76% of patients. Moreover, we found no significant elevation of liver enzymes during atorvastatin treatment; and no adverse effects were observed. Taken together, these results serve that atorvastatin has efficacy in patients with NASH accompanied by dyslipidemia.

Kiyici et al [17] have demonstrated the usefulness of atorvastatin in NASH patients with hyperlipidemia, and other reports have also demonstrated the improvement of liver enzymes in NAFLD patients with hyperlipidemia by atorvastatin [18-20]. Our study was in accordance with these reports and included multiple end point measurement (liver enzymes, adipocytokines, IR, lipid profile, glucose metabolism, and histologic changes before and after treatment). In experimental models, the decrease in hepatic triglyceride secretion without an increase in hepatic triglyceride concentration, the reduction of hepatic FFA, and the reduction of cholesteryl ester availability derived from newly synthesized cholesterol that limits the secretion of very low-density lipoprotein by statins including atorvastatin have been demonstrated [35,36]. Furthermore, a recent report by Kainuma et al [37] has shown that an animal fed a high-cholesterol diet exhibits hepatic steatosis, inflammation, ballooning, and fibrosis, histologic features of NASH. Thus, atorvastatin could be beneficial; and controlling the excess cholesterol might be useful for the treatment of NASH with dyslipidemia.

Oxysterols and other cholesterol oxidation products are physiologic ligands of nuclear liver X receptor (LXR). The LXR plays an important role in cholesterol homeostasis (serves as molecular sensors of cellular cholesterol concentrations and effectors of tissue cholesterol reduction), glucose metabolism, and fatty acid synthesis as well [38-40]. The LXR regulates lipogenic gene expression (eg, fatty acid synthase) by controlling sterol regulatory element–binding protein 1c (SREBP-1c) [38-40]. Several reports have demonstrated that activation of LXR leads to hepatic steatosis through activation of SREBP-1c in an animal model [41]. Furthermore, statins have been reported to decrease SREBP-1 [42,43]. Taken together, controlling cholesterol levels by statins, for example, atorvastatin, could be effective and reasonable in the treatment of NASH with dyslipidemia.

Another mechanism of atorvastatin is to induce peroxisome proliferator–activated receptor (PPAR) α and PPARγ [44-47]. The PPARα activation increases β-oxidation of fatty acids, followed by the decrease of fatty acids available for triglycerides synthesis, and thus decreases the content of triglycerides in the liver. The PPARγ has been demonstrated to attenuate the inflammatory response by inhibiting the production of TNF-α in monocytes [47]; to reduce interleukin-6 [46], a powerful inducer of CRP; and to reduce profibrogenic and proinflammatory actions in hepatic stellate cells [48,49]. These mechanisms are in accordance with our findings.

Significant reduction in high-sensitivity CRP (−40%) might be relevant because this marker of inflammation has the ability to activate, complement, and recruit monocytes and up-regulate adhesion molecules and chemoattractant chemokines [50]. Furthermore, atorvastatin has a potent antioxidant effect [51], thus influencing the pathogenesis of NASH and its metabolic abnormalities.

Another important finding in this study was the decrease of long-chain fatty acids in plasma lipids (Table 5). Especially, a significant decrease was observed in n-6 series linoleic acid and its metabolite arachidonic acid.

Prostaglandins derived from arachidonic acid have a modulatory role on interleukin-6 and TNF-α production, thus participating in the pathobiology of inflammation [52]. These changes of fatty acids may be one of the beneficial effects of this treatment.

Imbalance of adipocytokines (decreased levels of adiponectin and increased levels of TNF-α) was improved after treatment without significant changes of visceral fat. Atorvastatin did not change the glucose and insulin levels during 75-g OGTT before and after treatment. In other words, postprandial high glucose levels and hypersecretion of insulin were evident at the end of treatment. Because glucose and/or insulin directly influence connective tissue growth factor to induce fibrosis in hepatic stellate cells [53,54], this observation might explain the different results of liver fibrosis changes. In this regard, by addressing high levels of postprandial glucose and insulin, further improvement of histologic changes might be possible.

In conclusion, atorvastatin was administered to NASH patients with dyslipidemia who did not respond adequately to diet and exercise therapy. As a result, lipid levels, liver function, adipocytokines levels, fibrosis markers, long-chain fatty acid composition, and liver histologic findings were improved. However, 4 of 17 patients had progression of fibrosis over the 2-year period, with 3 of them progressing to stage 3. It is unclear whether this divergent response represents sampling error, heterogeneity in the population, or untreated postprandial hyperglyceridemia. Controlled trials are needed to further investigate and resolve this, and caution is warranted in applying statin therapy to NASH.

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Advanced glycation end products enhance the proliferation and activation of hepatic stellate cells

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Background. Advanced glycation end products (AGEs), final reaction products of protein with sugars, are known to contribute to diabetes-related complications. We have recently demonstrated high levels of serum AGEs in patients with nonalcoholic steatohepatitis (NASH). However, direct evidence for the participation of AGEs in hepatic inflammation and fibrosis has not been shown. To explore the pathogenesis of NASH, we examined the biological influence of AGEs on hepatic stellate cells (HSCs) in vitro. ***Methods.*** An established human HSC line, LI90, was exposed to a glyceraldehyde-derived-AGE (glycer-AGE), and the phenotypical changes of the LI90 cells were investigated. Intracellular formation of reactive oxygen species (ROS) was measured using a fluorescent probe. Cell proliferation was examined by MTS assay. Fibrogenic marker gene expression was analyzed by quantitative real-time polymerase chain reaction. The production of monocyte chemoattractant protein 1 (MCP-1) was assessed by enzyme-linked immunosorbent assay. ***Results.*** The expression of AGE receptor was confirmed in LI90 cells at the mRNA and protein levels. In addition to increasing intracellular ROS generation, glycer-AGE upregulated fibrogenic genes such as those encoding for α-smooth muscle actin, transforming growth factor-β1, and collagen type Iα2. The expression of MCP-1 mRNA in LI90 cells as well as its secretion into the culture medium was significantly increased in response to AGEs. These changes were attenuated by treatment with the antioxidant *N*-acetylcysteine. ***Conclusions.*** These data indicate that AGEs induce ROS generation and intensify the proliferation and activation of HSCs, supporting the possibility that antioxidants may represent a promising treatment for prevention of the development of hepatic fibrosis in NASH.

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Introduction

Advanced glycation end products (AGEs) are a heterogeneous group of irreversible reactive derivatives formed by nonenzymatic glucose–protein condensation reactions. In addition to increasing during normal aging, AGEs are known to be elevated in the plasma and to accumulate in various tissues at an accelerated rate in diabetic patients and to contribute to diabetic complications. In fact, in vitro experiments have demonstrated biological effects of AGEs on retinal pericytes, vascular endothelial cells, and renal mesangial cells.[1–3]

Nonalcoholic steatohepatitis (NASH) is a spectrum of nonalcoholic fatty liver diseases (NAFLD),[4] with manifestations ranging from steatosis to cirrhosis and which may even provoke the development of hepatocellular carcinoma. NASH is also recognized as a component of metabolic syndrome, which has insulin resistance as a common feature. We recently reported that, among several types of AGEs, glyceraldehyde-derived-AGE (glycer-AGE) is significantly higher in the serum of patients with NASH, suggesting that glycer-AGE may play an important role in the pathogenesis of NASH.[5]

Hepatic stellate cells (HSCs), as the main extracellular matrix-producing cells, are a key player in hepatic fibrosis. HSCs undergo transdifferentiation to a myofibroblast-like phenotype when exposed to various stimuli. Once transdifferentiated, HSCs secrete various mediators, including transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1, which contribute to the additional progression of hepatic inflammation and fibrosis. Recently, the expression of the receptor for AGE (RAGE) was confirmed in rat

primary HSCs and was upregulated during activation of HSCs.[6]

To gain further insights into the pathogenesis of NASH, we examined the influence of AGEs on HSCs in vitro. We found that AGEs enhanced activation and proliferation of HSCs, which was accompanied by increased generation of intracellular reactive oxygen species (ROS). Furthermore, AGEs increased the production of proinflammatory cytokines from HSCs. These findings suggest a novel molecular mechanism by which AGEs contribute to hepatic fibrosis and inflammation in NASH.

Methods

Chemicals

N-Acetylcysteine (NAC), diphenylene iodonium chloride (DPI), indomethacin, allopurinol, and 2',7'-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium cyanide (KCN) was obtained from Nakalai Tesque (Kyoto, Japan).

Cell cultures

We utilized HSC line LI90 (JCRB0160, Japan Health Science Foundation, Tokyo, Japan), established by Murakami et al.,[7] which exhibits characteristics compatible with normal activated HSCs. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) on uncoated plastic dishes, and then starved in FBS-free media for 48 h before the start of all experiments.

Preparation of AGE proteins

Glycer-AGE was prepared as described previously.[8] Control nonglycated bovine serum albumin (BSA), without reducing sugars, was made under the same conditions as the glycer-AGE.

Western blot analysis

Cells were lysed in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Denver, CO, USA) for 20 min on ice, followed by centrifugation at 13000 *g* for 5 min. The extracted proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 90 min, and then incubated overnight at 4°C with primary antibody of rabbit anti-receptor for AGEs (RAGE)[9] at a 1:1000 dilution, or mouse anti-β-actin (Sigma, Tokyo, Japan) at a 1:10000 dilution. Following incubation with a horseradish peroxidase-linked secondary antibody, the bands were visualized using an ECL Plus Western blotting analysis system (Amersham Biosciences, Buckinghamshire, UK).

Measurement of intracellular ROS generation

Intracellular ROS generation[10] was investigated by using the fluorescent probe DCFDA to detect the liberation of dichlorofluorescein (DCF). Subconfluent LI90 cells in 96-well plates were preloaded with 10 μM DCFDA for 30 min and washed twice with phosphate-buffered saline (PBS). Cells were then exposed to 100 μg/ml glycer-AGE for 24 h with either 10 mM NAC or a series of inhibitors of ROS-producing systems: DPI (25 μM), KCN (500 μM), allopurinol (100 μM), and indomethacin (100 μM), as previously reported.[11-13] The fluorescence intensity was measured by a fluorescence spectrophotometer (ARVO sx 1420 Multilabel Counter, Wallac, Waltham, MA, USA) at the wavelength of 490 nm for excitation and 520 nm for emission.

Cell proliferation assay

A methyl tetrazolium salt (MTS) assay (Promega, Madison, MI, USA) was utilized to examine LI90 cell proliferation. LI90 cells (5×10^3 cells/well) were seeded into 96-well culture plates. After 48 h of incubation with 100 μg/ml of glycer-AGE, 20 μL/well of MTS solution was added and cells were incubated for another 2 h. Fluorescent intensity was analyzed by measuring absorbance at 490 nm with a microplate reader (ImmunoMini NJ-2300 Inter Med, Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated with an RNeasy Mini-kit (Qiagen, Hilden, Germany). Single-stranded complementary DNA was synthesized from 1 μg of RNA using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and subjected to the following PCR reactions with specific primer sets (Table 1).[14-17] To examine the mRNA expression of RAGE, we conducted semi-quantitative PCR using a GeneAmp Gold RNA PCR Core Kit (Applied Biosystems). To quantify the mRNA encoding α-smooth muscle actin (α-SMA), TGF-β1, MCP-1, and collagen type Iα2 (COL1A2), real-time PCR was performed with a LightCycler-FastStart DNA Master SYBR Green I kit and LightCycler 5.32 software (Roche Molecular Biochemicals, Mannheim, Germany). The relative expression

Table 1. Polymerase chain reaction primers used in this study

Gene	Primer sequences (sense/antisense)	GenBank accession number	Annealing temperature (°C)
RAGE	5'-ATGGAAACTGAACACAGGCC-3' 5'-CACACATGTCCCCACCTTAT-3'	NM_001136	59
TGF-β1	5'-GCCCTGGACACCAACTATTGC-3' 5'-GCTGCACTTGCAGGAGCGCAC-3'	NM_000660	60
α-SMA	5'-ACTGGGACGACATGGAAAAG-3' 5'-TAGATGGGGACATTGTGGGT-3'	NM_001613	59
COL1A2	5'-ACCTGGTCAAACTGGTCCTG-3' 5'-GTGTCCCCTAATGCCTTTGA-3'	NM_000089	60
MCP-1	5'-GACCACCTGGACAAGCAAAC-3' 5'-CTCAAAACATCCCAGGGGTA-3'	NM_002982	55
β-actin	5'-GAGCGGGAAATCGTGCGTGACATT-3' 5'-GATGGAGTTGAAGGTAGTTTCGTG-3'	NM_001101	55

RAGE, advanced glycation end product receptor; TGF-β1, transforming growth factor β1; α-SMA, α-smooth muscle actin; COL1A2, collagen type Iα2; MCP-1, monocyte chemoattractant protein 1

levels were calculated according to the formula $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycle (Ct) values between the target and the reference gene, β-*actin*.

Quantification of MCP-1 protein by enzyme-linked immunosorbent assay

To quantify the protein levels of MCP-1 in media, LI90 cells (1×10^4 cells/well) were grown in a six-well dish and stimulated by 100 μg/ml of glycer-AGE with or without 10 mM NAC for 48 h. The supernatant of the conditioned media was subjected to analysis with a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between two groups were performed with the Mann-Whitney *U* test. The level of significance was set at $P < 0.05$ in all cases.

Results

RAGE expression in LI90

We first investigated the expression of RAGE in LI90 cells. RT-PCR identified RAGE mRNA (Fig. 1A), and the presence of RAGE protein was confirmed by Western blot analysis (Fig. 1B).

AGE-induced intracellular ROS generation

Increased ROS formation and resulting oxidative stress in HSCs are considered to contribute to the pathogenesis of various liver diseases.[18] Therefore, we examined whether HSCs generate ROS in response to AGEs. We observed significant ROS induction following exposure of cells to glycer-AGE (Fig. 2). Furthermore, cotreatment with NAC was found to reduce AGE-induced ROS generation. To elucidate the source of ROS, we then examined the inhibitory effect of the following

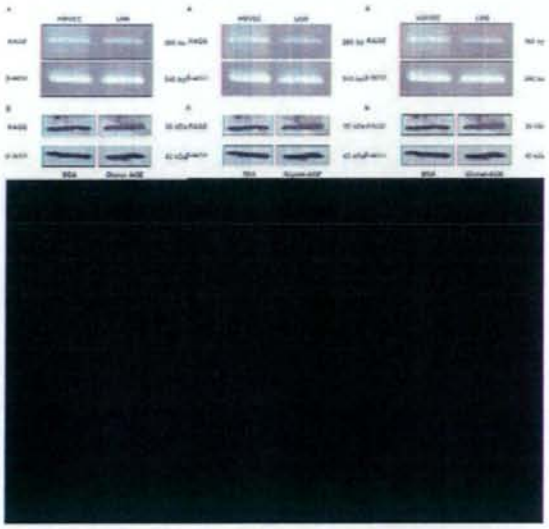

Fig. 1A, B. Expression of advanced glycation end product receptor (*RAGE*) in LI90 cells. **A** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that RAGE mRNA is expressed in LI90 cells. *HMVEC*, human dermal microvascular endothelial cells (positive control). **B** Western blot analysis showed that RAGE protein is expressed in LI90 cells, and exposure to glyceraldehyde-derived advanced glycation end product (*glycer-AGE*) for 24 h did not affect its expression level