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Erythrocyte ribavirin concentration for assessing hemoglobin reduction in interferon and ribavirin combination therapy

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Abstract

Background: Ribavirin-induced hemolytic anemia is one of the important adverse effects for the premature cessation of interferon and ribavirin combination therapy for hepatitis C virus clearance. To elucidate the mechanism of this matter, we examined the effects of plasma and erythrocyte ribavirin concentration on hemoglobin (Hb) reduction to assess hemolytic anemia in this combination therapy.
Method: Nineteen patients, treated with the interferon alpha-2b and ribavirin combination therapy, were included. Plasma and erythrocyte ribavirin concentrations were monitored for the first 28 days of the combination therapy, in relation to changes in hematological parameters. Hb and hematocrit values. The initial dose of ribavirin was 11.5 ± 1.5 mg/kg/day.
Results: Steady-state plasma and erythrocyte ribavirin concentrations were 8.9 ± 2.6 and 1218 ± 270 μM, respectively. Significant correlation was observed between erythrocyte ribavirin and Hb reduction ($r = 0.360$, $p < 0.05$), but not between plasma ribavirin and Hb reduction. The patients with higher levels of erythrocyte ribavirin (≥1000 μM) had greater Hb reduction compared to those with lower levels (<1000 μM) (3.8 ± 1.2 g/dL versus 2.6 ± 0.9 g/dL, $p < 0.05$). Nine cases out of 12 patients who developed anemia within the first 28 days of the combination therapy had higher levels of erythrocyte ribavirin (≥1000 μM).
Conclusion: We confirmed that erythrocyte ribavirin was strongly associated with Hb reduction in interferon and ribavirin combination therapy.

Keywords: Erythrocyte ribavirin; Anemia; Hepatitis C virus; Interferon

1. Introduction

Ribavirin co-administration with interferon alpha-2b including pegylated derivatives plays an important role in hepatitis C virus (HCV) clearance, leading to improvements in sustained viral response [1–4]. However, a substantial population of the patients receiving this combination therapy suffers from severe hemolytic anemia, which sometimes requires ribavirin dose reduction and cessation of the treatment [5–7].

Although the mechanism of ribavirin-induced anemia has remained unclear, it has been speculated that highly accumulated ribavirin in erythrocytes reduces erythrocyte life span [8–10]. Once incorporated into erythrocytes via the equilibrate nucleoside transporter 1 (ENT-1), ribavirin is converted into phosphorylated metabolites by intracellular phosphorylation [9,11,12]. Since the phosphorylated metabolites

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are not substrates for ENT-1, they cannot be effluxed out of the erythrocytes and so accumulate internally. Phosphorylated ribavirin (mono- and tri-phosphates) is thought to exert antiviral effects on HCV in hepatocytes [13]. On the other hand, after entering erythrocytes it impairs erythrocyte integrity by reducing intracellular ATP levels, resulting in accelerated erythrophagocytosis in the reticuloendothelial system [8].

Thus, ribavirin disposition in erythrocytes may influence the occurrence of anemia in interferon and ribavirin combination therapy. We previously observed that marked elevation (around 1000 μM) of erythrocyte ribavirin including phosphorylated metabolites associated with hemoglobin (Hb) reduction in the combination therapy [14]. However, we could not assess the threshold of ribavirin concentration in blood (plasma and erythrocyte) leading severe Hb reduction, because of the limited number of patients. In the present study, we report the impact of blood ribavirin concentration, especially 1000 μM of erythrocyte ribavirin, on Hb and hematocrit (Ht) reduction in further conducted the therapeutic drug monitoring of ribavirin under the combination therapy.

2. Patients and methods

2.1. Patients

Nineteen patients with chronic hepatitis C under the interferon alpha-2b and ribavirin combination therapy were examined. The dosage regimen for interferon and ribavirin was observed in accordance with the standard dosing instructions set for Japanese HCV patients. Patients received 10 million IU of interferon alpha-2b daily for the first 2 weeks followed by 6 million IU three times weekly for 22 weeks. The initial dose of oral ribavirin was adjusted in accordance with their body weight, 600 mg/day for patients weighing less than 60 kg, and 800 mg/day for patients weighing over 60 kg. The mean initial dose of ribavirin was 11.5 ± 1.5 mg/kg/day. The patients' profile is indicated in Table 1.

Venous blood samples for determining blood ribavirin concentration were collected from the patients. Heparinized blood collection (10 mL) was done at 0, 1, 3, 7, 14, 21, and 28 days after starting the combination therapy. Hematological parameters, such as Hb, Ht, white blood cell and platelet counts, and biochemical parameters, such as HCV-RNA, aspartate aminotransferase and alanine aminotransferase (ALT), blood urea nitrogen, and serum creatinine were also measured on each sampling day. Anemia was defined as blood hemoglobin level less than 13.5 g/dL in male and less than 11.5 g/dL in female patients in this study. Informed consent was obtained from the patients and the study was approved by the ethical committee of our University.

Table 1
Patients' profile and response to interferon and ribavirin combination therapy

	Baseline	Day 28
Age (year)	49.1 ± 14.1	
Sex (M/F)	14/5	
Body weight (kg)	61.9 ± 11.4	
Ribavirin (mg/kg/day)	11.5 ± 1.5	11.2 ± 1.2
Hepatitis C virus RNA (kIU/mL)*	1963 ± 3409	24 ± 57
Sero group: 1/2/unknown	12/5/2	
White blood cell ($\times 10^3$ μL^{-1})*	5.2 ± 0.6	3.1 ± 1.3
Hemoglobin (g/dL)**	15.0 ± 1.5	11.4 ± 1.3
Hematocrit (%)**	44 ± 5	34 ± 4
Platelet ($\times 10^9$ L^{-1})	134 ± 29	118 ± 45
Aspartate aminotransferase (U/L)*	60 ± 27	35 ± 14
Alanine aminotransferase (U/L)**	94 ± 47	36 ± 18
Blood urea nitrogen (mg/dL)	15.8 ± 3.5	13.4 ± 1.6
Serum creatinine (mg/dL)	0.77 ± 0.16	0.69 ± 0.17
Ribavirin concentration (μM)		
Plasma		8.9 ± 2.6
Erythrocyte		1218 ± 270

Data are expressed as mean ± S.D. or number of patients.
* Significant difference was observed between baseline and day 28 at $p < 0.05$.
** Significant difference was observed between baseline and day 28 at $p < 0.0001$.

2.2. Quantification of blood ribavirin

Quantification of plasma and whole blood ribavirin concentration was carried out by high-performance liquid chromatography (HPLC) developed by us [10]. Briefly, a 20 μL of whole blood supplemented with a six-fold volume of ice-cold distilled water was subjected to acid phosphatase (2 units, Sigma–Aldrich Co., St. Louis, MO) digestion to convert phosphorylated metabolites into free ribavirin. The resulting mixture, spiked with an internal standard (3-methylcytidine methosulfate, Sigma–Aldrich Co.), was treated by phenyl boronic acid (PBA) column (Bond Elute PBA; Varian, Palo Alto, CA) extraction followed by reverse-phase HPLC analysis. The dephosphorylation step was omitted when unchanged ribavirin was determined in plasma and whole blood. Since phosphorylated ribavirin was undetectable in plasma [10], plasma samples were not treated with acid phosphatase. The concentration of erythrocyte ribavirin was calculated with the following formula:

$$C_{rbc} = \frac{[C_w - C_p(1 - Ht)]}{Ht},$$

where C_{rbc} is the erythrocyte ribavirin concentration; C_w the concentration in whole blood; C_p the concentration in plasma; and Ht is the hematocrit.

The HPLC apparatus used in this study was the model 8020 system (Tosoh Corp., Tokyo, Japan) equipped with a UV detector, an auto-sampler, and a pump. A C18 reverse-phase column (TSK-Gel ODS-80Ts, Tosoh Corp.) was used for separation of ribavirin from other contaminants. The detection wavelength was set at 225 nm. The mobile phase solvent, 10 mM ammonium phosphate buffer (pH 2.5), was pumped out at a flow rate of 1.0 mL/min. All chemicals for the assay

were of HPLC or regent grade (Wako Pure Chemicals Ind., Osaka, Japan or Sigma–Aldrich Co.).

2.3. Statistical analysis

Changes in Hb and ALT from the baseline after starting the combination therapy were analyzed by the Dunnett test. Correlation coefficients between ribavirin concentrations and hematological parameters were determined by linear regression analysis. Student's *t*-test was used to assess the difference in ribavirin concentrations and the reduction of hematological parameters between the two groups, patients with <1000 and ≥1000 μM of erythrocyte ribavirin concentration. A *p*-value less than 0.05 was considered to be significant.

3. Results

The change in ALT, Hb, plasma-, and erythrocyte ribavirin concentrations during the first 28 days of the combination therapy is shown in Fig. 1. ALT and Hb were gradually decreased and significant reductions were observed on days 7 and 14, respectively (Fig. 1). Steady-state plasma and erythrocyte ribavirin concentrations reached levels of 8.9 ± 2.6 and 1218 ± 270 μM, respectively, 28 days after starting the combination therapy (Table 1). Phosphorylated metabolites (1133 ± 234 μM) accounted for 93% of erythrocyte ribavirin, whereas the metabolites were not detected in plasma (data not shown). These concentrations observed in the present study agreed with our previous study of a small number of HCV patients [10,14]. The erythrocytes/plasma ratio of ribavirin concentration was gradually increased, reaching a plateau of 149 ± 45 within 2 weeks (data not shown). Hb levels decreased correspondingly with the raise of ribavirin concentrations, and bottomed out at around day 28. We confirmed that ribavirin concentrations and Hb did not change significantly after day 28 (data not shown).

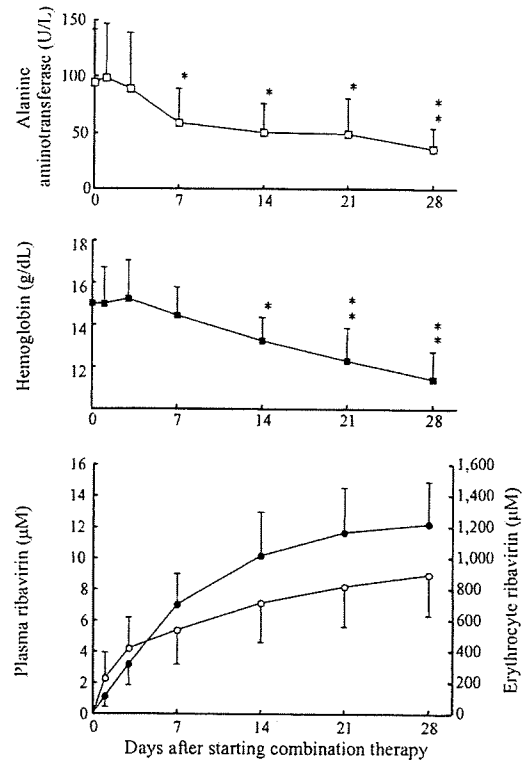

Fig. 1. Change in plasma (○) and erythrocyte (●) ribavirin concentrations, and consequent reduction of hemoglobin (■) and alanine aminotransferase (□) levels after starting interferon alpha-2b and ribavirin combination therapy. Significant differences were observed at $^{*}p < 0.05$ and $^{**}p < 0.0001$.

There was no correlation between Hb reduction and the daily dose of ribavirin (mg/kg/day) (data not shown). Significant correlation was not observed between Hb reduction and ribavirin concentration on day 14 (Fig. 2) when significant Hb reduction from baseline was firstly observed after starting the combination therapy (Fig. 1). However, weak correlation

Table 2
Ribavirin concentrations and changes in hemoglobin, hematocrit and alanine aminotransferase levels 28 days after starting combination therapy

Groups	Steady-state ribavirin concentration (μM) of	
	<1000 ($n = 7$)	≥1000 ($n = 12$)
Ribavirin dose (mg/kg/day)	12.1 ± 1.5	11.2 ± 1.4
Ribavirin concentration (μM)		
Plasma	7.5 ± 1.9	9.8 ± 2.7
Erythrocyte**	941 ± 63	1377 ± 197
Hemoglobin (g/dL)	11.8 ± 1.6	11.3 ± 1.0
ΔHemoglobin*	2.6 ± 0.9	3.8 ± 1.2
Hematocrit (%)	35 ± 5	33 ± 3
ΔHematocrit*	7 ± 4	12 ± 4
Alanine aminotransferase (U/L)	33 ± 20	38 ± 19
ΔAlanine aminotransferase	71 ± 54	43 ± 32

Data are expressed as mean ± S.D. (Δ) Difference in the values between baseline and day 28.
* Significant difference between two groups was observed at $p < 0.05$.
** Significant difference between two groups was observed at $p < 0.001$.

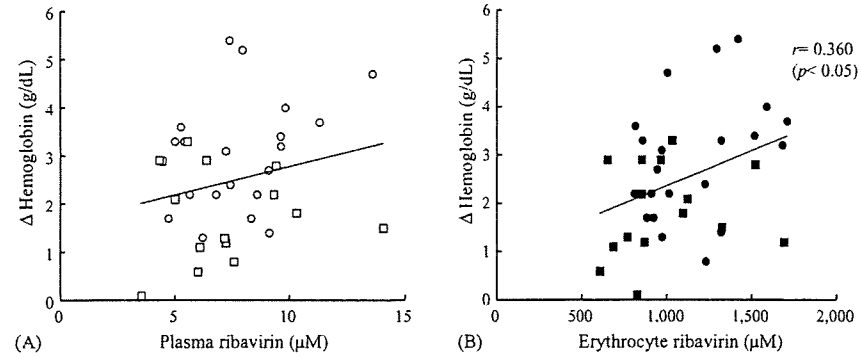

Fig. 2. Correlation between hemoglobin reduction from the baseline and plasma (A, open symbol) and erythrocyte (B, closed symbol) ribavirin concentration 14–28 days after starting the combination therapy. Square symbol and circle symbol represented data on day 14 and data from days 21 to 28, respectively. (Δ) Difference in the values from baseline.

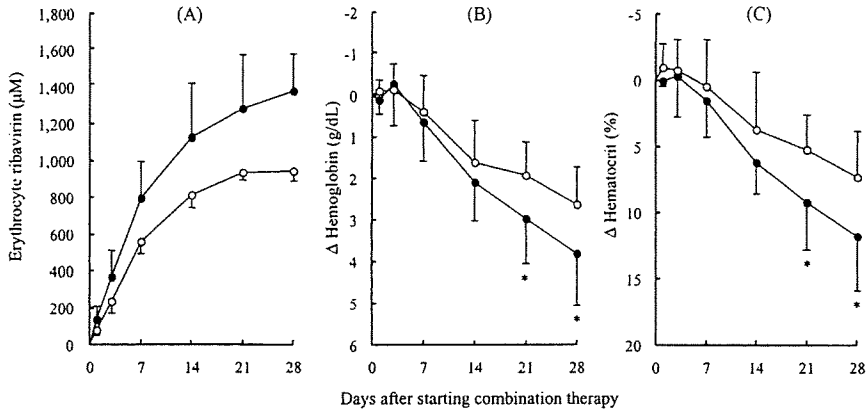

Fig. 3. Change in erythrocyte ribavirin concentrations (A), Δhemoglobin (B) and Δhematocrit (C). (Δ) Difference in the values from baseline. Closed circle (●) and open circle (○) represented higher steady-state erythrocyte ribavirin (≥1000 μM) group and lower (<1000 μM) group, respectively. Significant differences were observed between the two groups: $^{*}p < 0.05$.

was found between Hb reduction and erythrocyte ribavirin 14–28 days after starting the combination therapy ($r = 0.360$, $p < 0.05$), but not with plasma ribavirin (Fig. 2).

The patients with higher levels of erythrocyte ribavirin (≥1000 μM) at steady-state had greater Hb and Ht reduction compared with those of the patients with lower levels (<1000 μM) (Fig. 3). Significant differences in Hb and Ht reduction from the baseline were observed between the groups with higher and lower levels of erythrocyte ribavirin ($p < 0.05$) (Table 2). Nine cases out of 12 patients who developed anemia within the first 28 days of the combination therapy had higher levels of erythrocyte ribavirin (≥1000 μM). There was no significant difference in ribavirin dose and ALT reduction between the two groups (Table 2).

4. Discussion

Marked elevation of erythrocyte ribavirin concentration including its phosphorylated metabolites (149-fold versus plasma ribavirin concentration) was observed in 19 HCV patients following interferon alpha-2b and ribavirin combination therapy. Steady-state erythrocyte ribavirin concentration was 1218 ± 270 μM within 28 days after starting the combination therapy (Fig. 1; Table 1). These pharmacokinetic data were almost same with our previous study of a small number of patients [10,14]. We confirmed that erythrocyte ribavirin concentration showed significant correlation with Hb reduction (Fig. 2). Significant correlation between erythrocyte ribavirin (not plasma ribavirin) and Hb reduction ($r = 0.360$, $p < 0.05$) suggested that the erythrocyte ribavirin would be a preferable parameter for assessing ribavirin-induced hemolytic anemia. We further evaluated erythrocyte ribavirin levels over 1000 μM, which induced intracellular ATP reduction in in vitro [8]. Our finding in practical HCV treatment, which erythrocyte ribavirin levels over 1000 μM induced greater Hb reduction and developing anemia (Fig. 3; Table 2), also supports that a possible mechanism of ribavirin-induced anemia, confirmed in in vitro study [8].

Several researchers determined plasma ribavirin concentration and found the correlation between plasma ribavirin

and viral response [15–18]. Arase et al. suggested that the desirable serum ribavirin concentrations for higher sustained viral response were 12.3–14.3 μM at a steady state [15]. We support the determining plasma levels to conduct therapeutic drug monitoring of ribavirin for sufficient clinical outcome of the combination therapy. We further emphasize the importance of the determination of erythrocyte ribavirin concentration to achieve safe combination therapy in addition to the determination of plasma ribavirin. Significant correlation was not observed between Hb reduction and ribavirin dose (mg/kg/day) (data not shown). This observation was compatible with the report by Van Vlierberghe et al. [19]. Since the degree of Hb and Ht reduction might be dependent on erythrocyte ribavirin concentration and a possible threshold of the onset of Hb and Ht reduction might be around 1000 μM (Fig. 3), dose modification of oral ribavirin might be considered when the erythrocyte ribavirin concentration over 1000 μM and severe Hb reduction are observed simultaneously. Optimal dose modification leads to the enhancement of adherence to the combination therapy, resulting in higher virological response as well as avoiding severe anemia [20].

In conclusion, erythrocyte ribavirin was strongly associated with Hb reduction in interferon and ribavirin combination therapy. It was confirmed that the higher erythrocyte ribavirin concentration (≥1000 μM), in which impairing erythrocyte integrity had been observed in in vitro study for the action mechanism of ribavirin-induced anemia, produced severe Hb reduction in patients under interferon and ribavirin combination therapy.

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