Analysis of Erythrocyte Elements in Chronic Hepatitis C Patients Receiving PegIFN Monotherapy, Dual Therapy, and Triple Therapy using in-air MicroPIXE

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Abstract
Background and Aim: The aim of the present study was to elucidate the pathogenesis of anemia associated with pegylated interferon (PegIFN) -based regimens using in-air micro particle-induced X-ray emission (PIXE).
Methods: Four chronic hepatitis C (CHC) patients, three CHC patients receiving PegIFN monotherapy, five CHC patients receiving PegIFN+ribavirin (dual therapy), five CHC patients receiving PegIFN+ribavirin+telaprevir (triple therapy), and four healthy controls were enrolled in this study. Elemental distribution in erythrocytes was analyzed using in-air microPIXE.
Results and Conclusions: Erythrocyte shrinkage was observed in CHC patients receiving triple therapy. Cl, S, and K dots spread patchily in the erythrocytes of CHC patients receiving monotherapy, and this was more prominent in CHC patients receiving triple therapy. Ca dots formed small dense granules in CHC patients receiving dual and triple therapies. Zn and Cu levels were higher in CHC patients receiving dual and triple therapies. The number of Mn dots was higher in CHC patients receiving triple therapy.
In conclusion, PegIFN, ribavirin, and telaprevir may alter the membrane structure of erythrocytes, thereby contributing to anemia associated with PegIFN-based regimens. Furthermore, derangements in the distribution of Ca may play roles in ribavirin-induced anemia.

Introduction
Hepatitis C virus (HCV) infection has been identified as a major cause of chronic liver disease, affecting 170 million individuals worldwide.¹ ² Chronic infection with HCV leads to progressive hepatic fibrosis and cirrhosis in up to 20% of patients, and approximately 10-20% of cirrhotic patients will develop hepatocellular carcinoma (HCC) within 5 years.³ Approximately thirty thousand individuals die annually from liver cancer in Japan. HCV is a major cause of HCC in Japan, with 70% of cases being related to HCV.⁴ ⁵ In order to prevent progression to chronic liver disease, the continuous virus burden must be interrupted.
Antiviral therapy for chronic hepatitis C (CHC) has advanced since the discovery of HCV in 1989 and the introduction of interferon (IFN) as an antiviral agent against HCV in the early 1990s. IFN alpha was initially used as monotherapy.⁶ The next step involved the application of pegylated interferon (PegIFN) in order to adapt its pharmacokinetics and allow for better efficacy with a more tolerable dosing schedule: a once weekly instead of thrice weekly subcutaneous injection. Ribavirin (RBV), a nucleoside analog with large antiviral properties, will most likely be required
in combination with PegIFN (dual therapy), and achieves a sustained virological response (SVR) rate of 40–52% in interferon-resistant cases.7,8

Telaprevir (TVR), an inhibitor of HCV NS3/4A protease, has shown promising treatment outcomes in combination with Peg IFN+RBV (triple therapy). The SVR rate improved to more than 70% in HCV genotype 1 patients treated with PegIFN-based triple therapy.9–11

However, the incidence of hematological side effects is high with this therapeutic regimen. Anemia is the most frequently reported hematological abnormality. Although problematic with dual therapy, the addition of TVR as triple therapy has markedly exacerbated the extent of this side effect.10,11 The precise mechanism responsible for anemia associated with PegIFN-based regimens has not yet been fully determined.

Micro particle-induced X-ray emission (PIXE) is a powerful tool for a 2-dimensional elemental analysis with high spatial resolution. This method is based on a scanning nuclear microprobe technique. An external beam system has many advantages, such as requiring no special sample preparations against a vacuum environment, the easier handling and observation of samples, and reductions in the damage induced by heating and charging. Therefore, the combination of micro-PIXE and the external beam system is inferred to be more powerful. Sakai et al. developed an external ion-beam system combined with a light ion-microbeam system, which has been designed for a microPIXE analysis of biological samples in an air environment (in-air microPIXE) (Fig. 1).12,13 The in-air microPIXE system enables the detection and visualization of the 2-dimensional distribution of elements in a single cell with a resolution of the sub-micron order; therefore, we have utilized this method to analyze elemental distribution in cultured liver cells.14 Since a mature erythrocyte is 8 μm in diameter, this cell is suitable as a sample material for an in-air microPIXE analysis.15 In order to elucidate the pathogenesis of PegIFN, RBV, and TVR-induced anemia, we herein analyzed elemental distribution in the erythrocytes of CHC patients receiving monotherapy, dual therapy, and triple therapy using in-air microPIXE.

**Subjects and Methods**

Fourteen patients with CHC and four healthy volunteers were enrolled in this study (Table 1). All patients had positive HCV antibodies (Abbott Japan, Tokyo, Japan), detectable HCV RNA (quantitative RT-PCR, Abbott Real time HCV, Abbott Japan) in their sera, and elevated alanine aminotransferase (ALT) levels. CHC patients consisted of 4 cases without PegIFN therapy, 3 cases receiving PegIFN monotherapy (monotherapy), 5 cases receiving PegIFN+RBV (dual therapy), and 5 cases receiving PegIFN+RBV+TVR (triple therapy).

**Table 1** Subjects

<table>
<thead>
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<th>Subjects</th>
<th>male</th>
<th>female</th>
<th>age</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3</td>
<td>1</td>
<td>22-61</td>
</tr>
<tr>
<td>CHC patients</td>
<td>2</td>
<td>2</td>
<td>58-68</td>
</tr>
<tr>
<td>CHC patients receiving monotherapy</td>
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<td>61-70</td>
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<tr>
<td>CHC patients receiving dual therapy</td>
<td>3</td>
<td>2</td>
<td>57-73</td>
</tr>
<tr>
<td>CHC patients receiving triple therapy</td>
<td>2</td>
<td>3</td>
<td>56-72</td>
</tr>
</tbody>
</table>

CHC: Chronic hepatitis C

CHC patients were positive for HCV antibody

monotherapy: PegIFN
dual therapy: PegIFN + ribavirin
triple therapy: PegIFN + ribavirin + telaprevir

PegIFN+RBV (dual therapy), and 5 cases receiving PegIFN+RBV+TVR (triple therapy).

Whole blood was collected via a peripheral vein into a container with EDTA-2Na between 4 and 6 weeks of therapy. The sample used in the PIXE analysis was prepared by our method.16 In brief, blood was added to equal volumes of physiological saline, centrifuged (1,400 rpm, 5 min), and the supernatant
was discarded. Residual erythrocytes were used for sample preparation. Erythrocytes were dropped on a 5-μm polycarbonate film, which was then sunk into isopentane chilled with liquid nitrogen to its melting point (−160°C) and lyophilized by vacuum evaporation at 1.0×10⁻² torr (Fig. 2). Three point zero MeV proton beams with a diameter of 1 μm were generated by the TIARA single-ended accelerator at the Japan Atomic Energy Agency, Takasaki. Total scan counts were fixed at one hundred thousand, and two scan dimensions were set as 25×25 μm² (Fig. 1 and 4).

This study was performed under the approval of the Ethics Committee of Gunma University. Following an explanation of the study and its aims, written informed consent was obtained from each patient.

Results

1. X-ray spectra derived from erythrocytes in healthy controls (Fig. 3).

The characteristic X-ray spectrum of erythrocytes was a high peak of iron (Fe), which corresponded to the abundance of this metal.

In healthy controls, the shape of erythrocytes was spherical and dented at the center, and was, thus, described as donut-like. Chloride (Cl) dots distributed around a limb of the erythrocyte fit well with the donut-like appearance in healthy controls. Hence, the shape of erythrocytes was demarcated by the contour plot of the Cl map in this study (Fig. 4).
Phosphorus (P), sulfur (S), and potassium (K) dots were distributed in a similar manner to Cl dots. Calcium (Ca) was distributed granularly in erythrocytes. Fe aggregated focally, dividing into 3-4 pieces. Manganese (Mn), copper (Cu), and zinc (Zn) dots were faintly detected in the erythrocytes of healthy controls.

2-1. **X-ray spectra derived from erythrocytes of healthy controls, CHC patients, and CHC patients receiving monotherapy**

X-ray spectra patterns were approximately equal among healthy controls, CHC patients, and CHC patients receiving Peg-IFN monotherapy (Fig. 5).

2-2. **Elemental maps of erythrocytes in healthy controls, CHC patients, and CHC patients receiving monotherapy (Fig. 6).**

No significant differences were observed in elemental maps between healthy controls and CHC patients. In CHC patients receiving PegIFN monotherapy, Cl, S, and K dots aggregated patchily in the erythrocytes, concealing their dented appearance. Dots of the other elements, such as P, Ca, and Fe, were distributed in a similar manner to those of the healthy controls and CHC patients.
Fig. 6 Representative elemental maps derived from erythrocytes of healthy controls (upper panel), CHC patients (middle panel), and CHC patients receiving PegIFN monotherapy (lower panel).

Fig. 7 Representative X-ray spectra derived from erythrocytes of healthy controls, CHC patients receiving dual and triple therapies. monotherapy: PegIFN, dual therapy: PegIFN+RBV, triple therapy: PegIFN+RBV+TVR

3-1. X-ray spectra derived from erythrocytes of healthy controls and CHC patients receiving dual and triple therapies (Fig. 7)

X-ray spectra derived from erythrocytes were similar among healthy controls and CHC patients receiving PegIFN+RBV (dual therapy) and PegIFN+RBV+TVR (triple therapy).

3-2. Elemental maps of erythrocytes of CHC patients receiving dual and triple therapies

Erythrocyte shrinkage was moderate in CHC patients receiving dual therapy and severe in CHC patients receiving triple therapy (Fig. 8).

In CHC patients receiving dual therapy, Cl, S, and K dots were distributed thickly and spread patchily over most erythrocytes, leading to the disappearance of the donut-like shape (Fig. 8, 11, and 12). Alternations in Cl and K maps were more conspicuous in CHC patients receiving triple therapy than in those receiving dual therapy. Ca dots formed small dense granules in CHC patients receiving dual and triple therapies (Fig.
4. Metal levels in erythrocytes

Metal levels in erythrocytes were calculated as follows. Three cells were selected arbitrarily, and the quantity of each element was calculated based on the counts of the irradiation loading dose (Dose) in addition to the counts of characteristic X rays, which were corrected using the analytical sensitivity of each element (Fig. 16).

Cu levels were high in the order of healthy controls, CHC patients, CHC patients receiving dual therapy, and CHC patients receiving triple therapy. Zn levels were reduced in CHC patients, and increased by dual and triple therapies. Fe levels were higher in CHC patients and CHC patients receiving dual therapy than in healthy controls, and were the highest in CHC patients receiving triple therapy (Fig. 17).
Discussion

IFN was initially introduced as monotherapy against HCV in the early 1990s. Current IFN-based regimens for the treatment of CHC patients consist of PegIFN in combination with RBV (dual therapy), with the addition of TVR (triple therapy). Dual and triple therapies have achieved SVR rates of 50% and more than 70% for genotype 1 HCV, respectively. However, the incidence of hematological side effects is high with this therapeutic regimen. The main adverse effects of IFN that cause the discontinuation of therapy are varied. Although the most common adverse effects are the ‘flu-like symptoms of fatigue, myalgia, fever, and lassitude, these are typically managed easily and rarely lead to treatment discontinuation. Cytopenia, particularly anemia, has emerged as perhaps the most problematic side effect. IFN mainly contributes to anemia through bone marrow suppression.

The present study showed that Cl, S, and K dots aggregated patchily in the erythrocytes of CHC patients receiving PegIFN monotherapy, concealing the dented appearance of erythrocytes. These results indicate alternations in erythrocyte membrane struc-
Fig. 12 K maps derived from erythrocytes of healthy controls (upper panel) and CHC patients receiving dual therapy (middle panel) and triple therapy (lower panel).

Fig. 13 Ca maps derived from erythrocytes of healthy controls (upper panel) and CHC patients receiving dual therapy (middle panel) and triple therapy (lower panel).

...tuates. Thus, the disturbance of erythrocyte membranes has been speculated as a factor contributing to IFN-associated anemia; further studies are needed in order to clarify the relationship between alterations in erythrocyte membranes and IFN-associated anemia, including its direct suppression of bone marrow.

RBV is necessary when using IFN to achieve SVR rates greater than those achieved with monotherapy. On the other hand, RBV causes dose-dependent and reversible hemolytic anemia. After entering red blood cells, RBV is phosphorylated into its active form, leading to the depletion of adenosine triphosphate. This leads to impaired antioxidant mechanisms, resulting in membrane oxidative damage and subsequent extravascular red blood cell removal by the reticuloendothelial system.⁶,¹⁹

As shown in Fig. 8, 11, and 12, Cl, S, and K dots aggregated patchily over the erythrocytes of CHC patients receiving dual therapy, resulting in the disappearance of the donut-like shape, and were more apparent than in those receiving PegIFN monotherapy. As described above, alternations in these elemental maps may reflect membrane oxidative damage.

RBV stimulates not only hemolysis, but also Ca²⁺...
entry with the subsequent triggering of cell membrane scrambling and cell shrinkage and, thus, the suicidal death of human erythrocytes. Ca dots characteristically formed small dense granules in the erythrocytes collected from patients receiving dual and triple therapies (Fig. 13). Ca\(^{2+}\) induced by RBV may aggregate in erythrocytes. Furthermore, erythrocyte shrinkage was observed in patients receiving RBV-based regimens (Fig. 8). Based on the changes induced by RBV, derangements in the distribution of Ca may be associated with RBV-induced cryptosis, which is suicidal erythrocyte death.

In a pivotal study of RBV and TVR, hemoglobin levels in 34% of patients treated with TVR-based therapy decreased to <10 g/dl and to 14% in those treated with dual therapy.\(^{6-11}\) Anemia in patients treated with triple therapy is caused by all 3 drugs and likely occurs due to multiple mechanisms. While these mechanisms have not yet been completely elucidated, evidence suggests that RBV-induced hemolytic anemia is partly responsible.

Cl and K maps were altered more in CHC patients receiving triple therapy than in those receiving dual therapy, suggesting that TVR also damages erythrocyte
membranes (Fig. 8 and 12). This effect may be compounded due to bone marrow suppression by PegIFN and TVR. Therefore, the use of TVR and PegIFN appears to inhibit the compensatory increases observed in natural erythropoiesis-stimulating growth factor when RBV alone is used.

Alterations in iron homeostasis in HCV-infected patients are receiving increasing attention. Approximately 40% of patients have elevated levels of iron and ferritin in their sera, and 10% of patients have elevated levels of iron in their livers. Thus, iron overload is a common finding in CHC patients and elevated iron indices have been correlated with the progression of liver disease. An excess of iron induces the formation of reactive oxygen species that activate hepatic stellate cells, which contribute to hepatic fibrogenesis.

Consistent with these findings, Fe levels were increased in the erythrocytes of CHC patients in the present study. Fiel et al. previously reported that iron deposition in the liver is enhanced during treatments with RBV. As shown in Fig. 17, Fe levels in the erythrocytes of CHC patients were not changed by dual therapy, but were increased by triple therapy. The Fe map was not altered by PegIFN-based regimens. Taken together with the Fe map and Fe levels, the ferropenic type of anemia was unlikely to have participated in anemia associated with PegIFN-based regimens.

Hepatic Zn levels were found to be decreased, while Cu levels were conversely increased with the
progression of liver disease. The present study found low Zn levels and high Cu levels in the erythrocytes of CHC patients (Fig. 17), indicating that elemental distribution in erythrocytes may reflect that in the whole body of living subjects. We previously reported that Zn supplements in combination with PegIFN monotherapy was effective for patients with Genotype 1b HCV, but were not useful in combination with PegIFN + RBV (dual therapy). As shown in Fig. 17, Zn and Cu levels in erythrocytes were increased by dual and triple therapies, but not by monotherapy. Increments in Zn levels by RBV may be the reason why Zn supplements were not effective in combination with PegIFN and RBV.

Mn dots were clearly observed in the erythrocytes of patients receiving triple therapy (Fig. 14). Furthermore, Mn levels in erythrocytes were increased by triple therapy, but not by dual therapy (data not shown). Since Mn$^{2+}$ has been reported to serve as a co-factor of the HCV NS5B RNA-dependent RNA polymerase inhibitor, Mn may play a role in the high antiviral effectiveness of triple therapy.

Conclusion

The results of the present study suggest that membrane damage to erythrocytes is a possible factor for anemia associated with PegIFN-based regimens. RBV increased Ca$^{2+}$ concentrations in erythrocytes, and Ca dots formed small dense granules in the erythrocytes of patients receiving dual and triple therapies. Furthermore, erythrocyte shrinkage was detected in these patients. These results may be associated with eryptosis, which is suicidal erythrocyte death.

The clinical relevance of Zn, Cu, and Mn metabolism to HCV infection has not yet been clarified in detail; therefore, further studies are needed in order to elucidate the relationship between trace elements and the efficacy of PegIFN-based regimens.

The in-air microPIXE method appears to be a powerful tool for investigating anemia associated with IFN-based regimens.

References