Changing profile of platelet activity and turnover indices during treatment response of immune thrombocytopenia

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Abstract

Both platelet count and function change after treatment of immune thrombocytopenia. Platelet function can be measured by plasma markers, including platelet activity [e.g., soluble P-selectin (sP-selectin) and soluble CD40 ligand (sCD40L)] and platelet turnover markers [e.g., glycocalicin (GC)]. Patients were classified into no response (NR, including new diagnosis), partial response (PR) and complete response (CR). One hundred and sixteen samples (29 CR, 32 PR, 55 NR) from 79 patients were collected. Plasma markers (sP-selectin, sCD40L and GC) were measured by ELISA. Platelet counts and mean platelet volume (MPV) were obtained in the clinical laboratory using GenS System-2. The results showed that responsive patients (PR + CR) had higher levels of sP-selectin (P = 0.026) and sCD40L (P = 0.001). Although there was no difference in MPV (P = 0.077) or GC (P = 0.078), there was a marked decrease of GC index (P < 0.001) in responsive patients. Paired sample analysis showed no difference in sP-selectin, sCD40L, MPV or GC but significant difference in GC index (P = 0.017) between NR and PR. Another paired sample analysis showed no difference in sP-selectin, sCD40L, MPV or GC but significant difference in GC index (P = 0.029) between PR and CR. Patients with refractory and newly diagnosed disease had a significant difference in GC (P = 0.020) and sCD40L (P = 0.001), despite similarly low platelet counts. In conclusion, platelet activity markers (sP-selectin and sCD40L) and GC indices change in parallel with treatment response. Plasma levels of GC and sCD40L may be predictors of treatment response.

Keywords P-selectin · CD40 ligand · Glycocalicin · Immune thrombocytopenia

Introduction

Severity of bleeding symptoms is highly variable among patients with immune thrombocytopenia (ITP). Such variability in bleeding manifestations may result from platelet functions. The classical platelet function assay, light transmittance platelet aggregometry, cannot be reliably performed in ITP due to its relatively low platelet counts [1]. Alternative assays, such as impedance aggregometry [2, 3] and plasma markers of platelet activity [e.g., soluble P-selectin (sP-selectin) and soluble CD40 ligand (sCD40L)], may be used [4, 5]. Platelet activities may change along with treatment response [5, 6]. In addition to platelet activity, ITP is also characterized by high turnover of platelets. Platelet turnover can be directly measured by the radioisotope-labeling method [7, 8]. It can also be indirectly measured by flow cytometry or plasma markers [e.g., glycocalicin (GC)] [9, 10].

It is unclear whether change of platelet activity and turnover markers may predict change of platelet counts in ITP. Herein, we presented our data of evolutional profiles of plasma sP-selectin, sCD40L and GC in various phases of ITP treatment.

Materials and methods

Patients were screened in the outpatient clinic of hematology department, Chang Gung Memorial Hospital at Linkou. The diagnosis of ITP was made by exclusion of other etiologies of thrombocytopenia. Only patients requiring treatment...
were included in this study. Treatment response of ITP was classified according to the international consensus criteria [11]. The categories included no response (NR, including new diagnosis), partial response (PR) and complete response (CR). In addition to patients with ITP, 17 healthy donors (5 male and 12 female) were recruited for control.

After the diagnosis of ITP was established, an informed consent was obtained from each patient. Blood samples were collected from antecubital veins into tubes containing EDTA. The sample tubes were centrifuged for 15 min at 1710 g. The supernatant was retrieved and cryopreserved at −70 °C until assays. Plasma levels of sP-selectin and sCD40L, GC, were measured by enzyme-linked immunosorbent assays (ELISA) using commercial kits according to the manufacturer’s instructions (R&D systems, Minneapolis, MN, USA). A GC index was calculated by a formula [GC × 250 × 10⁶/mL]/[individual platelet count], as described in the previous literature [9]. Platelet counts and mean platelet volume (MPV) were obtained in the clinical laboratory using GenS System-2. According to the package inserts of the manufacturers, the sensitivity (minimum detectable dose) was 0.5 ng/mL for sP-selectin, 2.1–10.1 (mean 4.2) pg/mL for sCD40L, and 7.5 pg/mL for GC. All data were presented as median ± standard error of the mean (SEM).

Statistical analyses were performed using GraphPad Prism version 7.00 for Windows (San Diego, California, USA). All data are expressed as mean ± standard error of the mean (SEM). The Student’s t-test was used to determine statistical significance when two groups of data were compared. A paired t-test was used when results of paired samples were compared between NR, PR and between PR, CR. The difference was considered significantly different if P < 0.05. This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Chang Gung Memorial Hospital.

Results

Comparison of ITP and bone marrow disease

One hundred and sixteen samples (29 CR, 32 PR, 55 NR) from 79 patients were collected for this study. For control, 15 subjects with severe thrombocytopenia due to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) were recruited and samples collected in the same manner. To compare the platelet activity and turnover indexes, data of ITP patients with NR (N = 55, including new diagnosis) were compared to patients with MDS/AML (N = 15). For reference, in the normal control group, the platelet count was 264.0 ± 13.2 × 10⁹ per liter. The MPV was 8.00 ± 0.23 femtoliter. The plasma level of sP-selectin was 30.97 ± 3.08 ng/mL, sCD40L was 248.5 ± 16,702 ng/mL, and GC was 0.408 ± 0.046 μg/mL. The platelet counts were similar between ITP and MDS/AML groups (19.0 ± 1.6 × 10⁹ vs 11.0 ± 4.1 × 10⁹ per liter, P = 0.980). Patients with ITP and patients with MDS/AML were not significantly different in sCD40L (127.0 ± 29.2 vs 132.0 ± 22.9 ng/mL, P = 0.5332), MPV (9.20 ± 0.27 vs 8.25 ± 0.28 femtoliter, P = 0.2613), GC (0.402 ± 0.042 vs 0.167 ± 0.102 μg/mL, P = 0.5864) and GC index (19.000 ± 1.661 vs 11.000 ± 4.103, P = 0.6179).

However, in ITP patients, plasma levels of sP-selectin were significantly different from subjects with MDS and AML (24.87 ± 1.20 vs 24.90 ± 3.91 ng/mL, mean level 24.80 vs 31.80 ng/mL, P = 0.0271). Although results of the normal control group were not compared to ITP or MDS/AML, the data were shown in the figure as a reference. The results are presented in Fig. 1.

Comparison of new diagnosis and refractory disease

Patients with NR was subdivided into new diagnosis and refractory ITP patients. Platelet counts were different between these two groups (25.0 ± 2.7 × 10⁹ vs 15.6 ± 1.8 × 10⁹ per liter, P = 0.004). Patients with new diagnosis of ITP have higher levels of sCD40L (127 ± 56.7 vs 5.52 ± 9.8 ng/mL, P = 0.001) and lower levels of GC (0.150 ± 0.053 vs 0.480 ± 0.063 μg/mL, P = 0.020) compared with patients with refractory ITP. Patients with new diagnosis and refractory ITP were not different in blood levels of sP-selectin (29.2 ± 2.2 vs 23.5 ± 1.2 ng/mL, P = 0.253), GC index (2.421 ± 2.774 vs 7.881 ± 1.777, P = 0.369) or MPV (9.35 ± 0.46 vs 9.20 ± 0.35 femtoliter, P = 0.832). The results are presented in Fig. 2.

Comparison of patients with and without response

To characterize platelet activity and turnover indexes in different phases of ITP, patients were divided into no response (NR) and response (PR + CR) groups. The platelet counts were significantly lower in the no response group (18.0 ± 1.7 × 10⁹ vs 95.0 ± 13.7 × 10⁹ per liter, P < 0.0001). Patients with no response had lower levels of sP-selectin (24.9 ± 1.23 vs 31.4 ± 2.15 ng/mL, P = 0.0008) and sCD40L (126.7 ± 29.2 vs 238.2 ± 84.9 ng/mL, P = 0.0014) than those with PR or CR. There was a trend toward difference in platelet turnover markers MPV (9.0 ± 0.27 vs 9.58 ± 0.19 femtoliter, P = 0.0771) and GC (0.404 ± 0.418 vs 0.159 ± 0.033 μg/mL, P = 0.0784). After correction of GC by platelet counts, the GC index of the NR group was markedly higher (5.186 ± 1.573 vs 0.430 ± 0.122, P < 0.0001), compared to patients with PR and CR. Although results of the normal control group were not compared to ITP or MDS/AML, the data were shown in the figure as a reference. The data are presented in Fig. 3.
Fig. 1 Comparison of platelet count, platelet activity and turnover markers between ITP and MDS/AML patients, and healthy donors. Comparable platelet counts (a). Plasma sP-selectin significantly different between ITP and MDS/AML (b). No significant difference found in plasma levels of sCD40L (c), MPV (d), GC (e), or GC index (f).

Fig. 2 Comparison of platelet count, platelet activity and turnover markers between ITP at the time of new diagnosis and refractory disease. Lower platelet count in refractory disease (a). No significant difference in plasma sP-selectin levels (b). Significant lower levels of sCD40L in refractory disease (c). No difference in MPV (d). Higher plasma levels of GC in refractory disease (e). No difference in GC indexes (f).
To evaluate the plasma platelet activity marker level contributed by each individual platelet on the average, we analyzed the sP-selectin/platelet count and sCD40L/platelet count ratios to evaluate the average platelet function of each individual platelet. The results showed both P-selectin/platelet count (1.36 ± 0.683 vs 0.24 ± 0.029, \( P = 0.0003 \)) and sCD40L/platelet count ratios (6.45 ± 4.99 vs 1.82 ± 0.631, \( P = 0.0032 \)) were significantly higher in patients with NR than patients with CR or PR. The data are presented in Fig. 4.

**Comparison between complete, partial and no response**

The patients were divided into three groups (NR, PR and CR), and the platelet counts were different (NR: 18.0 ± 1.7 \( \times 10^9 \),...
PR: $66.0 \pm 2.5 \times 10^9$, CR: $201.0 \pm 20.6 \times 10^9$ per liter, $P < 0.001$). In all patients, viral hepatitis [hepatitis B (HBV) and C (HCV)] was screened and concurrent autoimmune diseases surveyed. Autoimmune diseases were found in 3 patients of NR [2 systemic lupus erythematosus (SLE) and 1 rheumatoid arthritis (RA)], patients of PR (2 SLE and 1 RA) and 1 patient with CR (SLE). Two patients had HBV (one in NR and the other in PR group), and none had HCV. The demographic data and platelet counts are summarized in Table 1. In comparing platelet activity markers, the 3 groups of patients had significantly different levels of sP-selectin (NR: 24.9 ± 1.23, PR: 26.0 ± 2.87, CR: 35.6.0 ± 3.1 ng/mL, $P = 0.002$) and sCD40L (NR: 126.7 ± 29.2, PR: 123.4 ± 66.5, CR: 469.8 ± 151.4 ng/mL, $P < 0.0001$). In comparison of platelet turnover markers, the 3 groups of patients were not significantly different in plasma GC levels (NR: 0.403 ± 0.042, PR: 0.191 ± 0.050, CR 0.157 ± 0.044 μg/mL, $P = 0.2399$) and MPV (NR: 9.2 ± 0.27, PR: 9.6 ± 0.27, CR 9.05 ± 0.25 ng/mL, $P = 0.1675$). After correction of platelet count, the GC indexes were significantly different among the 3 groups (NR: 5.186 ± 1.573, PR: 0.698 ± 0.203, CR 0.241 ± 0.057, $P < 0.0001$).

The data are presented in Fig. 5. The P-selectin/platelet count ratio (NR: 1.36 ± 0.68, PR: 0.39 ± 0.04, CR

| Table 1 | Platelet count and demographic data of ITP patients in study |
|---------|-----------------|----------------|----------------|----------------|
|         | NR              | PR             | CR             | $P$ value      |
| Age     | 54.0 ± 16.7     | 41.0 ± 17.2    | 49.0 ± 17.4    | 0.3326         |
| Gender  |                 |                |                | 0.0376         |
| Male    | 6               | 12             | 6              |                |
| Female  | 23              | 15             | 29             |                |
| Platelet count ($\times 10^9$ per liter) | 14.00 ± 9.00 | 66.00 ± 14.68 | 201.00 ± 110.80 | $< 0.0001$ |
| Autoimmune disease | 3 (2 SLE, 1 RA) | 3 (2 SLE, 1 RA) | 1 (SLE) | 0.4396 |
| Viral hepatitis | 1 (HBV) | 1 (HBV) | 0 | 0.5381 |

NR no response, PR partial response, CR complete response, SLE lupus erythematosus, RA rheumatoid arthritis, HBV hepatitis B virus

Fig. 5 Comparison of platelet count, platelet activity and turnover markers among patient samples in NR, PR and CR. Different platelet counts among NR, PR, and CR (a). Different plasma sP-selectin and sCD40L levels among NR, PR, and CR with the main difference in the CR group (b and c). No significant difference in MPV and GC levels among NR, PR, and CR (d and e). Significant difference of GC index among NR, PR and CR and also significant between NR and PR and between PR and CR (f)
0.17 ± 0.014, \( P < 0.0001 \) were significantly different among NR, PR and CR patients. However, for the sCD40L/platelet count ratio, despite the significant overall difference (NR: 6.45 ± 4.99, PR: 1.69 ± 1.10, CR 2.50 ± 0.56, \( P < 0.0001 \)) and difference between NR and PR \( (P < 0.001) \), there was no significant difference between patients with PR and CR.

The data are presented in Fig. 6.

### Analysis of paired samples

Thirteen patients had paired samples in NR and PR. Paired analysis showed no difference in sP-selectin (26.15 ± 4.48 vs 26.75 ± 3.81 ng/mL, \( P = 0.234 \)), sCD40L (121.4 ± 47.9 vs 159.8 ± 125.1 ng/mL, \( P = 0.081 \)), MPV (10.1 ± 0.59 vs 9.8 ± 0.32 femtoliter, \( P = 0.256 \)) or GC (0.148 ± 0.118 vs 0.554 ± 0.118 μg/mL, \( P = 0.091 \)) but significant difference in GC index (4.957 ± 1.407 vs 1.676 ± 0.529, \( P = 0.017 \)) (Fig. 7). Twelve patients had paired samples in PR and CR. Analysis showed no difference in sP-selectin (28.09 ± 2.39 vs 28.67 ± 4.14 ng/mL, \( P = 0.234 \)), sCD40L (258.6 ± 99.8 vs 476.0 ± 326.7 ng/mL, \( P = 0.071 \)), MPV (9.55 ± 0.45 vs 9.00 ± 0.39 femtoliter, \( P = 0.148 \)) or GC (1.005 ± 0.146 vs 1.019 ± 0.119 ng/mL, \( P = 0.230 \)) but significant difference in GC index (3.036 ± 0.601 vs 1.717 ± 0.217, \( P = 0.037 \)) (Fig. 7).

### Discussion

ITP is characterized by increased platelet activity and rapid platelet turnover. Platelet activities can be measured by aggregation tests [1, 12], flow cytometry [13–15] or representative plasma markers [4]. Platelet turnover can be measured by kinetic studies [9], flow cytometry [16, 17] or representative plasma markers [18–20]. Compared to other laboratory methods, ELISA measurement of plasma markers is an easy, less labor-intensive and relatively standardized method.

The clinical relevance of platelet function in ITP has been well demonstrated. Most of such studies reported platelet function is correlated with bleeding manifestations and severity. The platelet function in the majority of such studies was measured by flow cytometry-based assays [13, 14, 21]. Clinical utility of plasma markers related to platelet activities, on the other hand, has been rarely reported. In some studies, platelet-related plasma markers were used to
distinguish ITP from hypoplastic etiologies of thrombocytopenia (e.g., acute leukemias, MDS and aplastic anemia) and thereby providing diagnostic values [4, 5, 22]. Such distinction was also found in the present study, which showed significantly different sP-selectin plasma levels between ITP and MDS/AML, while their platelet counts were comparable. Despite the clear and reproducible difference of platelet function between ITP and hypoplastic thrombocytopenia, application of such assays appears to be limited in clinical medicine. The plasma marker of choice, the standard method of measurement and the optimal cutoff levels have not been well defined. On the other hand, such distinction by plasma markers is less important when bone marrow aspirations or biopsies are done and the morphological diagnosis is made definitively.

In addition to the utility in clinical differential diagnosis at the initial phase, the dynamic change of platelet function markers is interesting but relatively under-investigated. In general, ITP is characterized by high platelet activities (represented by sP-selectin) and increased turnover rates (represented by GC, reticulated platelets or immature platelet fractions) at initial presentation [4]. In previous studies, abnormalities of platelet function were reversed or normalized after treatment [23]. While such normalization of platelet function seems to be reasonable in pathophysiology, persistent abnormality which is independent of platelet counts, has been reported [24].

In the present study, we performed multiple analyses to explore the change of platelet function during various stages of clinical course. In platelet activity markers, both sP-selectin and sCD40L increased after treatment response. The difference is significant in analysis of responder versus non-responder and analysis of NR versus PR versus CR.

While it is certain that levels of platelet activity markers change with treatment response, the exact timing of change is not clear. If change of platelet activity markers precedes the change of platelet counts, such platelet activity markers may predict platelet count response in potential. The frequency of monitoring in the present study was insufficient to address this issue. However, in comparison of ITP patients with new diagnosis and refractory disease, we found significantly lower levels of sCD40L in patients with refractory disease. It suggests patients with refractory ITP and patients which later on responded to treatment may have different characteristics. In other words, a higher plasma level of sCD40L may predict treatment response. In the literature, the significance of sCD40L had been reported. Nagahama et al. found that sCD40L levels were significantly higher in untreated ITP than the control group, the non-immune thrombocytopenia patients and the treated ITP patients [25]. However, to our knowledge, difference of sCD40L between refractory and newly diagnosed ITP is a novel finding that has not been reported before. The predictive power of sCD40L should be investigated in further prospective, large-scale studies. It should be noted that not all sCD40L is derived from platelets. Human T cells are a rich source of sCD40L and indeed, T cells are involved in the pathogenesis of ITP [25, 26]. In fact, it had been shown that anti-CD40L antibody is a potential therapeutic agent for ITP [27]. As refractory and newly diagnosed ITP were distinguished by sCD40L but not sP-selectin, the possibility of immune mechanism, not platelet activity, should be considered.

Although platelet turnover markers are less frequently associated with bleeding symptoms, some studies suggested some markers, such as immature platelet fraction, is associated with bleeding risk [28]. On the other hand, platelet turnover markers are clinical relevant in their diagnostic value. For example, platelet kinetic studies revealed underproduction and relatively short half-lives of platelets in patients with ITP [7, 8, 29]. The unique features of immature platelet fractions and reticulated platelets measured by flow cytometry have been well demonstrated in ITP [17, 23]. In addition, platelet turnover can be indirectly assessed by MPV [19]. GC is a fragment of platelet glycoprotein Ib which is shed into plasma after cleavage of the platelet membrane protein. It is a common method of measuring platelet turnover [10]. It was found plasma GC levels were correlated with MPV in previous studies, suggesting both markers can be used to evaluate platelet turnover [19]. In practice, GC level is often corrected by platelet counts. GC index, therefore, is more commonly used in clinical correlation. Barsam et al. [23] had shown the difference of GC and GC index in ITP as opposed to controls. Such a difference is ameliorated at the time of platelet response of ITP treatment. Such a phenomenon illustrated the high platelet turnover in ITP and the change of turnover during response to treatment.

In comparison of ITP and MDS/AML with thrombocytopenia, none of the platelet turnover markers (GC, GC index and MPV) was significantly different. On the other hand, in comparison of various groups divided according to disease status and treatment response, GC index explicitly distinguishes patients with and without response. In further analysis of paired samples, GC index again showed explicit difference between NR and PR and patients, as well as between PR and CR patients. Such findings suggested ITP patients with new diagnosis or refractory disease had high platelet turnover, as indicated by GC index. Such high turnover was ameliorated after treatment response.

Further comparison between new diagnosis and refractory ITP showed the GC index was not significantly different between the two groups. The difference, however, can be observed for the uncorrected blood GC levels. Whereas a plausible explanation of such disparity is difficult, the distinguished blood GC level may suggest a difference in the biological nature, especially platelet turnover, of these two groups of patients, with higher GC levels among the
refractory disease patients. As a result, plasma GC level may be useful in predicting treatment response or outcome of ITP patients.

In summary, we have characterized the profile of platelet activity and turnover markers in patients with ITP. The sP-selectin levels are different from MDS/AML. Levels of sP-selectin, sCD40L and particularly GC indices, change with treatment response. Plasma levels of sCD40L and GC are different between newly diagnosed and refractory ITP patients. It suggests sCD40L and GC may be useful in predicting treatment response of ITP.

Authors' contribution CO and HC designed the study and wrote this manuscript. Y-SH, M-CK and T-LL collected patient information and revised the manuscript. P-LL conducted the experiment and analyzed the data. All authors reviewed and approved the contents of the manuscript.

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Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest All authors declared they have no conflict of interest.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication Patients signed informed consent regarding publishing their data.

Ethics approval This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Chang Gung Memorial Hospital.

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