INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease mainly affecting the peripheral joints, but extra-articular systems are often affected. Anemia is one of the common extra-articular manifestations in RA. The prevalence of anemia is 26.9%-77.6% in patients with RA (1). The leading cause of the anemia in RA is chronic inflammation, with the pattern of a normochromic and normocytic anemia. It is associated with decreased serum iron and total iron-binding capacity (TIBC), ferritin, and transferrin levels were measured. Serum concentration of pro-hepcidin, the prohormone of hepcidin, was measured using enzyme-linked immunosorbent assay (ELISA). Mean concentration of serum pro-hepcidin was 237.6 ± 67.9 ng/mL in 40 RA patients. The pro-hepcidin concentration was correlated with rheumatoid factor, CRP, ESR, and DAS28. There was a significant correlation between pro-hepcidin with tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. The pro-hepcidin concentration was significantly higher in the patients with active RA (DAS28>5.1) than those with inactive to moderate RA (DAS28≤5.1). However, the pro-hepcidin concentration did not correlate with the anemia profiles except hemoglobin level. There was no difference of pro-hepcidin concentration between the patients with anemia of chronic disease and those without. In conclusion, serum concentration of pro-hepcidin reflects the disease activity, regardless of the anemia states in RA patients, thus it may be another potential marker for disease activity of RA.
Serum Pro-hepcidin in Rheumatoid Arthritis

MATERIALS AND METHODS

Patients

Forty patients with RA were enrolled in this study and the patients all met the American College of Rheumatology 1987 revised criteria for the classification of RA (9). Informed consent was obtained from the patients before the study, and this study was approved by the Ethical Committees in Konkuk Medical Center (KUH1010061).

Disease activity evaluation in RA patients

When the sera were obtained, clinical assessments, such as tender joint counts, swollen joint counts, and 100 mm-visual analogue scale (VAS) were performed by one rheumatologist. Erythrocyte sedimentation rate (ESR), CRP, the titer of IgM rheumatoid factors (RF) and the titer of antibodies to cyclic citrullinated peptide (anti-CCP Ab) were measured. From these clinical and laboratory data, Disease Activity Score 28 (DAS28) was calculated.

Laboratory study for anemia

Laboratory analysis for anemia, such as complete blood count (CBC), hemoglobin (Hb), hematocrit, serum iron, TIBC, ferritin, and transferrin levels, are measured using standard laboratory methods. Anemia was defined as Hb <13 g/dL in male and <12 g/dL in female. Anemia of chronic disease was defined as normocytic anemia with decreased serum iron concentrations, normal to decreased transferrin levels and normal or increased serum ferritin levels (10). Patients whose anemia did not result from anemia of chronic disease were excluded in this study.

Serum concentration of pro-hepcidin and proinflammatory cytokines (TNF-α, IL-1β, and IL-6)

Serum pro-hepcidin concentration was measured using the DRG® Hepcidin Prohormone Enzyme Immunoassay Kit (DRG Instruments, Marburg, Germany), according to the manufacturer’s instructions. Pro-hepcidin is the 84 amino acid precursor of the active hepcidin peptide.

RESULTS

The clinical characteristics of the 40 RA patients were as follows: age 57.6 ± 13.3 yr, disease duration 52.6 ± 79.6 months, duration of morning stiffness 45.4 ± 66.5 min, and DAS28 4.64 ± 18.4. RF was positive (>18 IU/mL) in 67.5% of the patients. 75% of the patients had positive anti-CCP Ab (>20 Unit/mL). Table 1 shows the additional clinical data of the RA patients included in the study.

The laboratory profiles relative to anemia were as follows; Hb 11.9 ± 1.5 g/dL, serum iron 63.9 ± 35.9 μg/dL, TIBC 291.6 ± 43.9 μg/dL, ferritin 116.4 ± 168.6 ng/mL, and transferrin 235.3 ± 37.9 ng/mL. When the patients were divided into anemic and non-anemic groups, the patients with ane-
mias of chronic disease had lower serum iron (78.9 ± 39.9 vs. 47.4 ± 20.7 μg/dL, P < 0.001), higher ESR (32.2 ± 23.9 vs. 44.8 ± 24.3 mm/hr, P = 0.02), and higher CRP (1.3 ± 1.8 vs. 3.4 ± 5.3 mg/dL, P = 0.03), than the patients without anemia. Other clinical and laboratory parameters (disease duration, morning stiffness, 100 mm VAS, DAS28, tender/swollen joint counts), RF and anti-CCP Ab titers were not different between anemic and non-anemic groups.

In 40 RA patients, the mean concentration of serum pro-hepcidin was 237.6 ± 67.9 ng/mL. Serum pro-hepcidin concentration negatively correlated with serum iron (r = -0.23, P = 0.04), but it did not correlate with TIBC, ferritin and transferrin. There was no difference of pro-hepcidin concentration between the patients with anemic and non-anemic groups.

Next, we determined the relationship between serum pro-hepcidin concentration and RA disease activity parameters. The pro-hepcidin concentration correlated with DAS28 (r = 0.4, P < 0.001), tender joint count (r = 0.3, P = 0.003), RF titer (r = 0.51, P < 0.001), ESR (r = 0.53, P < 0.001), and CRP (r = 0.22, P = 0.05). When the patients were divided into two groups using DAS28 score: patients with active RA (DAS28 > 5.1, n = 14) and those with inactive to moderate RA (DAS28 ≤ 5.1, n = 26), the serum pro-hepcidin concentration was higher in patients with active disease than in those inactive to moderate disease (271.7 ± 95.7 ng/mL vs. 219.3 ± 37.6 ng/mL; P = 0.01, Table 2). There is no difference of pro-hepcidin concentration in the groups with inactive and moderate diseases. However, the pro-hepcidin concentration was not dependent of current medication including steroid, disease modifying anti-rheumatic drugs (DMARDs), and biologics.

Finally, we analyzed the relationship between pro-hepcidin with proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. Mean concentrations of serum TNF-α, IL-1β, and IL-6 were 197.8 ± 22.6 pg/mL, 252.5 ± 5.8 pg/mL, and 262.3 ± 17.3 pg/mL, respectively. There was significant correlation between the serum concentration of pro-hepcidin with the serum concentrations of TNF-α (r = 0.58, P = 0.001), IL-1β (r = 0.59, P < 0.001), and IL-6 (r = 0.4, P = 0.01).

**DISCUSSION**

Hepcidin synthesis is regulated by extrinsic or intrinsic iron loading, anemia, and hypoxemia. Moreover, the hepcidin production is induced by a particular cytokine, IL-6, during inflammation (7). In a previous study, IL-6 is induced within 3 hr after injection of lipopolysaccharide (LPS) and urinary hepcidin peaks within 6 hr, followed by decrease in serum iron (12). IL-6 is one of the proinflammatory cytokines, which has central role in the pathogenesis of RA, a representative inflammatory disease. IL-6 induces acute phase response, and differentiates B and T cells, and promotes joint destruction in RA (13-15). Serum levels of IL-6 are correlated with RA disease activity and clinical symptoms. Because anemia is a common extra-articular manifestation of RA, we hypothesized IL-6-hepcidin could be the possible link in RA to the anemia.

A recent study showed that RA patients have higher serum concentration of pro-hepcidin than patients with SLE and healthy volunteers, but the pro-hepcidin concentration doesn’t correlate with RA disease activity scores, TNF-α, or IL-6 (14). However, in our study, serum pro-hepcidin concentration correlated with RA disease activity parameters such as DAS28, tender joint count, ESR and CRP. The serum concentration of pro-hepcidin also correlated with proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. In the previous study, serum concentrations of TNF-α, IL-1β, and IL-6 are correlated with RA disease activity indexes, such as DAS28, ESR, and CRP (16-18). The roles of these proinflammatory cytokines in the RA pathogenesis are well known and the inhibitors of each cytokines are used universally as the therapy for active RA. Correlation of pro-hepcidin with proinflammatory cytokines suggests that pro-hepcidin was induced by the proinflammatory cytokines, especially IL-6. Moreover,

| Table 1. Clinical characteristics of the 40 patients with rheumatoid arthritis |
|-----------------|------------------|
| Parameters      | Findings         |
| Age (yr)        | 57.6 ± 13.3      |
| Sex (male/female)| 4:36             |
| Disease duration (months) | 52.6 ± 79.6 |
| Morning stiffness (min) | 45.4 ± 66.5 |
| DAS28           | 4.64 ± 1.84      |
| Visual analogue scale (mm) | 34.5 ± 1.2   |
| Tender joint counts | 7.9 ± 6.6      |
| Swollen joint counts | 4.6 ± 6.3      |
| Erythrocyte sedimentation rate (mm/hr) | 38.2 ± 24.9    |
| C-reactive protein (mg/dL) | 2.3 ± 4.0       |
| Rheumatoid factor (IU/mL) | 90.1 ± 157.4    |
| Anti-CCP antibody (unit/mL) | 73.6 ± 91.9    |

Data were expressed by mean ± SD.

DAS28, Disease Activity Score 28; Anti-CCP antibody, anti-cyclic citrullinated peptide antibody.

| Table 2. Laboratory parameters relative to anemia and the serum concentrations of pro-hepcidin in the 14 patients with active RA (DAS>5.1) and the 26 patients with inactive to moderate RA (DAS ≤5.1) |
|-----------------|-----------------|
| Parameters      | DAS>5.1 (n=14)  | DAS≤5.1 (n=26) |
| Hemoglobin (g/dL) | 11.5 ± 1.7      | 12.1 ± 1.3      |
| Iron (μg/dL)     | 57.5 ± 26.0     | 67.4 ± 40.4    |
| TIBC (μg/dL)     | 269.6 ± 43.4    | 303.5 ± 40.1   |
| Ferritin (ng/mL) | 197.8 ± 247.5   | 73 ± 82.5      |
| Transferrin (ng/mL) | 219.8 ± 45.3  | 250.8 ± 23.1   |
| Pro-hepcidin (ng/mL) | 271.7 ± 96.9 | 219.3 ± 36.4   |

Data were expressed by mean ± SD.

TIBC, total iron binding capacity; DAS, Disease Activity Score.
when the patients were divided two groups by disease activities, the RA patients with active disease had higher concentration of serum pro-hepcidin than those with inactive disease. This result suggested that serum pro-hepcidin concentration could be another useful marker for RA disease activity as acute phase reactants, active joint counts or proinflammatory cytokines. Our results showed different results from the previous study in RA (14), it is plausible that the patients in our study were older Asian with more active RA than the patients who were enrolled in the previous study (mean age: 57.6 vs. 46.4 yr, and mean DAS28: 4.64 vs. 3.59). These differences of characteristics of patients could be the cause of different results. Further study should be warranted to elucidate this contradictory result.

On the other hand, pro-hepcidin did not correlate anemia profiles, except for negative correlation with serum iron concentration. It was postulated that serum pro-hepcidin could not represent the bioactive hepcidin (12), thus serum pro-hepcidin concentration did not reflect actual anemic parameters, as supported by a previous studies (19, 20). After injection of LPS, serum pro-hepcidin levels don’t change significantly, although pro-inflammatory cytokine induction, urinary hepcidin excretion, and serum iron are decreased (12). No difference of serum pro-hepcidin between the RA patients with or without anemia may be explained that serum pro-hepcidin could not reflect the iron state exactly in human disease setting. Another explanation is that hepcidin is overproduced by the inflammatory response in RA, and the over-expressed hepcidin might disturb the normal physiologic response in the iron metabolism. Many arguments on the correlation between pro-hepcidin and anemia indicate that measurement of serum pro-hepcidin could not substitute bioactive hepcidin. Therefore, available method for measurement of hepcidin is needed to reveal accurate relationship of inflammatory diseases and anemia. We postulate that there is different clinical significance between hepcidin and pro-hepcidin, the former might regulate iron metabolism and the latter might regulate inflammatory process. More research is needed to serial measurement of serum pro-hepcidin in same patients as the disease activity changes.

In conclusion, proinflammatory cytokines play a major role in the development of anemia of RA, as they inhibit erythropoiesis, and hepcidin might involve in minor mechanism of anemia in the inflammatory disease, as it regulates iron metabolism inappropriately. Hepcidin or pro-hepcidin might play an important role in RA, as the mediator linking anemia and the inflammatory cytokines. Pro-hepcidin could be another useful marker for RA disease activity.

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