Serum Prohepcidin Level in Behçet's Disease

Behçet Hastalığında Serum Prohepsidin Düzeyi

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ABSTRACT Objective: The aim of this study was to determine the serum levels of the prohormone form of hepcidin, prohepcidin, and its relations with iron status, acute phase reactants, cytokines, and its possible role in the development of anemia of chronic disease in patients with Behçet's disease (BD). **Material and Methods:** The study included 37 BD and 28 rheumatoid arthritis (RA) patients and 30 healthy controls (HC). Hematocrit, serum iron status, TNF-α, IL-6 and prohepcidin levels were determined. **Results:** The increases in TNF-α level in the BD group and IL-6 level in the RA group were prominent. Serum prohepcidin level in the BD group was lower than the levels in the RA and HC groups ($p< 0.01$, $p> 0.05$, respectively). prohepcidin level did not correlate with any parameters in the BD group, but correlated with hematocrit and ferritin levels in the RA group ($r= 0.401$, $p< 0.05$, $r= 0.595$, $p< 0.01$ respectively), and TNF-α, IL-6 and ferritin levels in the HC group ($r= 0.474$, $p< 0.05$, $r= 0.526$, $p< 0.01$, $r= -0.603$, $p< 0.01$ respectively). **Conclusion:** Serum prohepcidin concentrations vary widely within the groups as do iron parameters and cytokines. Despite these wide variations the correlations are not significant. The absence of expected correlations indicates that serum prohepcidin is not a useful biomarker for clinical or research purposes.

Key Words: Anemia; Behçet syndrome; prohepcidin

ÖZET Amaç: Bu çalışmada, Behçet hastalığı (BH)'nda hepsidinin bir prohormon formu olan prohepsidinin serum düzeylerinin araştırılması ve prohepsidinin demir parametreleri, akut faz reaktanları, sitokinler ile ilişkisi ve kronik hastalık anemisi gelişimindeki olasılı rolünün değerlendirilmesi amaçlandı. **Gereç ve Yöntemler:** Çalışmaya 37 BH ve 28 romatoid artrit (RA) hastaları ve 30 sağlıklı kontrol (SK) alındı. Hematokrit, serum demir parametreleri, TNF-α, IL-6 ve prohepsidin düzeyleri belirlendi. **Bulgular:** BH grubunda TNF-α, RA grubunda ise IL-6 düzeyindeki artış daha belirgindi. Serum prohepsidin düzeyi RA ve SK grupları ile karşılaştırıldığında, BH grubunda düşüktü (sırasıyla; $p< 0.01$, $p> 0.05$). prohepsidin düzeyi, RA grubunda hematokrit ve ferritin düzeyleri ile (sırasıyla; $r= 0.401$, $p< 0.05$, $r= 0.595$, $p< 0.01$); SK grubunda TNF-α, IL-6 ve ferritin düzeyleri ile koreleydi (sırasıyla; $r= 0.474$, $p< 0.05$, $r= 0.526$, $p< 0.01$, $r= -0.603$, $p< 0.01$); ancak, BH grubunda çalışılan parametrelerden hiçbiri ile korele değildi. **Sonuç:** Demir parametreleri ve sitokinlerde olduğu gibi serum prohepsidin konsantrasyonları, çalışma grupları arasında farklılıklar göstermektedir. Bu farklılıklara karşın, prohepsidin ile demir parametreleri, akut faz reaktanları ve sitokinler arasında beklenilen korelasyonların bulunmayışı prohepsidinin klinik ve çalışma amaçlı kullanışlı bir biyomarker olmadığını göstermektedir.

Anahtar Kelimeler: Anemi; Behçet hastalığı; prohepsidin

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Behçet's disease (BD) is a systemic inflammatory disorder that progresses with spontaneous relapses and remissions.[1-4]

The anemia of chronic disease (ACD) is an acquired disorder occurring in patients with a variety of inflammatory disorders. The pathogenesis of

this anemia was attributed to deficiencies in multiple steps in erythropoiesis, including a blunted response to erythropoietin (EPO), decreased survival of mature red blood cells, and decreased iron availability.[5-7] However, the molecular basis of the ACD has not been fully elucidated.[5-7] Cytokines including IL-1, IL-6, TNF-α, and interferons are hypothesized to be involved in the maintenance of red blood cell production or stability.[5-7]

A recently discovered anti-microbial, cysteine-rich cationic peptide, hepcidin, influences intestinal iron absorption and release from stores.[6,8,9] Hepcidin appears to act by inhibiting cellular iron export by binding directly to the iron exporter ferroportin and inducing its internalization and degradation.[10] Hepcidin supplementation was shown to lead to hypoferremia in both *in vitro* and *in vivo* studies.[11] Production of hepcidin was reported to increase in patients diagnosed with ACD.[9,12-14] Furthermore, hypoferremia and development of fatal anemia was reported in a study involving transgenic mice with excessive hepcidin production.[15]

Reports suggest that recently discovered hepcidin, synthesized by the liver, known as type II acute-phase reactant, may play a key role in the pathogenesis of ACD and iron metabolism.[9,12-14]

In the present study, we aimed to assess the level of serum prohepcidin, an immature form of hepcidin, and its relation with iron status, acute-phase reactants, cytokines, and its possible role in the development of ACD in a cohort of BD patients.

MATERIAL AND METHODS

Patients: Thirty-seven patients with BD according to the International Study Group for BD criteria, 28 with RA according to the American Rheumatism Association criteria and 30 age- and sex-matched HC were included in this study.[16,17] All the participants were of Turkish origin, from the Upper Euphrates region of Turkey.

Detailed histories of all the participants were obtained, and their systemic and rheumatologic examinations were carried out. We excluded patients with other concomitant hematological diseases like thalassemia and sickle-cell anemia, heart, lung, kidney and liver diseases (cirrhosis or alcoholic hepatitis and those with AST or ALT levels increased more than two folds) and acute or chronic infection, malignancy and current pregnancy or delivery within 6 months. Participants who received blood transfusion, EPO and iron treatment and who had recent history of bleeding were also excluded.

The study was approved by the institutional ethics committee, and all participants gave informed consent before entering the study.

Laboratory analysis: Blood samples were drawn from all participants who had fasted overnight. The samples were centrifuged at 3.000 rpm for 10 minutes to obtain sera and were stored at –20°C until they were analyzed. Laboratory tests including complete blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), total protein, albumin, hepatic and renal function tests, serum iron, total iron binding capacity (TIBC) and ferritin were assessed using standard laboratory methods. Transferrin saturation (TfS) was calculated and expressed as follows:

TfS%=serum iron/TIBC ×100.

Serum TNF-α and IL-6 levels were measured using appropriate commercial kits (Medgenix, Biosource International, Camarillo, USA) by the ELISA method. Serum prohepcidin concentration was determined by ELISA using a commercially available kit (DRG Instruments, GmbH, Marburg;, Germany), according to the manufacturer's instructions, and as described previously.[18]

Determination of anemia subgroups: In all participants, peripheral blood slides were examined. Anemia was determined as hemoglobin (Hb) levels <13.5 g/dL for men, and <12 g/dL for women.[19,20] Of the anemia patients, those with serum ferritin level <60 ng/mL were diagnosed with iron deficiency anemia (IDA), and those with serum ferritin level >60 ng/mL were interpreted as ACD.[21-23] This cut off value of 60 ng/mL is generally accepted for all inflammatory diseases.

Statistical analysis

Statistics were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS,

Chicago, IL, USA). The results were expressed as mean ± SD. The Student's t test was used to compare group results and the Kruskal-Wallis variance analysis and for dual comparisons the Mann Whitney U test were used to compare anemia subgroups. Correlation analysis was performed using the Pearson product moment test. Chi-square test was used to compare categorical variables. Differences were considered significant at $p< 0.05$.

RESULTS

The demographics and clinical laboratory data for the BD, RA and HC groups are summarized in Table 1. The levels of ESR, CRP and TNF-α and IL-6 were higher in the BD and RA groups, than in the HC group (Table 1). The increase in TNF-α level in the BD group and IL-6 level in the RA group were prominent, the difference being insignificant. The serum prohepcidin level of the RA group was higher than the level in the BD and HC groups. Serum iron levels in the BD and RA groups, and hematocrit and TfS levels in the RA group were lower than the levels in the HC group (Table 1).

TABLE 1: Demographics and clinical laboratory data in the BD, RA and HC groups.

	BD (n= 37)	RA (n= 28)	HC (n= 30)
Age (years)	32.9 ± 10.9	35.6 ± 9.1	33.9 ± 8.4
Disease duration (years)	2.3 ± 4.3	3.8 ± 5.1	-
Sex (M/F)	18/19	12/16	16/14
ESR (mm/h)	21.8 ± 19.7*	29.7 ± 25.4	9.7 ± 8.9
CRP (mg/L)	19.8 ± 27.9*	28.5 ± 43.2*	3.7 ± 2.3
TNF-α (pg/mL)	10.2 ± 12.6*	6.5 ± 5.9†	3.3 ± 1.6
IL-6 (pg/mL)	5.6 ± 6.5*	10.4 ± 14.4*	1.5 ± 1.5
Total protein (g/dL)	7.5 ± 0.4	7.3 ± 0.6†	7.6 ± 0.5
Albumin (g/dL)	4.3 ± 0.3**	4.1 ± 0.4**	4.6 ± 0.3
WBC ($10^3/\mu L$)	9.1 ± 3.2*	9.9 ± 3.3*	7.1 ± 1.4
Hb (g/dL)	13.6 ± 1.9	12.9 ± 2.1†	14.6 ± 1.9
Ht (%)	41.5 ± 5.2	39.8 ± 5.8	43.3 ± 4.9
MCV (fL)	80.1 ± 8.9	82.7 ± 8.4	83.9 ± 4.7
RDW (%)	13.4 ± 0.9	14.1 ± 1.9	13.9 ± 6.01
Serum iron (μg/dL)	76.4 ± 42.1†	66.2 ± 42.5*	91.9 ± 37.8
Ferritin (ng/mL)	127.1 ± 182.4	109.8 ± 129.5	68.4 ± 55.8
TIBC (μg/dL)	263.8 ± 96.7	263.1 ± 75.7	235.2 ± 59.1
TfS (%)	37.1 ± 37.3	30.7 ± 29.5†	41.8 ± 20.3
Prohepcidin (ng/mL)	148.6 ± 57.7§	238.7 ± 202.7†	158.4 ± 44.2

BD: Behçet's disease, RA: Rheumatoid Arthritis, HC: Healthy controls, M: Male, F: Female, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin-6, WBC; White blood cell count, Hb: Hemoglobin, Ht: Hematocrit, MCV: Mean corpuscular volume, RDW: Red cell distribution width, TIBC: total iron binding capacity, TfS: transferrin saturation.
When compared with HC group; †p< 0.05, *p< 0.01, **p< 0.001.
When compared with RA group; §p< 0.01.

Serum prohepcidin level did not correlate with hematocrit, serum iron status and cytokine levels in the BD group, but it positively correlated with hematocrit and ferritin levels in the RA group, and had a negative correlation with ferritin level and positive correlations with TNF-α and IL-6 levels in the HC group.

In the BD group, 8 cases (21.6%) had anemia, of whom 3 (8.1%) were ACD, and 5 (13.5%) had IDA, whereas in the RA group, 11 patients (39.3%) had anemia, of whom 6 (21.4%) had ACD, and 5 (17.9%) had IDA. Serum prohepcidin levels were insignificantly lower in the ACD subgroups of the BD and RA groups than in their IDA and non-anemic subgroups.

In the BD group, all cases (100%) had oral ulcer, 28 (75.7%) had skin lesions, 17 (45.9%) had genital lesions and/or scars, 13 (35.1%) had uveitis, 10 (27.1%) had vascular involvement, and 8 (21.6%) had positive pathergy test result. Mean DAS-28 score was 3.14 ± 1.3 in the RA group, and it correlated with the CRP level ($r= 0.504$, $p< 0.05$) but not with the level of prohepcidin ($r= -0.127$, $p> 0.05$).

DISCUSSION

Hepcidin was suggested to be responsible for the development of anemia in inflammation models induced by turpentine and the same study showed that hepcidin mRNA expression in the liver was decreased about threefold by phlebotomy or phenylhydrazine induced anemia.[9] Serum prohepcidin and hepcidin levels in cases with accompanying anemia were lower, relative to non-anemic cases in chronic renal failure (CRF) patients.[24,25] In our study, ACD subgroups of the BD and RA groups had insignificantly lower serum prohepcidin levels than their non-anemic subgroups. Hepatic hepcidin expression is shown to be induced by iron overload and inflammation and is suppressed by hypoxia and anemia.[8,9] Reduced serum

prohepcidin levels in the anemic cases and the positive correlation between prohepcidin and hematocrit in the RA group in our study may support this view.

In our study, positive correlations were found between serum prohepcidin and TNF-α and IL-6 levels in the HC group. Although TNF-α was reported to inhibit hepcidin expression in an in vitro study, it is a pro-inflammatory cytokine, and is known to pioneer an increase in the production of many other cytokines.[26,27] In our study, the positive correlations between serum prohepcidin and TNF-α and IL-6 levels may have resulted from the indirect effects of TNF-α.

In the present study, prohepcidin level positively correlated with ferritin level in the RA group. Hepcidin is also known as a type II acute-phase protein, and in chronic inflammatory conditions, increased hepcidin levels are correlated with increased ferritin levels.[13] Similarly, ferritin had a positive correlation with CRP and a negative one with albumin in the BD and RA groups in our study.

Assays for hepcidin detection in plasma or urine are not readily available, and the development of reagents has been hampered by technical difficulties.[18,28] Serum prohepcidin was measured by the ELISA technique as a surrogate parameter of hepcidin in our study. However, analysis of prohepcidin may determine inactive propeptide but not its mature-active form, since it has never been validated that prohepcidin reflects the level of active-mature hepcidin. Furthermore, Kemna et al. reported that LPS injection showed a remarkable difference in which prohepcidin levels remained constant, while bioactive urinary hepcidin increased dramatically, and this study indicates that measurement of prohepcidin with the currently available commercial assay is not very reliable as the substitute for bioactive hepcidin analysis.[29] Thus, if we verified the prohepcidin data by measuring bioactive hepcidin in serum or urine using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) measurement method, suspicions on prohepcidin measurement method could be removed.[25,30]

In addition to the unreliability of the prohepcidin assay, the low number of patients in ACD and IDA subgroups may be the limitations of our study. Thus, our study is inadequate to determine the possible relation between prohepcidin and ACD, since there are only 3 ACD patients in the BD group. Other limitations of the study may be the failure to use indicators that are more pertinent such as TfR to distinguish between ACD and IDA and the lack of data on the hemochromotosis gene (HFE) which is an upstream regulator of hepcidin. Furthermore, lack of data on the effects of therapy given to the patients on the prohepcidin levels in another major limitation.

In conclusion, serum prohepcidin concentrations were not correlated with the degree of inflammatory activity in the patients groups. Moreover, whether the analysis of serum prohepcidin reflects mature-active hepcidin is unclear. Further studies that determine active hepcidin instead of prohepcidin are necessary to evaluate the role of hepcidin in these systemic inflammatory diseases.

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