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AYSUN TOKER

HÜLYA AKSOY

BÜNYAMİN BÖREKÇİ

ASUMAN ÖZKAN

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Correlations of Serum IL-6 Levels and Prolidase Activity Between Bone Turnover Markers and Bone Mineral Density in Postmenopausal Women With and Without Osteoporosis

Aysun TOKER¹
Hülya AKSOY²
Bünyamin BÖREKÇİ³
Asuman ÖZKAN²

Aim: The purpose of this study was to measure serum prolidase activity and interleukin-2 and 6 (IL-2, IL-6) levels and to investigate the relationship of these parameters with bone mineral density (BMD) and bone turnover markers in postmenopausal women with and without osteoporosis.

Materials and Methods: 59 postmenopausal women were included in this study (21 women with osteoporosis and 38 without). Serum IL-2 and IL-6 levels, prolidase activity, osteocalcin (OSC), calcium (Ca), alkaline phosphatase (ALP), urinary deoxypyridinoline (DPD) and BMD were measured in postmenopausal women with and without osteoporosis.

Results: Serum prolidase activity was slightly higher in postmenopausal nonosteoporotic women than in postmenopausal osteoporotic women, but the difference did not reach a statistical significance. Mean values of OSC and IL-6 levels were significantly higher in postmenopausal osteoporotic than in nonosteoporotic women ($P < 0.05$). There were significant correlations between OSC with IL-6 ($r = 0.545$, $p = 0.01$) and urinary DPD ($r = -0.513$, $p = 0.01$) in postmenopausal osteoporotic women.

Conclusions: Serum prolidase activity in postmenopausal osteoporotic women was not correlated with the bone turnover markers and BMD. Elevated levels of IL-6 in postmenopausal osteoporosis might have an important role in the pathogenesis of postmenopausal osteoporosis.

Key Words: Bone turnover, IL-6, IL-2, prolidase activity, postmenopausal osteoporosis

¹ Department of Biochemistry, Numune State Hospital, Erzurum - TURKEY

² Department of Biochemistry, Faculty of Medicine, Atatürk University, Erzurum - TURKEY

³ Department of Obstetrics and Gynecology, Faculty of Medicine, Atatürk University, Erzurum - TURKEY

Postmenopozal Osteoporozu Olan ve Olmayan Kadınlarda Serum IL-6 Seviyeleri ve Prolidase Aktivitesinin Kemik Döngü Belirteçleri ve Kemik Mineral Dansitesi ile İlişkileri

Amaç: Bu çalışmanın amacı; postmenopozal osteoporozu olan ve olmayan kadınlarda serum prolidaz aktivitesinin ve interleukin-2, 6 (IL-2, IL-6) seviyelerinin ölçülmesi ve bu parametrelerin kemik mineral dansitesi (BMD) ve kemik döngü belirteçleri ile ilişkilerinin araştırılmasıdır.

Yöntem ve Gereç: Çalışmaya 59 postmenopozal kadın dahil edildi (21 osteoporozu olan, 38 osteoporozu olmayan). Serum IL-2 ve IL-6 seviyeleri, prolidaz aktivitesi, osteokalsin (OSC), kalsiyum (Ca), alkalin fosfataz (ALP), idrar deoksipridinolin (DPD) düzeyleri çalışıldı ve BMD ölçümleri yapıldı.

Bulgular: Serum prolidaz aktivitesi postmenopozal osteoporozu olmayan kadınlarda osteoporozu olanlara göre yüksek olarak bulundu, fakat bu yükseklik istatistiksel olarak anlamlı değildi. OSC and IL-6 seviyelerinin ortalama değerleri postmenopozal osteoporozu olan kadınlarda olmayanlara göre belirgin olarak yüksek bulundu ($P < 0.05$). Postmenopozal osteoporozu olan kadınlarda OSC ile IL-6 ($r = 0.545$, $p = 0.01$) ve idrar DPD ($r = -0.513$, $p = 0.01$) arasında anlamlı korelasyon saptandı.

Sonuç: Postmenopozal osteoporozu olan kadınlarda serum prolidaz aktivitesi, kemik döngü belirteçleri ve BMD ile korelasyonu yoktur. Postmenopozal osteoporozda IL-6'nın yükselmiş seviyeleri postmenopozal osteoporozun patogeneğinde önemli rol oynayabilir.

Anahtar Sözcükler: Kemik döngüsü; IL-6, IL-2, prolidaz aktivitesi; postmenopozal osteoporoz

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Correspondence

Aysun TOKER
Department of Biochemistry Lab.,
Yoncalı Hydrotherapy and Physical
Treatment Hospital,
Kütahya - TURKEY

draysuntoker@hotmail.com

Introduction

Osteoporosis is a common disease that is characterized by decrease in mineral density and increase in fracture risk. Laboratory studies are contributing each day to the growing accumulation of knowledge about osteoporosis. Recent studies have indicated that osteoporosis is caused by complex interactions among local and systemic regulators of bone cell function (1).

Morphological studies and some changes in biochemical markers have shown that bone turnover becomes faster in menopause, and both formation and resorption marker levels increase. In bone turnover, the resorption phase is much shorter than the time needed for osteoblastic changes. Hence, any factor increasing bone turnover may lead to bone loss (2).

Prolidase (E.C.4.3.13.9) is a cytosolic exopeptidase and takes a mediating role in the last period of collagen degradation (3), although extracellular collagenases initiate the breakdown. Prolidase is effective on collagen, which contains proline or hydroxyproline on C terminal. Thus, the increase in the enzyme activity may correlate with increased collagen degradation and its serum activity measurement may be a useful tool in the diagnosis of osteoporosis. Liver prolidase exhibits two forms (prolidase I and II) after ion exchange chromatographic separation. Only prolidase I activity is present in human plasma (4). The investigations on serum prolidase activity showed that it changes in various diseases such as liver cirrhosis (5) and breast cancer (6).

Interleukin (IL)-6 is one of the potent regulators of bone resorption and is produced by immune and non-immune cells and plays an important role in the pathogenesis of osteoporosis. Estrogen changes the dependency of bone cells on several cytokines, including IL-6, IL-1 and tumor necrosis factor (TNF) (7). It was demonstrated that 17 beta estradiol inhibits IL-6 production in several types of osteoblastic cells (8).

Determination of pyridinoline and deoxypyridinoline (DPD), nonreducible cross-links that stabilize the mature collagen chains within the extracellular matrix, provides improved specificity over hydroxyproline in assessing bone resorption. DPD is restricted to bone and dentine, whereas pyridinoline is widespread in hard connective tissues, with high levels in cartilage. DPD formed during collagen maturation is a specific and sensitive product of bone formation, because it is not formed during

biosynthesis and is not metabolized before excretion in urine. Thus, urinary DPD is a specific bone resorption marker and is a good marker in conditions such as postmenopausal osteoporosis (9).

Osteocalcin (OSC) is a specific product of osteoblasts in bone turnover. OSC synthesis is stimulated by $1,25(\text{OH})_2\text{D}$. In bone, OSC is one of the important proteins aside from collagen. It contains 49 amino acids and 3 glutamyl residues, which are carboxylated by post-translational enzymatic carboxylation. OSC levels are found to be increased in patients with increased bone formation (9).

The aim of this study was to measure serum prolidase activities and IL-6 levels and to investigate the relationship between bone mineral density (BMD) and bone turnover markers in postmenopausal women with and without osteoporosis.

Materials and Methods

This study was conducted at Atatürk University, Faculty of Medicine, Department of Biochemistry and Clinical Chemistry and Department of Obstetrics and Gynecology, in 2005. Informed consent was obtained from all patients according to the Helsinki Declaration. All female participants were postmenopausal. Our exclusion criteria were: treatment for osteoporosis, use of medicine that could affect bone metabolism such as calcium (Ca), vitamin D and other medications that alter bone metabolism such as in endocrine diseases and hepatorenal disorders. Fifty-nine women were included in the study (21 with postmenopausal osteoporosis and 38 without). All women had natural menopause and no menstrual cycle in the last one year.

Venous blood samples were collected into plain tubes between 9:30 and 11:00 a.m. after 10 to 12 h fasting. After centrifugation at 2000 g at 4 °C, serum samples were separated. Serum Ca and alkaline phosphatase (ALP) were analyzed immediately and three aliquots were stored at -80 °C for serum prolidase activity, and IL-6 and IL-2 levels. Urinary DPD in first morning urine specimens was analyzed in the same day.

Bone mineral density was assessed in both groups. BMD was obtained from antero-posterior spine L2-L4 scanning with dual energy X-ray absorptiometry (Hologic model QDR-4500A). Subjects with T scores lower than -2.5 were accepted as osteoporotic.

For measurement of prolidase activity, serum was diluted 40-fold with 2.5 mmol/l Mn^{2+} , 40 mmol/L Trizma HCl buffer (pH 8.0) and preincubated at 37 °C for 2 h. The reaction mixture containing 30 mmol/L gly-pro, 40 mmol/L Trizma HCl buffer (pH 8.0) and 100 μ L of preincubation serum in 1 ml was incubated at 37 °C for 30 min. Addition of 0.5 ml of 20% trichloroacetic acid solution then stopped the incubation reaction. The supernatant was used for the measurement of proline using the method proposed by Myara (10), which is a modification of Chinard's method. All reagents were of analytical grade and obtained from Sigma (St. Louis, USA) and Merck (Darmstadt, Germany). Intra- and interassay precision performances of the assay were determined from a serum pool on 10 replicates in a single run, and in 10 different runs yielded coefficients of variation of 3.8 and 9.0%, respectively.

An enzyme linked immunosorbent assay (ELISA) test was conducted to determine levels of the cytokines (IL-2 and IL-6) using the Biosource ELISA kit (Biosource International, Camarillo, CA) according to recommendations of the manufacturers. Urinary DPD was determined by chemiluminescent immunometric assay (Immulite 2000). Serum Ca and ALP levels were determined by Olympus (Japan) using commercially available kits.

Statistical Evaluation

The results are given as mean \pm SD. Differences in the parameters between the two groups were compared with independent t-test. Pearson's correlation coefficient was estimated to quantify the strength of the association between serum prolidase activity, IL-2 and IL-6 with bone turnover markers and BMD. A p value of <0.05 was accepted as significant.

Results

As expected from the definition criteria, mean BMD was significantly higher in the nonosteoporotic than in the osteoporotic group ($P < 0.001$). While mean serum prolidase activity in postmenopausal osteoporotic women (0.85 ± 0.11 U/L) was not significantly different from postmenopausal nonosteoporotic women (0.90 ± 0.06 U/L, $P > 0.05$), mean values of OSC and IL-6 levels were significantly higher in postmenopausal osteoporotic than in nonosteoporotic women ($P < 0.05$). Serum total ALP activity, Ca, IL-2 and prolidase and urinary DPD levels in the postmenopausal osteoporotic women were similar to values in postmenopausal nonosteoporotics (Table 1).

No correlations were determined between serum prolidase activity and bone turnover markers or between serum prolidase and BMD in either group.

Table 1. Patient characteristics, bone turnover markers and BMD values in postmenopausal women with and without osteoporosis.

	Osteoporotic women (n = 21)	Nonosteoporotic women (n = 38)
Age (years)	49.66 \pm 5.84	50.15 \pm 5.22
BMI (kg/m ²)	30.11 \pm 4.22	31.45 \pm 5.25
BMD (g/cm ²)	-2.84 \pm 0.28*	-1.36 \pm 1.10
Total ALP (U/L)	133.42 \pm 56.65	128.83 \pm 59.90
Ca (mg/dl)	9.24 \pm 0.53	9.36 \pm 0.56
OSC (ng/ml)	10.31 \pm 2.96*	7.70 \pm 2.44
Urinary DPD (nmol/mmol creatine)	8.74 \pm 4.34	8.14 \pm 2.94
IL-2 (pg/L)	26.54 \pm 8.42	28.67 \pm 8.61
IL-6 (pg/L)	6.69 \pm 1.30*	5.57 \pm 1.19
Prolidase (U/L)	0.85 \pm 0.11	0.90 \pm 0.06

Data are given as mean \pm SD; *: $P < 0.05$.

BMI: Body mass index. BMD: Bone mineral density. ALP: Alkaline phosphatase. Ca: Calcium. OSC: Osteocalcin. DPD: Deoxypyridinoline.

There was a positive correlation between serum IL-6 levels and OSC levels in postmenopausal osteoporotic women ($r = 0.545, p = 0.01$, Figure 1). Serum OSC levels exhibited a negative correlation with urinary DPD levels in the same group ($r = -0.513, p = 0.017$, Figure 2). No correlation was found between the bone turnover markers (urinary DPD, OSC, total ALP) with serum IL-6 and IL-2 levels in postmenopausal nonosteoporotic women.

Discussion

Estrogens are known to change collagen metabolism. The increase in serum prolidase I enzyme activity is correlated with increased rates of collagen turnover (11). Prolidase is a highly specific peptidase which catalyzes the hydrolysis of compounds that involves the imino nitrogen of proline or hydroxyproline (4). This enzyme plays an important role in the recycling of proline for collagen synthesis. However, the mechanism and endpoints by which this enzyme is regulated remain great unknowns.

IL-6 production is affected by estrogens. Serum IL-6 levels do not change between the follicular phase and luteal phase of the menstrual cycle in healthy premenopausal women (12). However, there is no consensus about serum IL-6 levels in menopause. Some previous studies failed to demonstrate a regulatory effect of ovarian steroids on IL-6 levels in human bone cells and animal (13-15). Furthermore, McKane et al. (16) suggested that there was no difference in serum IL-6 levels between pre- and postmenopausal women. On the

other hand, Rachon et al. (17) found that estrogen deprivation might result in IL-6 production by the peripheral blood mononuclear cells in postmenopausal women. They reported that the mean serum levels of IL-6 in postmenopausal women were significantly higher than in young women. The effect of estrogen in IL-6 production was observed in *in vitro* conditions. After the incubation period with 17β estradiol in the cultured cells, it was found that IL-6 production significantly decreased in the culture containing estradiol. *In vivo* studies also showed that women using hormone replacement therapy have significantly lower serum IL-6 levels compared with women not using hormone replacement therapy (17,18). Scheidt-Nave et al. (19) reported that IL-6 was an important factor during the first accelerated phase of bone loss; however, different mechanisms may be more relevant to bone loss in the later menopausal period.

Although the defined cytokines increase over time, the functions of cytokines are not fully understood. It was shown that IL-1, IL-6, TNF and IL-11 have important roles in the pathogenesis of osteoporosis (7,20). In one study, it was emphasized that not only IL-1, IL-6, TNF and IL-11 but also IL-2, IL-8 and IL-10 may have roles in the pathogenesis of osteoporosis (21).

We found that serum OSC and IL-6 levels were higher in postmenopausal osteoporotic than in non-osteoporotic women. Serum prolidase activity was found to be slightly decreased in the postmenopausal osteoporotic group, but it was not statistically different from the nonosteoporotic group. Serum prolidase and IL-2 did not show any correlations with the other parameters in either group. In

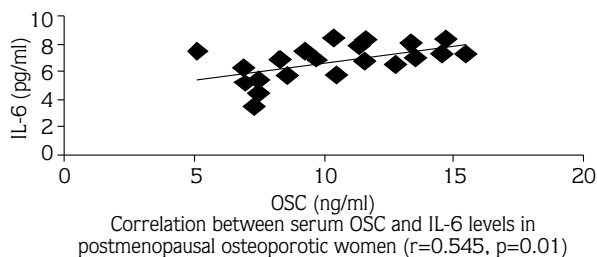


Figure 1. Correlation between serum IL-6 and OSC in postmenopausal osteoporotic women.

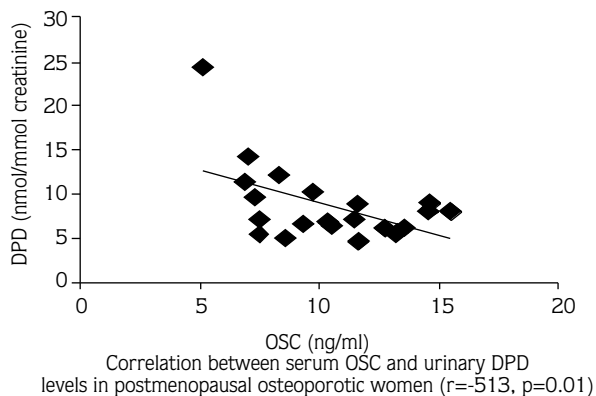


Figure 2. Correlation between serum OSC and urinary DPD in postmenopausal osteoporotic women.

our study, unchanged serum prolidase activity might be the result of lack of estrogen in postmenopausal women, even if in osteoporosis, collagen turnover is increased, in line with the findings of Verit et al. in 2006 (22). It has been reported that collagen content in various tissues decreases in menopause with loss of estrogen (23). However, some studies have shown that prolidase activity and collagen synthesis were increased by estrogen in cultured cells (6,24). Although it is well-described that lack of estrogen is the major cause of postmenopausal osteoporosis, we think that the role of estrogen, which prevents bone loss, is very complex. Some studies suggest that estrogen may regulate many factors that play an important role in bone remodeling in the bone microenvironment (25,26). In high turnover

osteoporosis, it was reported that serum OSC was elevated (9) and in postmenopausal osteoporosis it was shown that IL-6 levels increase (17). In our study, IL-6 levels showed no correlation with BMD and the other biochemical bone turnover markers except OSC. This finding is in agreement with other conclusions in the literature (27-29). Prolidase is a marker of bone turnover. In the menopause patient, its serum activity may not help to determine bone turnover alteration. While the underlying mechanisms of osteoporosis are largely unknown, it is certain that loss of estrogen is the principal factor, and cytokines have a role in this cycle. Further longitudinal studies are required to explain the exact roles of cytokines in the pathogenesis of osteoporosis.

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