Biomedical Research (Tokyo) **37** (3) 215–220, 2016

Steroid changes adipokine concentration in the blood and bone marrow fluid

Tatsuya Fukushima, Akira Hozumi, Masato Tomita, Akihiko Yonekura, Noriaki Miyata, Takashi Miyamoto, Kenji Taguchi, Hisataka Goto, Keiichi Tsuda, and Makoto Osaki
Department of Orthopaedic Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Japan

(Received 8 April 2016; and accepted 26 April 2016)

ABSTRACT

Our previous study has shown that plasminogen activator inhibitor 1 (PAI-1) gene expression and secretion from bone marrow adipocytes increased markedly with dexamethasone administration. The purpose of the present study was to measure the secretion of various adipokines from human bone marrow and blood, and investigate how adipokine secretion changes in a steroid environment. Human blood and bone marrow fluid were collected from a steroid treatment group and a control group during hip replacement surgery, and an enzyme-linked immunosorbent assay (ELISA) was used to measure the adiponectin, leptin, and PAI-1 levels. Adiponectin and leptin showed no significant differences between bone marrow and blood levels, but PAI-1 was significantly higher in bone marrow. The steroid treatment group had higher levels of leptin and PAI-1 in both the blood and bone marrow than the control group. PAI-1 was present at high concentrations in the bone marrow and increased by steroid treatment. High levels of PAI-1 in bone marrow may influence intraosseous hemodynamics and may induce necrotic bone disorders.

Osteonecrosis of the femoral head (ONFH) is a common complication caused by high-dose administration of glucocorticoids. Glucocorticoid hormone has diverse activities in multiple organs throughout the body, and hypercortisolism causes various disorders, including ONFH. Several studies have shown that micro-vascular thrombosis and subsequent local blood flow are principle features in the development of glucocorticoid-induced osteonecrosis (4). Other reports presented that vascular endothelial cell disturbance due to various oxidative stresses or abnormal lipid metabolism was important cause of ONFH (12, 27).

Recent reports have shown that visceral adipocytes secrete various physiologically active substances known as adipokines. In the bone marrow space, there is a large quantity of mature adipocytes that are possible candidates for adipokine secretion. Considering the enclosed nature of the bone marrow space, intramedullary adipocytes may be involved in bone metabolism, as we previously reported that bone marrow adipocytes express receptor activator of nuclear factor kappa-B ligand (RANKL) and support osteoclast differentiation (5, 8).

Plasminogen activator inhibitor-1 (PAI-1), one of the adipokines secreted by adipocytes (1), suppresses fibrinolysis by binding tissue-type plasminogen activator (t-PA), and a relationship between PAI-1 and thrombosis or hypercoagulation has been suggested. Furthermore, blood coagulation in the femoral head is thought to be associated with osteonecrosis. There are several reports regarding increased PAI-1 secretion in serum of patients with ONFH (11, 16, 25, 26). We also previously reported dexamethasone-induced PAI-1 secretion from human bone marrow adipocytes (9). Its secretion with steroid administration was increased about 2.5-fold for 24 h, but inhibited by simvastatin, which is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase

Address correspondence to: Akira Hozumi, MD. PhD
Department of Orthopaedic Surgery, Graduate school of Biomedical Sciences, Nagasaki University, 1-7-1, Sakamoto Nagasaki, 852-8501, Japan
Tel: +81-95-819-7321, Fax: +81-95-849-7325
E-mail: akira45512@gmail.com

inhibitors (21). These results imply the possibility that PAI-1 that has been increased by steroids affects intraosseous hemodynamics and is involved in the development of ONFH.

The purpose of the present study was to examine and compare the adipokine concentration in blood and bone marrow fluid from patients with or without steroid treatment.

MATERIAL AND METHODS

The subjects were 35 patients who underwent hip arthroplasty, consisting of 15 cases receiving steroid administration from among patients with idiopathic femoral head necrosis (S group), and 20 cases with hip osteoarthritis without steroid treatment as a control group (C-group). Patients with rheumatoid arthritis or abnormal lipid metabolism, or who were positive for hepatitis B or C virus, or had a C-reactive protein (CRP) of 2.1 mg/dL or higher were excluded from this study. The S-group had 3 men and 12 women, with a mean age of 57 (29 to 80) years, body mass index (BMI) of 22.9 (16.7 to 29.1) kg/m^2, and mean steroid dosage of 10.5 (2.5 to 22.5) mg prednisolone equivalent; all cases had idiopathic femoral head necrosis. The C-group had 4 men and 16 women, with a mean age of 63 (43 to 76) years and BMI of 23.5 (18.2 to 28.5) kg/m^2; all cases had hip osteoarthritis.

Venous blood and bone marrow fluid were collected at the same time during the surgery, which was performed at a constant time of day; 1.8 mL of blood or bone marrow fluid were then mixed into 0.2 mL of 3.2% sodium citrate. Each specimen was promptly plasma-separated by centrifugation at 4°C (3000 rpm, 15 min) and stored frozen at −80°C. The specimens were thawed when it was time for testing, and an enzyme-linked immunosorbent assay (ELISA) was used to measure the secretion levels of various adipokines (PAI-1, adiponectin, and leptin). PAI-1 was measured using a t-PA/PAI-1 complex (test method: EIA; kit reagent: LPIA/tPAI test; measurement equipment: ELISA·F300, Sysmex Corp., Kobe, Japan) and total PAI-1 (test method: latex agglutination method; kit reagent: LPIA·tPAI test; measurement equipment: STACIA, Mitsubishi Chemical Medience Co., Ltd. Tokyo, Japan). Adiponectin (test method: turbidimetric latex agglutination; kit reagent: human adiponectin latex; measurement equipment: JEOL BM-9030, Mitsubishi Chemical Medience Co., Ltd.) and leptin (test method: double antibody assay; kit reagent: Human Leptin RIA kit; measurement equipment: γ-counter AccuFLEX γ-7010, LINCO RESEARCH, St Charles, MO) were also measured by ELISA. There were 13 cases in the S-group and 19 cases in the C-group where only the t-PA/PAI-1 complex was studied, due to discontinuation of sales of the ELISA kit in the middle of the experiment. Considering the possibility that PAI-1 is affected by inflammation, only CRP-negative cases (< 0.17 mg/dL) in the C (13 cases) and S (6 cases) groups were investigated and compared.

The Wilcoxon signed-rank test was used for comparisons between blood and bone marrow fluid, and the Mann-Whitney U test was used for comparisons between the C- and S-groups. A P-value <0.05 was considered significant.

RESULTS

Adiponectin levels in the C group were 9.3 (2.8–17.3) μg/mL for blood and 8.9 (2.6–19.1) μg/mL for bone marrow fluid, and in the S-group, 9.3 (3.1–22.8) μg/mL for blood and 8.4 (3.0–22.8) μg/mL for bone marrow; no significant differences were found between the blood and bone marrow and between the C- and S-groups (Fig. 1).

Leptin levels were 5.6 (1.1–25.9) ng/mL for blood and 4.8 (1.4–20.8) ng/mL for bone marrow fluid in the C group, and 13.2 (3.4–40.5) ng/mL for blood and 11.2 (4.2–32.8) ng/mL for bone marrow fluid in the S group. The leptin level of the S-group was significantly higher, by two-fold or more, in both blood and bone marrow (Fig. 2). A significant difference was not found between blood and bone marrow in either the C-group or the S-group.

PAI-1 (measured with t-PA/PAI-1 complex) was 12.2 (2.0–23.6) ng/mL for blood and 43.8 (24.6–91.5) ng/mL for bone marrow fluid in the C group, and 21.4 (7.1–45.4) ng/mL for blood and 67.8 (23.3–100) ng/mL for bone marrow in the S group. PAI-1 levels in the bone marrow fluid were markedly higher, by about three-fold, compared to levels in the blood for both the C-group and the S-group. Comparing the C- and S-groups, PAI-1 levels were significantly higher in the S-group, with the PAI-1 in the bone marrow fluid of the S-group were the highest (Fig. 3a).

Total PAI-1 in the C-group was 17.4 (10–50) ng/mL for blood and 81.7 (46–118) ng/mL for bone marrow, while it was 35.1(10–79) ng/mL for blood and 90.8(42–173) ng/mL for bone marrow in the S group. Similar to the t-PA/PAI-1 complex, the total PAI-1 levels in the bone marrow fluid were markedly higher, by about four-fold, than levels in the blood. Furthermore, PAI-1 levels in the bone mar-

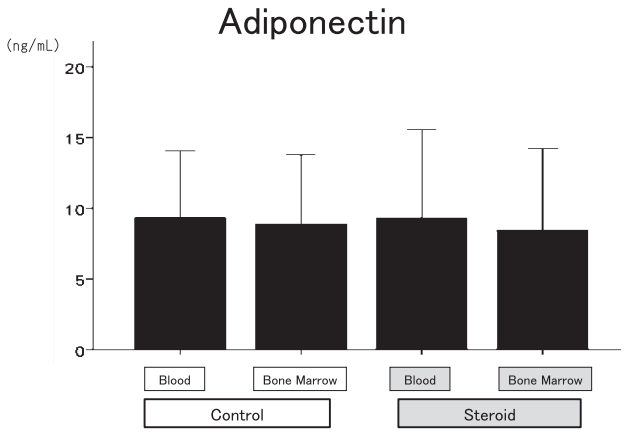

Fig. 1 Adiponectin levels in the blood and bone marrow fluid. Significant differences in adiponectin levels are not observed in the blood and bone marrow fluid in the C- and S-groups.

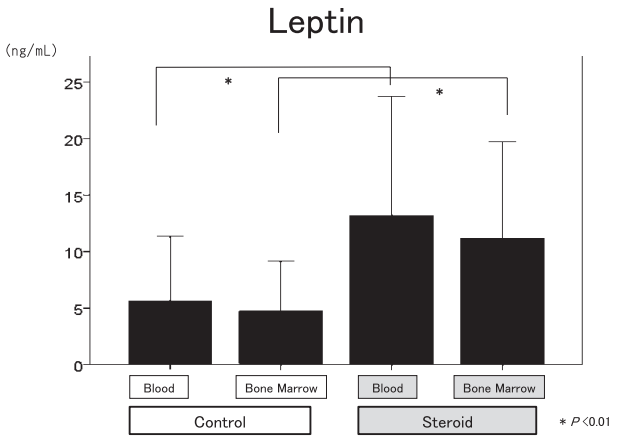

Fig. 2 Leptin levels in the blood and bone marrow fluid. A significant difference is not observed in leptin levels in the blood and bone marrow fluid for either the C- or S-group. Leptin levels are higher in the S-group than in the C-group for both the blood and the bone marrow fluid.

row fluid of the S-group were the highest (Fig. 3b).

In the CRP-negative group, due to the smaller number of samples, a significant difference in the PAI-1 level was not found between the C- and S-groups, but it was significantly elevated in the bone marrow of both groups. These were essentially the same results as when CRP was not considered (Fig. 4a). Total PAI-1 in the CRP-negative group was significantly elevated similar to the t-PA/PAI-1 complex (Fig. 4b).

DISCUSSION

In recent years, there have been various reports on the physiological effects of various adipokines, such as adiponectin, leptin, and PAI-1. Adiponectin is a secretory protein that is specifically expressed in adipocytes; it inhibits the occurrence of type 2 diabetes and arteriosclerosis (28), and it has been shown to be associated with metabolic syndrome (14, 20). In bone metabolism, adiponectin has been shown to inhibit the differentiation of osteoclasts and promote the differentiation of osteoblasts (18). However, adiponectin also has a negative effect on bone mass, and high levels of adiponectin increase the risk of bone fracture; adipocyte differentiation is enhanced by steroids, and some reports suggest an association between steroids and osteoporotic fractures. Leptin is a typical appetite-regulating hormone that is secreted by adipocytes, and it exhibits an appetite-suppressing effect and energy consumption-increasing effect (6). In bone metabolism, similar to adiponectin, it is thought to promote calcification and growth differentiation by acting on osteoblasts (19). PAI-1 is thought to be associated with thrombosis and cardiovascular disease (7), and as regards its effects on bone, it is a risk factor for ONFH (3); it has been reported that, when glucocorticoid levels are higher, PAI-1 increases and becomes a cause of bone necrosis (12).

In the present study, neither adiponectin nor leptin was different between blood and bone marrow, and we infer that there is no major difference between the environments in the blood and in the bone. However, leptin did show clearly higher levels in the S-group, in both blood and bone marrow fluid, implying the possibility that steroids are responsible for some form of systemic effect. In the periphery, leptin acts to promote the production of reactive oxygen species and has a neovascularization effect and an arteriosclerosis promotion effect (2). It has been demonstrated that leptin levels in the blood and vitreous body are elevated in diabetic retinopathy which is one of the representative microangiopathy (24). Though the present study does not represent especially high leptin levels, the possibility cannot be ruled out that, in the bone marrow, which is an enclosed environment similar to the vitreous body, steroid-induced effects and various intercellular interactions are involved in the micro-arterial dynamics. The mechanism of its action will need to be further investigated.

PAI-1 levels, comparing blood and bone marrow fluid, were markedly higher in the bone marrow fluid as compared to other adipokines for both tPA/PAI-1 complex and total PAI-1; they were also higher in the S-group than in the C-group. In obese rats, PAI-

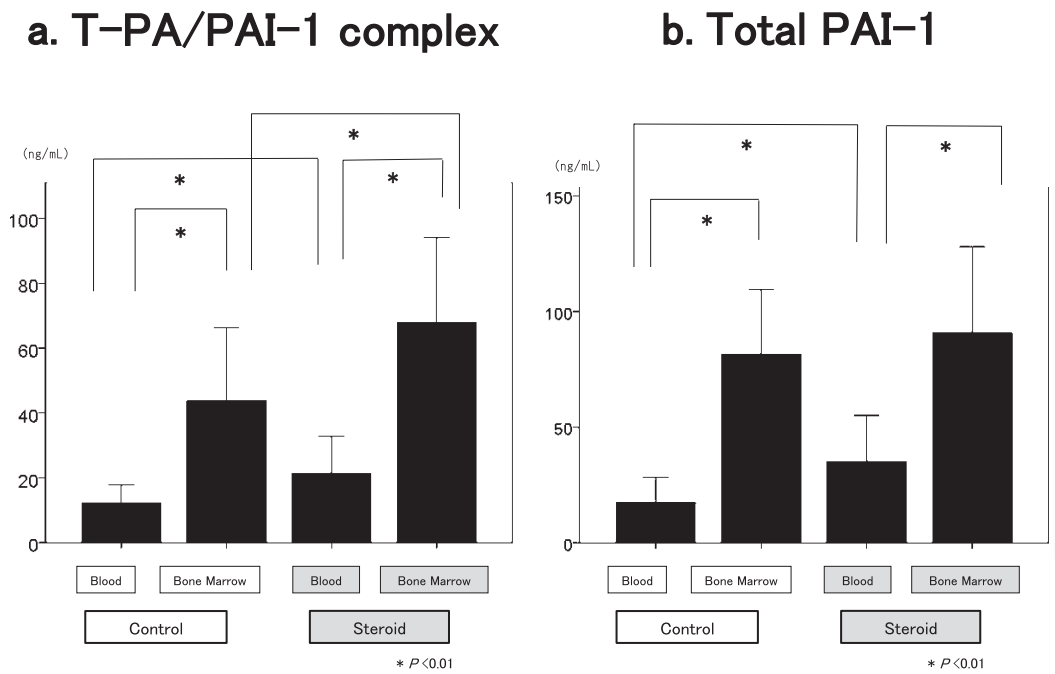

Fig. 3 PAI-1 levels in the blood and bone marrow fluid. **a**) tPA/PAI-1 complex levels. tPA/PAI-1 complex levels are higher in bone marrow fluid than in blood for both the C- and S-groups, which both show significant differences. t-PA/PAI-1 complex levels are higher for the S-group in both the blood and bone marrow fluid, and values are highest for the S-group bone marrow fluid. **b**) Total PAI-1 levels. Total PAI-1 levels in bone marrow fluid are higher than blood for the C-group and S-group. Though comparison of bone marrow fluid between the C- and S-groups does not show a significant difference, the highest values are those for bone marrow fluid in the S-group.

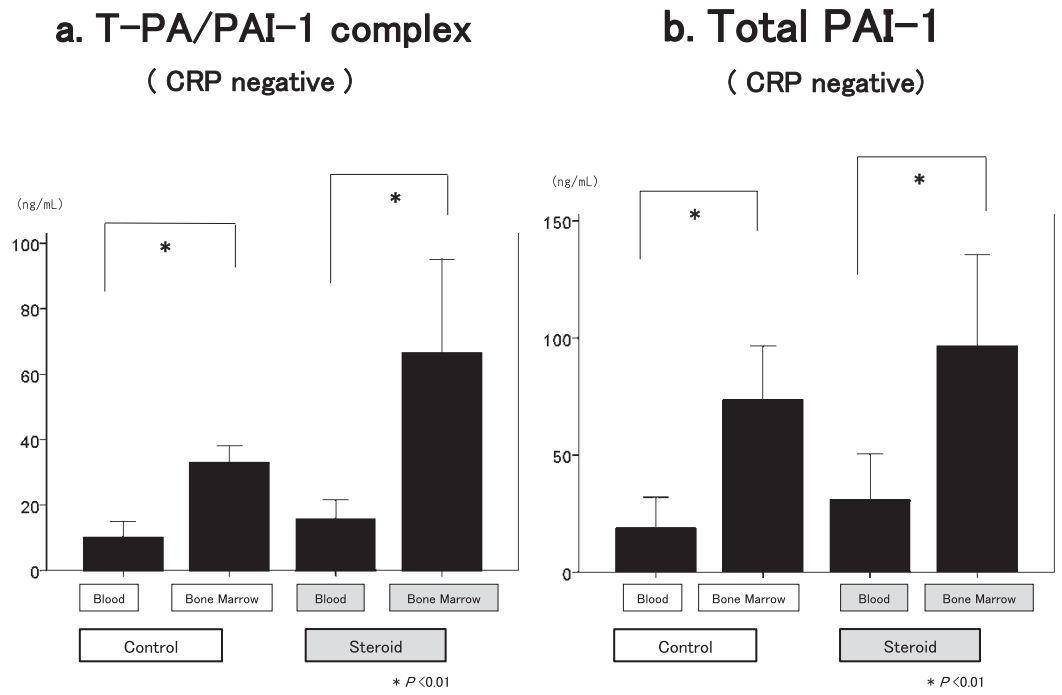

Fig. 4 PAI-1 levels in blood and bone marrow fluid in the CRP-negative group. **a**) tPA/PAI-1 complex levels; **b**) Total PAI-1 levels. In the CRP-negative group, tPA/PAI-1 complex levels and total PAI-1 levels both show significant differences in comparisons between blood and bone marrow fluid in the C-group and the S-group. Comparison between the C- and S-groups does not reveal a significant difference, but the highest values are exhibited by the S-group bone marrow fluid.

1 levels in the blood are reportedly more reflective of visceral fat accumulation than subcutaneous fat (22), but the results of the present study demonstrated that PAI-1 levels were markedly higher in bone marrow than blood, suggesting that PAI-1 from bone marrow adipocytes has a major effect on bone metabolism. It has been reported that glucocorticoid increases the size of bone marrow adipocytes (13), induces a high level of intraosseous pressure and capillary compression (29), and leads to the development of ONFH (17). Considering the enclosed nature of the bone marrow space, intramedullary adipocytes may be involved in bone metabolism. A high level of PAI-1 in bone marrow, which is mainly secreted from bone marrow adipocytes, is closely associated with the onset of ONFH. The results of the present study are consistent with this hypothesis. Ibrahim *et al.* investigated active PAI-1 levels after irradiation in the bone marrow and plasma of mice, and they showed that the bone marrow had a markedly higher rate of increased PAI-1 than the plasma (10); this also supports our hypothesis.

The limitations of the present study include the differences of sex, BMI, diurnal variation, and inflammation values because of the small sample size. However, large differences were not observed between specimens due to sex or BMI. Diurnal variation was reduced as much as possible by collecting blood and bone marrow fluid at substantially the same time in all cases.

Inflammatory diseases are also known to affect PAI-1 levels, and the involvement of inflammation was excluded as much as possible by excluding patients with rheumatoid arthritis, those who were positive for hepatitis B or C virus, those with a CRP of 2.1 mg/dL or higher, and those with bone marrow edema on preoperative MRI. Furthermore, PAI-1 levels in the bone marrow were highest in the S-group in a comparison of only the CRP-negative groups (<0.17 mg/dL) (Fig. 4a, b). Therefore, though inflammation is involved in elevated PAI-1 levels, we believe that this does not rule out our hypothesis that PAI-1 is susceptible to the effects of steroids in bone marrow, which is an enclosed space.

In terms of technical issues regarding the collection of bone marrow fluid, blood may have been mixed into the bone marrow fluid due to bleeding from other tissue. However, if blood had diluted the bone marrow fluid concentration, pure PAI-1 levels in bone marrow should be higher. PAI-1 is also secreted from vascular endothelial cells and other sources (15, 23), not only from bone marrow adipocytes. However, considering the presence of fat in the bone marrow and several previous studies, the primary origin of PAI-1 is considered to be from bone marrow adipocytes.

In conclusion, levels of various adipokines were compared between human bone marrow and blood. In humans, adiponectin and leptin had equivalent levels in the blood and bone marrow, but PAI-1 levels were significantly higher in the bone marrow. We think that an increase of PAI-1 levels caused by steroids is an important factor that is associated with the development of ONFH.

REFERENCES

1. Alessi MC, Poggi M and Juhan-Vague I (2007) Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. *Curr Opin Lipidol* **18**, 240–245.
2. Chiba T, Shinozaki S, Nakazawa T, Kawakami A, Ai M, Kaneko E, Kitagawa M, Kondo K, Chait A and Shimokado K (2008) Leptin deficiency suppresses progression of atherosclerosis in apoE-deficient mice. *Atherosclerosis* **196**, 68–75.
3. Ferrari P, Schroeder V, Anderson S, Kocovic L, Vogt B, Schiesser D, Marti HP, Ganz R, Frey FJ and Kohler HP (2002) Association of plasminogen activator inhibitor-1 genotype with avascular osteonecrosis in steroid-treated renal allograft recipients. *Transplantation* **74**, 1147–1152.
4. Fukui K, Kominami R, Shinohara H and Matsumoto T (2006) Glucocorticoid induces micro-fat embolism in the rabbit: a scanning electron microscopic study. *J Orthop Res* **24**, 675–683.
5. Goto H, Osaki M, Fukushima T, Sakamoto K, Hozumi A, Baba H and Shindo H (2011) Human bone marrow adipocytes support dexamethasone-induced osteoclast differentiation and function through RANKL expression. *Biomed Res (Tokyo)* **32**, 37–44.
6. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK and Friedman JM (1995) Weight-reducing effects of the plasma protein encoded protein by the obese gene. *Science* **269**, 543–546.
7. Halleux CM, Declerck PJ, Tran SL, Detry R and Brichard SM (1999) Hormonal control of plasminogen activator inhibitor-1 gene expression and production in human adipose tissue: stimulation by glucocorticoids and inhibition by catecholamines. *J Clin Endocrinol Metab* **84**, 4097–4105.
8. Hozumi A, Osaki M, Goto H, Sakamoto K, Inokuchi S and Shindo H (2009) Bone marrow adipocyte support dexamethasone-induced osteoclast differentiation. *Biochem Biophys Res Commun* **382**, 780–784.
9. Hozumi A, Osaki M, Sakamoto K, Goto H, Fukushima T, Baba H and Shindo H (2010) Dexamethasone-induced plasminogen activator inhibitor-1 expression in human primary bone marrow adipocyte. *Biomed Res (Tokyo)* **31**, 281–286.
10. Ibrahim AA, Yahata T, Onizuka M, Dan T, Strihou CVYD, Miyata T and Ando K (2014) Inhibition of plasminogen activator inhibitor type-1 activity enhances rapid and sustainable hematopoietic regeneration. *Stem Cells* **32**, 946–958.
11. Kang P, Shen B, Yang J and Pei F (2008) Circulating platelet-derived microparticles and endothelium-derived microparticles may be a potential cause of microthrombosis in patients with osteonecrosis of the femoral head. *Thromb Res* **123**, 367–373.
12. Kerachian MA, Seguin C and Harvey EJ (2009) Glucocorti-

coids in osteonecrosis of the femoral head: A new understanding of the mechanisms of action. *J Steroid Biochem Mol Biol* **114**, 121–128.
13. Kitajima M, Shigematsu M, Ogawa K, Sugihara H and Hotokebuchi T (2007) Effects of glucocorticoid on adipocyte size in human bone marrow. *Med Mol Morphol* **40**, 150–156.
14. Lara-Castro C, Fu Y, Chung BH and Garvey WT (2007) Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease. *Curr Opin Lipidol* **18**, 263–270.
15. Laug WE, Aebersold R, Jong A, Rideout W, Berqman BL and Baker J (1989) Isolation of multiple types of plasminogen activator inhibitors from vascular smooth muscle cells. *Thromb Haemost* **61**, 517–521.
16. Miyanishi K, Kamo Y, Ihara H, Naka T, Hirakawa M and Sugioka Y (2006) Risk factors for dysbaric osteonecrosis. *Rheumatology (Oxf)* **45**, 855–858.
17. Miyanishi K, Yamamoto T, Irisa T, Yamashita A, Jingushi S, Noguchi Y and Iwamoto Y (2002) Bone marrow fat cell enlargement and a rise in intraosseous pressure in steroid-treated rabbits with osteonecrosis. *Bone* **30**, 185–190.
18. Oshima K, Nampei A, Matsuda M , Iwaki M, Fukuhara A, Hashimoto J, Yoshikawa H and Shimomura I (2005) Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. *Biochem Biophys Res Commun* **331**, 520–526.
19. Reseland JE, Syversen U, Bakke I, Qvigstad G, Eide LG, Hjertner O, Gordeladze JO and Drevon C (2001) Leptin is expressed in and secreted from primary cultures of human osteoblasts and promotes bone mineralization. *J Bone Miner Res* **16**, 1426–1433.
20. Ryo M, Nakamura T, Kihara S, Kumada M, Shibazaki S, Takahashi M, Nagai M, Matsuzawa Y and Funahashi T (2004) Adiponectin as a biomarker of the metabolic syndrome. *Circ J* **68**, 975–981.
21. Sakamoto K, Osaki M, Hozumi A, Goto H, Fukushima T, Baba H and Shindo H (2011) Simvastatin suppresses dexamethasone-induced secretion of plasminogen activator inhibitor-1 in human bone marrow adipocytes. *BMC Musculoskelet Disord* **12**, 82.
22. Shimomura I, Funahashi T, Takahashi M, Maeda K, Kotani K, Nakamura T, Yamashita S, Mirua M, Fukuda Y, Takemura K, Tokunaga K and Matsuzawa Y (1996) Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* **2**, 800–803.
23. Sprengers ED and Kluft C (1987) Plasminogen activator inhibitors. *Blood* **69**, 381–387.
24. Suganami E, Takagi H, Ohasi H, Suzuma K, Suzuma I, Oh H, Watanabe D, Ojima T, Suganami T, Fujio Y, Nakao K, Ogawa Y and Yoshimura N (2004) Leptin stimulates ischemia-induced retinal neovascularization: possible role of vascular endothelial growth factor expressed in retinal endothelial cells. *Diabetes* **53**, 2443–2448.
25. Tan X, Cai D, Wu Y, Liu B, Rong L, Chen Z and Zhao Q (2006) Comparative analysis of serum proteomes: discovery of proteins associated with osteonecrosis of the femoral head. *Transl Res* **148**, 114–119.
26. Van Veldhuizen PJ, Neff J, Murphey MD, Bodensteiner D and Skikne BS (1993) Decreased fibrinolytic potential in patients with idiopathic avascular necrosis and transient osteoporosis of the hip. *Am J Hematol* **44**, 243–248.
27. Weinstein RS (2012) Glucocorticoid-induced osteonecrosis. *Endocrine* **41**, 183–190.
28. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R and Kadowaki T (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J Biol Chem* **278**, 2461–2468.
29. Zizic TM, Marcoux C, Hungerford DS and Stevens MB (1986) The early diagnosis of ischemic necrosis of bone. *Arthritis Rheum* **29**, 1177–1186.