Dexamethasone-induced plasminogen activator inhibitor-1 expression in human primary bone marrow adipocytes

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ABSTRACT
Several studies have demonstrated the association of plasminogen activator inhibitor-1 (PAI-1) with osteonecrosis, but the underlying mechanism of osteonecrosis and its relationship with local PAI-1 is not clear. The objective of this study was to evaluate PAI-1 production by primary human bone marrow adipocytes and the effects of glucocorticoid administration. Bone marrow was obtained from 25 individuals during prosthetic insertion. Mature adipocytes were cultured for 24 h with or without dexamethasone. PAI-1, adiponectin, tumor necrosing factor-α (TNFα) expression were measured by latex photometric immunoassay or RT-PCR. Adiponectin, TNFα and PAI-1 were detected in all culture media. PAI-1 expression was significantly increased by treatment with $10^{-6}$ mol/L dexamethasone up to 24 h in protein and mRNA levels, while the levels of other adipokines did not change by dexamethasone. These results suggest that bone marrow adipocytes may play important roles for the development of glucocorticoid-induced osteonecrotic diseases by enhancing PAI-1 expression.

Osteonecrosis of the femoral head is a common complication caused by high-dose administration of glucocorticoid (10). Glucocorticoid hormone exhibits diverse activities in multiple organs, and hypercortisolism causes various disorders including osteonecrosis. The pathogenesis of osteonecrosis seems to be multifactorial and has not fully understood yet (13). Several studies in human and animal models have shown that micro-vascular thrombosis and subsequent impaired local blood flow are principle features in the development of glucocorticoid-induced osteonecrosis (5, 7). PAI-1 is a physiological inhibitor of plasminogen activators, which is secreted from endothelial cells, platelets and adipocytes. Particularly large amount of PAI-1 from adipocytes is known to be closely associated with the development of several disorders, including type 2 diabetes, arteriosclerosis and cardiovascular diseases (1). Several studies showed the association of systematic PAI-1 with idiopathic osteonecrosis of the femoral head. High serum PAI-1 level and certain homozygous polymorphisms in PAI-promoters have been identified in idiopathic osteonecrosis (4, 6, 19). However, there is few report mentioned about relevance between osteonecrosis and local PAI-1 secretion.

In the bone marrow space, there is large quantity of mature adipocytes which are candidate provider of PAI-1 and the other adipokines. Considering the closed bone space, intramedullary adipocytes may be involved in bone metabolism. In the previous reports we demonstrated that bone marrow mature adipocytes express NFκ-B ligand (RANKL) and support osteoclast differentiation (7). We think that mature human bone marrow adipocytes secret biological active substances, for example adipokines, which might play the important roles in the bone metabolism.
marrow metabolism and be attributed to the development of several bone disorders. In this report we evaluated the direct role of glucocorticoid in the expression of PAI-1 from bone marrow adipocytes, and raised the possibility that augmented local PAI-1 secretion from bone marrow adipocyte may be one of pathogenetic factor of glucocorticoid-induced osteonecrosis.

MATERIALS AND METHODS

Isolation and primary culture of bone marrow adipocytes. During prosthetic replacement surgery of the hip joint, 10 mL of bone marrow fluid was aspirated from 25 patients with femoral neck fracture. The subjects consisted of twenty females and five males, with a mean age of 69 years (range: 42–82). We excluded patients with underlying diseases, such as diabetes mellitus, rheumatoid arthritis, metabolic bone disorders, and those with a history of glucocorticoid therapy. Before surgery, informed consent was obtained from all patients. The study protocol was approved by the Institutional Review Board. Bone marrow fluids were mixed with 40 mL of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY), and treated with 0.1% collagenase A (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. Digested cells were then centrifuged at 200 × g for 5 min, and the adipocyte layer was carefully aspirated from the upper lipid phase. To purify the isolated adipocytes, cells were filtered through a nylon mesh of 200 μm diameter, and filtered cells were washed three times with fresh medium. With these procedures, mature adipocytes were almost completely isolated without stromal cells such as preadipocytes, fibroblasts and endothelial cells (16, 18). Adipocytes were counted and 5 × 10^6 cells were then suspended in 3 mL serum-free DMEM in a 15 mL Falcon tube, and then subjected to suspension culture in 5% CO₂ at 37°C.

Histological examination of cultured adipocytes. After enzymatic digestion, the isolated adipocytes were smeared onto a glass slide and fixed with formaldehyde. Oil red O staining was performed to examine the composition of a cell population consisting of isolated adipocytes.

Measurement of adiponectin, TNFα and PAI-1 secretion. Human bone marrow adipocytes from 25 independent individuals were cultured in 5 mL of serum-free DMEM with or without dexamethasone (10⁻⁶ mol/L) for 24 h. The concentrations of adiponectin, TNFα and PAI-1 in culture medium were determined by human adiponectin ELISA kit (Ohtsuka Pharmaceutical Co., Ltd., Tokyo, Japan), Quanti Glo Human TNFα Immunoassay 2nd Generation kit (R&D system, USA) and LPIA tPAI-1 test (Mitsubishi Chemical Iatron, Tokyo, Japan) respectively, according to each manufacturer’s instruction. Dose dependent study was also performed from five independent individuals which were randomly selected from 25 individuals.

Measurement of PAI-1 mRNA expression. PAI-1 gene expression was measured by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from adipocytes using an RNeasy kit (Qiagen, Hilden, Germany). The expression of PAI-1 mRNA (100 ng) was quantified by real-time PCR using ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan). Specific primers for PAI-1 (Hs00243519_m1), and the amplification protocol were provided by the TaqMan Gene Expression Master Mix (Applied Biosystems). The expression of each PAI-1 was normalized by GAPDH expression. Dose-dependent and time-course study up to 24 h was also performed in five independent individuals randomly selected from 25 individuals.

Statistical analysis. All data are expressed as the means ± SD. Differences between two or more groups were tested by the Wilcoxon rank test. *P*-value < 0.05 was considered significant.

RESULTS

After enzymatic digestion, the isolated cells were stained with oil red O. A large bulbar structure and a small granular structure suggested fatty droplets. These structures were stained with oil red O (Fig. 1), confirming the presence of fatty droplets in the cell population.

Bone marrow adipocytes secreted all of adiponectin, TNFα and PAI-1, although the secretion levels of adiponectin and TNFα were not significantly changed by dexamethasone administration (Fig. 2). Concerning about PAI-1 secretion, its secretion increased in all individuals with an average 2.4-fold increase (range, 1.11 to 5.25 fold increase, *P* = 0.0004) with the treatment with 10⁻⁶ mol/L dexamethasone for 24 h and mRNA expression changed with an average 4.7-fold increase (range 1.88 to 5.72 fold increase, *P* = 0.001) (Fig. 3A, B). Incubation with various concentrations of dexamethasone from 10⁻⁸
PAI-1 from bone marrow adipocytes

The precise mechanism, to explain the pathogenesis of steroid-induced osteonecrosis, remains unclear. A consensus etiopathogenesis has been recently unified on both intravenous thrombosis and extravascular lipid-deposition induced by abnormal fat metabolism. Activated oxidative stress induced by glucocorticoid leads to endothelium injury and abnormal fat metabolism, which cause the imbalance between coagulation and fibrinolysis, leading to impairment of intra-osseous blood supply. These reactions might be important factor in onset of osteonecrosis of femoral head. Several studies reported that a combination of anticoagulant and lipid-lowering agent showed a significant decrease in the onset of osteonecrosis compared with an anticoagulant or a lipid-lowering agent alone in human and animal models (14, 15, 20). Another report suggested that a high serum PAI-1 level may be a risk factor for the development of osteonecrosis of the femoral head by studying thrombophilis and hypofibrolysis patients (4, 6).

Fat tissue is now postulated as a multifunctional organ besides its central role of lipid storage, and has a major endocrine function secreting several hormones, notably leptin and adiponectin, PAI-1, and a wide range of other protein factors (12, 17). The association of adipokines with vascular and metabolic disorders has been extensively studied, but few studies have investigated the physiological function of adipocytes present in bone marrow.

To 10^{-5} mol/L demonstrated that PAI-1 secretion was augmented at 10^{-7} mol/L and peaked at 10^{-6} mol/L dexamethasone administration (Fig. 4A). Consistent with PAI-1 secretion into the condition media, a significant expression of PAI-1 mRNA was observed at 10^{-7} mol/L and peaked at 10^{-6} mol/L dexamethasone administration with an average 3.7-fold increase from the basal mRNA level (Fig. 4B). Time-course study performed for adipocytes with 10^{-6} mol/L dexamethasone up to 24 h demonstrated that PAI-1 mRNA expression was significant at 6 h and peaked at 12 h with an average 9-fold increase from the start point (Fig. 4C).

**DISCUSSION**

The precise mechanism, to explain the pathogenesis of steroid-induced osteonecrosis, remains unclear. A consensus etiopathogenesis has been recently unified on both intravenous thrombosis and extravascular lipid-deposition induced by abnormal fat metabolism. Activated oxidative stress induced by glucocorticoid leads to endothelium injury and abnormal fat metabolism, which cause the imbalance between coagulation and fibrinolysis, leading to impairment of intra-osseous blood supply. These reactions might be important factor in onset of osteonecrosis of femoral head. Several studies reported that a combination of anticoagulant and lipid-lowering agent showed a significant decrease in the onset of osteonecrosis compared with an anticoagulant or a lipid-lowering agent alone in human and animal models (14, 15, 20). Another report suggested that a high serum PAI-1 level may be a risk factor for the development of osteonecrosis of the femoral head by studying thrombophilis and hypofibrolysis patients (4, 6).

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**Fig. 1** Histological appearance of culture adipocytes. The cytoplasm of the cells collected from the floating layer of the bone marrow fluid was stained with oil red O.

**Fig. 2** Effects of dexamethasone (10^{-6} mol/L) on adiponectin and TNF-α production. Adiponectin and TNF-α secretion into the condition media from primary human bone marrow adipocytes was evaluated by ELISA. Bone marrow adipocytes were prepared from 25 patients with femoral neck fracture, and cultured for 24 h in the presence or absence of 10^{-6} mol/L dexamethasone (DEX) under a serum-free condition. Bone marrow adipocytes secreted both of adiponectin and TNF-α, although their secretion levels were not significantly changed by dexamethasone admistration.
To evaluate the biology of mature bone marrow adipocyte and to assess the direct role of glucocorticoid on the expression of adipokines, we utilized primary culture of bone marrow adipocytes instead of cloned cell lines or mesenchymal cells capable of differentiating into adipocyte under adipogenic cocktails. In this study, we treated the suspended adipocytes with or without dexamethasone, an analog of glucocorticoid, for 24 h, and measured the levels of certain adipokines in the condition media. We demonstrated here that likewise visceral and subcutaneous adipocytes, bone marrow adipocytes express adiponectin, TNFα and PAI-1, and that among the examined adipokines, dexamethasone induces the secretion of PAI-1 to around 2.4-fold of control in 24 h. In PAI-1 expression, there seems to be the...
difference between visceral and subcutaneous adipocytes. PAI-1 induction by dexamethasone is prominent in visceral fatty tissue. However, the PAI-1 induction rate seems to be still lower than that of bone marrow adipocytes. Halleux et al. reported dexamethasone (50 nmo/L) induced PAI-1 secretion from visceral adipose tissue to around 1.7 fold for 24 h (9).

The PAI-1 induction by dexamethasone was very quick. The PAI-1 mRNA induction by dexamethasone was in 6 h and peaked at 12 h with more than 9 fold induction then decreased at 24 h. The concentration of dexamethasone sufficient for maximum induction was $10^{-6}$ mol/L, which is almost the same amount as the pharmacological concentration used for intravenous pulse therapy. We think that remarkable PAI-1 expression from bone marrow adipocytes may be very important extravenous factor which will induce intravenous thrombus formation by a paracrine manner. In regard to the increase rate of PAI-1 secretion by glucocorticoid administration, there was variability among individuals (range, 1.11 to 5.25 times). We postulate that there was inter-individual variation in the number of glucocorticoid receptors or 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) activity, which interconverts active cortisol and inactive cortisone as reported by Cooper et al. (2, 3). These could potentially influence the response to the dexamethasone, and the relative susceptibility of individuals to the onset of osteonecrosis and other bone diseases induced by glucocorticoid.

Adiponectin and TNFα are very important endocrine factors which are regulating whole body metabolism. Previous study reported that glucocorticoid induces the expression of adiponectin particularly in visceral adipocytes obtained from obese individuals (8), and that TNFα expression is also suppressed by dexamethasone (22). In our study, however, dexamethasone did not modify the levels of adiponectin and TNFα, indicating a distinct regulatory mechanism in bone marrow adipokines compared with other fatty tissues. Thus, it is conclusively that adiponectin and TNFα secreted from bone marrow adipocytes do not directly involve in the underlying mechanism of acute onset of steroid-induced osteonecrosis.

In conclusion, we think that remarkable PAI-1 expression from bone marrow adipocytes may be a very important extravenous factor which will induce intravenous thrombus formation by a paracrine manner of cell to cell interaction. Dexamethasone-induced PAI-1 production by bone marrow adipocytes could be one of the pathogenesis of osteonecrosis. Glucocorticoid might also affect the set of adipokine secretion from human bone marrow adipocytes and influence various bone disorders including osteoporosis. Further investigation to clarify detailed roles of bone marrow adipocytes will provide exciting new insights into bone metabolism and various bone disorders.

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