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Dexamethasone treatment alters function of adipocytes from a mesenchymal stromal cell line ☆

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ABSTRACT

Osteoporosis and osteonecrosis are associated with corticosteroid treatment, but the pathophysiologies are unclear. We hypothesized that mature adipocytes present within the bone marrow compartment play a key role in the development of both diseases. Adipocytes have recognized regulatory effects on bone viability and healing by releasing signaling molecules called adipokines. Our purpose was to evaluate whether dexamethasone alters adipokine expression in differentiated bone-marrow-derived adipocytes. Adipocytes differentiated from mouse D1 mesenchymal stromal cells were treated with dexamethasone (10^{-5}, 10^{-6}, 10^{-7}, or 10^{-8} M) or with diluent alone (controls) for up to 6 days. Using real-time polymerase chain reaction and enzyme-linked immunosorbent assay analyses, six key adipokines and the transcription factor HIF-1α were evaluated. Dexamethasone treatment increased PAI-1 protein expression with increased mRNA expression at 4 days, while decreasing HIF-1α mRNA expression and protein concentrations. VEGF A mRNA expression was increased at 4 days for most dexamethasone concentrations, with minimal changes in protein levels. Dexamethasone increased adiponectin mRNA expression and protein levels at 4 and 6 days and decreased leptin, interleukin-6, and tumor necrosis factor α mRNA expression at all time periods. Dexamethasone treatment of bone-marrow-derived adipocytes resulted in detectable changes in mRNA expression and protein levels of adipokines and HIF-1α. The detected adipokine alterations could be important early events in the pathogenesis of steroid-induced osteonecrosis and osteoporosis.

1. Introduction

Osteonecrosis and osteoporosis have been associated with corticosteroid use [1,2]. Osteonecrosis is an uncommon disorder associated with intravascular disruption of blood flow, local edema, fat cell hypertrophy, and inability to heal subchondral bone fractures [3]. There is a high disease burden on patients with lupus erythematosus, transplants, and other disorders, requiring high-dose corticosteroid treatment. Although the radiographic and pathologic changes associated with late osteonecrosis are well documented [4,5], early changes at the cellular level in the femoral head as a result of corticosteroids have not been defined. Glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis. It is a critical public health problem, with one study indicating that fractures may occur in 30–50% of patients receiving chronic corticosteroid therapy [6].

Although corticosteroid-induced osteoporosis and osteonecrosis are different clinical entities, they have several similarities, most notably, an increase in the relative number and volume of adipocytes in bone marrow and weakening of the bone [7]. Osteoporosis results from an imbalance of bone-forming osteoblast activity and bone-resorbing osteoclast activity, leading to vertebral compression fractures and fragility fractures. Corticosteroid-associated osteonecrosis has been associated with apoptosis of osteocytes [8], prevention of osteoblast differentiation [9], limited angiogenesis, and resultant inability to heal stress fractures in the subchondral bone [10].

Adipocytes may play a key early role in both disorders by signaling adjacent bone and vascular tissue in response to corticoste-

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roids. Adipose tissue, now recognized as an endocrine organ with the ability to signal surrounding cells [11,12], is not simply a reservoir for stored energy, releasing adipokines that have substantial effects on local tissues such as altering bone turnover and healing [13]. The more than 50 known adipokines range from cytokines and growth factors to proteins of the alternative complement system with broad-ranging effects on metabolism, inflammation, vascular tissue, and angiogenesis [14].

We hypothesized that dexamethasone alters adipokine expression in differentiated bone-marrow-derived adipocytes. Six specific adipokines (PAI-1, adiponectin, leptin, IL-6, and the transcription factor HIF-1α) were investigated with regard to adipogenesis, angiogenesis, and inflammation.

2. Materials and methods

Early-passage D1 cloned bone marrow MSCs from the BALB/c mouse [15] were purchased from ATCC (Manassas, VA). Cells were thawed, grown for 2 days, split with trypsin 0.05% with ethylenediaminetetraacetic acid (Gibco, Grand Island, NY), and plated at 1×10^5 cells/mL on 12-well Costar plates (Costar Cat#3513) precoated with laminin (BD Biosciences, Bedford, MA) overnight. D1 cells were maintained in standard media: Dulbecco's modified Eagle's medium with L-glutamine (ATCC) supplemented with 10% v/v fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA), 1% v/v penicillin (10,000 u/mL), and streptomycin (10,000 μg/mL) (Gibco). Cells were grown until 80% confluent in a 5% CO_2-humidified incubator at 37 °C.

MSCs were differentiated with rosiglitazone as described [16]. In our study, when D1 MSCs were approximately 80% confluent, rosiglitazone (Cayman Chemicals, Ann Arbor, MI) dissolved in dimethyl sulfoxide (10 μg/mL) (Sigma Aldrich Co., St. Louis, MO) was added. Cells were grown in the rosiglitazone medium for 48 h and returned to the standard media for 5 more days. The medium was exchanged every 48 h. During this period, cellular triglyceride stores increased in size (Fig. 1A); the cells expressed markers of mature adipocytes (Fig. 1B). Once the adipocyte phenotype was confirmed in parallel cultures with Oil Red O staining (Sigma Aldrich Co.), defined as Day 0, dexamethasone treatment was initiated. Western blot analysis of cells showed that the rosiglitazone treatment caused expression of aP-2 and peroxisome proliferator-activated receptor gamma, markers of terminal adipocyte differentiation [17] (Fig. 1B). In one experiment (data not shown), the expression of these differentiation markers did not change over the course of 2 or 3 weeks.

Mature adipocytes were used for the experiment after assuring terminal differentiation. The cells were divided into control (no treatment) or treatment (10^{-8} M, 10^{-7} M, 10^{-6} M, or 10^{-5} M dexamethasone) (Sigma Aldrich Co.) groups for 2, 4, or 6 days. The media (with fresh dexamethasone as appropriate) were changed every 2 days for the 4- and 6-day treatments. At the termination of the study, the cells were isolated for RNA extraction and analysis with RT-PCR; the supernatants were collected for ELISA analysis of secreted proteins.

In a parallel experiment, Oil Red O staining was performed before and after the dexamethasone treatment experiment to analyze the adipocytes' morphologic response to dexamethasone and to obtain a semiquantitative measure of the adipocytes' triglyceride content. Cells were washed once in room-temperature phosphate-buffered saline and fixed for 1 h in 10% formaldehyde. They were then stained with filtered Oil Red O per the manufacturer's instructions, washed twice with distilled water, and left under water for photographs using an inverted light microscope (Leica Microsystems, Wetzlar, Germany) and a digital camera. The cells were then removed from the plate and analyzed using a triglyceride assay kit (Wako Chemicals, Richmond, VA).

All wells were treated with 1 mL of Trizol (Invitrogen Life Technologies, Grand Island, NY), scraped, triturated, and stored at −80 °C. All samples were processed in one batch to reduce variability. Initial samples were electrophoresed on a 2% agarose gel in ribonuclease-free buffer to ensure RNA integrity before complementary deoxyribonucleic acid production. Samples were dissolved in diethylpyrocarbonate-treated water and quantitated using the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA).

One microgram of RNA was used for each reaction. Samples were treated with deoxyribonuclease in 10x buffer (Invitrogen Life Technologies) for 15 min at room temperature. The reaction was stopped with EDTA acid and by heating to 65 °C for 10 min. The complementary deoxyribonucleic acid was generated in a thermocycler with a Thermoscript RT-PCR kit (Invitrogen Life Technologies) with random hexamers, 10 mM deoxyribonucleotide

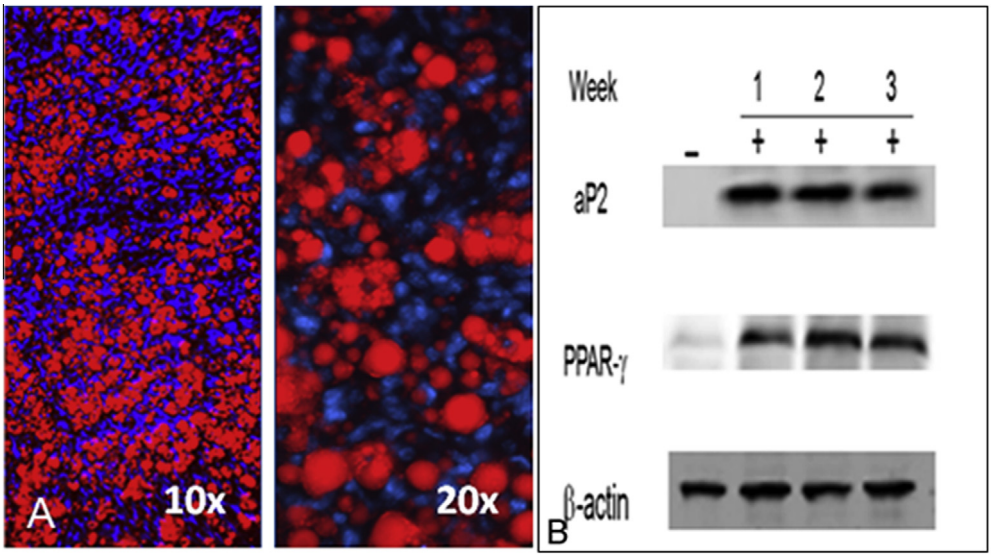

Fig. 1. Confirmation of differentiation of adipocytes. (A) D1 cells, differentiated into adipocytes, were stained with Oil Red O, counterstained with 4′-6-diamidino-2-phenylindole, and photographed using the Leica Texas red filter. (B) Western blot analysis of differentiated cells showed expression of peroxisome proliferator activated receptor gamma (PPAR-γ) and adipocyte Protein 2 (aP2): both markers of terminal adipocyte differentiation.

Table 1
Sequence of gene-specific primers used for RT PCR analysis of adipokine mRNA.

Mouse mRNA	Primer sequence
Adiponectin	Fwd 5′ AAGGACAAGGCCGTTCTCT 3′
Adiponectin	Rev 5′ TATGGGTAGTTGCAGTCAGTTGG 3′
HIF-1α	Fwd 5′ ACCTTCATCGGAAACTCCAAAG 3′
HIF-1α	Rev 5′ ACTGTTAGGCTCAGGTGAACT 3′
IL-6	Fwd 5′ AACGATGATGCACTTGCAGA 3′
IL-6	Rev 5′ GAGCATTGGAAATTGGGGTA 3′
Leptin	Fwd 5′ GAGACCCCTGTGTCGGTTC 3′
Leptin	Rev 5′ CTGCGTGTGTGAAATGTCATTG 3′
PAI-1	Fwd 5′ GGGGGATGAAAGAGACAGC 3′
PAI-1	Rev 5′ CCGGTGGAGACATAACAGAT 3′
TNF-α	Fwd 5′ ATGAGAGGGAGGGCCATTTG 3′
TNF-α	Rev 5′ CAG CCTCTTCTCATTCCTGC 3′
VEGF-A	Fwd 5′ GTGCACTGGACCCTGGCTTTA 3′
VEGF-A	Rev 5′ GGTCTCAATCGGACGGCAGTA 3′
PPAR-γ	Fwd 5′ GCCTTGCTGTGGGGATGTC—3′
PPAR-γ	Rev 5′ TCCTTGGCCCTCTGAGATGAG 3′
GAPDH	Fwd 5′ AAATGGTGAAGGTCGGTGTG 3′
GAPDH	Rev 5′ TGAAGGGGTCGTTGATGG 3′

triphosphate's 5x first-strand buffer, 0.1 M dithiothreitol, and RNAseOut ribonuclease inhibitor (Invitrogen Life Technologies).

The RT-PCR of reverse transcribed complementary deoxyribonucleic acid was performed using a Biorad iCycler and Biorad iQ sYBR Green supermix (Bio-Rad Laboratories, Inc., Munchen, Germany) in 25 μL of reaction volume. The PCR primers were synthesized at our institution's deoxyribonucleic acid synthesis core facility. Primer sequences were identified from peer-reviewed literature individual to each gene (Table 1). Each primer pair was quality controlled by finding a single major band on melting temperature analysis on the iCycler. PCR products were run on a 2% agarose gel with ethidium bromide and evaluated for a single band under ultraviolet light. All primers were selected to have an annealing temperature of 60 °C to allow higher throughput. PCR conditions were 95 °C for 3 min, then 50 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase was used as the control for normalization. The number of PCR cycles to logarithmic expansion was exported for each gene product into a spreadsheet and compared among samples using the delta-delta-Ct method for relative quantity calculation [18]. All experiments were done with triplicates wells; the RT-PCR of each sample was also done in triplicate.

Using kits (R & D Systems, Inc., Minneapolis, MN), ELISA was performed to assay for mouse VEGF A, adiponectin, leptin, HIF-1α, IL-6, and TNFα. The ELISA plates were precoated with 2.0 μg/ml capture antibody overnight at room temperature. Plates were then washed three times with washing buffer and blocked with 1% bovine serum albumin in phosphate-buffered saline and

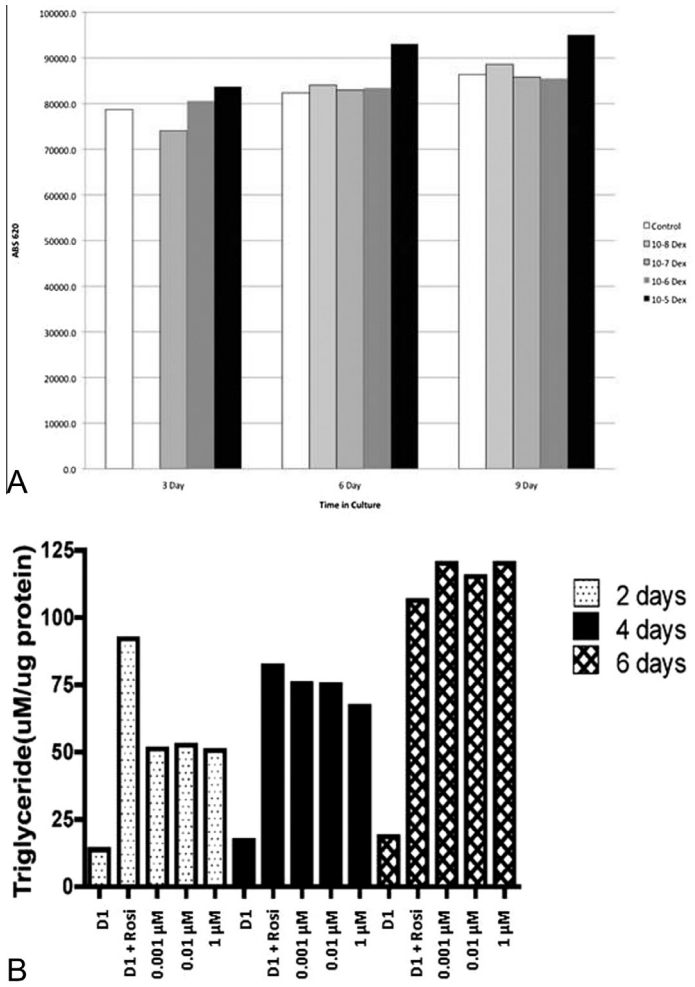

Fig. 2. The effect of dexamethasone on cell viability and triglyceride production. (A) Viability studies showed equivalent survival of cells in control and treated cells at 3, 6, and 9 days of treatment. ABS, absorbance at 620 nm; Dex, dexamethasone. (B) Triglyceride production was normalized to protein concentration in dexamethasone-treated cells at 2, 4, and 6 days compared with undifferentiated D1 cells and differentiated D1 adipocytes without dexamethasone treatment. Triglyceride production increased with differentiation into adipocytes and treatment with dexamethasone. Rosi, rosiglitazone.

100 μl of cell culture supernatant (cell lysates for HIF-1α); standards were added to appropriate wells and incubated for 2 h at room temperature. After washing, detection antibody was added for another 2 h. Plates were rinsed again and incubated with streptavidin horseradish peroxidase for 1 h. The plates were then washed, 100 μl of the enzyme substrate were added for 20 min, and 50 μl of stop solution were added to each well. Absorbance was read at 450 nm using a Wallac Vitor2 plate reader (PerkinElmer, Shelton, CT). PAI-1, adiponectin, Leptin, IL-6, and TNFα were assayed using the mouse adipocyte panel and mouse panel 2B multiplex immunoassay system (Millipore Corp., Billerica, MA), which performs multianalyte analysis from a single sample. Aliquots of medium from non-dexamethasone-treated D1 cells were used as ELISA control samples. Experiments were done in triplicate wells, and multiple runs were conducted to ensure reproducibility of results. Values are expressed as picograms per milliliter, except for adiponectin and PAI-1, which are expressed in nanograms per milliliter.

To ensure that the changes observed in RT-PCR and ELISA were not simply attributable to death of the adipocytes caused by dexamethasone treatment, parallel experiments were conducted with trypan exclusion (data not shown), and calcein-ethidium bromide live-dead tests were conducted [19].

The results were analyzed using JMP statistical software (SAS Institute Inc., Cary, NC). Normality and equal variances were evaluated before ANOVA. ANOVA was performed for each of the conditions; the Dunnett's test was used for comparisons to controls. The Tukey Honestly Significant Difference test was used for between-group comparisons (significance, $P \leqslant 0.05$).

3. Results

Live-dead analysis showed equivalent survival in varying concentrations and durations of dexamethasone treatment (Fig. 2A). No statistically significant difference was noted between the dexamethasone-treated groups and controls. Triglyceride production increased with differentiation into adipocytes (Fig. 2B). Treatment of differentiated adipocytes with dexamethasone decreased triglyceride levels at 2 days, with a return to differentiated adipocyte control level (no dexamethasone) at 4 and 6 days.

Dexamethasone treatment significantly decreased mRNA expression of PAI-1 at 2 days for all dexamethasone dosages

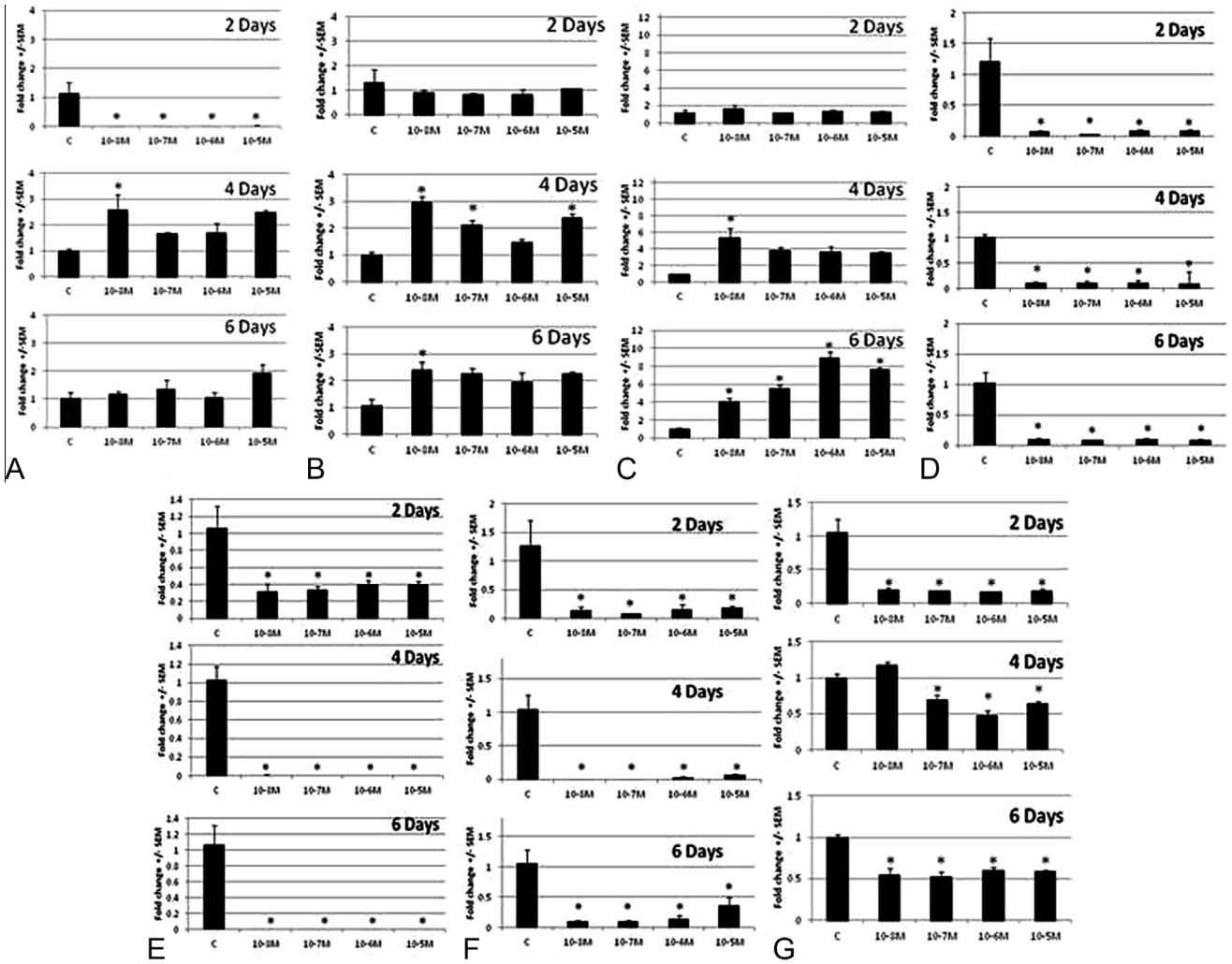

Fig. 3. Expression of adipokines and HIF-1α in differentiated adipocytes treated with 10^{-5} M, 10^{-6} M, 10^{-7} M, or 10^{-8} M dexamethasone for 2, 4, or 6 days compared with untreated controls. Experiments were done in triplicate wells. The RT-PCR fold changes were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. All RT-PCR was also done in triplicate, and standard error of the mean bars are shown; asterisks indicate significance at $P \leqslant 0.05$. (A) Plasminogen activator inhibitor-1 (PAI-1). (B) Vascular endothelial growth factor A (VEGF-A). (C) Adiponectin. (D) Leptin. (E) IL-6. (F) Tissue necrosis factor-α (TNF-α). (G) Hypoxia-inducible factor 1α (HIF-1α).

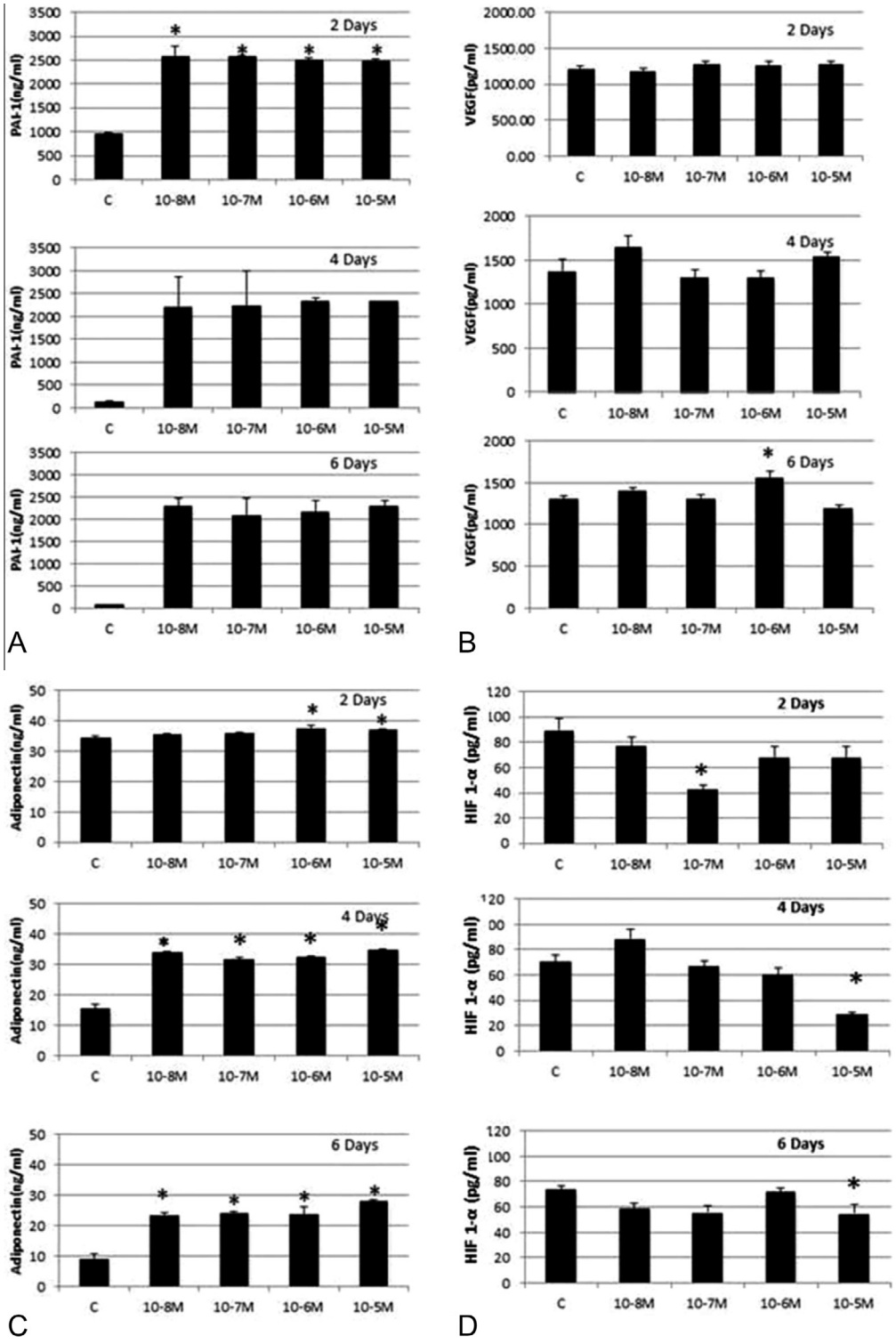

Fig. 4. Analysis of proteins within the supernatants of differentiated adipocytes treated with 10^{-5} M, 10^{-6} M, 10^{-7} M, or 10^{-8} M dexamethasone for 2, 4, or 6 days. Experiments were done in triplicate wells. Multiple runs were conducted to ensure reproducibility of results. Standard error of the mean bars are shown; asterisks indicate significance at $P \leqslant 0.05$. (A) Plasminogen activator inhibitor-1 (PAI-1). (B) Vascular endothelial growth factor A (VEGF-A). (C) Adiponectin. (D) Hypoxia-inducible factor 1α (HIF-1α).

($P \leqslant 0.05$) (Fig. 3A). Small, nonsignificant increases in mRNA expression were observed for most treatment groups at 4 and 6 days, except for a significant elevation for10^{-8} M dexamethasone at Day 4 ($P \leqslant 0.05$). PAI-1 protein concentrations were notably increased after all doses of dexamethasone treatment at 2, 4, and 6 days (Fig. 4A), although this difference was significant only at Day 2 ($P \leqslant 0.05$).

Increased VEGF-A mRNA expression was observed at 4 and 6 days. The differences were significant for most of the treatment groups (except 10^{-6} M) at Day 4 ($P \leqslant 0.05$) and for 10^{-8} at Day 6 ($P \leqslant 0.05$) (Fig. 3B). A small, nonsignificant increase in VEGF-A was observed in the supernatants at Day 4 for cells treated with 10^{-8} and 10^{-5} M dexamethasone; it was significantly increased for 10^{-6} M dexamethasone at Day 6 ($P \leqslant 0.05$) (Fig. 4B).

Adiponectin mRNA expression significantly increased for cells treated with 10^{-8} M dexamethasone at Day 4 ($P \leqslant 0.05$) and for all treatment groups at Day 6 ($P \leqslant 0.05$) (Fig. 3C). Significant increases of adiponectin were detected in cells treated with 10^{-6} and 10^{-5} M dexamethasone at 2 days and for all 4- and 6-day treatment groups ($P \leqslant 0.05$) (Fig. 4C).

Leptin mRNA expression was significantly decreased at 2, 4, and 6 days at all dexamethasone concentrations compared with controls ($P \leqslant 0.05$) (Fig. 3D). Leptin protein was not detected in any of the supernatants (including controls) (data not shown).

Regarding the inflammatory cytokines, IL-6 (Fig. 3E) and TNF-α (Fig. 3F), mRNA expression decreased significantly for all dexamethasone concentrations at each time period ($P \leqslant 0.05$). Protein expression for either of the cytokines was not detected in the supernatants for any of the groups studied.

Dexamethasone treatment resulted in significantly decreased HIF-1α mRNA expression at 2, 4, and 6 days after treatment at nearly all dexamethasone concentrations ($P \leqslant 0.05$) (Fig. 3G). Small decreases of HIF-1α protein levels were observed, with significant differences for 10^{-7} M dexamethasone at Day 2 and for 10^{-5} M dexamethasone at Days 4 and 5 ($P \leqslant 0.05$) (Fig. 4D).

4. Discussion

Increased numbers of adipocytes and adipocyte hypertrophy have been observed in patients with osteonecrosis and osteoporosis who have been treated with corticosteroids [20,21]. However, little is known about the effect of corticosteroid therapy on the function of these cells. Furthermore, many studies have focused on pre-adipocytes and differentiation pathways of these cells. Our study found that dexamethasone treatment alters the synthesis and release of a number of proteins from mature adipocytes. These adipokines have been shown to affect other cell types present within the bone marrow compartment and may play a vital role in the early pathophysiology of glucocorticoid-associated osteonecrosis and osteoporosis.

PAI-1 is associated with decreased fibrinolysis and an increased risk of thrombosis [10,22]. Our study showed that, after an initial decrease, mRNA expression increased slightly at 4 and 6 days, with reproducible 10-fold increases in protein expression in response to dexamethasone. Therefore, PAI-1 is likely to be a link among steroids, adipocytes, and the related pathology that results. Increased PAI-1 protein levels are consistent with previous studies linking PAI-1 released from adipocytes with osteonecrosis of the femoral head [10,23] and may be important in intravascular coagulation in osteonecrosis. Interestingly, a recent study showed that PAI-1 secretion by bone marrow adipocytes in response to dexamethasone could be prevented by pretreatment with simvastatin, indicating a potential role for these lipid-lowering medications in preventing osteonecrosis [24].

For VEGF-A, increased mRNA expression was observed at 4 and 6 days of dexamethasone treatment, associated with variable secretion of VEGF-A into the supernatants at 4 and 6 days. VEGF A induces angiogenesis in bone, which is critical in bone healing [25]; therefore, if VEGF-A is a mediator in steroid-induced osteonecrosis, it would be hypothesized to decrease in response to dexamethasone. In fact, Li et al. [26] have shown VEGF-A to be significantly down-regulated in response to dexamethasone. However, they used undifferentiated D1 MSCs, whereas we used differentiated mature adipocytes. Therefore, although dexamethasone may influence VEGF-A expression in undifferentiated cells, it is unlikely to be a critical adipokine mediator of steroid-induced osteoporosis or osteonecrosis when considering mature signaling of adipocytes.

Adiponectin mRNA expression increased 4- to 8-fold and the protein levels doubled after 4 and 6 days of dexamethasone treatment. Adiponectin has been shown to negatively impact bone mass, and high levels of adiponectin can lead to lower bone mass [27,28] and may be an essential independent risk factor for fractures. A recent cohort study of elderly patients showed a 94% higher risk of fracture in those with high serum adiponectin levels than in those with lower adiponectin levels [29]. Our results indicate that dexamethasone treatment of mature adipocytes is a notable source of adiponectin, which may be a key link between steroid treatment and osteoporotic fractures.

In our study, leptin mRNA levels were significantly decreased by dexamethasone treatment to approximately 10% of controls, whereas the leptin protein was not detected. Leptin has been shown to have an anabolic effect on osteoblasts, encouraging MSCs to become osteoblasts and limiting adipogenesis [30,31]. Leptin inhibits receptor activator of nuclear factor κB ligand production while increasing osteoprotegerin production, which limits osteoclast development [32]. Furthermore, leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice [33]. The decreased leptin expression after dexamethasone treatment that we observed could result in a diminished anabolic effect on osteoblasts and in increased osteoclast production.

IL-6 and TNF-α mRNA expression decreased significantly in response to dexamethasone at 4 and 6 days (with undetectable protein levels). Both are pro-inflammatory cytokines with wide-ranging effects. Previously, IL-6 has been shown to cause osteoclast differentiation and bone resorption [34]. Chronic inflammation is associated with bone loss, but the exact mechanism is poorly understood [35]. The decrease in IL-6 and TNF-α expression levels we have shown in response to dexamethasone could be interpreted as being protective of bone. However, IL-6 and TNF-α protein levels in our study were undetectable even in the untreated controls, which would indicate that the mature adipocytes in bone are not a notable source of IL-6 or TNF-α. Given these results, IL-6 and TNF-α are not likely to be critical adipokines released by mature adipocytes in steroid-induced osteoporosis and osteonecrosis.

Hypoxia-inducible factor-1α, which is an inducible transcription factor that regulates a cell's response to hypoxia, showed a more than 50% decrease in mRNA expression after dexamethasone treatment by RT-PCR. Decreased HIF-1α levels in response to dexamethasone may impair the adipocytic response to hypoxia. This effect has been shown previously in cultured hepatocytes treated with dexamethasone [36], but to our knowledge, never before in adipocytes.

Our study adds to the understanding of the pathophysiology of steroid-induced osteoporosis and osteonecrosis. Most noteworthy, the adipokines adiponectin and PAI-1 are upregulated in mature adipocytes in response to dexamethasone and could be key early changes in osteonecrosis and osteoporosis. Additionally, dexamethasone decreases HIF-1α, which could inhibit the normal response to hypoxic ischemic event in the femoral head. VEGF-A was increased in response to dexamethasone. Increased VEGF-A expression has been noted in the tissue surrounding osteonecrotic lesions [37]. However, the implications of these findings need further exploration.

We limited our study to the evaluation of only six adipokines and the transcription factor HIF-1α. It is possible, even likely, that the expression of other proteins may be affected by exposure of adipocytes to dexamethasone. Another limitation of the study is that it is an *in vitro* study using a mouse cell line. However, hopefully, this research, and future efforts, will help direct the development of treatments to minimize the effects of corticosteroids on bone. Identifying genes and pathways associated with these dis-

eases could lead to novel therapies limiting the disabling effects of osteonecrosis in young patients and limiting fragility fractures in patients with glucocorticoid-induced osteoporosis. In addition, future screening panels may include adipokines as biomarkers for disease and for the evaluation of the effectiveness of treatment.

Conflict of interest

The authors have no conflicts of interest with the topic of this manuscript. This was a basic science research project; IRB approval is not required.

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