[Frontiers in Bioscience 16, 2747-2755 , June 1, 2011]

The effect of dexamethasone and hypoxic stress on MC3T3-E1 cells

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1. ABSTRACT

Osteonecrosis of the femoral head (ONFH) can be caused by a decrease in the activity or numbers of osteoblasts, a process in which apoptosis may play an essential role. We investigated the effect of dexamethasone (Dex) combined with hypoxic stress on murine osteoblastic MC3T3-E1 cells. Flow cytometry, western blot and real-time quantitative PCR analyses revealed that hypoxia significantly enhanced Dex-induced apoptosis. Further data demonstrated that both the death receptor and the mitochondria-mediated pathway were involved in Dex-induced apoptosis under hypoxic conditions. However, the death receptor pathway had only a minor effect on this process. The expression levels of Bcl-2 and Bax, which regulate the mitochondria-initiated apoptotic cascade signaling pathway, were significantly different in response to Dex and hypoxia. The mitochondrial membrane potential collapsed, and the inhibitor brain- derived neurotrophic factor (BDNF) conferred effective protection against apoptosis. In summary, the mitochondria-mediated apoptotic pathway functions in osteoblast apoptosis that is induced by Dex in a hypoxic environment, and the present study may help us to gain further insight into the molecular mechanisms of steroid-induced ONFH.

2. INTRODUCTION

Avascular necrosis is defined as the cellular death of bone tissues caused by an interruption of the blood supply. A number of theories about different risk factors have been proposed to account for the cause of osteonecrosis of the femoral head (ONFH) (1-3). However, the precise molecular mechanisms have not been fully elucidated. Epidemiological data have suggested that excessive steroid medication is closely associated with the initiation and development of ONFH (4). Steroid hormones have a bivalent effect, and they can function as both blood coagulants and anti-inflammatory drugs (5). However, prolonged use or a high dose of steroid hormones may lead to bone necrosis (6). Steroid hormones have been shown to induce apoptosis of osteoblasts, which are critical to bone disease. Weinstein has demonstrated that glucocorticoids can specifically induce osteoblasts to apoptose (7), and a number of apoptosis-related caspases were activated in this process. It remains unclear whether steroid hormones are involved in the process of apoptosis *in vivo*. The emerging data suggest that steroid hormones can induce blood coagulation and result in subsequent tissue ischemia of the femoral head (8). Thus, many hypotheses about the effect of hypoxic stress on apoptosis exist, and it is necessary to

Table 1. Primers used for real-time quantitative RT-PCR

	Forward	Reverse
FasL	TGAATTACCCATGTCCCCAG	AAACTGACCCTGGAGGAGCC
Fas	ACAGAAAGCATGATCCGCG	GCCCCCCATCTT TTGGG
Bcl-2	GGTGGTGGAGGAACTCTTCA	ACGCTCTCCACACACATGAC
Bax	ATGGAGCTGCAGAGGATGAT	GAAGTTGCCATCAGCAAACA
Actin	TTCGTTGCCGGTCCACA	ACCAGCGCAGCGATATCG

determine whether hypoxic stress contributes to the osteoblast apoptosis that is induced by steroid hormones and whether the combined effect is critical to the progression of ONFH.

In the present study, we sought to demonstrate the effects of a glucocorticoid (Dex) combined with hypoxic stress on osteoblasts and explore the sophisticated molecular mechanisms of steroid-induced ONFH. Prevention of osteoblast apoptosis is an extremely important clinical goal, and this study will be beneficial to new therapeutic approaches.

3. MATERIALS AND METHODS

3.1. Cell culture and materials

Murine osteoblastic MC3T3-E1 cells were cultured in a modified MEM (Invitrogen™ by Life Technologies) supplemented with 10% fetal bovine serum (FBS, Invitrogen™ by Life Technologies), 2 mM L-glutamine, 2.0 mg/ml $NaHCO_3$ and 25 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2. When the cells were approximately 65% confluent, the dishes were placed in a normoxic (20% O_2) or hypoxic (1% O_2) environment for 24 h. Oxygen was supplanted by infusion of a 5% CO_2/95% nitrogen gas in 37°C humidified hypoxia work stations (StemCell Technologies; Del Mar, CA). Dex was obtained from Sigma-Aldrich and used at 10^{-6} M throughout the study. Cleaved caspase-3, cleaved caspase-8, Fas and FasL antibodies were obtained from Sigma-Aldrich. Bax, Bcl-2 and cleaved caspase-9 were purchased from Cell Signaling Technology (Beverly, MA). The cleaved caspase-12 antibody was purchased from Sigma-Aldrich. The cytochrome *c* antibody was purchased from BD Pharmingen.

3.2. Apoptosis assay

The apoptotic cells were analyzed using the Annexin V-FITC assay (Abcam). The cells were washed twice with cold PBS and then incubated in binding buffer (10 nM HEPES, pH 7.4, 5 nM $CaCl_2$, and 140 nM NaCl). Following incubation, 0.1 ml of the solution was transferred to a new tube, and 5 μl of Annexin V-FITC and 10 μl of propidium iodide were added. After 15 min at room temperature in the dark, the cells were analyzed immediately using flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence, X-axis) and the non-vital dye propidium iodide (red fluorescence, Y-axis) allows the discrimination of intact cells (FITC-PI-), early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+). Brain-derived growth factor (BDNF), Z-VAD-fmk and Salubrinal (SAL) were used as inhibitors to determine different pathways involved in Dex and hypoxia induced apoptosis in MC3T3-E1 cells.

3.3. Cytochrome *c* release

MC3T3-E1 cells were harvested at 24 h, and mitochondrial and cytosolic fractions were prepared using a mitochondria/cytosol fractionation kit (BioVision; San Francisco, CA) according to the manufacturer's protocol. Cytosolic fractions (20 μg) and mitochondria (5 μg) were separated using 10% SDS-PAGE, and a cytochrome *c* antibody was used to probe the protein.

3.4. Western blot analysis

Near-confluent cells were changed to MEM containing 0.5% FBS for 16 h before the addition of 10^{-6} M Dex for 24 h. MC3T3-E1 cells were lysed in a buffer containing 50 mM HEPES (pH 7.9), 2 mM EDTA, 0.1% SDS, 20 μg/ml leupeptin, and 10 μg/ml phenylmethanesulfonylfluoride (PMSF). The protein concentration was determined using a Coomassie dye binding assay (BioRad; Hercules, CA). Aliquots of 50 μg of lysate were analyzed on 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% (w/v) non-fat milk at room temperature for 1 h followed by overnight incubation with optimal dilutions of antibodies against caspase-3, -8, -9, and -12 and actin at 4°C. Appropriate secondary antibodies were added for 1.5 h. The antigen/antibody complex was detected using an ECL reagent (Amersham Pharmacia Biotech; Piscataway, NJ).

3.5. Measurement of mitochondrial transmembrane potential

MC3T3-E1 cells were harvested and washed twice in PBS, and 5 μg/ml 5,5,6,6-tetraethylbenzimidazocarbocyanine iodide (JC-1; Molecular Probes) was added for 15 min in the dark (9). Then, the stained cells were washed twice in PBS and analyzed using flow cytometry. The data were acquired on a BD Biosciences FACScan™. In cells with a healthy mitochondrial transmembrane, the dye JC-1 forms J-aggregates and emits at 590 nm (FL-2 channel, red fluorescence). However, JC-1 exists as a monomer and emits at 527 nm (FL-1 channel, green fluorescence) when the transmembrane is intact. The mitochondrial depolarization is characterized by a decrease in the red/green fluorescence intensity ratio.

3.6. Quantitative real-time PCR

Total RNA (2 μg) was extracted from MC3T3-E1 cells treated under different conditions using an RNeasy Protect Mini-Kit (Qiagen; Valencia, CA). cDNA was synthesized using an Omniscript RT Kit (Qiagen). Quantitative PCR was performed using an ABI PRISM 7900HT instrument and the primers in Table 1.

Amplification was performed under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s,

60°C for 30 s, and 72°C for 10 s and a final extension for 10 min at 72°C. The specificity of the product was confirmed using melt-curve analysis. Quantification was presented as ratios relative to the actin expression level. The actin gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of actin. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (*CT*; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the *CT* value of the sample for the target gene and the mean *CT* value of that sample for the endogenous control (actin). Relative expression was calculated as the difference (ΔC_T) between the ΔC_T values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-\Delta\Delta CT}$

3.7. Statistical analysis

The results were presented as means ± standard deviation (SD) of three or more independent determinations. Statistical analysis was performed using two-tailed Student t-test for unpaired data and ANOVA for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Effects of Dex and hypoxic stress on murine osteoblastic MC3T3-E1 cells

To investigate the effects of Dex and hypoxic stress on osteoblasts, flow cytometry was employed to analyze apoptosis in MC3T3-E1 cells treated with 10^{-6} M Dex or cultured under hypoxic conditions for varying times. As shown in Figure 1A, apoptosis in MC3T3-E1 cells increased from 0.2% in the control cells to 7.8% in Dex cells treated for 48 h in a time-dependent manner. The number of apoptotic cells increased significantly from 1.9% in control cells to 43% after 48 h of hypoxic stress (Figure 1B). To determine whether hypoxic stress could promote the osteoblast apoptosis induced by Dex, cells were treated with Dex and cultured in a hypoxic environment. As shown in Figure 1C, apoptosis increased significantly when cells were treated with both Dex and hypoxic stress compared to either treatment alone. The number of apoptotic cells increased linearly up to 45% at 48 h. However, excessive necrosis also occurred at this time point. Our observations indicate that the combination of Dex and hypoxic stress effectively induces apoptosis in MC3T3-E1 cells at 24 h post-treatment.

4.2. The mitochondria-mediated apoptotic pathway is induced by Dex and hypoxic stress in MC3T3-E1 cells

Caspase activation is required for the execution of apoptosis. Thus, we examined the effect of Dex and hypoxic stress on the activation of apoptosis initiators and effector caspases. Caspase-3 is a major apoptosis effector caspase. Western blotting demonstrated that Dex combined with hypoxia significantly increased the expression of cleaved caspase-3. Activation of the death receptor pathway effector caspase-8 and the mitochondria-mediated apoptosis effector caspase-9 was observed. Caspase-8 increased slightly, while caspase-9 increased significantly. Cleaved caspase-12 was not altered under any conditions. To investigate the effect of Dex and hypoxic stress on the death receptor pathway in MC3T3-E1 cells, the expression of Fas and FasL, which are members of the death receptor pathway, were examined using western blot and real-time PCR. As shown in Figure 2A, western blot results revealed that the total expression of Fas and FasL in MC3T3-E1 cells were higher under hypoxia stress; however, there were not significantly different between control and DEX or combined with hypoxia treated cells. The mRNA levels of Fas and FasL were slightly increased in cells treated with Dex and hypoxia compared to control cells (Figure 2B). These results demonstrate that the combination of Dex and hypoxia has significant effects on the signaling cascades in the mitochondria-mediated apoptotic pathway.

4.3. The mitochondria-mediated apoptotic pathway is essential for Dex-induced apoptosis under hypoxic conditions

Mitochondrial membrane potential disruption appears to be critical for apoptosis. JC-1, which is sequestered in the mitochondria of healthy cells and exists as monomers in apoptotic cells, was used to detect a change in the mitochondrial membrane potential. As shown in Figure 3A, the cells treated with Dex and hypoxic stress exhibited a collapsed mitochondrial membrane potential. The disruption of DYmt correlated with cytochrome *c* release. Thus, we examined whether cytochrome *c* was released from the mitochondria to the cytosol following the change in mitochondrial transmembrane potential. Western blot revealed that cytosolic cytochrome *c* appeared in cells cultured under hypoxic conditions, and protein levels increased when Dex was also added (Figure 3B). The activation of Bax is involved in mitochondria-mediated apoptosis (10, 11), and Bcl-2 is an important apoptosis-suppressing gene that regulates the process of apoptosis (12). Therefore, we examined the mRNA and protein levels of Bax and Bcl-2. As shown in Figure 3D-F, the combined effects of Dex and hypoxia resulted in a significant increase in Bax and the down-regulation of Bcl-2. These results demonstrate that mitochondria-mediated apoptosis plays an essential role in the Dex- and hypoxic stress-induced apoptosis of MC3T3-E1 cells.

4.4. The effects of different apoptotic inhibitors on MC3T3-E1 cells

To further characterize the apoptotic pathway, we used different apoptotic pathway inhibitors and analyzed the MC3T3-E1 cells using flow cytometry. As Figure 4 shows, brain-derived growth factor (BDNF) effectively blocked the apoptosis induced by Dex and hypoxic stress in MC3T3-E1 cells (13). Z-VAD-fmk, which is a cell-permeable caspase inhibitor, had little effect on inhibiting apoptosis (14). Salubrinal (SAL) is a selective inhibitor of cellular complexes that dephosphorylate eIF2α, and it protects cells from endoplasmic reticulum (ER) stress (15). Use of SAL did not result in a reduction in the number of apoptotic cells. These results provide evidence for the importance of the mitochondria-mediated apoptotic pathway in the process of apoptosis induced by Dex and hypoxic stress.

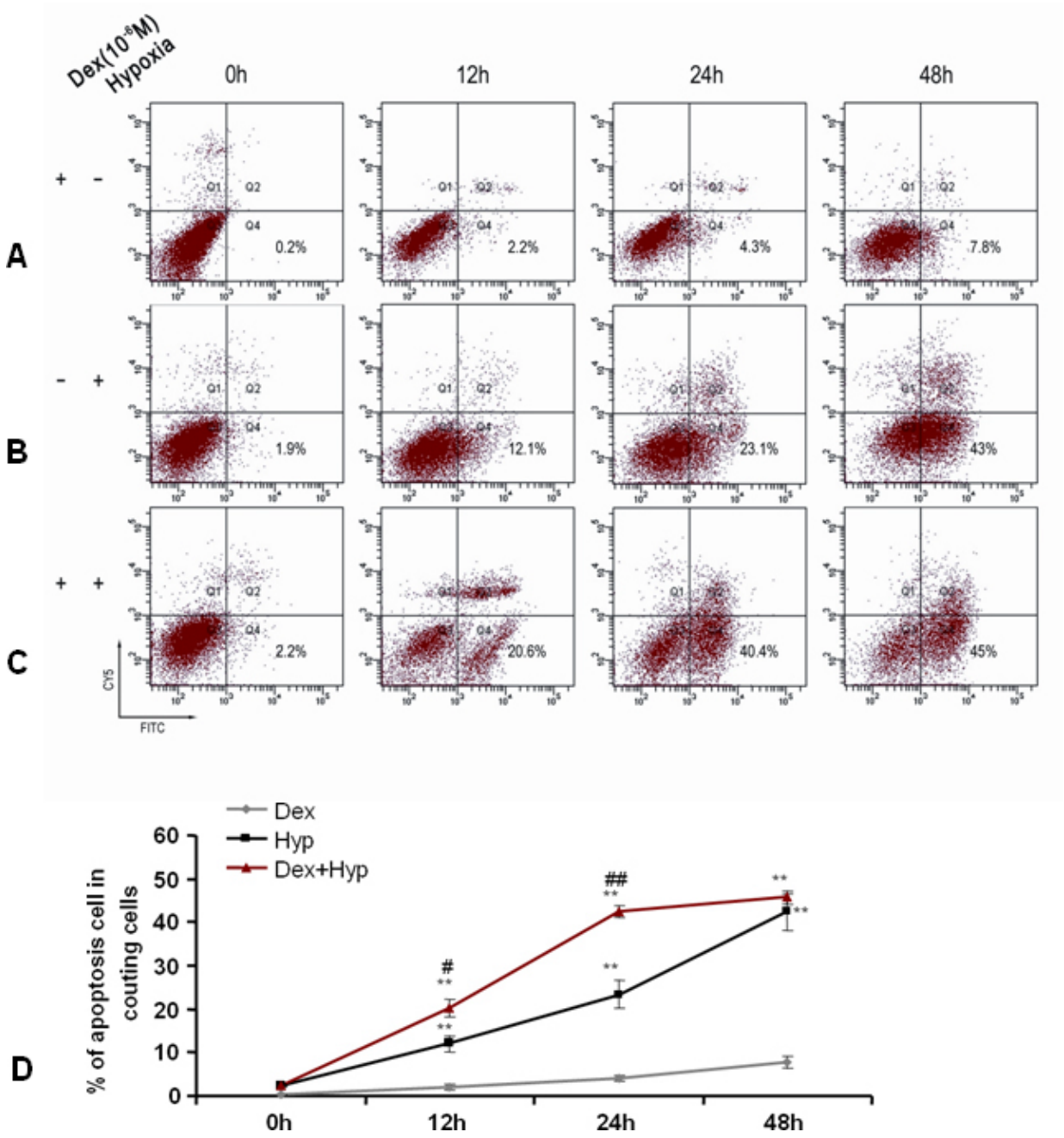

Figure 1. Flow cytometry analysis shows apoptosis induced by Dex and hypoxia in murine osteoblastic MC3T3-E1 cells. A: MC3T3-E1 cells were incubated without (control) or with Dex (10^{-6} M) for 12, 24, or 48 h. B: MC3T3-E1 cells were cultured in a normal environment (control) or under hypoxic conditions for 12, 24, or 48 h. C: MC3T3-E1 cells were incubated with Dex under hypoxic conditions for 0, 12, 24, or 48 h. All experiments were performed in triplicate, values were analyzed and presented as means ± standard deviation (SD).

5. DISCUSSION

Femoral head osteonecrosis refers to the death of bone and marrow cells due to interruption of blood supply, which occurs for various reasons, such as femoral neck fractures (16), alcohol abuse and long-term steroid medication. The disease will progress to femoral head collapse if left untreated. It is important to detect early molecular changes in ONFH before destructive changes become obvious. Recent studies have demonstrated that osteocyte apoptosis is essential to the process of ONFH (17). Previous findings have indicated that one high dose of steroids can induce apoptosis in bone and result in subsequent tissue ischemia (5, 18); however, the effects of hypoxia induced by steroids and the mechanisms underlying cell apoptosis are still poorly understood. In the present

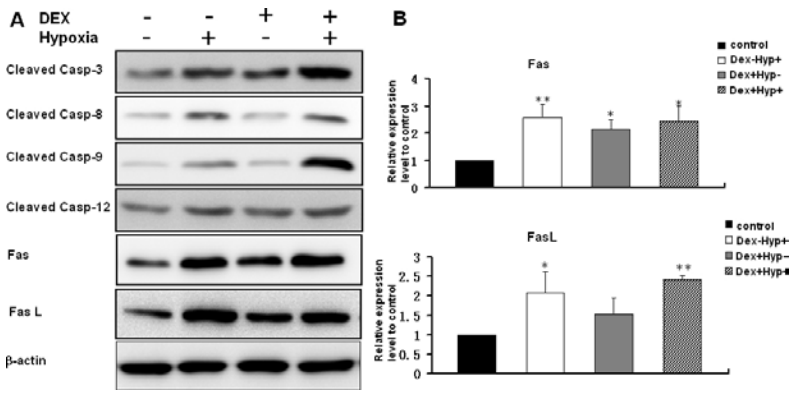

Figure 2. The apoptotic pathway of Dex-induced apoptosis under hypoxic conditions. A: The cleavage of caspase proteins, Fas and FasL expression in MC3T3-E1 cells was analyzed using western blot with actin as the control. B: Fas and FasL expression in MC3T3-E1 cells were analyzed using real-time quantitative PCR, and relative expression levels were calculated by comparison to the internal actin control. Results were means ± SD (n = 3). *P < 0.05 vs. control, **P < 0.01 vs. control.

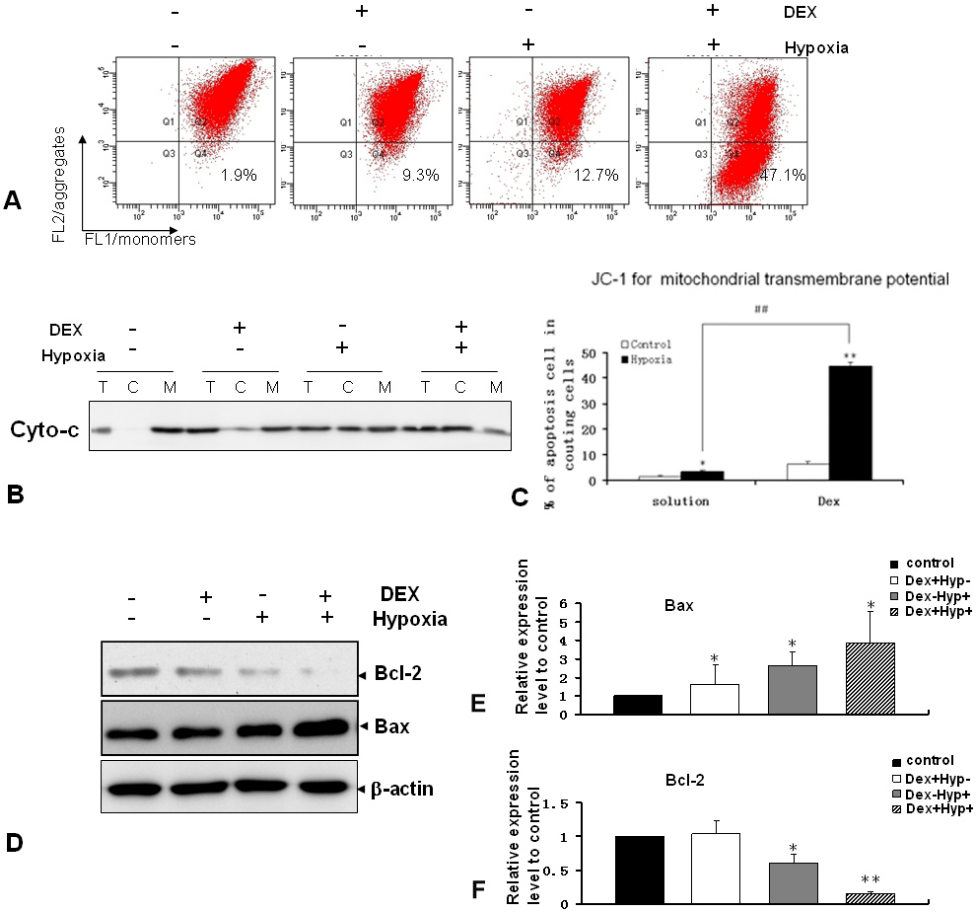

Figure 3. The effects of Dex and hypoxic stress on the mitochondria-mediated apoptosis signaling pathway. A: The reduction of DYmt was detected using flow cytometric analysis using the probe JC-1 in MC3T3-E1 cells treated under different conditions. The relative intensity of green (FL1) versus red fluorescence (FL2) is plotted. The results were consistent in triplicate experiments and values were presented as means ± standard deviation (SD) in fig S2. B: The release of cytochrome *c* from mitochondria to the cytosol was analyzed using western blot (T-total, C-cytosol, M-mitochondria). E-F: The expression of Bax and Bcl-2 proteins was analyzed using western blot, and actin was used as a control. D: The pro-apoptotic Bax and anti-apoptotic Bcl-2 expression levels were analyzed using real-time quantitative PCR, and relative expression levels were calculated by comparison to the internal actin control. All experiments were performed in triplicate, and values are presented as means ± standard deviation (SD). *P < 0.05 vs. control, **P < 0.01 vs. control.

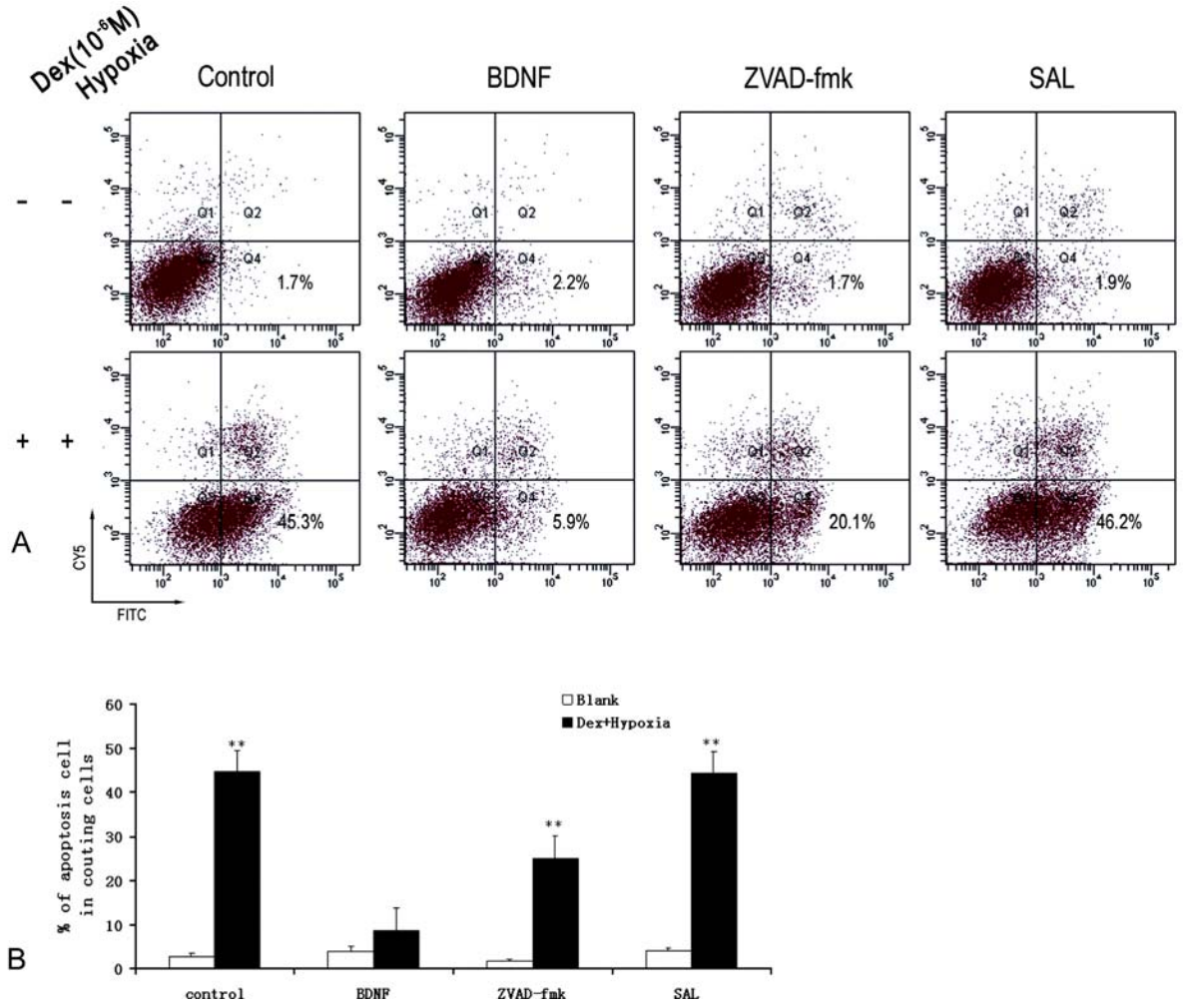

Figure 4. The effect of specific apoptotic pathway inhibitors was analyzed using flow cytometry. BDNF inhibits the mitochondria-mediated pathway, ZVAD-fmk inhibits the Fas/FasL-induced pathway, and SAL inhibits the ER stress-induced pathway. All experiments were performed in triplicate, values were analyzed and presented as means ± standard deviation (SD).

study, hypoxic stress enhanced the apoptosis induced by a single dose of Dex in osteoblasts. The mechanisms that underlie this phenomenon might explain the pathogenesis of ONFH induced by high-dose steroid hormones.

Apoptosis occurs in both physiological and pathological situations and is critical for the maintenance of normal cell functions. This process is controlled by proteins that participate in cross-talk between various organelles and signaling pathways (19). The death receptor pathway and the mitochondrial pathway have been proposed as the two major pathways for initiating apoptosis (20, 21). The extrinsic pathway is associated with death receptors, and their ligands induce apoptosis by activation of the Fas receptor system (22). Fas/FasL complexes recruit adaptor molecules following activation of the apoptosis-related caspase-8 (23). In this study, the expression levels of both Fas and FasL protein and mRNA levels were consistent using western blotting analysis and RT-PCR techniques; however, there was a slight increase in Fas and FasL protein levels between Dex and the control. Moreover, hypoxic stress did not increase the expression of Fas or FasL protein. These results confirm that hypoxic stress had little effect on the activity of the Fas/FasL signaling pathway in cells treated with Dex. The intrinsic pathway (mitochondria-mediated pathway) involves the release of cytochrome *c* from the mitochondria into the cytosol. The apoptosome cleaves pro-caspase-9, which results in activation of effector caspases (24, 25). Previous findings have demonstrated that dysfunctions in mitochondria are involved in many diseases (26-29). The present study indicates that the Dex- and hypoxic stress-induced apoptosis in MC3T3-E1 cells is accompanied by the collapse of the mitochondrial membrane potential and the release of cytochrome *c* from the mitochondria to the cytosol. Interestingly, the disruption of the mitochondrial membrane potential was not obvious in cells treated with Dex or hypoxia alone comparing to the combination. However, treatment with either Dex or hypoxia alone altered the distribution of cytochrome *c*, which is required for the formation of the Apaf-1/caspase-9 complex (30). This result implicated that it needed detailed study for the correlation of

cytochrome *c* release and the collapse of the mitochondrial membrane potential. The effects of hypoxia on the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax are still unknown. In this model, hypoxic stress caused a significant decrease in Bcl-2 protein levels and an increase in Bax protein levels in Dex-induced apoptotic MC3T3-E1 cells showing that the mitochondria-mediated pathway played a more important role in this process. The BDNF could effectively inhibit caspase-3 activation (31) and increased the Bcl-2 anti-apoptosis protein expression (32). This may explain the inhibition of the apoptosis induced by hypoxia and DEX. However, the comprehensive mechanism underlying this inhibition need to be further investigated.

Emerging findings have shown that the apoptotic pathway activated by endoplasmic reticulum (ER) stress is associated with multiple diseases; however, the molecular mechanisms of this pathway have not been fully elucidated (33). Caspase-12 is specifically involved in the ER stress apoptotic pathway (34, 35). In the present study, neither Dex nor hypoxic stress activated this caspase protein, and a specific inhibitor of the ER stress pathway did not decrease the number of apoptotic cells induced by Dex or hypoxia. However, many studies have shown that cross-talk exists between ER stress and other apoptotic pathways. Shiraishi *et al.* found that the mitochondria-initiated apoptotic pathway was involved in ER stress-induced apoptosis (36, 37). In the current study, the mitochondria pathway was triggered and played an essential role in apoptosis; however, it was unclear whether the subsequent effects activated the ER stress pathway *in vivo*. Further studies are needed to systematically investigate this hypothesis. Torres-Roca's group found that an early oxygen-sensitive pathway existed in the Dex-induced apoptosis of immature mouse thymocytes (38). Another group found that anti-apoptotic genes were upregulated in the early stages of hypoxia after exposure to a high dose of steroid hormones (8). These results suggest that hypoxic stress is closely associated with mitochondria-mediated apoptosis and provide evidence that hypoxic stress might inhibit apoptosis induced by Dex at an early stage. Our results showed that hypoxic stress promoted the apoptosis induced by Dex for a longer time compared to the results of others. Thus, we speculate that various signaling pathways are activated at different stages and that the pro-apoptotic effect at later stages may contribute to the decreased number or necrosis of osteoblasts.

In conclusion, we found that hypoxic stress enhanced Dex-induced apoptosis and that the mitochondria-mediated pathway played an essential role in the process of apoptosis. This mechanism may be critically associated with the steroid-induced osteonecrosis of the femoral head.

6. ACKNOWLEDGMENT

The authors declare that there are no conflicts of interest. The authors thank Dr Xiaolong Cai for technical assistance during this study.

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Abbreviations: ONFH: Osteonecrosis of the femoral head; BDNF; brain-derived neurotrophic factor; Dex: dexamethasone; FBS: fetal bovine serum; PVDF: polyvinylidene difluoride; SAL: Salubrinal; ER:

endoplasmic reticulum

Key Words: Osteonecrosis, Of The Femoral Head; Glucocorticoid; Apoptosis; Hypoxic Stress

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