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Apoptosis of primary osteoblasts induced by dexamethasone: cellular and molecular mechanisms

Deksametazonun primer osteoblast apopitozundaki rolü: Hücresel ve moleküler mekanizmalar

Nirong Bao, M.D., Jianning Zhao, M.D., Yurong Wang, M.D., Sujia Wu, M.D., Xiaofeng Zeng, M.D., Junhua Wang, M.D.

Department of Orthopaedics, Jinling Hospital, Nanjing, Jiangsu Province, China.

Objectives: We investigated the effect of glucocorticoids on cell viability of osteoblasts and explored the cytological and molecular mechanisms of osteoblast apoptosis induced by glucocorticoids.

Materials and methods: Dexamethasone was used to induce apoptosis of primary cultured osteoblast-like cells from skulls of suckling mouse. Surviving rate of osteoblasts, apoptosis rate, activity of caspase-3, and DNA binding activity of nuclear factor-kappa B were assessed using MTT-dye reduction microassay, flow cytometry, colorimetric substrate assay and electrophoretic mobility shift assay. The surviving rates of osteoblasts and fibroblasts were also compared to assess the differences in dexamethasone-induced effects on cells.

Results: Dexamethasone significantly decreased the survival rate of osteoblast-like cells through an apoptotic process, activated cellular caspase-3, and inhibited the activity of nuclear factor-kappa B, in a concentration- and time-dependent manner ($p<0.05$). However, dexamethasone did not exert any apoptotic effect on fibroblasts.

Conclusion: The results suggest that dexamethasone induces apoptosis of primarily cultured and non-transformed osteoblasts, which is caspase-3 dependent, while nuclear factor-kappa B may play a protective role through inhibition of caspase-3.

Key words: Apoptosis/drug effects; bone density/drug effects; bone marrow cells/drug effects; glucocorticoids/adverse effects; NF-kappa B/metabolism; mice; osteoblasts/metabolism/drug effects.

Amaç: Glikokortikoidlerin osteoblast hücrelerinin canlılığı üzerine etkisi incelendi ve osteoblast apopitozu sürecinde hücresel ve moleküler mekanizmalardaki rolü araştırıldı.

Gereç ve yöntem: Deksametazon, yenidoğan farelerin kranyal kemiklerinden elde edilen primer kültürde üretilen osteoblast-benzeri hücrelerde apopitoz oluşturmak amacıyla kullanıldı. Osteoblastların canlı kalma ve apopitoz oranları, *caspase-3* etkinliği ve nükleer faktör-kappa B'nin DNA bağlanma aktivitesi MTT-boya redüksiyonu mikrotesti, akım sitometrisi, kolorimetrik substrat testi ve elektroforetik mobilite şift testi ile ölçüldü. Deksametazonun hücrelere etkisindeki farklılıkları değerlendirmek amacıyla osteoblast ve fibroblastların canlı kalma oranları karşılaştırıldı.

Bulgular: Deksametazonun, yoğunluk ve zamana bağlı olarak, apopitoz sürecinde osteoblast-benzeri hücrelerin canlı kalma oranını anlamlı derecede düşürdüğü, hücresel *caspase-3* aktivasyonunu artırdığı ve nükleer faktör-kappa B etkinliğini baskıladığı gözlendi ($p<0.05$). Buna karşın, deksametazonun fibroblastlarda apopitotik etki oluşturmadığı görüldü.

Sonuç: Bulgularımız, deksametazonun *caspase-3* üzerinden transforme olmamış primer osteoblast kültüründe apopitoza neden olduğunu, buna karşın nükleer faktör-kappa B'nin *caspase-3*'ü baskılayarak bunu önleyebildiğini göstermektedir.

Anahtar sözcükler: Apopitoz/ilaç etkisi; kemik yoğunluğu/ilaç etkisi; kemik iliği hücresi/ilaç etkisi; glukokortikoid/yan etki; NF-kappa B/metabolizma; fare; osteoblast/metabolizma/ilaç etkisi.

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• *Correspondence: Nirong Bao, M.D. Department of Orthopaedics, Jinling Hospital, 305 East Zhong Shan Road, Nanjing, Jiangsu province, PRC 210002, China.*
Tel: +025 - 4826808 / 58016 Fax: +025 - 84829224 e-mail: nirong_bao@yahoo.com.cn

Hypercortisolism, cortisone *in vivo* exceeding the physiological level, leads to bone loss and decreased bone formation. In patients receiving glucocorticoids therapy for rheumatoid arthritis, asthma, and collagen vascular diseases, yearly bone loss may be 0.6% to 6%, resulting in osteoporosis.[1] Approximately 50% of patients with Cushing's syndrome and 30% to 50% of patients taking long-term glucocorticoids have at least one atraumatic fracture due to osteopenia. Histologic studies suggest that glucocorticoids treatment leads to a decrease in the number of osteoid seams, a low mineral apposition rate, and an increase in bone absorption markers.

Many of the *in vivo* effects of glucocorticoids on bone formation have been confirmed by *in vitro* studies. In bones cultured for 24 hours, glucocorticoids stimulated collagen synthesis, but at a later time they inhibited collagen, fibronectin, and DNA synthesis. In cultured rat osteoblastic cells, they inhibited cell division and DNA replication. Another mechanism by which glucocorticoids may affect osteoblasts is through the control of programmed cell death, apoptosis. They have been shown to decrease the number of osteoblasts and osteocytes by inducing apoptosis.[2-4] Glucocorticoids also induce apoptosis in mouse thymocytes and lymphocytes by a receptor-mediated process.[4]

In this study, we investigated the effect of glucocorticoids on cell viability of osteoblasts and the cytological and molecular mechanisms of apoptosis induced by glucocorticoids.

MATERIALS AND METHODS

Study design

In this study, dexamethasone was used to induce apoptosis of primary cultured osteoblast-like cells from skulls of suckling mouse after obtaining the approval of the Animal Experiment Administration to conduct experiments on animals. Surviving rate of osteoblasts, apoptosis rate, activity of caspase-3 and DNA binding activity of nuclear factor-kappa B were assessed using MTT-dye reduction microassay, flow cytometry, colorimetric substrate assay and electrophoretic mobility shift assay. The surviving rates of osteoblasts and fibroblasts were also compared to assess the differences in dexamethasone-induced effects on cells.

Materials

ICR mice were obtained from the Animal Center of Nanjing Jinling Hospital (Jiangsu, PRC). Alpha-minimal essential media (α-MEM) and fetal bovine serum (FBS) were obtained from Gibco (Gaithersburg, MD). Collagenase-I, MTT, dexamethasone, PDTC (Pyrrolidine dithiocarbamate) and the bicinchoninic acid protein assay kit were obtained from Sigma (St. Louis, MO). TNF-alpha was obtained from PeproTech (Rocky Hill, NJ); Electrophoretic mobility shift assay (EMSA) and caspase-3 colorimetric assay kits were obtained from Promega (Madison, WI).

Cell culture

I. Osteoblasts: Calvariae were removed from neonate mice, washed in DMEM medium, and minced with scissors. Osteoblasts were obtained by sequential digestion of the calvariae with 0.1% collagenase and 0.25% trypsinogen in culture flasks. Osteoblasts harvested were centrifuged at 500 x g for 5 minutes. The centrifugation was repeated for 10 minutes after suspension in DMEM medium containing 20% FBS. The cells harvested were plated in 50 ml culture flasks at a density of 10.000 cells/cm^2 in α-MEM medium containing 10% FBS. Osteoblasts have been shown to have increased procollagen I and alkaline phosphatase messenger RNA, and increased collagen synthesis compared with fibroblasts.[3] At confluence, the cells were trypsinized and subcultured. Finally, the cells were treated with varying doses of dexamethasone (1nM–10μM).

II. Fibroblasts: Scalps were removed form the mice during the same procedure and minced with scissors. Fibroblasts were separated by sequential digestion of the minced scalp with 0.1% collagenase alone. The harvest of fibroblasts was treated similarly to that of osteoblasts described above.

MTT cell viability assay

Cell viability was determined by the MTT-dye reduction microassay for each concentration of dexamethasone tested, for each pretreated hours of dexamethasone, and for TNF-α and DEVD-CHO incubation. Briefly, after 48-hour incubation, 15 μl MTT (5mg/ml) was added for 3 hours to 96-well microplates, and the absorbance was read at 492 nm on a multiscan MicroELISA read-

er. Cell viability was calculated as the ratio of optical densities in wells with dexamethasone in the presence or absence of TNF-α, DEVD-CHO, or PDTC.

Analysis of cell apoptosis rate

The harvested cells, incubated with various agents for certain periods, were fixed in 70% ethanol, treated with RNAase, and stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry and the labeled normal cells and apoptotic cells were quantified.

Colorimetric substrate assay for caspase-3 activity

The cells were counted and 2 x 10^6 were centrifuged at 400 x g for 10 minutes. The cells were resuspended in 50 µl of chilled Cell Lysis Buffer (1·L^{-1} HEPES pH 7.9, 10 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.5 mmol·L^{-1} DTT, 0.5 mmol·L^{-1} PMSF, and 5 ml·L^{-1} NP-40), then were incubated on ice for 10 minutes. Thereafter, the cell lysate was centrifuged in a microcentrifuge tube at 14,000 rpm for 3 minutes at 4 °C to precipitate cellular debris. The supernatant was transferred to another microcentrifuge tube and 50 µl of reaction buffer/1M DTT mix at a ratio of 100:1 was added to each reaction. Then 5 µl of 1 mM caspase-3 substrate (DEVD-pNA) was added to each tube in the presence or absence of caspase-3 inhibitor DEVD-CHO. Then each reaction was incubated at 37 °C in a water bath for an hour and the samples were read at 405 nm in a microplate reader.

Electrophoretic mobility shift assay (EMSA)

Nuclei from 3.3 x 10^6 TNF-α or dexamethasone-treated osteoblasts were extracted using a nuclear protein extracting kit. The cells were lysed with a hypotonic buffer (l·L^{-1} HEPES pH 7.9, 10 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.5 mmol·L^{-1} DTT, 0.5 mmol·L^{-1} PMSF, and 5 ml·L^{-1} NP-40). After centrifugation, the nuclear pellets were resuspended in an extraction buffer (20 mM HEPES pH 7.9, 25% [vol/vol] glycerol, 0.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol). The nuclear proteins were recovered after centrifugation at 14,000 rpm, quantified using a bicinchoninic acid protein assay kit, and used to carry out EMSA. To measure the activation of transcription factors, NF-kappa B, the oligonucleotide probes of NF-κB containing the IgG chain-binding site (NF-κB, 5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') were used. Two complementary strands of oligonucleotides were annealed and labeled with (-32P) deoxy-CTP using a random primer labeling kit (Rediprime, Amersham Pharmacia Biotech). Nuclear extracts (5 µg) were reacted with 2-5 ng of the radiolabeled NF-κB (50,000–100,000 cpm/ng). The reaction was performed in the presence of 10 mM

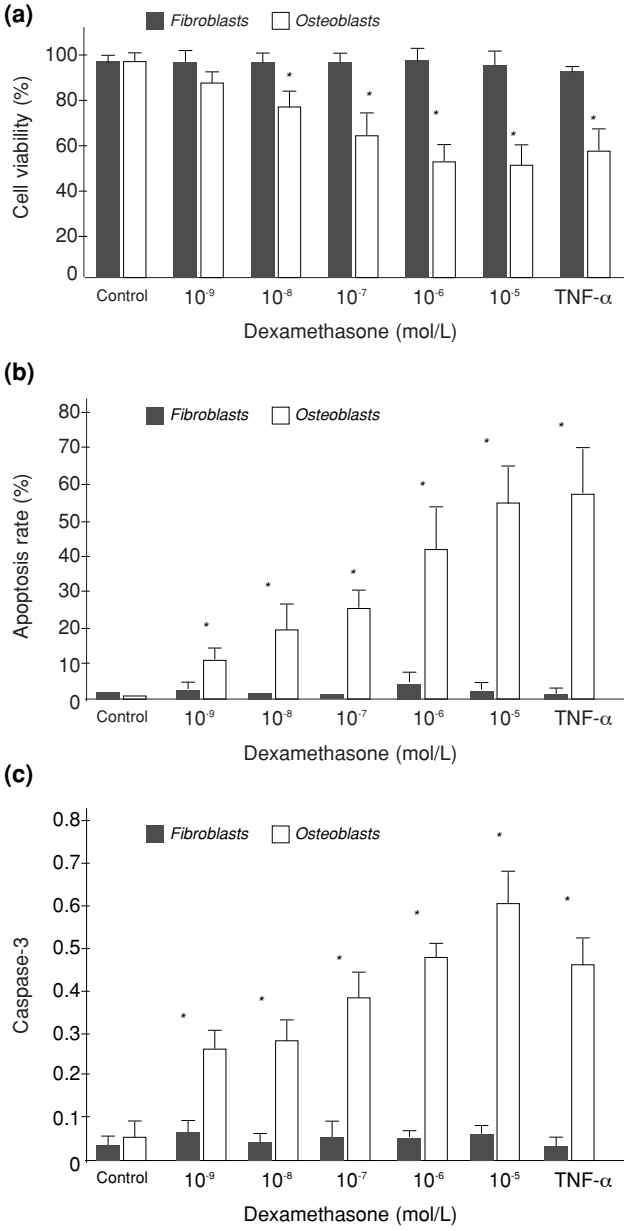

Fig. 1. Dexamethasone **(a)** decreased cell viability, **(b)** induced apoptosis of osteoblasts and **(c)** caspase-3 activity in a dose-dependent manner ($p<0.05$).

Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 4% glycerol (final volume 25 µl) at room temperature for 30 minutes. The reaction products were subjected to 4% PAGE in 0.5 x TBE buffer (50 mM Tris-HCl pH 8.5, 50 mM borate, and 1 mM EDTA). The gels were dried under vacuum for an hour. DNA-binding activity for NF-κB was measured using a PhosphoImager analyzer (BAS, Fuji Photo Film Co. Ltd.).

Statistical analysis

Statistical analyses were made by analysis of variance ANOVA in dose-response experiments and by two-tailed Student's t-test.

RESULTS

Dexamethasone decreased viable osteoblasts

To explore whether dexamethasone induced apoptosis of primarily cultured osteoblasts, the cells were treated with varying concentrations of dexamethasone for 24 hours. Figure 1 shows the effect of dexamethasone [1 nM to 10 µM] on cell viability measured using the MTT assay. Dexamethasone evidently decreased cell viability after incubation. This effect became increasingly significant at concentrations above 100 nM dexamethasone and peaked at 10 µM (Fig. 1a). When osteoblasts were incubated with TNF-α (20 ng/ml) for 48 hours for positive control, a significant decrease in cell viability was observed, which was consistent with the findings in dexamethasone incubation.

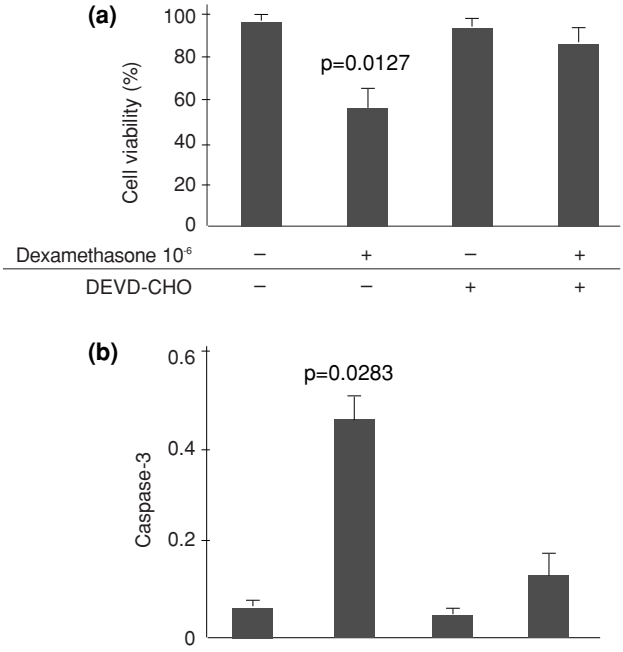

Fig. 2. (a) Cell viability in the presence and absence of DEVD-CHO and dexamethasone. **(b)** DEVD-CHO blocked dexamethasone-induced increase in Caspase-3 activity.

To determine the cell specificity of the glucocorticoids-associated effect, fibroblasts and osteoblasts were treated with 1 µM dexamethasone for 48 hours. In contrast to osteoblasts, the fibroblast did not undergo apoptosis (Fig. 1b).

Dexamethasone induced apoptosis of osteoblasts

We investigated by flow cytometry the DNA content in cells incubated with dexamethasone [1 nM to 10 µM] for 24 hours, which showed a remarkable increase in apoptotic cells in a trend with concentration, consistent with a decrease in viable osteoblasts (Fig. 1b).

Effects on activity of caspase-3

In the first set of experiments assaying caspase-3 activity in dexamethasone-induced cell death in osteoblasts, the colorimetric intensity of the caspase-3 protease cleavage product AMC was monitored at various concentrations of dexamethasone incubated for 24 hours. Caspase-3-like protease activity increased in a dose-dependent manner and peaked at 10 µM (Fig 2). In addition, to examine the role of caspase-3 activation in dexamethasone-induced apoptosis of osteoblasts, primary osteoblasts were pretreated with the specific caspase-3 inhibitor DEVD-CHO. As shown in Fig. 2, a constitutive level of caspase-3 is followed by non-affected cell viability, and addition of dexamethasone induced caspase-3 activity, interfering with the balance. Pretreatment of osteoblasts with 100 µM DEVD-CHO largely blocked the dexamethasone induction and prevented death that was seen after dexamethasone treatment at 1µM. Our data suggested that caspase-3-like cysteine protease activity was required to induce apoptosis in primary osteoblasts and that dexamethasone, a glucocorticoid hormone, exerted a regulatory role in apoptosis via the induction of caspase-3-like cysteine protease activity in primarily cultured osteoblasts.

Dexamethasone blocked TNF-α-induced NF-kappa B activation

It has been demonstrated that NF-κB, a nuclear transcription factor, is inhibited by glucocorticoids

in apoptotic process of thymocytes.[9] Therefore, we examined whether NF-κB could be inhibited in dexamethasone-treated primarily cultured osteoblasts. The binding activity of nuclear extracts from dexamethasone-treated cells to the oligonucleotide of NF-κB consensus binding sequences was observed using an electrophoretic gel mobility shift analysis (Fig. 3). NF-κB was found to be downregulated far below the constitutive level after incubation with dexamethasone. Furthermore, the inducing effect imposed by TNF-α was dramatically impaired by dexamethasone, suggesting that dexamethasone-induced apoptosis of osteoblasts occurred via inhibition of NF-κB activation.

NF-kappa B protected cells from TNF-α-induced apoptosis

NF-κB was reported to be an essential component in the signal transduction pathway of TNF-α-induced apoptosis.[11] In order to determine indirectly the putative role of NF-κB in osteoblast death, we investigated the impact of its inhibition on cell viability following TNF-α exposure. For this purpose, PDTC, an inhibitor of NF-κB activation, was used. NF-κB was evidently upregulated after TNF-α 20 ng/ml exposure. Treatment with PDTC decreased NF-κB activation induced by TNF-α in a dose-dependent manner. While 50% of cells survived after a 48-hour exposure to TNF-α, addition of 10 μM of PDTC resulted in a decrease in cell viability by 23%, and this killing effect became more apparent in the presence of 20 μM of PDTC (Fig. 4).

DISCUSSION

We have demonstrated that glucocorticoids induce apoptosis of osteoblast cells isolated from calvariae of mice. Glucocorticoid-induced apoptosis was dependent on the concentration and duration, with the maximum effect being associated with 10 μM dexamethasone and 48 hours of incubation. Glucocorticoids progressively induced caspase-3 activity after consecutive periods of incubation, suggesting a dose-dependent relationship. Caspase-3 level was significantly decreased by pretreatment with DEVE-CHO, inhibitor of caspase-3, which resulted in a remarkable increase in cell viability. Glucocorticoids imposed an effective inhibition of DNA binding activity of NF-κB, which was associated with a dramatic loss of osteoblasts.

Although fibroblasts and osteoblasts have many similarities in characteristics, glucocorticoid-induced apoptosis appears to be specific for osteoblasts. Glucocorticoids failed to induce apoptosis in fibroblasts derived from mouse scalp, as demonstrated by the MTT assay. In contrast, osteoblasts underwent apoptosis during incubation with dexamethasone. The targeting of only osteoblasts by glucocorticoid-induced apoptosis was also confirmed *in vivo*, in that, in response to glucocorticoids, only osteoblasts lying along the bone demonstrated an increase in the number of apoptotic cells. Glucocorticoids appear to have no significant effect on periosteal cells *in vivo*.[2] The finding that the effect of glucocorticoids is purely on osteoblasts and not on fibroblasts has been supported by experimental

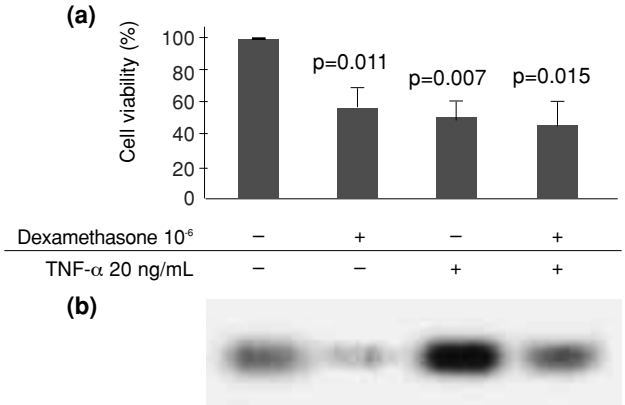

Fig. 3. (a) Cell viability in the presence and absence of TNF-α and dexamethasone. **(b)** NF-κB activity was evidently impaired by dexamethasone (assayed with EMSA).

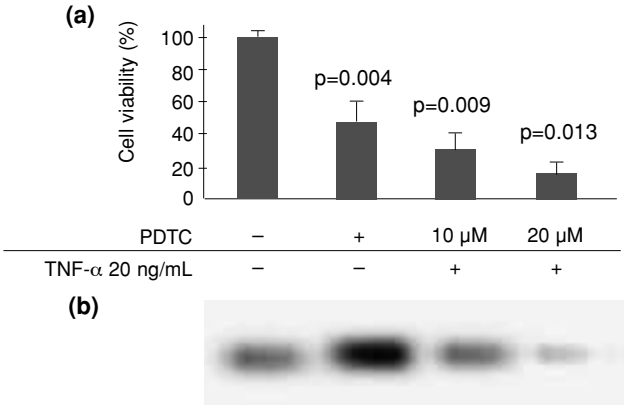

Fig. 4. (a) Cell viability in the presence and absence of TNF-α and PDTC. **(b)** PDTC decreased NF-κB activation (assayed with EMSA).

data demonstrating that glucocorticoids decrease cell attachment and fibronectin levels in osteoblastic F3 cells, but not in fibroblastic F1 cells.[5] On the other hand, glucocorticoids treatment also failed to induce apoptosis in transformed or immortalized osteoblast-like lines such as SaOS-2 cells, ROS 17/2.8 cells, and MC3T3 cells, which suggest that the effect of glucocorticoids is specific for primary, untransformed osteoblasts. Thus, it appears that normal cell function and signaling are necessary for glucocorticoid-induced apoptosis in osteoblasts.

In our study, we attempted to further characterize the biochemical pathways leading to signaling molecules directly involved in dexamethasone-induced apoptosis. Some experiments regarding the role of specific caspases demonstrated the pivotal role of caspase-9 in glucocorticoid-induced apoptosis;[6,7] however, caspase-3, a well-known executive member of the family, has not been well defined in glucocorticoid-induced apoptosis, especially in primary osteoblasts. Our data indicated that caspase-3 showed a dose-dependent and dramatic increase in osteoblasts after incubation with dexamethasone, suggesting a temporal and perhaps casual relationship. Therefore, we further assayed the role of caspase-3 in osteoblasts treated with dexamethasone using DEVD-CHO, inhibitor of the protease. The pretreatment of DEVD-CHO significantly blocked the apoptotic induction of dexamethasone. This suggests that caspase-3 plays an essential role in apoptosis of osteoblasts induced by glucocorticoids. In addition, it has been well-documented that caspase-8 is one of the major initiating caspases, upstream initiator of caspase-3. Therefore, we hypothesize that glucocorticoids may induce apoptosis by stimulating caspase-8, the caspase-3 pathway in primary osteoblasts.

It has been demonstrated that, in thymocytes, glucocorticoids inhibit NF-κB activity in two different mechanisms: one is through IκB, a specific inhibitor of NF-κB in cytoplasm; the other is by direct inhibition resulting from the combination of glucocorticoids with NF-κB. In this study, we found that dexamethasone significantly affected the activity of transcriptional factor NF-κB and impaired the NF-κB level in primary osteoblasts. In addition, the downregulation of NF-κB was accompanied by a decrease in cell viability as measured by the MTT assay.

The involvement of NF-κB in apoptosis has been suggested by other investigators and, very recently, it has been demonstrated that several oncogenic proteins synergize with NF-κB.[8,9] Mayo et al.[10] reported that NF-κB activation was required for oncogenic Ras to block transformation-induced programmed cell death. Chae et al.[11] suggested that inhibition of NF-κB would increase apoptosis of ROS 17r2.8 osteoblasts. Evidence from these studies strongly supports the hypothesis that NF-κB may work anti-apoptotically through the activation of various kinases. In our study, NF-κB was demonstrated to be involved in the dexamethasone-induced apoptosis of primary osteoblasts. To understand whether it prevents or enhances the process, we performed indirect assays: addition of TNF-α was found to induce the activity of NF-κB, resulting in decreased cell viability. Moreover, PDTC, an inhibitor of NF-κB, impaired NF-κB activity constitutively and reversed the effect of TNF-α on NF-κB activity, which resulted in a significant loss of viable osteoblasts with a dramatic increase in caspase-3 activity. Thus, NF-κB seems to have a protective effect on primary osteoblasts through inhibition of caspase-3. Considering this, we may hypothesize that dexamethasone-induced apoptosis may occur through a mechanism whereby NF-κB activity is inhibited in primary osteoblasts.

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