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ORIGINAL ARTICLE

Dynamic expression of Runx2, Osterix and AJ18 in the femoral head of steroid-induced osteonecrosis in rats

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Objective: To study dynamic changes in gene expression and protein synthesis of runt-related transcription factor-2 (Runx2), Osterix and AJ18 in the femoral head of steroid-induced osteonecrosis in rats.

Methods: Forty mature Wistar rats, 250–270 g (mean, 260 g) in weight, were randomly divided into model (30 rats) and control groups (10 rats). An early rat model of femoral head necrosis (FHN) was created by weekly injections of dexamethasone (20 mg/kg) into alternate sides of the gluteus maximus and twice-weekly training on a laboratory animal treadmill for 8 weeks. Hematoxylin and eosin (HE) staining was used to determine whether osteonecrosis had been successfully induced, and the model was then divided into equal 8, 10 and 12 week groups. At the end of the appropriate time period, total RNA and total protein were extracted from the femoral heads, and then real-time quantitative polymerase chain reaction and Western blot were performed to detect dynamic changes in the expression of Runx2, Osterix and AJ18 and protein synthesis in femoral heads with steroid-induced osteonecrosis in rats.

Results: At the post-modeling eighth, tenth and twelfth week, expression of Runx2 mRNA, Osterix mRNA and related protein synthesis were significantly down-regulated compared to that of the control group, which showed a downward trend with time; while expression of AJ18 mRNA and protein synthesis in the model group was much higher than in the control group, which showed an upward trend with time.

Conclusion: Glucocorticoids may induce femoral head osteonecrosis by down-regulating Runx2/Osterix mRNA and up-regulating AJ18 mRNA.

Key words: AJ18; Femoral head necrosis; Glucocorticoids; Osterix; Runt-related transcription factor-2

Introduction

Recently, several domestic and foreign scholars have reported extensive research on the pathogenesis of steroid-induced femoral head necrosis (FHN) at the cellular, molecular and genic levels. From the point of view of molecular biology and genetics, they have explained that glucocorticoids affect the balance of bone metabolism by regulating expression and activity of transcription factors. Some scholars believe that hormones can not only change the direction of bone marrow stromal cell differentiation by inducing bone marrow stromal cells into fat cells and inhibiting bone marrow stromal cells into osteoblasts[1], but that they also may affect osteoblast maturation and mineralization[2]. This theory has been confirmed by *in vitro* testing at both the cellular and genic levels. However, the induction function of hormones is rarely reported in *in vivo* studies. Runx2 is considered to be the most important transcription factor controlling the shape and differentiation of osteoblasts, its devitalization will stop bone marrow stromal cells from differentiating into osteoblasts. Osterix plays a role in the final stage of transformation of an immature to a mature osteoblast. Both of these are key genes for regulating osteoblast differentiation and maturation. *In vitro* experiments demonstrate that AJ18 can combine with the Runx2 binding sequence (Osterix), and that overexpression of AJ18 can inhibit the combination of Runx2 and Osterix and down-regulate osteocalcin promoter activity. AJ18 may serve as a transcription repressor protein to repress the combination of Runx2 and Osterix. The aim of this study was to induce a Wistar rats FHN model by using hormones, dynamically observe the expression of Runx2, Osterix and AJ18 mRNA and corresponding protein synthesis during different stages of steroid-induced FHN, and provide a basis for research on the pathogenesis of steroid-induced FHN.

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Materials and methods

Forty mature Wistar rats weighing 250–270 g (mean, 260 g) (provided by the Animal Experimental Center of the Medical Sciences Academy of the Chinese People's Liberation Army Military Academy) were used in the study. There were equal numbers of female and male rats. Seven to eight rats were housed in standard cages. All reagents and equipment used in the experiment are as follows: dexamethasone sodium 5 mg/ml (Tianjin Jinyao Amino Acid, Tianjin, China); rabbit anti-Runx2, anti-Osterix and anti-AJ18 (Boster Biological, Wuhan, China); rabbit anti-β-actin (Xaar Bio-Technology, Tianjin, China); RT Kit (Promega, Madison, WI, USA); platinum SYBR Green SuperMix-UDG (Invitrogen, Carlsbad, CA, USA); trizol reagent (Invitrogen); diethyl pyrocarbonate (DEPC, Promega); type TGL-16 high speed tabletop centrifuge (Mitsubishi, Tokyo, Japan); Himac CR22 Hitachi Eppendorf (Hitachi, Tokyo, Japan); computer controlled electrical stimulus laboratory animal treadmill (Huaibei Zhenghua Bio-Instrument Equipment, Huaibei, China); type DHG-9246A dry sterilization oven (Jing-hong Experimental Equipment, Shanghai, China); inverted microscopy (Olympus, Tokyo, Japan); GDS-8000 System (UVP, Upland, CA, USA); and the 7000 Sequence Detection System ABI Prism (Applied Biosystems, Foster, CA, USA).

Animal model and group

Forty rats were randomly divided into a normal control (10 rats) and a model group (30 rats), the latter being subdivided into three further groups of ten rats each: Group A, hormone injection and training for 8 weeks; Group B, hormone injection and training for 10 weeks; and Group C, hormone injection and training for 12 weeks. Group design *t*-test showed no statistically significant difference in body weights between groups ($t = 0.318$, $P > 0.05$). All model group animals received 20 mg/kg body weight of dexamethasone (Dex) by intramuscular injection into alternating sides of the gluteus maximus muscle once a week. After injection, animals were trained on a treadmill twice a week. Specimens were harvested from all ten animals in each group (control, A, B, C) per time point. The experimental protocol was approved by the Animal Experiment Ethics Committee of the authors' institute.

Harvesting of specimens

At the eighth (Group A), tenth (Group B) and twelfth (Group C) week, the animals were anesthetized by an intraperitoneal injection of 2 ml 10% chloral hydrate. Under strict aseptic surgery, both femoral heads were collected from each animal, and the shape of the femoral heads and color of their articular cartilage observed. The femoral heads were then washed with phosphate buffered saline (PBS) compounded with DEPC water. Next, one femoral head from each animal was split along the coronal plane and again washed with PBS. Then one half of each split femoral head was placed in formaldehyde fixation fluid for pathological examination and the other half, together with the contralateral intact femoral head, put into a nitrogen canister quickly, for polymerase chain reaction (PCR) and Western blot test.

Histological examination

All specimens were fixed with formaldehyde solution (0.1 mol/l, pH = 7.4) at 4°C for 4 days, washed with PBS for 5 seconds, placed in 10% ethylenediaminetetraacetic acid (EDTA)-Tris buffer solution, and decalcified at 37.4°C. The decalcifying fluid was changed once a week, and its degree of decalcification measured by a physical method. Once complete decalcification of the femoral heads had been achieved, they were progressively dehydrated in graded concentrations of ethanol. The specimens then underwent xylene transparent treatment for 2 h, after which they were embedded in paraffin wax and sections stained with HE for histological evaluation. An optical microscope was used to assess the structural changes in bone and marrow tissue.

Real-time fluorescence quantitative PCR

The femoral heads from the model and control groups were removed from the nitrogen canister, put in an aseptic mortar, and ground to powder in the presence of liquid nitrogen. Trizol reagent was then added and adequately mixed at room temperature. The mix was then transferred into Eppendorf (EP) tubes, and finally the general RNA was extracted according to the trizol procedure. After confirming by Agarose gel electrophoresis that RNA degradation had not occurred, reverse transcription was begun according to the manufacturer's instructions.

Primers were designed by Oligo 6.0 primer designing software and produced by Nanjing Jin Stewart Biological Technology, Nanjing, China (Table 1).

Using the Quant SYBR Green PCR Kit and following the manufacturer's instructions, the RT-PCR reaction system was as follows: cDNA 2 μl, double distilled water 8.5 μl, SYBR green PCR mix, 12.5 μl, primer one 1 μl (10 nmol/l), primer two (10 nmol/l), the total system was 25 μl. The reaction conditions were 95°C for 60 s, 95°C for 15 s, 60°C for 15 s, 72°C for 45 s, in all 36 cycles. Meanwhile a 65°C–95°C solubility curve was constructed.

Table 1 Primer details

Gene name	Primer	Sequence (5′-3′)	Length (bp)
Runx2	Primer F	ACA ACC ACA GAA CCA CAA G	105
(AF053950)	Primer R	TCT CGG TGG CTG GTA GTG A	
Osterix	Primer F	CTT TCC CCA CTC ATT TCC TG	89
(AF32187)	Primer R	CTA GGC AGG CAG TCA GAA G	
AJ18	Primer F	CCC CAA GGA AGT CAC CAG T	107
(AF32187)	Primer R	CTT TCT ATG GGA TCG GTC TCT T	
OC	Primer F	TGC AAA GCC CAG CGA CTC T	98
(X04141)	Primer R	AGT CCA TTG TTG AGG TAG CG	
β-actin	Primer F	CTT TCT ACA ATG AGC TGC GTG	127
(V01217)	Primer R	ATG GCT GGG GTG TTG AAG G	

The analysis of RT-PCR data were done by 7000Sequence Detection System own software, all of the sample's Ct values were computed by software, and data were analyzed by the relative quantitative method which means the use of $2^{-\Delta\Delta Ct}$ to compute the variance of gene expression between the model and control groups.

Western blot

Protein was extracted by the trizol method and after quantification, 60 μg of protein was subject to SDS-polyacrylamide gel electrophoresis, then switched to polyvinylidene fluoride membranes and chemiluminescence immunoassay carried out . The primary antibody working concentration of Runx2, Osterix and AJ18 was 1:300 and of β-actin was 1:1000, incubated overnight at 4°C. The secondary antibody was rabbit anti-mouse IgG (1:1000) marked by horse radish peroxidase, and was incubated at room temperature for 2 h.

After chemiluminescence of joined A and B (electrochemiluminescence system) liquid, the molecular weight and absorption optical density values of the target band was analyzed with a gel image processing system. The amount of target protein synthesis was expressed as the gray level ratio of target band and β-actin band.

Statistical analysis

Data were expressed as the mean ± standard deviation. The mean values of the HE stained optical microscope measurements in the four groups were compared by one-way analysis of variance (ANOVA) and the Student–Newman and Keuls (SNK) method, P-values < 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS statistical software program 16.0 (SPSS, Chicago, IL, USA).

Results

Histopathological findings

General findings in samples

In the control group, the morphological appearance of the femoral head was normal, and the articular cartilage surface smooth and ruddy. At the eighth week (experimental Group A), the morphological appearance of the femoral head was also normal and the articular cartilage surface smooth, but the color in the antero-lateral region of the articular cartilage was darker than in the other areas. At the tenth week (experimental Group B), the morphological appearance of the femoral head showed no obvious abnormalities, the articular cartilage surface was relatively smooth, and pale petechial changes were visible in the antero-lateral part of the articular cartilage. At the twelfth week (experimental Group C), the morphological appearance of the femoral head was still normal, but part of the articular cartilage surface was rough, and diffuse hemorrhage and large ecchymoses were observed. Moreover, the articular surface appeared off-white, and the bone was less hard and more fragile and was easy to chisel.

Optical microscopy findings

In the control group the bone trabeculae were intact and orderly. The osteocytes within them were clearly visible and empty lacunae were rare. Hematopoietic cells were abundant. There were relatively few fat cells and those seen were of normal morphology. The blood vessels in the bone marrow cavity were evenly distributed, no hemorrhage and only occasional macrophages were noted (Fig. 1a).

In the experimental groups the following was observed. At the eighth week, the bone trabeculae had become thinner than normal, the bone trabecular volume per unit area had decreased and they were arranged in a relatively ordered manner. Empty lacunae in bone cells were mildly

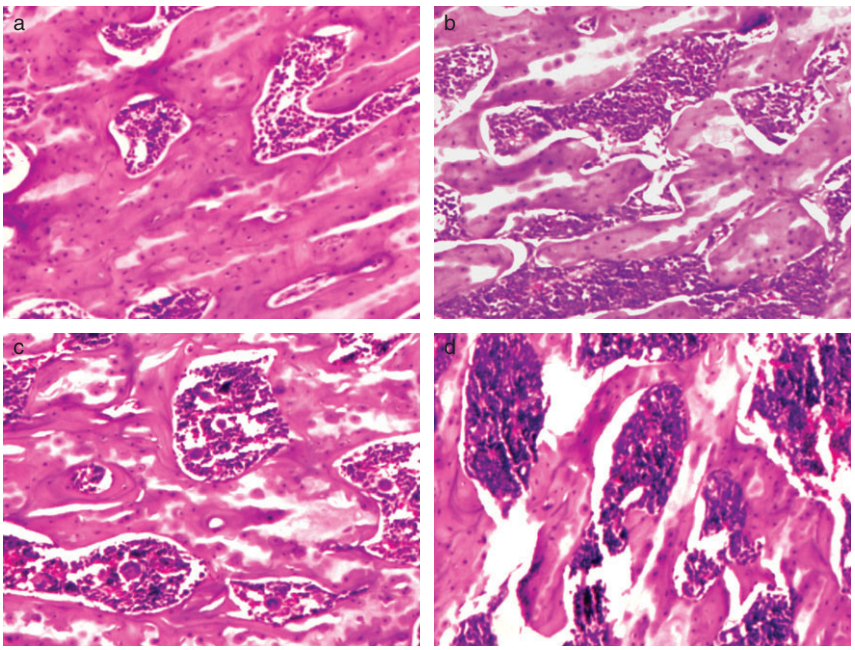

Figure 1 Light microscopic histological findings in specimens of femoral head (HE staining, ×100). (a) Normal control group: Bone trabeculae are intact and orderly, osteocytes in them are clearly visible, empty lacunae are rare, hematopoietic cells are abundant, and the few fat cells are of normal morphology. (b) At the eighth week (Group A): Bone trabeculae are thinner than normal, bone trabecular volume per unit area is decreased and their arrangement is relatively orderly, empty lacunae are increased mildly, the diameter of the bone marrow fat cells is larger, and hematopoietic tissue has decreased slightly. (c) At the tenth week (Group B): Bone trabeculae have thinned significantly, spaces have increased, the structure is mildly disordered, trabecular fracture is seen occasionally, the diameter of marrow fat cells is even greater, a large number of fat cells have accumulated, the proportion of hematopoietic tissue in the marrow cavity has become smaller, and empty lacunae have increased significantly. (d) At the twelfth week (Group C): Bone trabeculae are disordered, sparse and thin with extensive fractures, fat cells have increased markedly in size and quantity, hematopoietic cells are significantly reduced, and there are a large number of empty lacunae.

increased. The diameter of the bone marrow fat cells was larger and hematopoietic tissue had decreased slightly (Fig. 1b). At the tenth week, the bone trabeculae had thinned significantly, spaces had increased, the structure was mildly disordered, and occasional trabecular fractures were seen. The diameter of the bone marrow fat cells had increased further, a large number of fat cells had accumulated, the proportion of hematopoietic tissue in the marrow cavity had became smaller, and empty lacunae had increased significantly (Fig. 1c). At the twelfth week, the bone trabecular structure was disordered, being sparse and thin with extensive fractures, and there were a large number of empty lacunae. Fat cells had increased markedly in size and quantity, and the number of hematopoietic cells had reduced significantly (Fig. 1d). The image analysis system of Image-pro-plus was used to measure three indexes: ratio of empty lacunae per high power-field, ratio of bone trabecular area per unit field of view area and diameter of fat cells. At the eighth, tenth and twelfth week, there were statistically significant differences in these three parameters between the experimental and control groups (Table 2).

Table 2 Histopathology results at different times ($\bar{x} \pm s$)

Group	n	Fat cell diameter (μm)	Bone trabecular area (%)	Ratio of empty lacunae (%)
Control group	10	23.12 ± 1.44	60.48 ± 3.32	7.77 ± 1.84
Experimental group (8 week)	10	36.97 ± 1.42	47.00 ± 2.01	11.76 ± 2.80
Experimental group (10week)	10	48.54 ± 1.11	35.57 ± 2.11	17.36 ± 2.36
Experimental group (12week)	10	55.46 ± 2.58	28.58 ± 2.17	19.98 ± 2.21
F value		669.66	321.60	55.53
P value		<0.001	<0.001	<0.001

Table 3 The Ct and $2^{-\Delta\Delta Ct}$ values of Runx2, Osterix, and AJ18 gene in experimental and control groups ($\bar{x} \pm s$)

Time	Expressed gene	Model group Ct	Model group reference gene Ct	Normal group Ct	Normal group reference gene Ct	$2^{-\Delta\Delta Ct}$*
8 weeks	Runx2	20.44 ± 2.93	19.88 ± 1.55	19.41 ± 1.70	18.91 ± 1.91	0.9621
	Osterix	19.04 ± 2.18	19.88 ± 1.55	18.02 ± 1.84	18.91 ± 1.91	0.9661
	AJ18	25.30 ± 2.82	19.88 ± 1.55	26.16 ± 1.77	18.91 ± 1.91	3.5487
10 weeks	Runx2	22.17 ± 2.59	20.05 ± 1.41	19.32 ± 1.86	18.82 ± 1.73	0.3259
	Osterix	21.54 ± 2.45	20.05 ± 1.41	18.01 ± 1.84	18.82 ± 1.73	0.2026
	AJ18	24.53 ± 2.85	20.05 ± 1.41	25.16 ± 1.32	18.82 ± 1.73	3.6303
12 weeks	Runx2	22.43 ± 2.62	18.30 ± 0.72	19.30 ± 1.73	19.46 ± 1.26	0.0512
	Osterix	22.56 ± 2.73	18.30 ± 0.72	18.03 ± 1.83	19.46 ± 1.26	0.0194
	AJ18	23.11 ± 1.74	18.30 ± 0.72	26.17 ± 1.74	19.46 ± 1.26	3.7576

*$\Delta\Delta Ct = (Ct_{Model} - Ct_{Model\ Reference}) - (Ct_{Normal} - Ct_{Normal\ Rwference})$; $2^{-\Delta\Delta Ct} < 1$ indicates that genes expression in the experimental group is down-regulated; $2^{-\Delta\Delta Ct} > 1$ indicates that genes expression in the experimental group is up-regulated.

Real-time fluorescence quantitative PCR

At the eighth, tenth and twelfth week, expression of Runx2 and Osterix mRNA in the experimental groups (A, B and C) was less than that in the control group, and it showed a negative correlation with the duration of the experiment. Expression of AJ18 mRNA in the experimental group was higher than that in the control group (Table 3).

Western blot

At the eighth week, protein synthesis of Runx2 and Osterix in the experimental group was less than that in the control group, and there was a declining trend with time. Protein synthesis of AJ18 in the model group was far greater than that in the control group, and it was positively correlated to the time of drug administration and movement (Fig. 2).

Discussion

Since Pietrogrande and Mastromarino first reported in 1957 that the use of glucocorticoids led to FHN[3], steroid-induced FHN has gradually gained the attention of the medical profession. Glucocorticoids are the second most common cause of FHN, trauma being the leading cause, and the incidence of steroid-induced FHN has increased year by year. Many scholars have confirmed that a single large-dose of glucocorticoid can induce a typical animal model of FHN within a short term. Barrueco *et al.*[4] and Li Xiong *et al.*[5] injected prednisolone acetate intramuscularly into healthy SD rats to create animal models of FHN. After killing all animals at the 12th week, light microscopic examination of specimens showed that the bone trabeculae were sparse and broken, the ratio of empty lacunae had increased and sequestra had even appeared, marrow hematopoietic cells had been replaced by a wide range of fat vacuoles, and bone trabecular structures inside the head of femur were significantly damaged. Daniel *et al.* emphasized the important role of mechanical stress in the pathogenesis of steroid-induced FHN[6]. Some foreign scholars believe that rats are not the ideal animal to serve as a model of FHN because of they have so little activity in their cages, so in this experiment rats were forced to train periodically whil being given hormones. Studies have shown that the rate of empty lacunae in the subchondral zone of the femoral head in the normal adult rabbit is 8%–12% and that a greater rate indicates osteonecrosis[7,8]. In a pre-experiment for the present study, of 10 animals treated for 7 weeks, only two developed FHN, so the experimental time was extended to 8 weeks. In this study, two rats of 30 died at the eighth week (the causes of death being considered to be excess hormones and exercise induced weight loss, wasting and organ failure), making the success rate of the model 93.3% (28/30).

In the clinic, glucocorticoids are frequently used for the treatment of severe inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus.

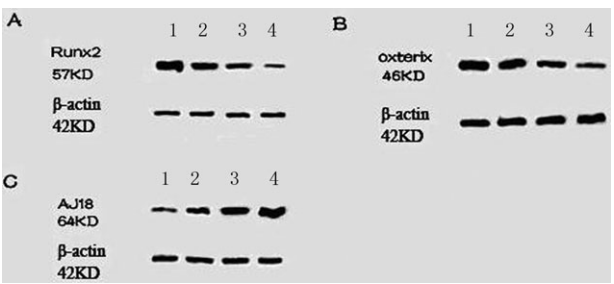

Figure 2 Protein expression of Runx2, Osterix, and AJ18 in the normal group and experimental groups at different times. 1, control group; 2, experimental group A (8 weeks); 3, experimental group B (10 weeks); 4, experimental group C (12 weeks).

However, long-term use of glucocorticoids causes severe osteoporosis and FHN as a secondary effect. In osteoblast cultures, Dex exhibits both negative and positive effects on osteoblast differentiation and mineralization, depending on the degree of cell maturity or cell density, drug concentration and time[9]. Moderate concentrations (1×10^8 mol/l) of Dex promote phenotypic markers of osteoblast differentiation, such as alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OC), and bone sialoprotein (BSP), while concentrations of Dex of $1 \times 10^6 - 1 \times 10^7$ mol/l inhibit phenotypic markers of osteoblasts[2]. Therefore, Dex is a potent stimulator of osteoblast differentiation *in vitro*. However, the mechanism(s) by which Dex promotes osteogenesis remain(s) poorly understood.

In the process of differentiation of bone marrow stromal cells into osteogenic lineage cells, they first differentiate into pre-osteoblasts, and this process requires Runx2. Pre-osteoblast cells express collagen I (Col I) and ALP, but do not express typical osteoblastic marker genes, such as OPN, OC, and BSP mRNA. Runx2 plays a positive regulatory role in the early stage of osteoblast differentiation, but in the later stages it plays a negative regulatory role[10]. Pre-osteoblast cells express Runx2 gene through one or several steps to differentiate into mature osteoblasts which can express the characteristic osteogenic maker genes OC and BSP; this process requires the presence of Osterix and its role[11].

Runx2 is also known as PEBP2αA/AML3/Cbfa1, belongs to the runt-domain gene family and has DNA-binding domains. Runx2 regulates bone marrow stromal cells to shape and differentiate into osteoblasts. Therefore, knocking out the Runx2 gene of rats will result in complete absence of bone formation. Genic mutation of Runx2 in the human is associated with an autosomal dominant genetic disease, namely cleidocranial osteogenesis imperfecta. Overexpression of exogenous Runx2 up-regulates ALP activity, and promotes mRNA expression of BSP and OC. This regulation is thought to occur when Runx2 binds to an osteoblast-specific *cis*-acting element, termed Osterix, in the promoter region of skeletal target genes.

Osterix is a zinc finger transcription factor and the absence of its phenotype has been identified to have greater impact than Runx2 on the late stage of osteoblast differentiation. It has been proved that Osterix is necessary in the late stage of differentiation of immature osteoblasts into mature osteoblasts. In the osteoblast differentiation signal pathway, Osterix plays a role downstream of Runx2[12]. The action of Osterix alone is sufficient to preventC3H10T1/2 and C2C12 cells from differentiating into osteoblasts. At the same time Osterix activates expression of OC and Col I and promotes differentiation of osteoblast-like calls. Overexpression of Osterix is effective at inducing mouse embryonic stem cells[13], bone marrow stromal cells[14] and adipose-derived stem cells[15] to differentiate into bone cells. In Osterix null mice, mesenchymal cells do not deposit bone matrix and no bone formation occurs.

During embryonic development of bone cells, appearance of Osterix occurs later than Runx2. Rat-related tests have found that Osterix is more specific than Runx2 in the role of bone formation. Nakashirna *et al*. found that pre-osteoblasts whose Osterix genes have been knocked out express cartilage cell marker genes, but without osteoblast characteristics[16]. During the process of differentiation of pre-osteoblast cells into osteoblast cells, the role of Osterix is necessary. At present, Runx2 and Osterix have gradually been recognized as the key factors in osteoblast differentiation and maturation as well as in bone cell metabolism, but changes in their expression in steroid-induced FHN have not been reported. In this experiment, it was found that in steroid-induced FHN, expression of Runx2 and Osterix mRNA is decreased, and the amount of corresponding protein is also significant lower than that in a control group.

Transcription factor AJ18, belonging to Krüeppel-associated box domain zinc finger protein, has been cloned from calvarial bone cells of fetus rats induced by BMP-7. *In vitro* experiments have demonstrated that AJ18 can combine with the binding sequence (Osterix) of Runx2, and that overexpression of AJ18 can inhibit the combination of Runx2 and Osterix and down-regulate OC promoter activity. Under stimulation by BMP-7, C3HT101/2 osteoblasts will induce overexpression of AJ18, which can further reduce the activity of ALP[17]. Moreover, in the process of osteoblast differentiation, the expression of AJ18 mRNA is earlier than that of Runx2 mRNA, which indicates that AJ18 may act as a transcription repressor protein to repress the combination of Runx2 and Osterix[18]. The present studies showed that expression of AJ18 was significant greater in the experimental group than in the control group, and this trend increased with lengthier drug administration.

On the basis of all of the above, the pathogenesis of steroid-induced FHN can be supposed to be as follows. Glucocorticoid down-regulates Runx2/Osterix mRNA and up-regulates AJ18 mRNA, which inhibits bone cell activity and bone formation and repair. Then osteoporosis occurs, bone strength decreases, and under stress microfractures occur in the bone trabeculae resulting in ultrastructural changes in them. Repeated such cycles occurring over time eventually lead to FHN.

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Disclosure

No benefits in any form have been, or will be, received from a commercial party related directly or indirectly to the subject of this manuscript.

References

1. Oshina H, Sotome S, Yoshii T, *et al*. Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells. Bone, 2007, 41: 575–583.
2. Mikami Y, Omoteyama K, Kato S, *et al*. Inductive effects of dexamethasone on the mineralization and the osteoblastic gene expressions in mature osteoblast-like ROS17/2.8 cells. Biochem Biophys Res Commun, 2007, 362: 368–373.
3. Pietrogrande V, Mastromarino R. Osteopatia da prolongato trattamen to cortisono. Orthop.Traumatol, 1957, 25: 791–810.
4. Barrueco JL, Gázquez A, Redondo E, *et al*. A histopathological and morphometrical study of femur head cartilage in Wistar rats treated with prednisolone. Histol Histopathol, 1989, 4: 317–323.
5. Li Xiong, Yuan H, Bei ML, *et al*. An experimental research of corticosteroids to ANFH used for a long time or a short time with an ultra-dose (Chin). Zhongguo Gu Yu Guangjie Sunshang Za Zhi, 1999, 14: 241–244.
6. Daniel M, Herman S, Dolinar D, *et al*. Contact stress in hips with osteonecrosis of the femoral head. Clin Orthop Relat Res, 2006, 447: 92–99.
7. Wang KZ, Mao LZ, Hu CG, *et al*. Experimental study on mechanism of steroid-induced avascular necrosis of femoral head. Chinese Journal of Surgery, 1994, 9: 515–517.
8. Wang YS, Mao KY, Li YB. An experimental study on the pathogenesis of alcohol induced avascular necrosis of the femoral head. Chinese Journal of Orthopaedics, 1998, 4, 231–233.
9. Ishida Y, Heersche JN. Glucocorticoid-induced osteoporosis: both *in vivo* and *in vitro* concentrations of glucocorticoids higher than physiological levels attenuate osteoblast differentiation. J Bone Miner Res, 1998, 13: 1822–1826.
10. Kern B, Shen J, Starbuck M, *et al*. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem, 2001, 276: 7101–7107.
11. Liu W, Toyosawa S, Furuichi T, *et al*. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol, 2001, 155: 157–166.
12. Nakashima K, de Crombrugghe B. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet, 2003, 19: 458–466.
13. Tai G, Polak JM, Bishop AE, *et al*. Differentiation of osteoblasts from murine embryonic stem cells by overexpression of the transcriptional factor Osterix. Tissue Eng, 2004, 10: 1456–1466.
14. Tu Q, Valverde P, Chen J. Osterix enhances proliferation and osteogenic potential of bone marrow stromal cells. Biochem Biophys Res Commun, 2006, 341: 1257–1265.
15. Wu L, Wu Y, Lin Y, *et al*. Osteogenic differentiation of adipose derived stem cells promoted by overexpression of Osterix. Mol Cell Biochem, 2007, 301: 83–92.
16. Nakashima K, Zhou X, Kunkel G, *et al*. The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. Cell, 2002, 108: 17–29.
17. Jheon A, Chen J, Teo W, *et al*. Temporal and spatial expression of a novel zinc finger transcription factor, AJ18, in developing murine skeletal tissues. J Histochem Cytochem, 2002, 50: 973–982.
18. Takahashi T, Kamiya N, Kawabata N, *et al*. The effect of retinoic acid on a zinc finger transcription factor, AJ18, during differentiation of a rat clonal preosteoblastic cell line, ROB-C20, into osteoblasts. Arch Oral Biol, 2008, 53: 87–94.