Dexamethasone induces osteogenesis via regulation of hedgehog signalling molecules in rat mesenchymal stem cells

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Abstract

Purpose Hedgehog signalling plays an important role during the development of tissues and organs, including bone and limb. Dexamethasone (DEX), a synthetic and widely used glucocorticoid, affects osteogenesis of bone marrow mesenchymal stem cells (MSCs), while the signalling pathway by which DEX affects osteoblast differentiation remains obscure. This study aimed to investigate expressions of hedgehog signalling molecules Shh, Ihh and Gli1 during DEX-induced osteogenesis of rat MSCs in vitro.

Methods DEX promoted osteoblast differentiation of MSCs at 10^{-8} mol/L from seven days to 21 days, demonstrated by enhancing alkaline phosphatase (ALP) activity and osteoblast-associated marker type I collagen expression during osteoblastic differentiation. Gene and protein expressions of hedgehog signalling molecules, Shh, Ihh and Gli1

were tested by RT-PCR and western blot analysis during osteoblast differentiation.

Results Shh expression was increased compared to the control while Ihh and Gli1 expressions were decreased on both mRNA and protein level during DEX-induced osteoblast differentiation of MSCs from seven days to 21 days. Altogether, these data demonstrate that DEX can enhance Shh expression via a Gli1-independent mechanism during osteoblast differentiation of MSCs.

Conclusions These results indicate that different patterns of hedgehog signalling are involved in DEX-induced osteogenesis and these findings provide insights into the mechanistic link between glucocorticoid-induced osteogenesis and hedgehog signalling pathway.

Introduction

Mesenchymal stem cells (MSCs) constitute a population of pluripotent cells within the bone marrow, which are capable of differentiating into a number of cell lineages including adipocytes, chondrocytes, myocytes and osteoblasts [1]. MSCs have been shown by in vivo transplantation to differentiate into osteoblasts [2, 3]. MSCs are considered as a promising candidate cell source for bone tissue engineering and regeneration. Small molecules that selectively induce osteoblast differentiation would provide useful tools to study the molecular mechanisms of osteoblast differentiation and ultimately might lead to useful therapeutic agents for the treatment of bone diseases, but the mechanisms have yet to be elucidated.

Dexamethasone (DEX), a synthetic form of glucocorticoid, can induce osteoporosis and even pathological fracture [4]. As normal bone turnover depends on a balance between

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osteoblasts and osteoclasts, it is suggested that DEX may suppress osteoblast differentiation both in vivo and in vitro [5]. However, many studies have shown that DEX in vitro enhances the osteoblast differentiation, alkaline phosphatase (ALP) activity and bone mineralisation [6–8]. Whether DEX inhibits or promotes osteoblast differentiation and bone formation in MSCs remains controversial [9], and biological mechanism and signalling pathway by which DEX affects osteoblast differentiation remain obscure.

The hedgehog (Hh) family of signalling molecules is conserved throughout evolution with three secreted proteins that have been identified in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) [10]. The hedgehog developmental signalling pathway is essential for proper embryonic development and functions in distinct tissues during adult life [11]. Recently, the hedgehog pathway has been associated with organ-specific metastasis to the bone [12, 13]. However, there is little available data about the role of hedgehog signalling molecules in osteogenic differentiation of bone marrow mesenchymal stem cells induced by DEX. In this study, we study the expressions of Shh, Ihh and Gli1 during osteoblast differentiation induced by DEX in rat mesenchymal stem cells.

Materials and methods

Experimental animals and rat MSCs isolation

All experimental animals were approved by the Local Ethics Committee for Animal Care and Use of Shandong University in China. Twenty four-week-old Wistar male rats weighing approximately 90–100 g were purchased from Shandong University Animal Centre (Jinan, China). Rats were killed by the cervical dislocation method. Both femora and tibiae were dissected under aseptic conditions. The bone marrow cells were flushed out of the femora and tibiae with Dulbecco's modified Eagle's medium–low glucose (DMEM–LG, Gibco, NY, USA) using a five millilitre syringe. The marrow cells were seeded at a concentration of $5 \times 10^5/\text{cm}^2$ in 30 ml plastic flasks (Corning, USA) containing DMEM–LG supplemented with 10 % foetal calf serum (Gibco, Milan, Italy), 1 % glutamine (Sigma, St. Louis, MO), and 1 % penicillin–streptomycin (Sigma). The cells were then incubated in 5 % CO₂ at 37 °C, and the medium was changed every three days. When the cells reached 80–90 % confluency in the flasks, cells were trypsinised (0.25 % trypsin, GIBCO) and expanded into plates as passage.

Surface antigens of cultured cells

Surface antigens of cultured cells were detected as described previously [14]. The passage 3 MSCs were harvested and the

cell density was adjusted to 1×10^7 cells/ml with PBS. Then the cells were labelled with CD34-PE and CD44-FITC at 4 °C in the dark for 30 minutes, washed once with PBS and subjected to surface antigen assays by flow cytometry.

Induction of osteoblast differentiation in rat MSCs

Rat MSCs from passage 3 were seeded in six-well plates at the concentration of $1 \times 10^5/\text{cm}^2$. After pre-culture for 24 hours, the MSCs were allowed to culture in osteogenesis culture medium (including 10^{-8} mol/L Dexamethasone, 10 mmol/L β -glycerophosphate, 50 $\mu\text{g}/\text{ml}$ ascorbic acid and 50 $\mu\text{g}/\text{ml}$ vitamin C) according to the experimental requirements for up to 21 days [15]. All MSCs were incubated in 5 % CO₂ at 37 °C, and the medium was replaced every three days before harvest.

ALP activity assay

The MSCs were harvested and resuspended in 250 μl of culture supernatants, followed by cell breaking with an ultrasound breaker. Using the ALP Detection Kit (Millipore, German), the ALP activities in the cell supernatants were quantified after the catalytic reaction at 405 nm on a spectrophotometer (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry

MSCs were fixed with 4 % paraformaldehyde in PBS (pH 7.4), permeabilised with 0.5 % Triton X-100 in PBS, and washed with PBS at room temperature. After blocking with 5 % non-fat dry milk in PBS, cells were incubated with the goat anti-type I collagen antibody, one of the osteoblast-associated markers (1:100, Santa Cruz Biotechnology, CA, USA), at 4 °C for 12 hours. Cells proceeded to incubate with rat anti-goat IgG HRP (1:500, Santa Cruz Biotechnology, CA, USA) at 37 °C for one hour and then were washed with PBS three times. Then, the staining was developed with a DAB staining kit according to the manufacturer's protocol (Zhongshan Golden Bridge, Beijing, China) and visualised using a converted microscope.

RNA extraction and RT-PCR

Total RNA was isolated by using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RT-PCR was performed by M-MLV Reverse Transcriptase (Invitrogen, CA, USA) according to the manufacturer's specifications. Briefly, first strand cDNAs were synthesised at 37 °C for one hour in 20 μl reaction mixture using 2 μg isolated mRNA. The serially diluted first-strand cDNA samples were used as templates. β -actin was a normalisation control for RT-PCR synthesis of cDNAs. The primer sequences were listed as follows: Shh: 5'-CAATTACAACCCCGACATCA-3'

Fig. 1 Expression of type I collagen in osteoblast differentiation. MSCs were cultured in the presence of 10^{-8} mol/L Dexamethasone (DEX) for 7, 14 and 21 days. Immunocytochemistry was performed to

examine the expression of type I collagen (positive in brown) (X40). **a–c** Control of **d–f**, respectively. **d–f** The type I collagen positive cells in MSCs treated with 10^{-8} mol/L DEX for 7, 14 and 21 days

(forward) and 5'-AGTCACTCGAA GCTTCACTCC-3' (reverse); Ihh: 5'-TCAGCGATGTGCTCATTTTC-3' (forward) and 5'-CCTCGTGAGAGGAGCATAGG-3' (reverse); Gli1: 5'-TTCAACTCGATGACCCC ACC-3' (forward) and 5'-GGCACTAGAGTTGAGGAATT-3' (reverse). PCR amplification was performed for 30 cycles,

and the cycling conditions were as follows: 94 °C for 30 seconds, 59 °C (β -actin) or 53 °C (Shh, Ihh and Gli1) for 40 s, 72 °C for 45 s, with a final extension at 72 °C for ten minutes. PCR products were assayed by 1 % agarose gel electrophoresis and analysed under UVI gel-image analysis system (UVI, UK). Relative density of objective mRNA was indicated by the ratio of OD_{objective} to OD _{β -actin}.

Fig. 2 Effect of dexamethasone (DEX) on Shh, Ihh and Gli1 mRNA expression in MSCs. MSCs were induced by 10^{-8} mol/L DEX treatment for 0, 7, 14, and 21 days. Shh, Ihh and Gli1 mRNA expression in the cells was detected by RT-PCR (**a**) and was indicated as relative intensity (**b**). Induction by DEX increased Shh mRNA expressions while it decreased Ihh and Gli1 mRNA expression in MSCs. DEX induced Shh, Ihh and Gli1 mRNA expression in a time-dependent manner during osteoblastic differentiation. * $p < 0.05$ versus control group

Western blot analysis

Cells were suspended in standard sodium dodecyl sulphate (SDS) sample buffer. Protein concentrations were determined with a Bio-Rad protein assay kit, using bovine serum albumin (BSA) as reference. Proteins (50 ng) were separated on SDS-polyacrylamide gels electrophoresis (10 % acrylamide), transferred to nitrocellulose membranes, and then probed with goat anti-Shh (ab19897) (1:500, Abcam, USA), rabbit anti-Ihh (ab52919) (1:1000, Abcam, USA), rabbit anti-Gli1(ab49314) (1:500, Abcam, USA) and rabbit anti-GAPDH (1:2000, Sigma, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (ICN, USA). Proteins were visualised with a SuperSignal West Pico chemiluminescence kit (Pierce, USA).

Statistical analysis

Each experiment was performed at least in triplicate. Statistical significance was assessed using Student's *t*-test. Data were presented as mean $\pm$ SEM, and $p < 0.05$ was considered significant.

Fig. 3 Protein expressions of Shh, Ihh and Gli1 during osteoblast differentiation of rat MSCs induced by DEX. MSCs were treated with 10^{-8} mol/L DEX for 7, 14 and 21 days. **a** Induction by DEX increased

Shh expression in MSCs. **b** Induction by DEX decreased Ihh expression in MSCs. **c** Induction by DEX decreased Gli1 expression in MSCs. * $p < 0.05$, induction groups versus non-induction groups

Results

Surface antigen identification of MSCs and multilineage differentiation of MSCs

The isolated cells were identified by flow cytometry for assaying CD34-PE and CD44-FITC as previously described [16]. In the study, the results revealed that isolated cells were CD44⁺ (90.16 %) and CD34⁺ (2.27 %). The surface antigen identification and multilineage differentiation ability supported that these isolated cells were MSCs.

ALP activity and expression of type I collagen in MSCs during osteoblast differentiation induced by DEX

Cells in the mesenchymal condensation differentiate into osteoblast lineage based on simultaneous expression patterns for known osteoblast-associated markers (collagen type I, alkaline phosphatase, osteopontin, bone sialoprotein, PTH1R and osteocalcin) [17]. Here, ALP activity and type I

collagen expression were tested to determine osteogenesis in rat MSCs treated with 10^{-8} mol/L dexamethasone, 10 mmol/L β -glycerophosphate, 50 μ g/ml vitamin C for zero, seven, 14 and 21 days. Treatment with DEX osteoblast differentiation medium for seven, 14, and 21 days significantly enhanced ALP activity (28 ± 1.0 U/L, 78 ± 1.0 U/L, 92 ± 1.0 U/L) compared to the control (14 ± 1.0 U/L) in MSCs ($P < 0.01$). The number of type I collagen-positive cells after DEX treatment significantly increased compared to control ($P < 0.01$) (Fig. 1 a–f).

Shh, Ihh and Gli1 mRNA expression during DEX-induced osteoblast differentiation in MSCs

The hedgehog signalling pathway (Hh) has been associated with the proliferation of MSCs, and played a major role in the induction of osteogenic and chondrogenic differentiations. However, there is little available data about the role of hedgehog signalling molecules in osteogenic differentiation of bone marrow mesenchymal stem cells induced by DEX.

In the nucleus, signalling of hedgehog is mediated by transcription factors of the Gli family: Gli1, Gli2, and Gli3. Gli transcription factors activate or repress downstream targets that mediate hedgehog signalling. Gli3 has been shown to act as a repressor of Ihh target genes in chondrocytes, but the role of other Gli isoforms is less clear in osteogenesis [18]. Our results indicated that mRNA expression of Shh was up-regulated while that of Ihh and Gli1 were down-regulated obviously as compared with that of control cells after 10^{-8} mol/L DEX treatment ($P<0.05$; Fig. 2). Shh, Ihh and Gli1 mRNA expression levels at days zero, seven, 14 and 21 showed time-dependent increases or decreases in rat MSCs after treatment with DEX.

Shh, Ihh and Gli1 protein expression during DEX-induced osteoblast differentiation in MSCs

To confirm that DEX changed expression of genes in the hedgehog signalling pathway, western blot experiments were carried out on Shh, Ihh and Gli1 protein expression. We analysed Shh, Ihh and Gli1 expression in rat MSCs treated with 10^{-8} mol/L DEX for zero, seven, 14 and 21 days. This concentration of DEX significantly increased osteoblast expression of type I collagen. The expression level of Shh increased in the DEX induction group compared with the non-induction group (Fig. 3a) ($p<0.05$). Expressions of Ihh and Gli1 decreased in the induction group compared with the non-induction group (Fig. 3b and c) ($p<0.05$). The expression of the hedgehog target molecule Gli1 obviously decreased in osteogenesis after 21 days DEX induction ($p<0.05$).

Discussion

Dexamethasone (DEX) was identified to selectively induce osteogenesis in multipotent mesenchymal progenitor cells. It has been proven that differentiation induced by 10^{-8} mol/L DEX induces osteogenesis in MSCs [15]. Our study confirmed that DEX promoted osteoblast differentiation in rat mesenchymal stem cells at the concentration of 10^{-8} mol/L from seven days to 21 days, demonstrated by up-regulation ALP activity and expression of osteoblast-associated markers type I collagen.

Hedgehog (Hh) signalling has been implicated in the development of osteoblasts and osteoclasts whose balanced activities are critical for proper bone formation [19–21]. Transcription factors of the Gli family (Gli1, Gli2, and Gli3) activate or repress downstream targets that mediate hedgehog signalling. Gli3 has been shown to act as a repressor of Ihh target genes in chondrocytes, while the role of other Gli isoforms is less clear in osteogenesis. Here, we investigated the expressions of Hh signalling molecules, including Shh, Ihh and Gli1, during osteoblast differentiation induced by 10^{-8} mol/L DEX in MSCs. This study showed that DEX

increased Shh expressions while decreased Ihh and Gli1 expression on both RNA level and protein level during osteoblastic differentiation in MSCs. The expression level of Gli1 was significantly down-regulated after 21 days treatment with DEX, suggesting that DEX enhances Shh expression via a Gli1-independent mechanism during osteoblast differentiation of MSCs. The other Gli transcription factors, Gli2 or Gli3, need to be further studied during osteoblast differentiation of MSCs induced by DEX. Our results suggested that Shh and Ihh signalling has been implicated in the development of osteoblast differentiation induced by DEX whose balanced activities may be critical for proper bone formation.

Some clinical evidence showed that DEX was an effective adjuvant to bone healing [22]. Corticosteroid injection into unicameral bone cysts has been a standard treatment for many years and has been demonstrated to promote bony healing of the lesion. Patients with cranial injuries often receive high doses of DEX. They also frequently form heterotopic bone. Whether DEX administration contributes to this excess bone formation is not known; our results provided insights into the mechanistic link between glucocorticoid-induced osteogenesis and hedgehog signalling pathway, which is a subject of clinical interest.

In summary, these results demonstrate that DEX can affect Shh, Ihh and Gli1 expression during osteogenic differentiation of MSCs confirming and extending the findings of the hedgehog signalling pathway's involvement in osteoblasts differentiation [23–25]. These findings provide insights into the mechanistic link between the hedgehog signalling pathway and DEX-induced osteoblast differentiation of MSCs. Since it is still not known what the mechanism of the hedgehog signalling pathway's involvement in DEX-induced osteoblast differentiation of MSCs is, further studies will need to identify additional details about this association.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Beyer Nardi N, da Silva ML (2006) Mesenchymal stem cells: isolation, in vitro expansion and characterization. *Handb Exp Pharmacol* 174:249–282
2. Cordonnier T, Langonné A, Sohier J, Layrolle P, Rosset P, Sensébé L, Deschaseaux F (2011) Consistent osteoblast differentiation of human mesenchymal stem cells with bone morphogenetic protein 4 and low serum. *Tissue Eng Part C Methods* 17(3):249–259
3. Sivasubramaniyan K, Lehnen D, Ghazanfari R, Sobiesiak M, Harichandan A, Mortha E, Petkova N, Grimm S, Cerabona F, de

- Zwart P, Abele H, Aicher WK, Faul C, Kanz L, Bühring HJ (2012) Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. *Ann NY Acad Sci* 1266:94–106
4. Woolf AD (2007) An update on glucocorticoid-induced osteoporosis. *Curr Opin Rheumatol* 19(4):370–375
5. Canalis E (2005) Mechanisms of glucocorticoid action in bone. *Curr Osteoporos Rep* 3(3):98–102
6. Chen TL (2004) Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. *Bone* 35(1):83–95
7. Eijken M, Koedam M, van Driel M, Buurman CJ, Pols HA, van Leeuwen JP (2006) The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. *Mol Cell Endocrinol* 248(1–2):87–93
8. Liu Y, Porta A, Peng X, Gengaro K, Cunningham EB, Li H, Dominguez LA, Bellido T, Christakos S (2004) Prevention of glucocorticoid-induced apoptosis in osteocytes and osteoblasts by calbindin-D28k. *J Bone Miner Res* 19(3):479–490
9. Cordero D, Marcucio R, Hu D (2004) Temporal perturbations in sonic hedgehog signaling elicit the spectrum of holoprosencephaly phenotypes. *J Clin Invest* 114(4):485–494
10. Hong D, Chen HX, Ge RS, Li JC (2008) The biological roles of extracellular and intracytoplasmic glucocorticoids in skeletal cells. *J Steroid Biochem Mol Biol* 111(3–5):164–170
11. Soltanoff CS, Yang S, Chen W, Li YP (2009) Signaling networks that control the lineage commitment and differentiation of bone cells. *Crit Rev Eukaryot Gene Expr* 19(1):1–46
12. Sterling JA, Oyajobi BO, Grubbs B, Padalecki SS, Munoz SA, Gupta A, Story B, Zhao M, Mundy GR (2006) The hedgehog signaling molecule Gli2 induces parathyroid hormone-related peptide expression and osteolysis in metastatic human breast cancer cells. *Cancer Res* 66(15):7548–7553
13. James AW, Leucht P, Levi B, Carre AL, Xu Y, Helms JA, Longaker MT (2010) Sonic hedgehog influences the balance of osteogenesis and adipogenesis in mouse adipose-derived stromal cells. *Tissue Eng Part A* 16(8):2605–2616
14. Lenton K, James AW, Manu A, Brugmann SA, Birker D, Nelson ER, Leucht P, Helms JA, Longaker MT (2011) Indian hedgehog positively regulates calvarial ossification and modulates bone morphogenetic protein signaling. *Genesis* 49(10):784–796
15. Levi B, James AW, Nelson ER, Brugmann SA, Sorkin M, Manu A, Longaker MT (2011) Role of Indian hedgehog signaling in palatal osteogenesis. *Plast Reconstr Surg* 127(3):1182–1190
16. Wan XC, Liu CP, Li M, Hong D, Li DM, Chen HX, Li JC (2008) Staphylococcal enterotoxin C injection in combination with ascorbic acid promotes the differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts in vitro. *Biochem Biophys Res Commun* 373(4):488–492
17. Hong D, Chen HX, Xue Y, Li DM, Wan XC, Ge R, Li JC (2009) Osteoblastogenic effects of dexamethasone through upregulation of TAZ expression in rat mesenchymal stem cells. *J Steroid Biochem Mol Biol* 116(1–2):86–92
18. Luu YK, Capilla E, Rosen CJ, Gilsanz V, Pessin JE, Judex S, Rubin CT (2009) Mechanical stimulation of mesenchymal stem cell proliferation and differentiation promotes osteogenesis while preventing dietary-induced obesity. *J Bone Miner Res* 24(1):50–61
19. Vinatier C, Bouffi C, Merceron C, Gordeladze J, Brondello JM, Jorgensen C, Weiss P, Guicheux J, Noël D (2009) Cartilage tissue engineering: towards a biomaterial-assisted mesenchymal stem cell therapy. *Current Stem Cell Res Ther* 4(4):318–329
20. Kesper DA, Didt-Kozziel L, Vortkamp A (2010) Gli2 activator function in preosteoblasts is sufficient to mediate Ihh-dependent osteoblast differentiation, whereas the repressor function of Gli2 is dispensable for endochondral ossification. *Dev Dyn* 239(6):1818–1826
21. Warzecha J, Göttig S, Brünig C, Lindhorst E, Arabmollahagh M, Kurth A (2006) Sonic hedgehog protein promotes proliferation and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells in vitro. *J Orthop Sci* 11(5):491–496
22. Miller MA, Ivkovic A, Porter R, Harris MB, Estok DM II, Smith RM, Evans CH, Vrahas MS (2011) Autologous bone grafting on steroids: preliminary clinical results. A novel treatment for nonunions and segmental bone defects. *Int Orthop* 35(4):599–605
23. Omenetti A, Yang L, Li YX, McCall SJ, Jung Y, Sicklick JK, Huang J, Choi S, Suzuki A, Diehl AM (2007) Hedgehog mediated mesenchymal–epithelial interactions modulate hepatic response to bile duct ligation. *Lab Invest* 87(5):499–514
24. Hammond CL, Schulte-Merker S (2009) Two populations of endochondral osteoblasts with differential sensitivity to hedgehog signaling. *Development* 136(23):3991–4000
25. Mak KK, Bi Y, Wan C, Chuang PT, Clemens T, Young M, Yang Y (2008) Hedgehog signaling in mature osteoblasts regulates bone formation and resorption by controlling PTHrP and RANKL expression. *Dev Cell* 14(5):674–688