Original article

Is 1, 25-dihydroxyvitamin D_3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells *in vitro*?

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Keywords: *human adipose tissue-derived stromal cell; osteoblast; 1, 25-dihydroxyvitamin D_3; dexamethasone*

Background Human adipose tissue-derived stromal cells (hADSCs) can be induced to differentiate along an osteoblastic lineage under stimulation of dexamethasone (DEX). Recent studies, however, have questioned the efficacy of glucocorticoids such as DEX in mediating the osteogenesis process of skeletal progenitor cells and processed lipoaspirate cells. Is it possible to find a substitute for DEX? Therefore, this study was designed to investigate osteogenic capacity and regulating mechanisms for osteoblastic differentiation of hADSCs by comparing osteogenic media (OM) containing either 1, 25-dihydroxyvitamin D_3 (VD) or DEX and determine if VD was an ideal substitute for DEX as an induction agent for the osteogenesis of hADSCs.

Methods Osteogenic differentiation of hADSCs was induced by osteogenic medium (OM) containing either 10 nmol/L VD or 100 nmol/L DEX. Differentiation of hADSCs into osteoblastic lineage was identified by alkaline phosphatase (ALP) staining, von Kossa staining, and reverse transcription-polymerase chain reaction assays for mRNA expression of osteogenesis-related genes such as type I collagen (COL I), bone sialoprotein (BSP), osteocalcin (OC), bone morphogenetic protein (BMP)-2, BMP-4, BMP-6, BMP-7, runt-related transcription factor 2/core binding factor α1 (Runx2/Cbfa1), osterix (Osx), and LIM mineralization protein-1 (LMP-1).

Results von Kossa staining revealed that the differentiated cells induced by both VD and DEX were mineralized *in vitro*. They also expressed osteoblast-related markers, such as ALP, COL I, BSP, and OC. Runx2/Cbfa1, Osx, BMP-6, and LMP-1 were upregulated during VD and DEX-induced hADSC osteoblastic differentiation, but BMP-4, BMP-7 were not. BMP-2 was only expressed in VD-induced differentiated cells.

Conclusions VD or DEX-induced hADSCs differentiate toward the osteoblastic lineage *in vitro*. Runx2/Cbfa1, Osx, BMP-2, BMP-6, and LMP-1 are involved in regulating osteoblastic differentiation of hADSCs, but BMP-4, BMP-7 are not. VD, but not DEX, induces expression of BMP-2 during osteogenic induction of hADSCs. VD is an ideal substitute for DEX for osteogenic induction of hADSCs.

Chin Med J 2006; 119(15):1278-1286

Recent studies suggest that human adipose tissue contains pluripotent stem cells similar to bone marrow-derived stem cells.[1] Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains a stroma that is easily isolated. Compared with cells harvested from bone marrow stroma, human adipose tissue-derived stromal cells (hADSCs) are easier to obtain, have a relatively lower donor site morbidity, and can provide a large quantity of stem cells at harvest.[2] Because of their abundance and accessibility, hADSCs might be a novel and promising cell therapeutic for bone repair and regeneration.[3]

Dexamethasone (DEX)-containing osteogenic medium (OM) is normally used for osteoblastic differentiation of mesenchymal stem cells (MSCs) and hADSCs.[2,4] However, recent studies have questioned the efficacy of glucocorticoids such as DEX in mediating the osteogenesis process of skeletal progenitor cells[5] and processed lipoaspirate cells.[1] Therefore, 1, 25-dihydroxyvitamin D_3 (VD) was added to OM containing DEX.[6] Few studies,

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This study was supported by a grant from the National Natural Science Foundation of China (No. 30200319).

however, have used VD without DEX to induce osteoblastic differentiation.[7] Is there a substitute for DEX? Erben et al[8] reported that short-term VD treatment creates new bone remodeling units and augments osteoblast recruitment and osteoblast team performance in rat cancellous bone. Also, VD is used clinically for the prevention and treatment of osteoporosis.[9] This suggests that VD might be a promising inducer of hADSC osteogenic differentiation. However, the osteogenic potential of hADSCs and the regulating mechanisms remain unclear due to the complex processes and the lack of adequate studies.

Therefore, the purpose of this study was to confirm: (1) whether VD stimulation induces hADSCs to differentiate along an osteoblastic lineage, (2) whether the regulating factors related to bone formation and development are involved in hADSCs osteogenic differentiation under either VD or DEX stimulation, and (3) whether VD is an ideal substitute for DEX as an induction agent for osteogenic differentiation of hADSCs.

METHODS

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). TRIZOL Reagent and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Oligo dT Primer and RNase-Free DNase I were purchased from Promega (Madison, WI, USA). Taq DNA polymerase and dNTP were purchased from GIBCO-BRL (Grand Island, NY, USA).

Cell origin, isolation, and culturing of hADSCs

Human adipose tissue was obtained with informed consent from 6 healthy patients (age: 25—55 years) who had liposuction surgery for esthetic reasons in the Plastic Surgery Hospital Affiliated to the Chinese Academy of Medical Science as approved by the Ethics Committee of Peking University Health Science Center. No diabetes, hepatitis, metabolic diseases, or other systemic complications were reported for these donors.

Isolation and culturing of hADSCs was performed as described by Zuk et al.[2] Briefly, the liposuction tissue was digested with 0.075% type I collagenase and the stromal cell pellet was obtained by centrifugation. The pellet was then resuspended in 160 mmol/L NH_4Cl to lyse contaminating red blood cells. The stromal cells were collected again by centrifugation, then filtered through a 100-μm nylon mesh to remove cellular debris, and resuspended and plated in control medium (containing DMEM, 10% FBS, and antibiotics) in tissue culture flasks. The initial plating density was approximately 3.5×10^3 cells/cm^2. The primary cells were incubated overnight at 37°C /5%CO_2. Following the initial incubation, the flasks were washed extensively with phosphate-buffered saline (PBS) to remove residual nonadherent red blood cells.

The stromal cells were then cultured for 7 to 10 days until they reached confluence. The cells were then harvested by digestion with 0.5 mmol/L EDTA/0.05% trypsin, centrifuged at 1200 r/min for 5 minutes, resuspended in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and plated at a density of approximately 1×10^4 cells/cm^2 Cells were cultured to confluence before a second harvesting and replating procedure as described above. At this stage, cells were used immediately for assays or cryopreserved in liquid nitrogen. The majority of studies were performed using freshly isolated cells.

Osteogenic induction

After primary culture in the control medium (DMEM +10% FBS +100 U/ml Penicillin +100 μg/ml streptomycin) and expansion for two passages, the cells were trypsinized and replated onto 6-well tissue culture plates at a density of 10^5 cells per well. The cells were incubated in the control medium for 1 day to adhere them to the plates and then placed in OM containing either DEX (Control Medium +100 nmol/L DEX +0.2 mmol/L ascorbic acid +10 mmol/L β-glycerophosphate [β-GP]) or VD (Control Medium +10 nmol/L VD +0.2 mmol/L ascorbic acid +10 mmol/L β-GP). DEX-containing OM was used as a positive control. The OM was changed every 3 days. Osteogenic differentiation was assessed by von Kossa staining[4] and alkaline phosphatase (ALP) staining[4] from 1 to 3 weeks after initial osteogenic induction. Expression of osteoblast-associated genes such as type I collagen (COL I), bone sialoprotein

Table. Primers and cycling conditions for RT-PCR

Gene (GenBank accession No.)	Sequence (5'→3')	Product size (bp)	Denature (°C)	Annealing (°C)	Extension (°C)	Cycles (times)
GAPDH (BC013852)						
U	TGGTATCGTGGAAGGACTCATGAC	189	95	55	72	35
D	ATGCCAGTGAGCTTCCCGTTCAGC					
COL I (AB209597)						
U	TGACGAGACCAAGAACTG	599	95	55	72	35
D	CCATCCAAACCACTGAAACC					
OC (X53698)						
U	ATGAGAGCCCTCACACTCCTC	297	95	55	72	35
D	CGGGCCGTAGAAGCGCCGATA					
BSP (J05213)						
U	GCTCAGCATTTTGGGAATGGC	614	95	55	72	35
D	CTGCATTGGCTCCAGTGACAC					
Runx2/Cbfa1NM_004348)						
U	GTGGACGAGGCAAGAGTTTCA	698	95	60	72	35
D	TGGCAGGTAGGTGTGGTAGTG					
Osx (AF477981)						
U	CTTCAGTCTTCCCAACTTCTTACAC	486	95	51	72	35
D	ACAAATTGGGTTAGCTACATCTCTG					
BMP-2 (BC069214)						
U	TTGCGGCTGCTCAGCATGTT	315	95	55	72	30
D	CATCTTGCATCTGTTCTCGGAA					
BMP-4 (NM_001202)						
U	ACCTGAGACGGGGAAGAAAA	348	95	60	72	32
D	TTAAAGAGGAAACGAAAAGCA					
BMP-6 (NM_001718)						
U	ACATGGTCATGAGCTTTGTGA	528	95	55	72	30
D	GTAGAGCGATTACGACTCTGT					
BMP-7 (BC008584)						
U	CCAACGTCATCCTGAAGAAATAC	271	95	60	72	37
D	GCTTGTAGGATCTTGTTCATTGG					
LMP-1 (AF345904)						
U	CAGCCGGTTCAGAGCAAAC	214	95	60	72	35
D	GCCAGTCCTCTGTGTTCTCC					

All primer sequences were determined with established GenBank sequences. U: upstream; D: downstream.

(BSP), osteocalcin (OC), bone morphogenetic protein (BMP)-2, BMP-4, BMP-6, BMP-7, Runx2/Cbfa1, osterix (Osx), and LIM mineralization protein-1 (LMP-1) were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) on 3, 7, 14, and 21 days after initial osteogenic induction. ALP staining, von Kossa staining and all gene expression experiments were performed in duplicate and repeated by hADSCs from individual donors.

RNA isolation and RT-PCR

Uninduced and induced culture layers on 3, 7, 14, and 21 days after osteogenic induction were rinsed with cold PBS and immediately lysed using Trizol Reagent. Total RNA was isolated and treated by RNase-free DNase I, and quantified by UV spectrophotometry. For RT-PCR analysis of mRNA expression, 1.0 μg of total RNA (in 20 μl reaction volume) was reverse-transcribed using reverse transcriptase (Superscript II) and oligo-dT primers in a standard reaction. The resultant cDNA (1 μl) was then used as template for PCR amplification (in 25 μl reaction volume) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COL I, BSP, OC, Runx2/Cbfa1, Osx, BMP-2, BMP-4, BMP-6, BMP-7, and LMP-1. The primers used in this investigation are listed in Table and all primer sequences were determined through established GenBank sequences. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as a control for assessing PCR efficiency; 10-μl aliquots of each reaction were evaluated by 2% agarose gel electrophoresis. Ethidium bromide-stained gels were digitally photographed (Kodak, Rochester, NY, USA).

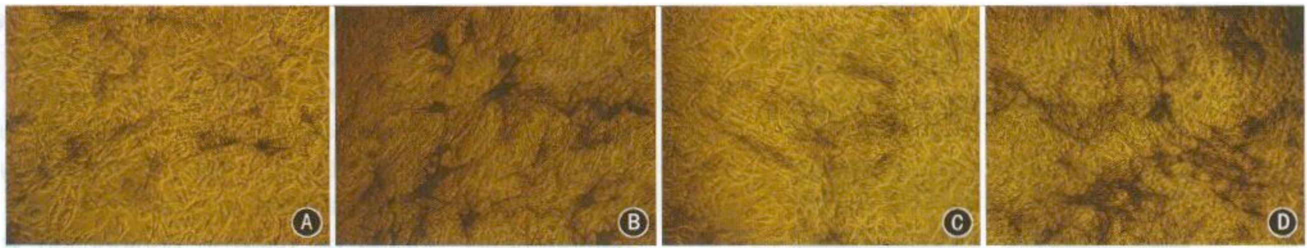

Fig. 1. Alkaline phosphatase (ALP) staining for induced human adipose tissue-derived stromal cells (hADSCs). Osteogenesis was confirmed with ALP staining. The cells stained positively for endogenous ALP activity after 7 or 14 days of culture in osteogenic media containing 1, 25-dihydroxyvitamin D_3 (VD) or dexamethasone (DEX). (**A**) hADSCs induced by DEX for 7 days; (**B**) hADSCs induced by DEX for 14 days; (**C**) hADSCs induced by VD for 7 days; (**D**) hADSCs induced by VD for 14 days. (Original magnification × 100).

RESULTS

hADSCs placed in OM containing either DEX or VD all exhibited changes in cell morphology after 3 days in culture. The cell morphology changed from a spindle-shaped fibroblastic appearance to a rounder, more cuboidal shape and the cells formed an extensive network of dense, multilayered nodules. The induced cells were all stained positively for membrane-bound ALP after 7 days of culture in both OM types, and the staining became stronger after 14 days of culture (Fig. 1). In contrast, undifferentiated hADSCs did not express ALP at any time point. Extracellular mineralization capacity was confirmed by von Kossa staining of the induced cells from both DEX and VD-containing OM after 14 and 21 days of culture (Fig. 2).

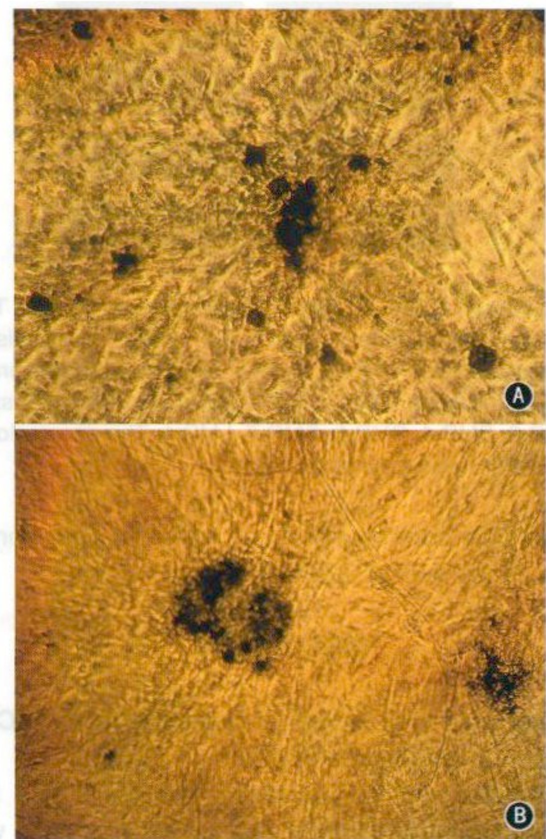

Fig. 2. von Kossa staining for induced human adipose tissue-derived stromal cells (hADSCs). Osteogenesis was also examined by von Kossa staining. Secretion of a calcified extracellular matrix was observed as black nodules: (**A**) hADSCs induced by dexamethasone for 21 days; (**B**) hADSCs induced by 1, 25-dihydroxyvitamin D_3 for 21 days. (Original magnification × 100).

To confirm osteogenesis, the cells were also examined by RT-PCR for the expression of several osteoblast-related genes including COL I, BSP, and OC (Fig. 3). DEX-induced differentiated hADSCs expressed COL I and BSP on 7, 14, and 21 days post-induction, and expressed OC only on day 21. VD-induced differentiated hADSCs expressed COL I on 7, 14, and 21 days post-induction, but expressed BSP and OC on days 14 and 21. To investigate genes that are involved in regulating the osteogenesis of hADSCs, transcription factors such as Runx2/Cbfa1 and Osx, growth factors such as BMP-2, BMP-4, BMP-6, and BMP-7, and the intracellular regulating factor, LMP-1 were also examined by RT-PCR (Fig. 3). DEX-induced differentiated cells expressed Runx2/Cbfa1 and BMP-6 at all time pointspost-induction, Osx at 7 and 14 days post-induction, and LMP-1 only on days 14 after osteogenic induction. BMP-2, BMP-4, BMP-7 expression was not observed during the induction course. VD-induced differentiated cells expressed BMP-6 from days 3 to 21, Runx2/Cbfa1 and LMP-1 from days 3 to 14, Osx from days 7 to 21, and BMP-2 only at 21 days post-induction. They did not express BMP-4, BMP-7, however, during the induction

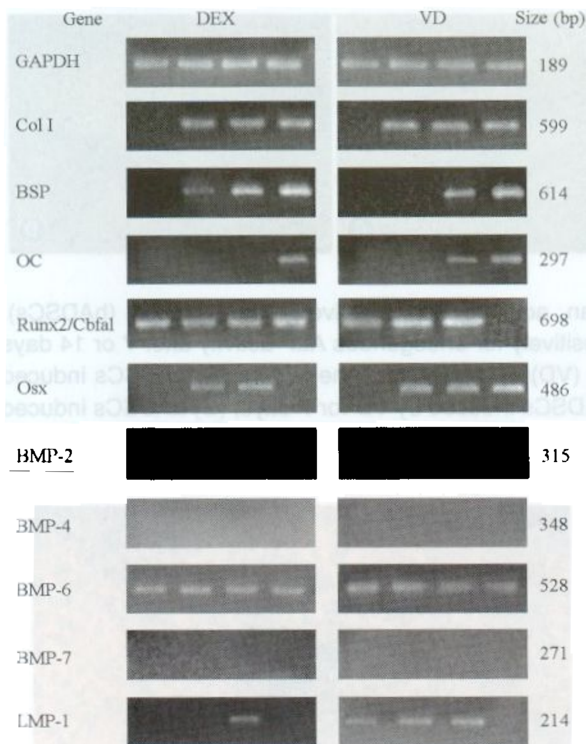

Fig. 3. RT-PCR of osteogenesis-related genes. To confirm osteogenesis, induced cells are also exmamined by RT-PCR for the expression of several genes including COL I, BSP, OC, Runx2/cbfa1, Osx, BMP-2, BMP-4, BMP-6, BMP-7, LMP-1. The expression of GAPDH was used as a control.

course. The control uninduced cells did not express these genes except GAPDH.

DISCUSSION

Comparison of osteogenic capacity of hADSCs cultured in two types of OM

Differentiation of bone marrow-derived MSCs into osteoblasts is induced *in vitro* by treating cells with very low concentrations of DEX, ascorbic acid, and β-glycerophosphate.[4] Their differentiation into osteoblasts is normally characterized by ALP enzyme activity, extracellular mineralization, and the expression of osteoblast-associated genes.[4,7] To confirm the osteogenic capacity of hADSCs, the cells were examined for these characteristics after osteogenic differentiaion. ALP is a membrane-bound enzyme abundant early in bone formation, and increased ALP levels correlate with increased bone formation.[10] After culturing in both types of OM for 7 and 14 days, the induced hADSCs formed an extensive network of dense nodules that stained positively for ALP. There was no significant difference in ALP expression between the two groups of hADSCs. Although ALP expression is strongly upregulated in osteogenic tissues, it is expressed in several nonosteogenic cell types and tissues such as cartilage, liver, and kidney.[11] Therefore, ALP expression, as an indicator of osteogenesis, should be used in conjunction with other osteogenesis-associated markers.

To further verify the osteogenic capacity of hADSCs, the expression of several osteoblast-related genes including COL I, BSP, and OC was also examined by RT-PCR assays. COL I, as an early osteogenic marker, is the most abundant ECM protein secreted by osteoblasts and is the basis for matrix mineralization.[12] Both VD and DEX-induced differentiated hADSCs expressed COL I from 7 to 21 days post-induction. There was no significant difference between cells from the two types of OM. Although COL I expression is strongly suggestive of osteogenesis, it is not considered to be a specific marker for osteogenic differentiation.[12]

Compared with COL I, BSP and OC are more specific for osteoblastic differentiation. BSP expression marks the middle stage of osteoblastic differentiation. BSP is reported to nucleate hydroxyapatite deposition and to represent an early stage of matrix mineralization.[12] In the present report, DEX-induced differentiated hADSCs expressed BSP at 7, 14, and 21 days post-induction; however, VD-induced differentiated hADSCs expressed BSP at 14 and 21 days post-induction. The reason for earlier BSP expression in OM containing DEX is that BSP expression might be more sensitive to DEX stimulation compared with VD stimulation. Beresford et al[13] reported that VD increased the expression of ALP and osteopontin mRNAs, but had no significant effect on BSP expression in human marrow stromal cells. OC is a late bone marker that is secreted only by osteoblasts and signals terminal osteoblastic differentiation and represents matrix maturation.[12] Human marrow stromal cells[12] or hADSCs[1] induced by DEX do not express OC during the induction course because DEX inhibits OC expression by induced marrow stromal cells and hADSCs. For human marrow stromal cells,[13] the addition of VD to DEX-containing OM recovers OC expression. In contrast, in the present study, very late OC expression was observed at 21 days post-induction

in DEX-containing OM, but in VD-containing OM, OC expression was observed at 14 and 21 days post-induction. This finding demonstrates that OC expression in induced hADSCs occurred earlier in VD-containing OM compared with DEX-containing OM indicating that the differentiated cells in VD-containing OM might mature earlier than those in DEX-containing OM. On the other hand, the reason that OC expression occurs earlier in VD might be that VD can specifically drive OC expression because there is a VD response element in the promoter region of OC that enhances OC transcription.[14] This indicates a special action of VD on OC expression in hADSCs osteogenic differentiation.

In addition to matrix secretion and matrix maturation mentioned above, osteogenic differentiation of hADSCs should also be examined by matrix mineralization which is the last phase in the bone developmental sequence.[15] The identification of a calcified ECM in culture layers is normally assessed by von Kossa staining.[4,7] Calcification nodules appear as black regions within the cell layers. Consistent with osteogenesis, several black nodules, indicative of a calcified ECM, were identified in hADSCs layers treated for 14 and 21 days in both types of OM, whereas there was no calcification observed in undifferentiated hADSCs. Because calcium deposition was not quantitatively measured in this study, no difference of calcification capacity between the two OM were detected.

Taken together, ALP, COL I, BSP, and OC expression in induced hADSCs and the production of a calcified ECM strongly suggest that these adipose tissue-derived cells can be induced toward an osteoblastic lineage by VD or DEX. There were no obvious differences detected in the osteogenic capacity of hADSCs with respect to BSP and OC expression during osteogenic induction by VD or DEX.

The effects of VD and DEX on the expression of regulating factors during osteoblastic differentiation of hADSCs

To investigate the genes involved in regulating hADSC osteogenesis, osteoblast-specific transcription factors such as Runx2/Cbfa1 and Osx were examined by RT-PCR. Runx2/Cbfa1 is the primary transcription factor for osteoblastic differentiation because osteoblast differentiation is arrested in both endochondral and intramembranous skeletons in Runx2/Cbfa1 null mice.[16] Runx2/Cbfa1 binding sites are present, sometimes multiple times, in the promoter region of most genes expressed in osteoblasts.[17] This results in the expression of osteoblast-specific genes such as OC and BSP in osteoblastic differentiation and indicates that Runx2/Cbfa1 positively controls OC and BSP expression.

Osx is another transcription factor that is specifically expressed in all developing bones.[18] In Osx null mice, no bone formation occurs. In Osx null mice, however, Runx2/Cbfa1 is expressed, suggesting that Osx acts downstream of Runx2/Cbfa1.[19] These findings indicate that one possible mechanism leading to the osteogenesis of hADSCs might be the upregulation of important transcription factors such as Runx2/Cbfa1 and Osx *in vitro*.

RT-PCR data demonstrated the upregulation of both Runx2/Cbfa1 and Osx during hADSC osteogenic induction in both OM. These observations indicate that induction of Cbfal and Osx mRNAs by DEX or VD stimulation might be followed by activation of osteoblast marker genes such as OC and BSP mRNAs to produce a bone-specific matrix that subsequently becomes mineralized. Therefore, the upregulated expression of Runx2/Cbfa1 and Osx in induced hADSCs, combined with the positive expression of OC, BSP, and COL I, signifies that the two transcription factors have very crucial roles in hADSC osteogenic differentiation induced by DEX or VD. This is consistent with what is found in osteogenic induction of MSCs.[17]

To further determine the regulating mechanism that acts upstream of Runx2/Cbfa1 in hADSC osteogenic differentiation, BMPs and the intracellular regulating factor LMP-1 were also examined by RT-PCR.

BMPs stimulate ectopic or orthotopic bone formation activity and have a very essential role in the formation of skeletal tissues.[20] They are the only growth factors with a potent ability to stimulate differentiation of human MSCs into an osteoblastic or chondrogenic orientation.[21] Recombinant human BMP-2, BMP-4, BMP-6, and BMP-7 stimulate osteogenesis *in vitro* and *in vivo*.[20, 22] Therefore, detection of BMP expression during hADSC

osteoblastic differentiation is very beneficial for understanding the osteogenesis process or mechanism

In this report, BMP-6 was observed at all time points post-induction in the differentiated hADSCs under DEX stimulation, whereas BMP-2, 4, and 7 were not expressed during the induction course. This result suggested a special role of BMP-6 in DEX-induced hADSC osteogenesis and was consistent with studies of rat calvarial osteoblast and human MSCs *in vitro*. Boden et al[23] reported that glucocorticoid-induced formation of bone nodules in fetal rat calvarial osteoblasts was mediated mainly by BMP-6. Glucocorticoids preferentially increased expression of BMP-6 mRNA, and the antisense oligonucleotide corresponding to BMP-6 strongly inhibited formation of bone nodules. Liu et al[24] reported that the addition of DEX to human MSCs resulted in a 16-fold increase in BMP-6 mRNA levels 24 hours after treatment and demonstrated that human BMP-6 transcription was regulated by glucocorticoids at the upstream transcription start site. This finding might account for the rapid and persistent increase in BMP-6 mRNA levels following DEX treatment of hADSCs in the present study.

Interestingly, the early and persistent expression of BMP-6 mRNA was also observed following VD treatment of hADSCs. This also indicates the special role of BMP-6 in hADSC osteogenesis under VD stimulation. In addition to the expression of BMP-6 at all time points post-induction, there was also late BMP-2 expression following VD treatment of hADSCs. Because BMP-2 is one of the most potent osteoinductive agents *in vitro* and *vivo*, BMP-2 expression following VD treatment of hADSCs, but not following DEX treatment of hADSCs, indicates that VD might be a more potent inducer of hADSC osteogenic differentiation *in vivo* and *in vitro* compared with DEX. In this study, the expression of other BMPs, like BMP-4 or BMP-7 was also examined. Although BMP-4 and BMP-7 are also effective growth factors in terms of osteogenesis and osseous defect repair,[20,22] their potential for hADSC osteogenesis is still in question because they are not expressed during the induction course.

LMP-1 was discovered as a novel intracellular protein in rats by Boden et al.[25] It is an intracellular regulator of osteoblast differentiation. When LMP-1 expression is blocked, differentiation and bone nodule formation do not occur in calvarial osteoblast cultures. When LMP-1 is overexpressed using the mammalian expression vector pCMV2, osteoblast differentiation is initiated in resting calvarial osteoblast cultures.[26] In the current study, the differentiated cells under DEX or VD stimulation expressed LMP-1. The data presented here strongly support a possible role of LMP-1 in the regulation of the complex program during osteoblast differentiation of hADSCs induced by DEX or VD. The precise intracellular mechanism of LMP-1, however, is unknown.

LMP-1 might work in conjunction with BMPs.[26] The association of LMP-1 with other proteins like BMP suggests another pathway of osteoblast differentiation. Boden et al[26] reported that in an osteoblast culture system glucocorticoids induced LMP-1 through the action of BMP-6. The reason that LMP-1 is regulated by BMP-6 and not by BMP-2, BMP-4, or BMP-7 might be consistent with its unique role early in osteoblast differentiation because BMP-6 is the earliest of these BMPs to be expressed during differentiation in their culture system.[23] These findings were consistent with our findings of hADSC osteogenesis under induction of DEX or VD. The present results demonstrate that only BMP-6, not BMP-2, BMP-4, or BMP-7, was expressed during the induction course and LMP-1 expression was positively detected, indicating that BMP-6 and LMP-1 have a very important role in osteoblastic differentiation of hADSCs and that LMP-1 might also be regulated by BMP-6. The relationship between BMP-6 and LMP-1 in the osteogenic program of hADSCs, however, was not examined in the current study and requires further investigation.

It is not known how LMP-1 regulates the osteogenic process downstream. LMP-1 seems to initiate a cascade of events. LMP-1 might upregulate the osteoblast transcription factor Runx2/Cbfa1,[26] suggesting a vital role of LMP-1 in the osteogenesis of hADSCs. The precise intracellular mechanism of LMP-1, however, requires further investigation. This protein appears to activate an intracellular switch that induces secretion of soluble factors like BMP-2 that initiate and promote osteoblast differentiation during embryogenesis and adult bone formation.[25]

Overexpression of LMP-1 in transfected cells might lead to elevated levels of BMP-2, BMP-4, BMP-6, and BMP-7 expression, which conveys its osteogenic effects on osteoblastic differentiation.[27] On one hand, LMP-1 is regulated by BMP-6, but on the other hand, LMP-1 induces the expression of many osteoinductive BMPs. Thus, LMP-1 is a very crucial coordinating factor that contributes to bone formation or osteoblastic differentiation.

In this study, there were differences in the hADSC expression of LMP-1 and BMP between the two types of OM. During the osteogenesis of hADSCs induced by VD, LMP-1 was expressed earlier and persisted longer compared with those induced by DEX. Stimulated by VD, these differentiated cells not only expressed BMP-6 early and persistently, but they also expressed BMP-2 in the late phase during which expression was not detected in cells induced by DEX. BMP-2 expression was detected just after LMP-1 expression and thus might have been switched on by LMP-1. This hypothesis, however, should be further verified.

In the osteogenesis of hADSCs induced by DEX, however, no BMP expression, except that of BMP-6, was observed. Furthermore, LMP-1 expression persisted transiently under DEX stimulation. Therefore, based on investigation of the expression of the above-mentioned regulating factors, VD is an ideal inducer of osteogenic differentiation of hADSCs compared with DEX. In terms of the relationship between LMP-1 and BMP during the osteogenesis of hADSCs, however, further studies are needed to elucidate this complex process.

Taken together, the present findings indicate that VD and DEX-stimulated hADSCs differentiate into osteoblasts. The upregulation of BMPs, LMP-1, Runx2/Cbfa1, and Osx might have a very important role in both osteogenic induction processes of hADSCs. The regulating mechanism of these factors, however, requires further investigation to improve its osteogenic efficiency and capacity. Comparison of the expression of regulating genes under VD or DEX stimulation, the early and persistent expression of LMP-1, and the positive expression of BMP-2 induced by VD indicates that VD might be an ideal inducing agent for the osteogenic differentiation of hADSCs instead of DEX.

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(Received December 18, 2005)
Edited by LIU Dong-yun