Journal of Cellular Biochemistry 96:533–542 (2005)

Induction of Osteogenic Differentiation of Human Mesenchymal Stem Cells by Histone Deacetylase Inhibitors

Hyun Hwa Cho,[1,2] Hyung Taek Park,[1] Yeon Jeong Kim,[1] Yong Chan Bae,[3] Kuen Taek Suh,[4] and Jin Sup Jung[1,2]*

[1]Department of Physiology, College of Medicine, Pusan National University, Pusan 602-739, Korea
[2]Medical Research Institute, Pusan National University, Pusan 602-739, Korea
[3]Department of Plastic Surgery, College of Medicine, Pusan National University, Pusan 602-739, Korea
[4]Department of Orthopedic Surgery, College of Medicine, Pusan National University, Pusan 602-739, Korea

Abstract Valproic acid (VPA) has been used as an anticonvulsant agent for the treatment of epilepsy, as well as a mood stabilizer for the treatment of bipolar disorder, for several decades. The mechanism of action for these effects remains to be elucidated and is most likely multifactorial. Recently, VPA has been reported to inhibit histone deacetylase (HDAC) and HDAC has been reported to play roles in differentiation of mammalian cells. In this study, the effects of HDAC inhibitors on differentiation and proliferation of human adipose tissue-derived stromal cells (hADSC) and bone marrow stromal cells (hBMSC) were determined. VPA increased osteogenic differentiation in a dose dependent manner. The pretreatment of VPA before induction of differentiation also showed stimulatory effects on osteogenic differentiation of hMSC. Trichostatin A (TSA), another HDAC inhibitor, also increased osteogenic differentiation, whereas valpromide (VPM), a structural analog of VPA which does not possess HDAC inhibitory effects, did not show any effect on osteogenic differentiation on hADSC. RT-PCR and Real-time PCR analysis revealed that VPA treatment increased osterix, osteopontin, BMP-2, and Runx2 expression. The addition of noggin inhibited VPA-induced potentiation of osteogenic differentiation. VPA inhibited proliferation of hADSC and hBMSC. Our results suggest that VPA enhance osteogenic differentiation, probably due to inhibition of HDAC, and could be useful for in vivo bone engineering using hMSC. J. Cell. Biochem. 96: 533–542, 2005.

Key words: valproic acid; adipose-tissue derived stromal cells; bone marrow stromal cells; histone deacetylase; osteogenic differentiation; BMP-2

Valproic acid (VPA; 2-propyl-pentanoic acid) is a short-chain branched fatty acid that has been initially discovered as an anticonvulsant and has been used today to treat a variety of seizure disorders as well as bipolar disorders (e.g., manic depressive illness). A number of targets for VPA action in bipolar disorder and epilepsy targets have been proposed [Johannessen, 2000; Gurvich and Klein, 2002]. VPA was shown to inhibit histone deacetylases (HDACs) at therapeutic concentrations [Gottlicher et al., 2001; Phiel et al., 2001]. Acetylation of the NH_2-terminal tails of the core histones is central to the specification of a "histone code" that influences the expression of target genes [Strahl and Allis, 2000; Thiagalingam et al., 2003]. Recruitment of histone acetyltransferases by transcription factor complexes is associated with a more open DNA conformation that, in general, facilitates transcription of target genes. Conversely, deacetylation of core histones by HDACs is associated with a "closed" chromatin conformation and repression of transcription. Therefore, inhibition of HDACs typically leads to derepression of transcription. In addition, inositol depletion

Grant sponsor: The Ministry of Health and Welfare; Grant number: (02-PJ10-PG8-EC01-0018).

*Correspondence to: Jin Sup Jung, MD, PhD, Department of Physiology, College of Medicine, Pusan National University, 1 Ga, Ami-Dong, Suh-Gu, Pusan 602-739, Korea. E-mail: jsjung@pusan.ac.kr

Received 13 February 2005; Accepted 3 May 2005

DOI 10.1002/jcb.20544

contributes to VPA inhibition of sensory neuronal growth cone formation in vitro [Williams et al., 2002].

HDAC inhibitors from diverse origins arrest cell growth and induce differentiation in various in vitro and in vivo models including acute promyelocytic leukemia and cell lines derived from colon, lung, prostate carcinomas [Marks et al., 2001], and cell lines derived from neuroblastoma, glioma, and teratocarcinoma [Cinatl et al., 1996; Blaheta and Cinatl, 2002]. Recently, VPA has been reported to inhibit cardiomyocyte differentiation of embryonic stem cells [Na et al., 2003] and differentiation of human adipocytes [Lagace and Nachtigal, 2004].

Mesenchymal stem cells (MSC), which can be isolated from bone marrow, adipose tissue, and cord blood, are defined as a multipotent cell with limited self-renewal capacity, and can be differentiated into adipocytes, chondrocytes, myoblasts, and osteoblasts [Caplan, 1991; Beresford et al., 1992; Pittenger et al., 1999; Zuk et al., 2001]. Therefore, MSC are an interesting target for use in gene and cell therapy [Banfi et al., 2000] and provide a unique model to better understand early differentiation events because they can differentiate into multiple mesenchymal lineages.

Conventional therapies of autogenous bone grafts, allograft implants, and prosthetic implants have been used to treat these problems. However, these methods are limited by supply and osteogenic potential. Recent advances in cell and molecular biology have enabled researchers in the bone tissue engineering field to incorporate cell and gene therapies. Stem cell therapy using adult-derived stem cells, specifically MSC, is an emerging field for bone regeneration. Genetic modification of MSC or the addition of BMP2 has been tried to improve in vivo bone regenerating ability of MSC in vivo [Lieberman et al., 2002]. Human adipose tissues also have multipotent progenitor cells, which can be differentiated to osteoblasts [Gimble and Guilak, 2003]. Therefore, adipose tissues-derived stromal cells (ADSC) are a good source for bone tissue engineering because of abundance of adipose tissues in human body.

In this study, we examined the effect of VPA on osteogenic differentiation of human ADSC (hADSC) and human bone marrow stromal cells (hBMSC). We find that the pretreatment of VPA increases osteogenic differentiation in hADSC and hBMSC, and that the effect on osteogenic differentiation is, at least in part, mediated by the increase of bone morphogenic protein 2 (BMP2) expression.

MATERIALS AND METHODS

Materials

VPA was dissolved in phosphate buffered saline (PBS), and valpromide (VPM) and trichostatin A (TSA) were dissolved in dimethylsulfoxide (DMSO), and applied as indicated in figure legends. DMSO levels were kept under 0.1%. VPM was a gift from Katwijk Chemie B.V. (Katwijk, The Netherlands).

Cell Culture

After informed consent, leftover materials (heparinized bone marrow cells and adipose tissues) were obtained from six individuals undergoing total hip arthroplasty and elective abdominoplasty. The patients for adipose tissues were 55-year-old male, 24-year-old female, and 43-year-old female. The patients for bone marrow samples were 45-year-old male, 48-year-old female, and 36-year-old female. hBMSC and hADSC were isolated according to the methods described in the previous studies [Lee et al., 2004a,b]. To isolate hADSC, adipose tissues were washed with PBS, and tissues were digested at 37°C for 30 min with 0.075% type I collagenase. Enzyme activity was neutralized with α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS) and centrifuged at 1,200*g* for 10 min to obtain a pellet. The pellet was incubated overnight at 37°C/5% CO_2 in control medium (α-MEM, 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove residual nonadherent cells and maintained at 37°C/5% CO_2 in control medium. Mononuclear cells from bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient (density = 1.077 g/cm^3; Sigma, St. Louis, MO), suspended in α-MEM containing 10% FBS and 100 U/ml of penicillin, 100 μg/ml of streptomycin and seeded at a concentration of 1×10^6 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. When the monolayer of adherent cells has reached confluence, cells were trypsinized (0.25% trypsin; Sigma), resuspended in α-MEM containing 10% FBS, and subcultured at a concentration of 2,000 cells/cm^2.

For the experiment, we used 3rd–5th passages of MSC.

Induction of Differentiation

Osteogenic differentiation was induced by culturing cells in osteogenic medium (OM, 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid in α-MEM) and extracellular matrix calcification was estimated by alizarin red S stain. To determine the effect of VPA, each 6-well plate contained a sample that was not treated with VPA as a control. Osteogenic differentiation was quantified by measurement of total signal of each well that represent stained area and its intensity by an image analysis program (Image Gauge ver 3.1, Fuji, Japan). The data in VPA-treated samples were normalized to the data in control samples of the same 6-well plate and presented as percentage control. Measurements were done in duplicate at each experiment and experiments were repeated from three different donors.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA was isolated from hADSC and reverse transcribed using conventional protocols. PCR amplification was performed using the primer sets. GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′, Runx2 5′-CTCACTACCACACCTACCTG-3′, 5′-TCAATATGGTCGCCAAACAGATTC-3′, alkaline phosphatase 5′-TGAAATATGCCCTGGAGC-3′, 5′-TCACGTTGTTCCTGTTTAG-3′, p21/WAF1 5′-GACACCACTGGAGGGTGACT-3′, 5′-GGCGTTTGGAGTGGTAGAAA-3′, osterix 5′-GCAGCTAGAAGGGAGTGGTG-3′, 5′-GCAGGCAGGTGAACTTCTTC-3′, osteopontin 5′-TTGCAGTGATTTGCTTTTGC-3′, 5′-ACACTATCACCTCGGCCATC-3′. All primer sequences were determined using established GenBank sequences. Duplicate PCR reactions were amplified using primers designed GAPDH as a control for assessing PCR efficiency and for subsequent analysis by agarose gel electrophoresis. The sequence of each PCR product was confirmed using automated sequencing. Non-induced hADSC were examined as a negative control.

Real-Time Polymerase Chain Reaction (RT-PCR)

To determine Runx2 and BMP-2 expression in hMSC, we used a real time PCR method. Briefly, cDNA was synthesized in a reaction containing 1 μg of DNase I treated total RNA of control hADSC and VPA-treated hADSC, oligo dT primer, dNTP, and avian myeloblastosis virus (AMV) reverse transcriptase. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA (no RT control). PCR primers were designed using software PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with published sequence data from the NCBI database. Primer sequences to be used in the experiment were as follows: β-actin, 5′-CTG GTG CCT GGG GCG-3′, 5′-AGC CTC GCC TTT GCC GA-3′; Runx2, 5′- TTA CTT ACA CCC CGC CAG TC-3′, 5′-CAG CGT CAA CAC CAT TC-3′; BMP-2, 5′-CCA CCA TGA AGA ATC TTT GG-3′, 5′-CCA CGT ACA AAG GGT GTC TC-3′.

Real-time quantitation was based on the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture for PCR with the LightCycler Instrument (Roche, Mannheim, Germany). The PCR reactions were carried out in a total volume of 20 μl, which included SYBR Green I with a Taq DNA polymerase reaction buffer, deoxynucleotide triphosphate mixture, 10 mM $MgCl_2$, PCR-grade water, 2 μl of template DNA, and primers. The amplification program consisted of one cycle of 95°C with 60 s hold ("hot start") followed by 45 cycles of 95°C with 0 s hold, specified annealing temperature with 5 s hold, 72°C with 12 s hold, and specified acquisition temperature with 2 s hold. All experiments were conducted three times, and negative and positive controls were included. While no template negative control (H_2O control) was run with every gene specific primer, the no RT-PCR control was run with only one primer pair which can amplify contaminated genomic DNA. Samples were analyzed in duplicate in three independent runs. Statistical significance was determined by ANOVA model. For each primer pair the linearity of detection was confirmed to have a correlation coefficient of at least 0.98 over the detection area by measuring a dilution curve with cDNA isolated from MSC. β-actin mRNA was amplified as an internal control. We used the comparative C_t method to quantify changes in gene expression between control and VPA-treated hADSC where C_t denotes the cycle at which fluorescence above background levels becomes significant. The fold-difference between control and VPA-treated samples is calculated according to $2^{-\Delta\Delta C_t}$. For each control

and VPA-treated sample, ΔC_t represents the difference between C_t of a target gene and the internal control (i.e., β-actin). The $\Delta\Delta C_t$ value represents the difference between ΔC_t values for a control sample and VPA-treated sample.

Evaluation of Cell Proliferation and Cell Viability

To determine the proliferation rate, cells were detached with Hank's balanced salt solution (HBSS) containing 0.5% trypsin and 0.02% EDTA. hADSC and hBMSC were plated at density of 2,000/cm^2 in a 6-well plate, and various concentrations of VPA were added the next day after plating. After 48 h, the cells were trypsinized and stained with 0.4% trypan blue (Sigma). The total cell number and the proportion of dead cells were measured with a hemocytometer. Cell death was determined by the presence of cytoplasmic trypan blue. This experiment was performed in triplicate.

Statistical Analysis

Comparisons of multiple groups were done with ANOVA with Bonferonni correction for multiple comparisons.

RESULTS

VPA Increases Osteogenic Differentiation

To determine whether VPA could affect osteogenic differentiation, hADSC and hBMSC were treated with various concentrations of VPA (0.5–3 mM) during the initial phase of osteogenic differentiation (the first 3 days). The treatment of VPA during the first 3 days of differentiation significantly increased osteogenic differentiation in hADSC and hBMSC in a dose-dependent manner (Fig. 1).

We assumed that the pretreatment of VPA is enough for the enhancement of osteogenic differentiation, because VPA-induced changes

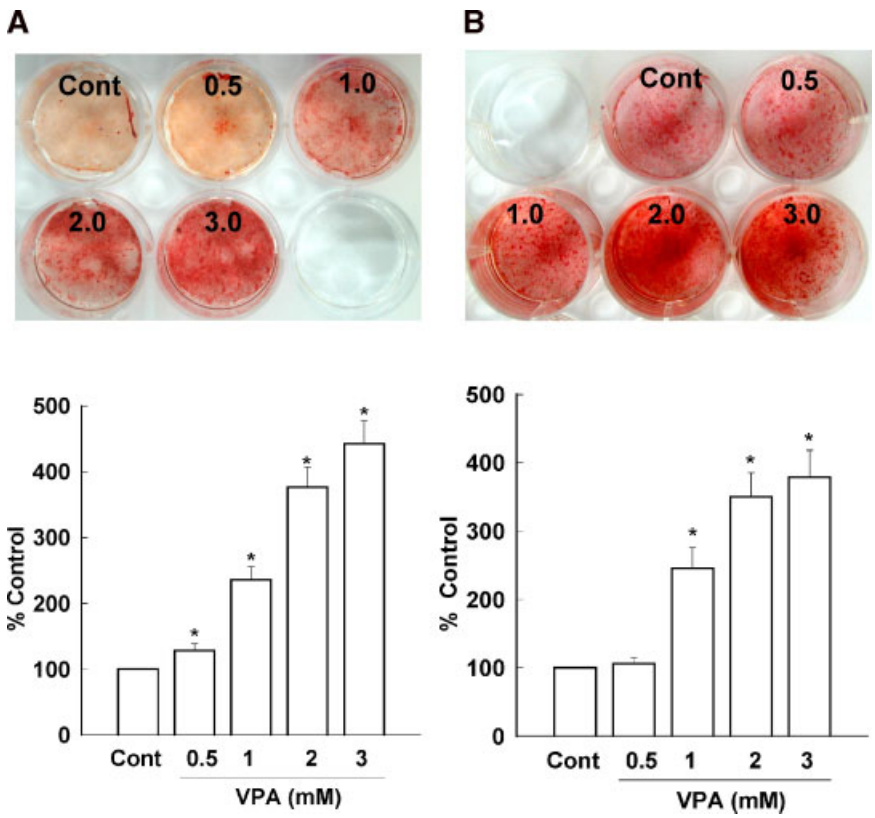

Fig. 1. **A**: Effect of valproic acid (VPA) on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSC) (A) and human bone marrow stromal cells (hBMSC) (**B**). Cells were grown to confluence and then induced osteogenic differentiation by differentiation medium. Differentiated cells were stained with alizarin red S at the 7th day after induction of differentiation in osteogenic medium. VPA was treated for the first 3 days of differentiation. The osteogenic differentiation was determined by quantitation of alizarin red S staining with an image analysis program. Data represent mean ± SEM of three different experiments. *$P < 0.05$ compared with control (Cont). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in chromatin conformations might be maintained after removal of the drug. To determine the period of the pretreatment of VPA, 1 mM VPA was pretreated for 1, 2, and 4 days, and the induction of osteogenic differentiation of hMSC was done without the addition of VPA. One day pretreatment of VPA could not show any effect on osteogenic differentiation. The pretreatment of VPA for 2 days markedly increased osteogenic differentiation, and the pretreatment for 4 days further increased it (Fig. 2). Therefore, MSC were pretreated with of 1 mM VPA for 4 days in the following experiments.

HDAC Inhibition and Osteogenic Differentiation

To verify if VPA treatment in our model was associated with HDAC inhibition, we determined if another HDAC inhibitor had similar effects on differentiation. hADSC were pretreated with the indicated concentrations of TSA and VPM for 4 days before induction of osteogenic differentiation. Similarly to VPA, TSA (75–500 nM) caused a dose-dependent increase of osteogenic differentiation (Fig. 3A). In contrast to VPA, VPM (0.5–3 mM), an amide analog of VPA that does not inhibit HDAC activity, did not significantly affect osteogenic differentiation (Fig. 3B).

Changes of Gene Expression in VPA-Treated hADSC

To understand VPA action on hADSC at molecular level, we determined the effects of VPA on gene expression. The treatment of VPA for 4 days increased p21, which is a well known target of histone deacetylase inhibitors, osterix and osteopontin. We further determined changes in expression of Runx2 and alkaline phosphatase genes during differentiation by RT-PCR. In control hADSC Runx2 and alkaline

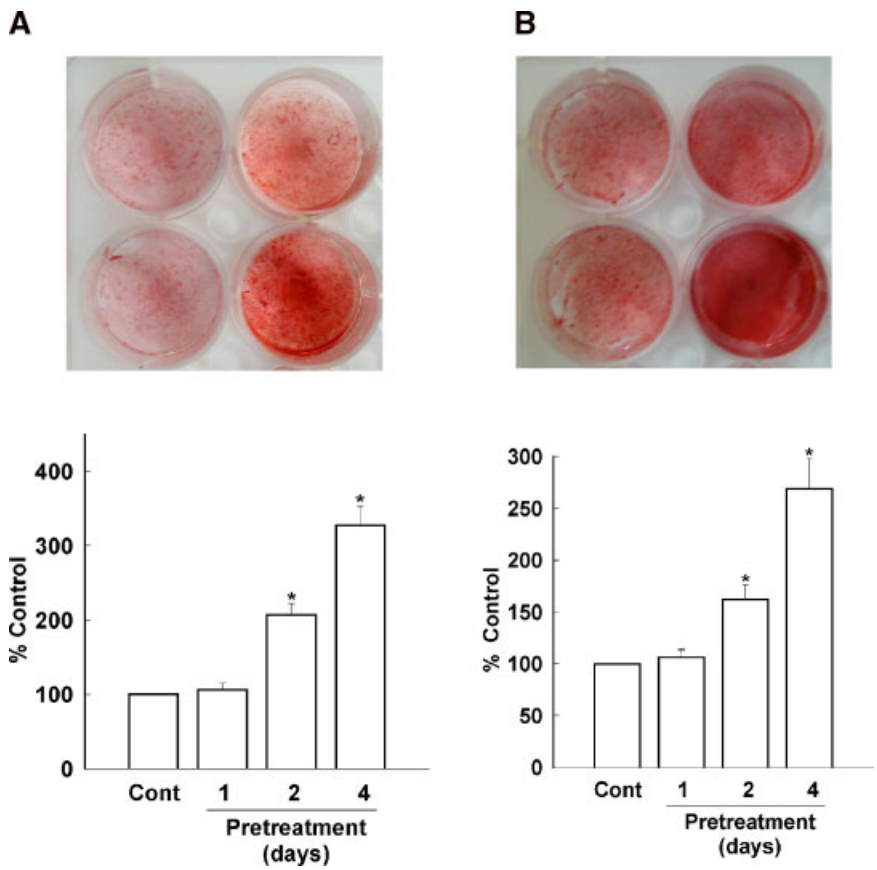

Fig. 2. Effect of VPA pretreatment on the osteogenic differentiation of hADSC (**A**) and hBMSC (**B**). hMSC were pretreated with 1 mM VPA for the indicated days before induction of osteogenic differentiation. The osteogenic differentiation was determined by quantitation of alizarin red S staining with an image analysis program at the 7th day after induction of differentiation. Data represent mean ± SEM of three different experiments. $*P < 0.05$ compared with control (Cont). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

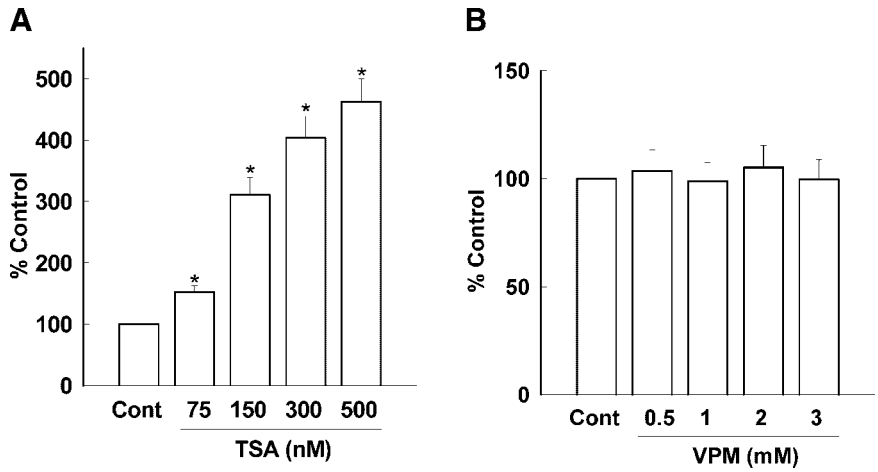

Fig. 3. Effect of trichostatin A (TSA) (**A**) and valpromide (VPM) (**B**) on osteogenic differentiation of hADSC. hADSC were pretreated with the indicated concentrations of TSA and VPM for 4 days before induction of osteogenic differentiation. The osteogenic differentiation was determined by quantitation of alizarin red S staining with an image analysis program. Data represent mean ± SEM of three different experiments.

phosphatase expression were increased at the 7the day after the induction of differentiation. The pretreatment of VPA for 4 days increased Runx2 expression, and VPA-pretreated cells showed increase in alkaline phosphatase expression at 3rd day after exposure of osteogenic medium and in Runx2 at 5th day after treatment (Fig. 4).

Osterix expression has been known to be controlled by BMP-2 and Runx2 [Celil et al., 2005] and BMP-2 has been shown to increase osteogenic differentiation in MSC [Dragoo et al., 2003]. The effect of VPA treatment on mRNA levels of BMP-2 and Runx2 gene were determined by real-time PCR analysis. Total RNAs were isolated from control or hADSC treated with 1 mM VPA for 4 days. Changes in mRNA in hADSC following the treatment of VPA were given in Table I for Runx2 and BMP2 genes relative to the internal control, i.e., β-actin (ΔC_t). The $\Delta\Delta C_t$ values were used to calculate fold-differences in mRNA levels. There was evidence of a 1.9-fold and 4.2-fold increase in mRNA levels for the Runx2 and BMP2, respectively (Table I).

Noggin binds to BMP 2 and BMP 4 with high affinity and blocks interaction with BMP receptor [Abe et al., 2000]. To determine the role of BMP2 on VPA-induced enhancement of osteogenic differentiation, control or VPA-pretreated cells were differentiated in the absence or presence of noggin (0.25 g/ml). The addition of noggin into differentiation media inhibited osteogenic differentiation of control hADSC, and significantly attenuated the VPA-induced potentiation of osteogenic differentiation of hADSC (Fig. 5).

Effect of VPA on Proliferation and Survival of hADSC and hBMSC

To determine the effect of VPA on the proliferation of hADSC and hBMSC, various concentrations of VPA were treated the next day after plating at density of 2,000 cells/cm^2. The treatment of VPA for 48 h inhibited proliferation of hADSC in a dose-dependent manner (Fig. 6). Although data were not shown, VPA has the same effect on the proliferation of hBMSC. Trypan blue staining showed that VPA at concentrations to be examined in this experiment did not affect survival of hADSC (Fig. 6).

DISCUSSION

VPA has multiple effects in diverse systems. For example, in humans VPA is an anticonvulsant, a mood stabilizer, and a potent teratogen. In most cases, however, the targets responsible for these effects have not been defined [Gurvich and Klein, 2002]. VPA can indirectly affect the function of a number of molecules, including PPAR, β-catenin, activator protein 1, protein kinase C, and MAPK [Blaheta and Cinatl, 2002]. VPA is also a direct inhibitor of several molecular targets, including HDACs, χ-amino butyric acid transaminase, and succinate semialdehyde dehydrogenase [Gurvich and Klein, 2002]. VPA has been shown to inhibit

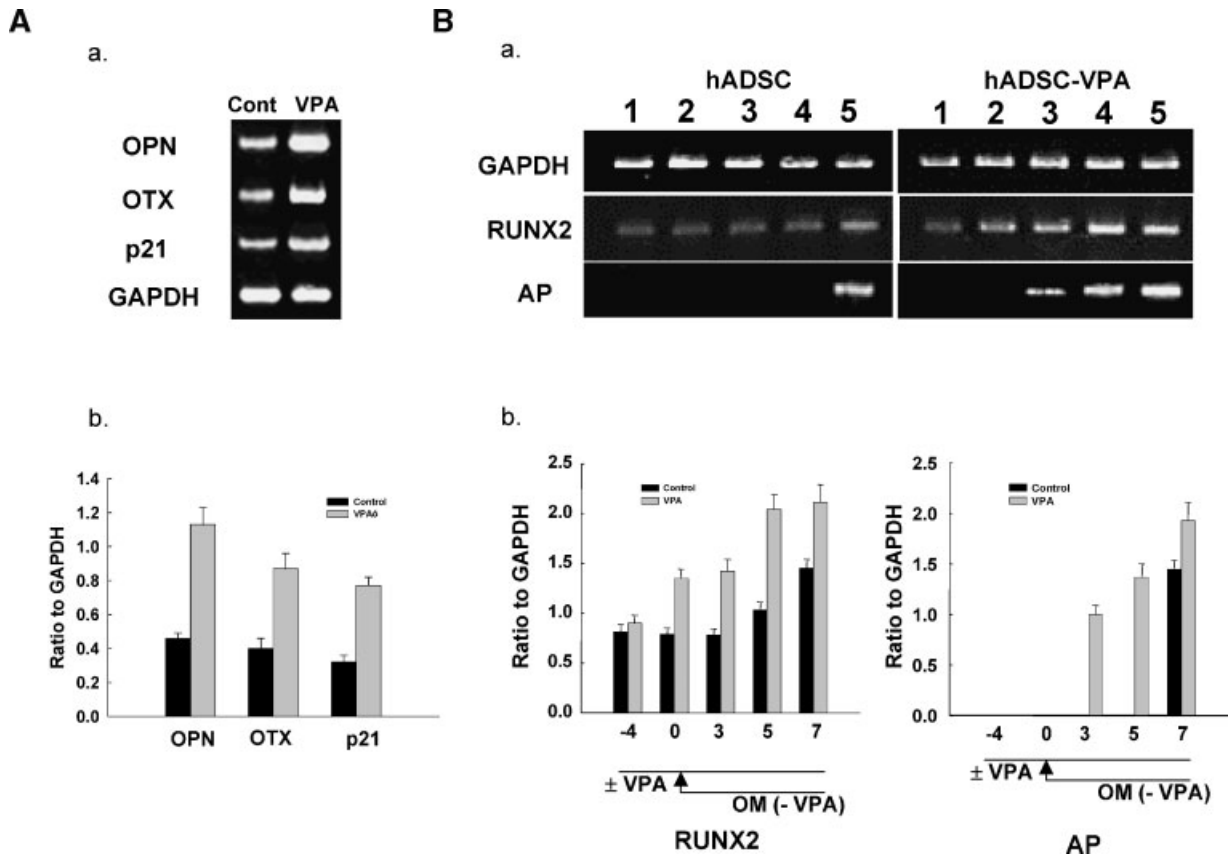

Fig. 4. RT-PCR analysis of osteogenic genes. Control hADSC or hADSC pretreated with 1 mM VPA for 4 days were induced for up to 1 week in osteogenic medium (OM) and analyzed by RT-PCR for the indicated genes. **A**: Changes in expression of p21/WAF1 (p21), osteopontin (OPN), and osterix (OTX) genes after treatment of VPA in hADSC. **a**: Representative photograph of RT-PCR products. Total RNA were isolated from hADSC before (**lane 1**) or after 4 days pretreatment of VPA (**lane 2**). **b**: Quantitation of PCR products. Quantity of amplified products were analyzed by an image analyzer (n = 3). Data represent relative value to GAPDH signal of the corresponding samples. **B**: Changes in expression of runx2 and alkaline phosphatase (AP) genes. **a**: Representative photograph of RT-PCR products. Total RNA were isolated from hADSC before (**lane 1**) or after 4 days pretreatment of VPA (**lane 2**). After then, hADSC were treated with osteogenic medium and total RNA were isolated at 3rd (**lane 3**), 5th (**lane 4**), and 7th day (**lane 5**) after induction of differentiation. **b**: Quantitation of PCR products. Quantity of amplified products were analyzed by an image analyzer (n = 3). Data represent relative value to GAPDH signal of the corresponding samples.

cell proliferation and adipogenic differentiation of preadipocytes, which is related to VPA-induced inhibition of HDAC [Lagace and Nachtigal, 2004]. In this study, we demonstrated that VPA increased osteogenic differentiation in hADSC and hBMSC. TSA, another well-known HDAC inhibitor, also showed the same effect on osteogenic differentiation of hADSC and hBMSC, whereas VPM, an amide analog of VPA that does not inhibit HDAC activity, did not. VPA increased expression of p21/WAF1 gene of which expression is controlled by HDAC activity [Han et al., 2001; Wang et al., 2004]. Furthermore, These data

TABLE I. C_t Values for BMP-2 and Runx2 in Control and VPA-Treated hADSC

Sample	BMP-2	Runx2	β-actin	ΔC_t of BMP2	ΔC_t of Runx2	$\Delta\Delta C_t$ of BMP2	$\Delta\Delta C_t$ of Runx2	Fold of BMP2	Fold of Runx2
Control	33.78 ± 0.04	32.19 ± 0.04	31.4 ± 0.09	2.38	0.79	0	0	1	1
VPA-treated	31.54 ± 0.05	31.12 ± 0.06	31.23 ± 0.03	0.31	−0.11	2.07	0.90	4.19	1.87

Values represent mean ± standard deviation of all RT-PCR replicates for n = 3 control samples or n = 3 VPA-treated samples. Control values are subtracted from VPA-treated values to obtain $\Delta\Delta C_t$ and fold-difference.

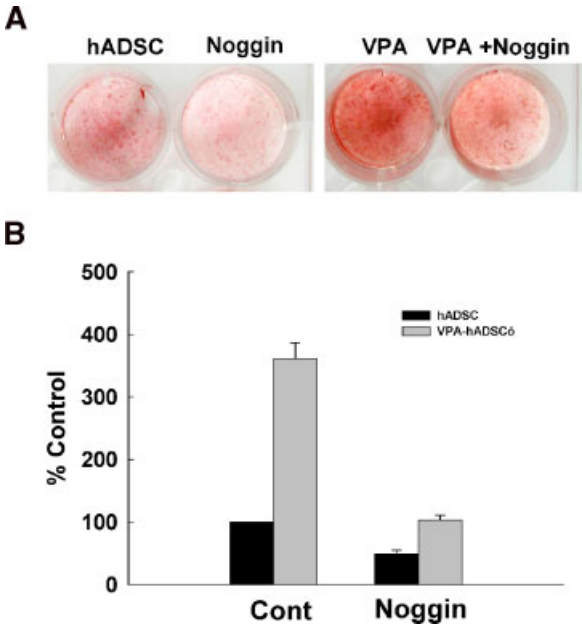

Fig. 5. Effect of noggin on VPA-induced osteogenic differentiation of hADSC. hADSC were pretreated with 1 mM VPA for 4 days before induction of osteogenic differentiation. The osteogenic differentiation of control or VPA-pretreated hADSC was induced in the absence or presence of 0.25 μg/ml noggin, and determined by quantitation of alizarin red S staining with an image analysis program in duplicate at the 14th day after induction of differentiation. Data represent mean ± SEM of three different experiments. $*P < 0.05$ compared with control (Cont). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

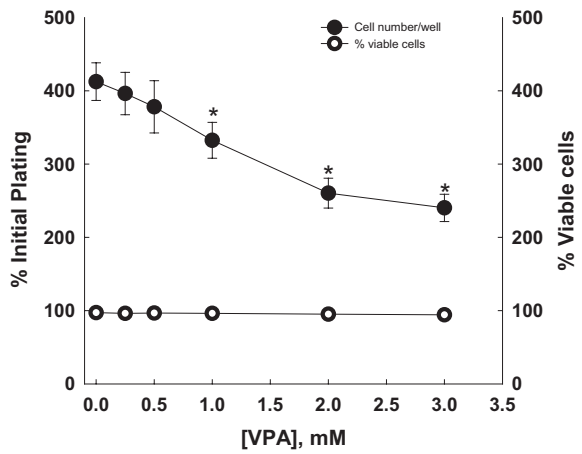

Fig. 6. Effect of VPA on the proliferation and survival of hADSC. The next day after plating hADSC (2,000 cells/cm^2), various concentrations of VPA were added. Proliferation of hADSC was determined by direct cell counting using a hemocytometer at the 2nd day after the addition of VPA. The percentage of dead cells was determined by trypan blue staining and data for cell proliferation were expressed as a percentage of the initial cell number (mean ± SEM of four experiments). $*P < 0.05$ compared with the data in the absence of VPA.

lead to the notion that the HDAC inhibitory properties of VPA may be responsible for enhanced osteogenic differentiation, although we did not make a direct measurement of HDAC activity in hADSC and hBMSC.

Because deacetylation of core histones by HDACs is associated with a "closed" chromatin conformation and repression of transcription, inhibition of HDACs can be supposed to lead to activation of transcription. In fact, HDAC inhibitors have been known to affect gene expression in various cells [Glaser et al., 2003; Tou et al., 2004; Yokota et al., 2004], suggesting that their pretreatment prior to the initiation of differentiation alone can affect on the properties of target cells. In models of muscle cell differentiation (C2C12 skeletal muscle cells and human skeletal myoblasts), pretreatment of HDAC inhibitors enhances myogenesis, whereas simultaneous addition of the inhibitors with the replacement of differentiation-inducing medium inhibits differentiation [Thiagalingam et al., 2003]. The data in the present study showed that the 4 days pretreatment of 1 mM VPA prior to induction of differentiation had a similar effect on osteogenic differentiation in hADSC and hBMSC compared with its treatment for the first 3 days of differentiation. Our RT-PCR and real-time PCR data demonstrated that VPA treatment itself increases osteogenic differentiation-related genes such as osterix, osteopontin, bmp2, and runx2 as well as p21/WAF1 in control culture conditions. The roles of these genes on osteogenic differentiation have been well defined in osteoblasts [Karsenty, 2001] and MSC [Olmsted et al., 2001; Diefenderfer et al., 2003; Dragoo et al., 2003; Shui et al., 2003], and expression of osterix has been shown to be controlled by BMP2 and Runx2 [Celil et al., 2005]. The role of BMP2 on the VPA-induced increase of osteogenic differentiation was confirmed by the finding that the treatment of noggin inhibited VPA-induced osteogenic differentiation. These results suggest that VPA induces expression of osteogenesis-related genes, which facilitates hADSC and hBMSC to respond to osteogenic signal.

VPA has been reported to stimulate proliferation of hematopoietic stem cells [Bug et al., 2005] and some reagents and cytokines have been reported to exert the effect on MSC differentiation by modulating the initial proliferating phase after induction of differentiation [Otto and Rao, 2004]. However, VPA-induced

enhancement of osteogenic differentiation could not be explained by cell proliferation, because VPA inhibited proliferation of hADSC and hBMSC in a dose-dependent manner.

It has been reported that growth and differentiation potential are similar between ADSC and BMSC [De Ugarte et al., 2003] and that their surface protein expression is similar [Gronthos et al., 2001]. We also have reported that hADSC isolated in our laboratory have similar characteristics with hBMSC [Kang et al., 2003; Lee et al., 2004a,b; Kim et al., 2005]. A similar response to VPA in differentiation and proliferation of hADSC and hBMSC in this study further support that MSC populations in bone marrow and adipose tissues share similar properties.

The autologous nature of mesenchymal stem cells, together with their putative multipotentiality, may make these cells an excellent choice for many future tissue engineering strategies and cell based therapies. MSC can differentiate into osteogenic and chondrogenic tissues in vivo [Benayahu et al., 1989; Krebsbach et al., 1997; Cowan et al., 2004] and preliminary data suggest that these cells can be used to repair bony and cartilagenous defects [Bruder et al., 1998; Johnstone and Yoo, 1999]. The findings in this study that the pretreatment of HADC inhibitors is enough for the enhancement of osteogenic differentiation indicate that HDAC inhibitors could be used for in vivo bone engineering using hMSC.

REFERENCES

Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, Stahl N, Jilka RL, Manolagas SC. 2000. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: Antagonism by noggin. J Bone Miner Res 15:663–673.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R. 2000. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. Exp Hematol 28:707–715.

Benayahu D, Kletter Y, Zipori D, Wientroub S. 1989. Bone marrow-derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo. J Cell Physiol 140:1–7.

Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 102:341–351.

Blaheta RA, Cinatl JJ. 2002. Anti-tumor mechanisms of valproate: A novel role for an old drug. Med Res Rev 22:492–511.

Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. 1998. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res 16:155–162.

Bug G, Gul H, Schwarz K, Pfeifer H, Kampfmann M, Zheng X, Beissert T, Boehrer S, Hoelzer D, Ottmann OG, Ruthardt M. 2005. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res 65:2537–2541.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9:641–650.

Celil AB, Hollinger JO, Campbell PG. 2005. Osx transcriptional regulation is mediated by additional pathways to BMP2/Smad signaling. J Cell Biochem 95:518–528.

Cinatl J, Jr., Cinatl J, Scholz M, Driever PH, Henrich D, Kabickova H, Vogel JU, Doerr HW, Kornhuber B. 1996. Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. Anticancer Drugs 7:766–773.

Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT. 2004. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol 22(5):560–567.

De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. 2003. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174:101–109.

Diefenderfer DL, Osyczka AM, Garino JP, Leboy PS. 2003. Regulation of BMP-induced transcription in cultured human bone marrow stromal cells. J Bone Joint Surg Am 85-A(Suppl 3):19–28.

Dragoo JL, Choi JY, Lieberman JR, Huang J, Zuk PA, Zhang J, Hedrick MH, Benhaim P. 2003. Bone induction by BMP-2 transduced stem cells derived from human fat. J Orthop Res 21:622–629.

Gimble JM, Guilak F. 2003. Differentiation potential of adipose derived adult stem (ADAS) cells. Curr Top Dev Biol 58:137–160.

Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. 2003. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: Defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2:151–163.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T. 2001. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20:6969–6978.

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. 2001. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189:54–63.

Gurvich N, Klein PS. 2002. Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. Pharmacol Ther 96:45–66.

Han JW, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, Lee YW, Lee HW. 2001. Activation of p21(WAF1/Cip1) transcription through Sp1 sites by histone deacetylase inhibitor apicidin: Involvement of protein kinase C. J Biol Chem 276:42084–42090.

Johannessen CU. 2000. Mechanisms of action of valproate: A commentary. Neurochem Int 37:103–110.

Johnstone B, Yoo JU. 1999. Autologous mesenchymal progenitor cells in articular cartilage repair. Clin Orthop 367:S156–S162.

Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS. 2003. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp Neurol 183:355–366.

Karsenty G. 2001. Transcriptional control of osteoblast differentiation. Endocrinology 142:2731–2733.

Kim SJ, Cho HH, Kim YJ, Seo SY, Kim HN, Lee JB, Kim JH, Chung JS, Jung JS. 2005. Human adipose stromal cells expanded in human serum promote engraftment of human peripheral blood hematopoietic stem cells in NOD/SCID mice. Biochem Biophys Res Commun 329: 25–31.

Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. 1997. Bone formation in vivo: Comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 63: 1059–1069.

Lagace DC, Nachtigal MW. 2004. Inhibition of histone deacetylase activity by valproic acid blocks adipogenesis. J Biol Chem 279:18851–18860.

Lee DH, Kang SK, Lee RH, Ryu JM, Park HY, Choi HS, Bae YC, Suh KT, Kim YK, Jung JS. 2004a. Effects of peripheral benzodiazepine receptor ligands on proliferation and differentiation of human mesenchymal stem cells. J Cell Physiol 198:91–99.

Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS. 2004b. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem 14:311–324.

Lieberman JR, Ghivizzani SC, Evans CH. 2002. Gene transfer approaches to the healing of bone and cartilage. Mol Ther 6:141–147.

Marks PA, Richon VM, Breslow R, Rifkind RA. 2001. Histone deacetylase inhibitors as new cancer drugs. Curr Opin Oncol 13:477–483.

Na L, Wartenberg M, Nau H, Hescheler J, Sauer H. 2003. Anticonvulsant valproic acid inhibits cardiomyocyte differentiation of embryonic stem cells by increasing intracellular levels of reactive oxygen species. Birth Defects Res Part A Clin Mol Teratol 67:174–180.

Olmsted EA, Blum JS, Rill D, Yotnda P, Gugala Z, Lindsey RW, Davis AR. 2001. Adenovirus-mediated BMP2 expression in human bone marrow stromal cells. J Cell Biochem 82:11–21.

Otto WR, Rao J. 2004. Tomorrow's skeleton staff: Mesenchymal stem cells and the repair of bone and cartilage. Cell Prolif 37:97–110.

Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276:36734–36741.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Shui C, Spelsberg TC, Riggs BL, Khosla S. 2003. Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. J Bone Miner Res 18:213–221.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. Nature 403:41–45.

Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. 2003. Histone deacetylases: Unique players in shaping the epigenetic histone code. Ann NY Acad Sci 983:84–100.

Tou L, Liu Q, Shivdasani RA. 2004. Regulation of mammalian epithelial differentiation and intestine development by class I histone deacetylases gene expression. Mol Cell Biol 24:3132–3139.

Wang S, Yan-Neale Y, Zeremski M, Cohen D. 2004. Transcription regulation by histone deacetylases. Novartis Found Symp 259:238–245; discussion 245–248, 285–288.

Williams RS, Cheng L, Mudge AW, Harwood AJ. 2002. A common mechanism of action for three mood-stabilizing drugs. Nature 417:292–295.

Yokota T, Matsuzaki Y, Miyazawa K, Zindy F, Roussel MF, Sakai T. 2004. Histone deacetylase inhibitors activate INK4d gene through Sp1 site in its promoter. Oncogene 23:5340–5349.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue engineering 7:211–227.