Parathyroid Hormone Enhances Osteoblast Differentiation from Human Skin Derived Precursor Cells *In Vitro*

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Parathyroid hormone (PTH), a new effective treatment for osteoporosis patients which promotes the anabolic effect in vivo, can enhance the differentiation of osteoblasts derived from the human skin-derived precursor cells (hSKPs) in vitro culture. This research investigated the effects of PTH by studying the gene expressions and other markers of osteoblast differentiation along with the induction of hSKPs to osteoblast in two experiment groups, i.e. the osteogenic induction medium (OM) only and the OM plus PTH (OM + PTH). The results of each type were compared between these two groups. Both groups expressed the Cbfa1 gene, a regulator of osteoblasts and also one of the most osteoblast specific genes. The findings were that the OM + PTH group showed more intense alkaline phosphatase staining than the other. The gene expressions of protein showing the mature osteoblasts like osteocalcin (OCN) and bone sialoprotein (BSP) in the OM + PTH group expressed higher and faster (Day 14) than the OM group. Moreover, the gene expression of osteoprotegerin (OPG) possessing the protein produced by the mature osteoblasts showed a higher level in the OM + PTH group on the same day as OCN and BSP occurred. This protein performs a function in inhibiting osteoclast maturation. The present study found that PTH enhanced the differentiation of osteoblasts derived from hSKPs by promoting the maturation of osteoblasts in vitro. It possibly concerns with the anabolic effect of PTH in a treatment for osteoporosis patients. Additionally, hSKPs are the interesting sources for osteoporosis treatments when combining with PTH.

Keywords: *Skin-derived precursors cell (SKPs), Stem cell, Parathyroid hormone (PTH), Osteoblast, Osteoblast differentiation*

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Stem cells can be differentiated into many cell types. The stem cells are found in various parts of adult human body. Recently, the stem cells from skin have shown their potential to be pluripontent. The authors have induced the stem cells from human skin that called human skin-derived precursor cells (hSKPs) to osteoblasts with the osteogenic gene expression[1]. The authors also demonstrated the physiological responses of osteoblast caused by the mesenchymal stem cells through inducing the strontium[2]. The present study has been designed for proving that osteoblasts developed by hSKPs can be induced and can perform their functions by the induction of parathyroid hormone (PTH).

PTH is a new agent for an osteoporosis treatment that provides wonderful effectiveness. The discovery of the parathyroid glands and their biological roles evolved later than that of the thyroid glands. The researches on PTH have undergone into four distinctive phases, starting before the turn of the 20th century. The realization of the PTH role resulted in comprehending the action of the glands in the area of calcium physiology. Then, the pharmacological uses of the amino acid Sequence 1 through 34 of the complete PTH molecule named teriparatide which is itself a recombination form utilized as an effective treatment

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for osteoporosis were submitted to FDA regulatory reviewed by the end of 2000. The efficacy of teriparatide was impressive; the treatments with this type of PTH to the postmenopausal woman group reduced risks of new vertebral fractures to nearly 70%[3-5].

The aim of the present study was to assess the effects of this hormone to the gene expressions and the characteristics of osteoblasts derived from hSKPs. Osteoblasts, in this regard, organized many types of bone matrix to form bone; therefore, the bone matrix gene expressions were analyzed in this study to evaluate the effects of PTH.

Material and Method

The human skin biopsies were obtained from the healthy male donors who were the medical staff in our group[1]. They also submitted the informed consent. The hSKPs were isolated and proliferated and then they were plated at a density of 1 x 105 cells/mL into 35 mm dish and cultured in the osteogenic induction medium called OM. The OM is minimum essential medium alpha medium (α-MEM; GibcoBRL) with 10% FBS, penicillin and streptomycin supplemented with ascorbic acid, dexamethasone, β-glycerophosphate, and L-glutamine (Sigma-Aldrich, St Louis, MO, USA). While a recombinant form PTH (teriparatide) was added to the OM at the concentration of 108 M called the OM plus PTH or OM + PTH group to evaluate the effects of PTH to the osteblasts that derived from hSKPs. The medium was replaced twice a week until the cells in both groups were harvested.

The undifferentiated cells (Day 0) and the cells that were cultured for 7, 14 and 21 days were prepared for further analysis through the alkaline phosphatase (ALP) activity, ALP staining and RNA extraction for studying the gene expressions by the real time polymerase chain reaction (PCR).

ALP activity

On the same day of doing the ALP staining, the cultivated hSKPs were detached by the enzymatic method and washed three times with PBS. The cells were lyzed by the lysis buffer containing 0.1% Triton x-100 (Sigma-Aldrich, St Louis, MO, USA). Twenty microliters of cell lysate were mixed with 100 µL Tris-glycine buffer pH 10.3 (50 mM Tris-HCl, 100 mM glycine (BioRad, Hercules, CA, USA) and 2 mM $MgCl_2$ (Sigma) and 100 µL of p-nitrophenyl phosphate (Sigma). The reaction mix was incubated at 37°C for 30 min and the reaction was stopped when adding 50 µL of 3 M NaOH. The absorbance was read at 405 nm in a microplate reader. The enzymatic activity was normalized to the total protein concentration by using bovine serum albumin (BSA; Roche, Basel, Switzerland) according to the standard of the Bradford (Sigma) protein detection method. The ALP activity was expressed as µM/mg protein/assay time.

ALP staining

The ALP staining was conducted in the cultured cells at the time mentioned above. It was washed twice with PBS containing calcium and magnesium. Then, the cells were fixed in the cold 10% neutral buffer formalin (NFB) for 15 min, and then washed by the deionized water and left to air dry. The fixed cells were incubated in the buffer containing 0.1 mg/µL naphthol AS-MX phosphate and 0.6 mg/µL fast red violet LB salt (Sigma-Aldrich, St Louis, MO, USA) for 45 min at room temperature in the dark. The cultured vessels were washed by the deionized water and were microscopically observed.

Ribonucleic acid (RNA) isolation and real-time PCR

The total RNA came from the hSKPs cultured in the OM and the OM plus parathyroid hormone. The RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). The real time reverse transcriptase PCR (real time RT-PCR) products were analyzed by the quantitative real-time PCR in TaqMan® Gene Expression. The assays of several target genes are as follows: ALP, bone sialoprotein (BSP), Cbfa1, osteocalcin (OCN), osteoprotegerin (OPG) and GAPDH as an endogenous control (Applied Biosystems; Foster City, CA, USA). All PCR reactions were performed in a real-time PCR 7300 system. The quantitative gene expressions using TaqMan® Gene Expression Assays was performed at the second step of the two-step real-time PCR.

The total RNA was reversely transcribed in a 20 µL reaction by using SuperscriptTM III reverse transcriptase based on the manufacturer's protocol (Invitrogen). One µL of cDNA was carried out by the real-time PCR. The assays were done in 25 µL two-step reactions containing TaqMan® Universal PCR Master Mix, 20X TaqMan® Gene Expression Assay Mix, and cDNA according to the manufacturer's instructions (Applied Biosystems). The reaction conditions consisted of pre-incubation at 50°C for 2 min, 95°C for 10 min, then cycling for 40 cycles of 95°C for 15 sec and 60°C for 1 min for the gene expressions of ALP, BSP, Cbfa1, OCN, OPG and GAPDH. The expression levels of each target gene were normalized to GAPDH, or the

house keeping gene. The real-time PCR was run in the 7300 Real-Time PCR System (Applied Biosystem). The increase in the PCR products was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding to dsDNA. The real-time PCR data were analyzed by using the 2 (ddct) method according to the manufacturer's directions. For each target gene, the mRNA levels were normalized to GAPDH and expressed as fold change values relative to the hSKPs undifferentiated cells.

Results

The hSKPs were isolated and passaged (Fig. 1) then cultured in the OM and the OM plus PTH groups for evaluating the gene expressions and other characteristics of osteoblasts that were affected by PTH. Two patterns of osteoblast differentiation were investigated by the gene expressions and the enzyme assay.

The ALP enzyme that was used as the osteoblast marker of differentiation and function when initiating the mineralization of bone tissues was assessed in both qualitative and quantitative gene expressions by the real-time PCR. The qualitative ALP was performed by staining the enzyme while the quantitative analysis was done by the ALP activity method. The qualitative process showing gross appearance of the OM plus PTH group had more intense color of red (the red color represented the ALP enzyme) than the OM group had on the same day and the color increasing was varied to the time (Fig. 2).

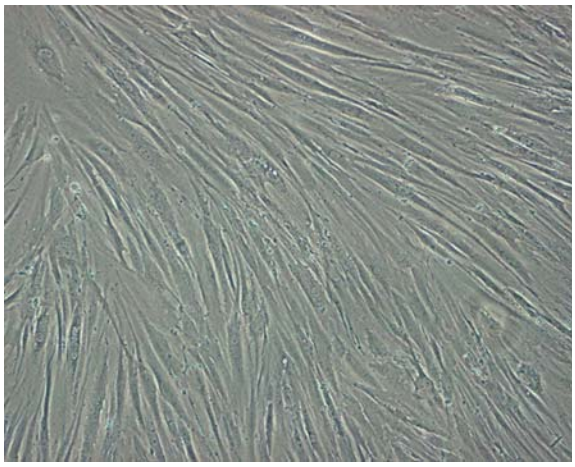

Fig. 1 Phase contrast micrographs of human skin-derived precursor cells (hSKPs) during expansion, 10x magnification

On the other hand, the ALP activity using the quantitative assay clearly showed the same patterns as the ALP gene expression analyzed by the real-time PCR technique did. Their patterns displayed the noticeable increase started from Day 0 to Day 14; they rose to the highest expressions on Day 14 and decreased on Day 21. The present study found that the expressions of this gene from both groups (the OM and the OM plus PTH) were early shown on Day 7 (Fig. 3).

The osteoblastic gene expressions including ALP, Cbfa1, BSP, OCN and OPG were analyzed by the real-time PCR. The Cbfa1 is the key role transcription factor in osteoblast differentiation. The gene expressions of Cbfa1 from osteoblasts derived from hSKPs induced in the OM were compared with the cells that were induced by the OM plus parathyroid hormone (OM + PTH) as shown in Fig. 4A. The Cbfa1 expressions in both groups of the experiment (the OM

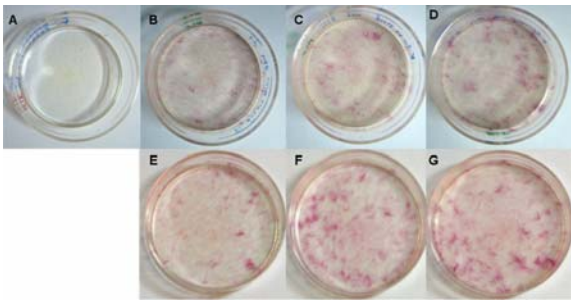

Fig. 2 ALP expressions of cultivated hSKPs, investigated by staining method, gross appearance. The upper panels are from the OM group on Day 0 (A), D 7, 14 and 21 (B, C and D) respectively, and the lower panels are from the OM + PTH group on Day 7, 14 and 21 (E, F and G)

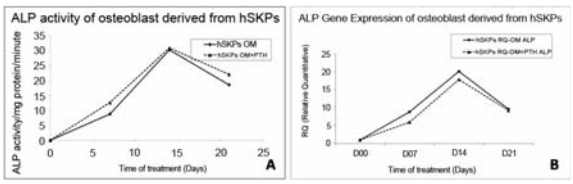

Fig. 3 ALP activity (A) and ALP gene expressions (B) of osteoblast derived from hSKPs in the OM and the OM + PTH groups showed the highest expressions on the same day (Day 14)

and the OM + PTH) were increased in the same direction of induction and early expressed on Day 7. On this day (Day 7), the expressions of the OM induction group were higher than those of the other. On the days later, the expressions had no difference.

The OCN gene expression of osteoblasts derived from hSKPs in the OM + PTH group was up-regulated on Day 14 while the OM group expressed on Day 21 (Fig. 4B). The gene expression of BSP showed the same trend as the one of OCN did (Fig. 4C). The OPG of the OM + PTH group exhibited a higher level of gene expression along with the differentiation of osteoblastic cells (Fig. 4D).

Discussion

PTH is a bone formation enhancer that has been licensed for use in the established postmenopausal osteoporosis. PTH and its analog, teriparatide [recombinant human PTH (1-34)] represent a new class of anabolic therapy for treatments of severe osteoporosis. The effects of PTH on human bone cells were demonstrated elsewhere[6]. This hormone can drive changes in gene expressions related to the activation of transcription factors. Cbfa1 is one of transcription factors that can be regulated by PTH; it is a regulator of osteoblast differentiation as well as the most osteoblast specific gene that appears to regulate the amount of bone matrix formed by differentiated osteoblast in postnatal animals.

hSKPs can be transdifferentiated into osteoblastic lineage both in the OM and the OM plus PTH conditions as shown in the results of the ALP staining, activity and gene expressions (Fig. 2, 3A and 3B respectively). It is widely accepted that ALP is a marker of osteoblast differentiation and function at the beginning of mineralization of bone tissues[1]. Thus, ALP and Cbfa1, which are both regulators of osteoblast differentiation and the most osteoblast specific genes[7-9], were presented in osteoblasts in the OM and the OM plus PTH groups. Even though the expressions of the OM + PTH group were lower than the OM group on Day 7, they were firstly higher on Day 14 and later on Day 21.

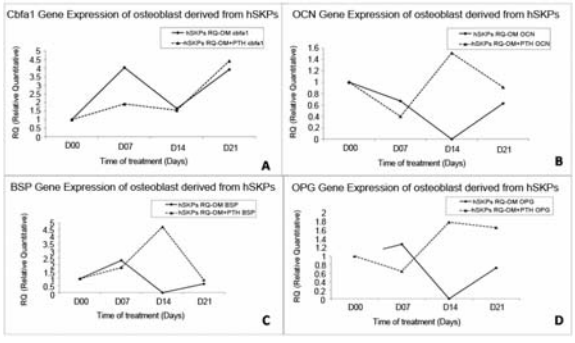

Fig. 4 Gene expressions of Cbfa1 (A), OCN (B), BSP (C) and OPG (D) from osteoblast derived from hSKPs

The present study also found that the gene expression of Cbfa1 with the expression of osteoblast specific-protein gene of OCN and BSP were increased by the effects of PTH in osteoblast derived from hSKPs. These proteins were mainly produced by osteoblasts. The Cbfa1 actually involved in regulating other genes of osteoblast specific protein including OCN[10-12] and BSP[13]. In the present study, the expressions of these genes were shown in the real-time PCR results (Fig. 4A, 4B and 4C respectively).

When the osteoblast differentiation markers derived from mesenchymal stem cells and then committed to osteoblasts with the expression of gene markers, *i.e.* Cbfa1 proliferated, the markers came to be osteopontin (OPN), collagen Type I (COL I) and histone. Then, the matrix maturation markers, that is, BSP appeared while COL I was still on when ALP rose up. Eventually, the mineralization process in the mature osteoblasts occurs with the gene markers called OCN, collagenase and OPN that display again as the late markers of osteoblast differentiation[14].

In Fig. 3 and 4, the Cbfa1, BSP and ALP on Day 7 were up-regulated. According to the changes of these gene markers, it can be concluded that the differentiation of hSKPs was developed to preosteoblast or immature osteoblast that is the stage of matrix maturation studied by Lian et al[14] and Stein et al[15]. When the differentiation progressed, the ALP gene expression raised higher the same as BSP and OCN. The last 2 genes were higher in the OM + PTH group only, but BSP and OCN in the OM group expressed the lower expressions than they had been on Day 7. However, their expressions increased on Day 21 the same as the OM + PTH group did. From these results, PTH showed an ability to promote BSP and OCN gene expressions in osteoblast derived from hSKPs. In addition, the OCN especially is one of the mature osteoblast markers and is the most specific marker associated with bone mineralization. Furthermore, BSP and OCN based on the studies of many researchers[14-16] were also increased at the beginning of mineralization stage.

Bone remodeling is a coupled process

concerning bone resorption and formation. Osteoblasts and osteoclasts are cells that involve this process. The osteoclast differentiation and function require binding to RANKL on osteoblasts by its receptor RANK on the osteoclast precursors. OPG is produced by mature osteoblasts which can bind to RANKL that acts as a decoy factor. This act obstructs the interaction between osteoblasts and osteoclast precursors[17]. The present study showed that osteoblasts derived from hSKPs in the OM + PTH group had the higher level of OPG gene than those in the OM group especially on Day 14. The cells on that day were the mature osteoblasts indicated by OCN, BSP, ALP and OPG up-regulated gene expressions in the OM + PTH group while the expressions in the OM group on the same day showed the lower level.

All these exhibited the effects of PTH that promoted the gene expressions for the maturation of osteoblasts derived from hSKPs. The hSKPs are the interesting sources for the stem cell treatments for bone diseases such as osteoporosis when combining with PTH. Moreover, this finding of promoting maturation of osteoblast *in vitro* can support the answer why PTH enhances the anabolic action in a treatment for osteoporosis patients.

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Potential conflicts of interest

None.

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การศึกษาผลของฮอร์โมนพาราไทรอยด์ที่ส่งเสริมการเปลี่ยนแปลงของออสติโอบาสต์ที่เจริญมาจากเซลล์ต้นกำเนิดจากผิวหนังในหลอดทดลอง

เกตน์สรี สุริยจันทร์, มลนิภา ศิลาอาสน์, ณรงค์ บุณยะรัตเวช, อานนท์ บุณยะรัตเวช

ฮอร์โมนพาราไทรอยด์เป็นยาตัวใหม่ ที่ใช้ในการรักษาผู้ป่วยโรคกระดูกพรุนได้อย่างมีประสิทธิผลในการสร้างกระดูกในร่างกาย สามารถส่งเสริมการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากผิวหนัง ให้เป็นเซลล์กระดูก (osteoblasts) ได้ในหลอดทดลอง การทดลองนี้ได้ศึกษาผ่านการแสดงออกของจีน และสัญลักษณ์ (markers) อื่นๆ ตลอดการเหนี่ยวนำเซลล์ต้นกำเนิดจากผิวหนังไปเป็นเซลล์กระดูกโดยการเปรียบเทียบระหว่าง 2 กลุ่ม คือ กลุ่มที่ใช้น้ำยาเหนี่ยวนำกระดูก (OM) เพียงอย่างเดียว กับกลุ่มที่เลี้ยงในน้ำยาเหนี่ยวนำกระดูกร่วมกับฮอร์โมน พาราไทรอยด์ ทั้ง 2 กลุ่มมีการแสดงออกของจีน Cbfa1 ซึ่งเป็นจีนควบคุม และจำเพาะที่สุดตัวหนึ่งของเซลล์กระดูกในกลุ่มที่ใช้น้ำยาเหนี่ยวนำกระดูกร่วมกับฮอร์โมพาราไทรอยด์มีการติดสีเอนไซม์อัลคาไลน์ฟอสฟาเตสเข้มกว่าอีกกลุ่มหนึ่ง ขณะเดียวกัน การแสดงออกของจีนที่แสดงถึงโปรตีนชนิดที่แสดงการเป็นเซลล์กระดูกตัวแก่ (mature osteoblast) ได้แก่ osteocalcin และ bone sialoprotein ในกลุ่มที่ใช้ฮอร์โมนพาราไทรอยด์มีค่าสูงกว่าและแสดงออก (ในวันที่ 14) เร็วกว่ากลุ่มที่ไม่ได้ใช้ ยิ่งไปกว่านั้น การแสดงออกของจีน osteoprotegerin (OPG) โปรตีนที่ผลิตโดยเซลล์กระดูกตัวแก่และมีหน้าที่ยับยั้งการเจริญของเซลล์สลายกระดูก (osteoclasts) มีค่าสูงกว่ากลุ่มที่มีฮอร์โมนพาราไทรอยด์ในวันเดียวกันกับ OCN และ BSP การศึกษาครั้งนี้แสดงให้เห็นว่า ฮอร์โมนพาราไทรอยด์สามารถส่งเสริมการเจริญเปลี่ยนแปลงจากเซลล์ต้นกำเนิดจากผิวหนังไปเป็นเซลล์กระดูก โดยการเหนี่ยวนำให้เกิดการเจริญแก่ตัวของเซลล์กระดูกได้ในหลอดทดลอง และอาจเกี่ยวข้องกับการที่ทำไมฮอร์โมนพาราไทรอยด์จึงส่งเสริมการสร้างกระดูกในการใช้รักษาผู้ป่วยโรคกระดูกพรุน อีกทั้งเซลล์ต้นกำเนิดจากผิวหนังก็เป็นแหล่งที่น่าสนใจในการใช้รักษาผู้ป่วยโรคกระดูกพรุนร่วมกับฮอร์โมนพาราไทรอยด์