Cellular ATP synthesis mediated by type III sodium-dependent phosphate transporter Pit-1 is critical to chondrogenesis

Atsushi Sugita1, 5, Shinji Kawai2, Tetsuyuki Hayashibara3, Atsuo Amano2, Takashi Ooshima3, Toshimi Michigami4, Hideki Yoshikawa5, Toshiyuki Yoneda1

1Department of Biochemistry, 2Department of Oral Frontier Biology, 3Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, 4Department of Bone and Mineral Research, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka 594-1101, Japan, 5Department of Orthopaedic Surgery, Osaka University Graduate School of Medicine, 1-8 Yamadaoka, Suita-Osaka 565-0871, Japan.

Running head: Pit-1 regulation of late chondrogenesis

Address correspondence to: Toshiyuki Yoneda, D.D.S., Ph.D. 1-8 Yamadaoka, Suita-Osaka 565-0871, Japan.
Fax: +81-6-6879-2890: E-mail: tyoneda@dent.osaka-u.ac.jp

Disturbed endochondral ossification in X-linked hypophosphatemia (XLH) indicates an involvement of phosphate (Pi) in chondrogenesis. We studied the role of the sodium-dependent Pi co-transporters (NPT), which are a widely-recognized regulator of cellular Pi homeostasis, and its downstream events in chondrogenesis using Hyp mice, the murine homologue of human XLH. Hyp mice showed reduced apoptosis and mineralization in hypertrophic cartilage. Hyp chondrocytes in culture displayed decreased apoptosis and mineralization compared with wild-type (WT) chondrocytes, while glycosaminoglycan synthesis, an early event in chondrogenesis, was not altered. Expression of the type III NPT Pit-1 and Pi uptake were diminished and intracellular ATP levels were also reduced in parallel with decreased caspase-9 and caspase-3 activity in Hyp chondrocytes. The competitive NPT inhibitor phosphonoformic acid (PFA) and ATP synthesis inhibitor 3-bromopyruvate (3-BrPA) disturbed endochondral ossification with reduced apoptosis in vivo and suppressed apoptosis and mineralization in conjunction with reduced Pi uptake and ATP synthesis in WT chondrocytes. Overexpression of Pit-1 in Hyp chondrocytes reversed Pi uptake and ATP synthesis and restored apoptosis and mineralization. Our results suggest that cellular ATP synthesis consequent to Pi uptake via Pit-1 plays an important role in chondrocyte apoptosis and mineralization and that chondrogenesis is ATP-dependent.

Endochondral ossification is critical to the development and growth of mammals. The process begins with condensation of undifferentiated mesenchymal cells and these cells differentiate into proliferating chondrocytes which express type II, type IX, type XI collagen and sulfated glycosaminoglycans (GAG) (1). Proliferating chondrocytes further differentiate into hypertrophic chondrocytes expressing type X collagen, undergo apoptosis, mineralize and ultimately replaced by bone. Disturbance of the endochondral ossification leads to a variety of skeletal disorders.

The genetic disease X-linked hypophosphatemia (XLH) is the most common form of inherited rickets in humans and is related to the dominant disorder of phosphate (Pi) homeostasis (2). XLH is shown to be caused by inactive mutations of the PHEX gene and characterized by hypophosphatemia secondary to renal Pi wasting, growth retardation due to disturbed endochondral ossification, osteomalacia resulting from reduced mineralization and abnormally-regulated vitamin D metabolism (3). Hyp mice also display similar biochemical and phenotypic abnormalities with human XLH including hypophosphatemia, osteomalacia and skeletal abnormalities. Hyp mice thus are a mouse homologue of the human XLH (4). Previous studies reported that Hyp mice exhibited disorganized hypertrophic cartilage with reduced apoptotic chondrocytes and hypomineralization (5). We have previously reported that osteoclast number was decreased in Hyp mice compared with wild-type (WT) mice and high-Pi diet partially restored this, showing that Pi influences osteoclastogenesis and suggesting this Pi effect on osteoclastogenesis may be associated with the pathogenesis of abnormal skeletogenesis in Hyp mice (6). However, it remains unclear whether disturbed Pi homeostasis influences endochondral ossification leading to abnormal skeletogenesis in Hyp mice. In this context, it is noted that intracellular Pi levels decrease and extracellular Pi
levels prominently increase from the proliferating to the hypertrophic zone during chondrogenesis (7-10), suggesting that cellular Pi levels are associated with chondrocyte differentiation.

Cellular Pi levels are controlled by the sodium-dependent Pi co-transporters (NPT) (11). Previous studies reported that the type III NPT (Pit-1) was expressed in hypertrophic chondrocytes during endochondral ossification in mice (12) and that the expression of the type IIa NPT (Npt2a) and Pit-1 was also detected in chick chondrocytes (13). Moreover, it has been demonstrated that Pi modulates chondrocyte differentiation (14-19) and apoptosis (13,20).

Based on these earlier results, we hypothesized that the NPT/Pi system plays a critical role in the regulation of chondrocyte differentiation. We found that Pit-1 expression in chondrocytes is decreased in Hyp mice compared to WT mice and that Pit-1 regulates apoptosis and mineralization in chondrocytes through modulating intracellular ATP synthesis and apoptotic signaling activity. On the other hand, Hyp chondrocytes showed no changes in GAG synthesis, which is an early event in chondrogenesis. Our findings suggest that ATP synthesis mediated by Pi influx via Pit-1 is critical in the regulation of late chondrogenesis including apoptosis and mineralization and that the differentiation of cartilage is an ATP-dependent event.

**EXPERIMENTAL PROCEDURES**

**Animals** - All mice used were of the C57BL/6J strain. Normal mice were purchased from Nihon-Dobutsu Inc. (Osaka, Japan). Hyp mice were initially obtained from The Jackson Laboratory (Bar Harbor, ME), and were produced by cross-mating homozygous Hyp females (Hyp/Hyp) to hemizygous Hyp males (Hyp/Y). All animal experiments were performed according to the guideline of the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry.

**Isolation and culture of mouse growth plate chondrocytes** - Growth plate chondrocytes were isolated from the ribs of 4-week-old normal and Hyp mice by sequential digestion with 0.2% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes and 0.2% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 3 hours as previously reported (21). Isolated cells were plated onto 100-mm tissue culture dishes at a density of 1x10⁶ cells in α-minimum essential medium (α-MEM: Sigma, St. Louis, MO) supplemented with 10% FCS (Valley Biomedical Inc., Winchester, VA), 2 mmol/L L-glutamine and 0.1 mg/mL kanamycin. Two days later, to induce chondrogenesis and cartilage nodule formation, cells were plated at 3x10⁵ cells/well onto 24-well plates or at 5x10⁴ cells/well on 96-well plates coated with type I collagen (Nitta Gelatin Inc., Osaka, Japan) and cultured in the differentiation medium consisting of Dulbecco’s modified Eagle medium (DMEM: Sigma) supplemented with 10% FCS, 50 μg/mL ascorbic acid and 100 ng/mL recombinant human bone morphogenetic protein-2 (rhBMP-2, Astellas Pharma Inc. Tokyo, Japan) for 7 days. From day 5 to day 7, to promote matrix mineralization, 5 mM β-glycerophosphate was added to the differentiation medium.

**Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR** - Total RNA from chondrocytes was prepared using RNAeasy kit (QIAGEN Inc., Valencia, CA) and reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). The primer sequences for mouse Npt1, mouse Npt2a, GAPDH and β-actin are available on request. PCR assays were performed using Taq DNA Polymerase (New England Biolabs Inc., Ipswich, MA) and dNTP Mix (Promega Corp. Madison, WI). Real-time PCR assays were performed using a LightCycler system (Roche Diagnostics) according to the manufacturer’s instructions. Each reaction was carried out with QIAGEN QuantiTect SYBR Green PCR Master Mix. The expression levels of mRNA are indicated as the relative expression normalized by GAPDH. The primer sequences are available on request. Each procedure was repeated at least four times to assess reproducibility.

**Measurement of Na-dependent Pi uptake** - Assay for Na-dependent Pi uptake by growth plate chondrocytes was performed essentially as described (22). Briefly, confluent cells cultured in 24 well Costar microtiter dishes were incubated in 2 ml of uptake solution (150 mmol/L NaCl, 1.0 mmol/L CaCl₂, 1.8 mmol/L MgSO₄, 10 mmol/L HEPES, pH 7.4) at 37°C for 5 min. Transport was then initiated by replacing the uptake solution with fresh uptake solution (2 ml), supplemented with 0.1 mmol/L KH₂PO₄, and containing 3 μCi/mL of KH₃PO₄ (MP Biomedicals Inc, Irvine, CA). Cells were then incubated for 5 min at 37°C, and the reaction was stopped by the addition of ice-cold uptake solution supplemented with 150 mmol/L choline chloride substitution for NaCl. The same solution was then used to wash the cells three times (2 ml per wash), dissolved in 0.2 N NaOH, and the ³¹P activity was counted on a scintillation counter. As a control, Na-independent Pi transport was measured in the same way, except that NaCl was
Reduced-Serum Medium. After adding the siRNA described (24). PFA from day 1 to day 7.

Chondrocytes were treated with phosphonoformic acid (PFA or foscarnet, Sigma), which is a competitive inhibitor of NPT (23), at concentrations of 10⁻⁵ M to 10⁻⁵ M respectively. In a separate tube 20 μL RNAi duplex oligoribonucleotides was added to 1 mL serum-free Opti-MEM I Reduced-Serum Medium (Invitrogen) were plated onto 24-well plates or 96-well plates to determine Pi-transport, intracellular ATP levels, caspase activity and apoptosis.

Knockdown of Npt2a and Pit-1 by siRNA - Chondrocytes were seeded at a density of 5x10⁵ cells in 100-mm tissue culture dishes in α-MEM supplemented with 10% FCS and 2 mmol/L L-glutamine. The sequences of Stealth RNAi duplex oligoribonucleotides for mouse Npt2a and mouse Pit-1 are available on request. Npt2a-targeted, Pit-1-targeted, or negative control (medium GC, siNEGATIVE, Invitrogen) Stealth RNAi duplex oligoribonucleotides was added to 1 mL serum-free Opti-MEM I Reduced-Serum Medium (Invitrogen) in a final concentration of 24 nM respectively. In a separate tube 20 μL Lipofectamine RNAiMAX (Invitrogen) were diluted in 1 mL serum-free Opti-MEM I Reduced-Serum Medium. After adding the siRNA solution to the Lipofectamine solution, the final transfection mixture was incubated for 20 min at room temperature. This transfection mixture was applied to the cells. After 48 hours, RNA extraction was performed for RT-PCR and transfected chondrocytes were plated onto 24-well plates or 96-well plates to determine Pi-transport, intracellular ATP levels, caspase activity and apoptosis.

Measurement of apoptotic cell death - DNA fragmentation was measured using Cell Death Detection ELISA PLUS kit (Roche Diagnostics Inc), which detects the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) by photometric enzyme-immunoassay. Briefly, after differentiation of chondrocytes in 96-well plates, cell lysates were used for ELISA procedure, following the manufacturer’s protocol. DNA fragmentation was quantified at 405 nm. Results were normalized to cellular protein concentration.
Measurement of caspase-9 and caspase-3 activity - Activity of caspase-3 and caspase-9 was measured using the Caspase-Glo 3/7 Assay kit (Promega) and the Caspase-Glo 9 Assay kit (Promega) according to the manufacturer’s instructions. Chondrocytes were cultured at a density of 5 x 10^3 cells/well for 5 days in 96-well plates in the differentiation medium and processed for caspase-9 and caspase-3 activity assays. The luminescence was measured using a luminometer (TD-20/20, Tuner Designs, Sunnyvale, CA). Results were normalized to cellular protein concentration.

Measurement of intracellular ATP levels - Intracellular ATP levels were measured by using ATP Assay kit (Calbiochem, Darmstadt, Germany). This assay utilizes luciferase to catalyze the formation of light from ATP and luciferin. Luminescence was measured using a luminometer (TD-20/20, Tuner Designs, Sunnyvale, CA). Results were normalized to cellular protein concentration.

Treatment with ATP synthesis inhibitor - In vitro: 3-bromopyruvate (3-BrPA, Sigma), a strong alkylation agent that abolishes cell ATP production via the inhibition of both glycolysis and oxidative phosphorylation (25-27), was added at 10^{-6}M–10^{-5}M in the differentiation medium from day 1 to day 7. In vivo: 3-BrPA (20 μg/kg of body weight) was intraperitoneal injected daily for 10 days into C57BL/6J mice. Control mice received vehicle PBS.

Statistical Analysis - Data were presented as mean +/- SEM. Raw data were analyzed by Mann-Whitney’s U test or one-way analysis of variance followed by a post hoc test (Fisher’s projected least significant difference) (StatView, SAS Institute Inc., Cary, NC) with a significance level of p < 0.05.

RESULTS

Reduced apoptosis and mineralization in growth plate cartilage in Hyp mice - It has been reported that apoptosis is a prerequisite to mineralization of chondrocytes (28). Previous studies including ours have reported that the growth plate cartilage in Hyp mice is hypominalized (5,6). We, therefore, examined apoptosis in the growth plate cartilage in Hyp mice compared to WT mice. Histological examination revealed that hypertrophic cartilage was elongated and disorganized in Hyp mice (Figure 1A, left). In conjunction with this, TUNEL staining showed decreased apoptosis in hypertrophic cartilage in Hyp mice (Figure 1A, right and Figure 1B). Consistent with these in vivo results, chondrocytes isolated from Hyp mice (Hyp chondrocytes) in culture showed decreased apoptosis assessed by DNA fragmentation using a commercially-available ELISA kit (Figure 1C) and mineralization determined by alizarin red staining (Figure 1D, bottom). Quantification of alizarin red staining was shown in Figure 1F. However, GAG synthesis that takes place at an early stage of chondrogenesis was not altered in Hyp chondrocytes as determined by alcian blue staining (Figure 1D, top) Alcian blue staining was quantified in Figure 1E.

Cellular events involved in reduced apoptosis in Hyp chondrocytes - Since activation of caspase-9 and caspase-3 is an important step that leads to apoptosis, the activity of caspase-9 and caspase-3 was next determined in WT and Hyp chondrocytes in culture. The activity of caspase-9 (Figure 2A) and caspase-3 (Figure 2B) was significantly decreased in Hyp chondrocytes. Adenosine triphosphate (ATP) has been reported to be critical in the activation of caspase-9 and caspase-3 (29, 30). Accordingly, we determined intracellular ATP levels in WT and Hyp chondrocytes and found that intracellular ATP levels in Hyp chondrocytes were significantly reduced compared with WT chondrocytes (Figure 2C). Collectively, these results suggest that decreased ATP levels impaired caspase signals and following apoptosis in Hyp chondrocytes.

Disturbed Pi homeostasis in Hyp chondrocytes - It has been described that Pi (polyphosphate) is a source of ATP (31). Accordingly, we next examined whether Pi uptake was changed in Hyp chondrocytes. As expected, we found that Pi uptake was significantly less in Hyp chondrocytes than WT chondrocytes (Figure 3A). Since cellular Pi uptake is under the control of NPT (11), NPT expression in Hyp chondrocytes was subsequently determined. RT-PCR showed that the type III NPT Pit-1 expression was decreased in Hyp chondrocytes (Figure 3B) and real-time PCR demonstrated Pit-1 expression was reduced at early stages of chondrocyte culture (Figure 3C). Consistent with our results, previous studies also reported that an increase in Pit-1 expression at early stage was associated with late chondrocyte differentiation (16, 18). On the other hand, there was no difference in the type II Npt2a expression between WT and Hyp chondrocyte (Figure 3B and Figure 3D). The type I Npt1 expression was not
detected in WT and Hyp chondrocytes (Figure 3B). These results suggest that Pi uptake via Pit-1 is specifically involved in the regulation of chondrogenesis including apoptosis and mineralization.

Suppression of chondrocyte differentiation by NPT inhibitor - To verify whether a decrease in Pi uptake due to reduced Pit-1 expression is responsible for a reduction in ATP levels in Hyp chondrocytes, we determined the effects of phosphonoformic acid (PFA or foscarinet), which is a competitive inhibitor of Pi uptake via NPT (23), on intracellular ATP levels. PFA (10^-3-10^-5 M) reduced Pi-uptake in chondrocytes in a dose-dependent manner (data not shown). PFA (10^-5 M) profoundly reduced intracellular ATP levels (Figure 4A). Of note, PFA treatment caused disorganization of growth plate cartilage (Figure 4B, left) and significantly decreased the number of TUNEL-positive chondrocytes in the hypertrophic cartilage (Figure 4B, right and Figure 4C) in a similar manner to those seen in Hyp mice. Consistent with these in vivo results, PFA markedly inhibited mineralization of chondrocytes in a dose-dependent manner (Figure 4D, bottom and 4E), while GAG synthesis was not affected by PFA treatment (Figure 4D, top). Furthermore, PFA also inhibited caspase-9 (Figure 4F) and caspase-3 activity (Figure 4G). We determined serum Pi levels in PFA-treated mice. There was a trend of decreased serum Pi levels in PFA-treated mice but it was not significantly different (Figure 4H). The results are consistent with the notion that Pi uptake via Pit-1 is closely associated with late chondrogenesis including apoptosis and mineralization through reducing ATP synthesis. These results also suggest an important role for intracellular Pi over extracellular Pi in the regulation of apoptosis and ATP synthesis in chondrocytes.

Suppression of chondrocyte differentiation by NPT siRNA - To further and more specifically verify the role of Pit-1 on chondrocyte differentiation, we performed knock-down experiments using small interfering RNA (siRNA) for Pit-1. As control, Npt2a was also knocked-down. siPit-1 and siNpt2a profoundly reduced Pit-1 and Npt2a mRNA levels in WT chondrocytes, respectively (Figure 5A). Pit-1 knock-down by siPit-1 significantly decreased Pi-uptake (Figure 5B), intracellular ATP levels (Figure 5C), caspase-9 (Figure 5D) and caspase-3 activity (Figure 5E). In parallel with these, apoptosis (Figure 5F) and mineralization (Figure 5G and 5H) were also suppressed. In contrast, knock-down of Npt2a by siNpt2a had no effects on apoptosis and mineralization and other determinations (Figure 5B, 5C, 5D, 5E, 5F, 5G and 5H). These data suggest that Pit-1 specifically controls Pi uptake and following cascades of ATP-dependent caspase signaling, apoptosis and mineralization in chondrocytes.

Recovery of differentiation in Hyp chondrocytes by Pit-1 overexpression - As an alternative approach to confirm a critical role of Pit-1 in apoptosis and mineralization in chondrocytes, we next examined the effects of Pit-1 overexpression on Hyp chondrocytes. Pit-1 overexpression significantly increased Pi uptake (Figure 6A) and intracellular ATP levels (Figure 6B) in Hyp chondrocytes. Furthermore, Pit-1 overexpression also stimulated caspase-9 (Figure 6C) and caspase-3 activity (Figure 6D), apoptosis (Figure 6E) and mineralization (Figure 6F and 6G). WT chondrocytes also showed significantly increased Pi uptake (Figure 6A), intracellular ATP levels (Figure 6B), caspase-9 (Figure 6C) and caspase-3 activity (Figure 6D), apoptosis (Figure 6E) and mineralization (Figure 6F and 6G) by Pit-1 overexpression. These results further suggest that Pit-1 is critical in the regulation of Pi uptake and following cascades of ATP-dependent caspase signaling, apoptosis and mineralization in chondrocytes.

Suppression of chondrocyte differentiation by ATP synthesis inhibitor - To further examine the role of intracellular ATP in chondrocyte differentiation, we studied the effects of the ATP synthesis inhibitor 3-bromopyruvate (3-BrPA). 3-BrPA (10^-5M) significantly reduced intracellular ATP levels in WT chondrocytes in culture (data not shown). Caspase-9 (Figure 7A) and caspase-3 activity (Figure 7B) were also significantly decreased in 3-BrPA-treated chondrocytes. More importantly, 3-BrPA treatment significantly decreased the number of TUNEL-positive chondrocytes in the hypertrophic zone in mice (Figure 7C and Figure 7D). 3-BrPA inhibited chondrocyte mineralization in a dose-dependent manner (Figure 7E and 7F). However, GAG synthesis was not affected by 3-BrPA (Figure 7E). The serum Pi levels in 3-BrPA-treated mice were not significantly different from control mice (Figure 7G), suggesting an important role for intracellular Pi over extracellular Pi. These results suggest that ATP synthesis is important for chondrocytes to undergo apoptosis via caspase signaling and advance to mineralization.
DISCUSSION

In the present study, we explored the role of Pi/NPT system in chondrogenesis using Hyp mice compared with WT mice. We found that Hyp mice exhibited a widened and disorganized hypertrophic zone with reduced chondrocyte apoptosis compared with WT mice. In addition, PFA (a competitive inhibitor of Pit-1) or 3-BrPA (an ATP synthesis inhibitor) markedly caused elongation and disorganization of hypertrophic cartilage with reduced apoptosis in WT mice in a similar manner to Hyp mice. It is noted that the disorders in the hypertrophic zone were most severe in Hyp mice compared with PFA- or 3-BrPA-treated mice, despite that the number of TUNEL-positive cells are comparable in these mice. We postulate that the disorders in Hyp mice are congenital and irreversible and thus most severe, whereas the disorders seen in PFA- and 3-BrPA-treated mice are due to transient exposure of these agents and reversible and thus less severe. Consistent with these in vivo results, Hyp chondrocytes in culture exhibited decreased activity of the apoptotic signaling including caspase-9 and caspase-3 and apoptosis and mineralization following to reduced Pi uptake and cellular ATP synthesis. Furthermore, PFA or 3-BrPA diminished caspase-9 and caspase-3 activity, apoptosis and mineralization in conjunction with a reduction in Pi uptake and ATP synthesis. Decreased Pit-1 expression and Hyp skeletal phenotype - Decreased Pi-uptake in Hyp chondrocytes is likely primarily due to reduced Pit-1 mRNA expression. Type Ila NPT expression was not diminished in Hyp chondrocytes and type I NPT was not expressed in chondrocytes. Earlier reports described that disturbed endochondral ossification was not rescued by Pi supplementation in Hyp mice (35-37), suggesting that intrinsic factors are involved. Miao et al (5) showed that reduced expression of PHEX and MMP-9 was associated with cartilage abnormalities in Hyp mice. Our results suggest that Pit-1 is one of these intrinsic factors responsible for the abnormal chondrogenesis seen in Hyp mice as well.

Regulation of Pit-1 expression - The mechanism underlying down-regulation of Pit-1 expression in Hyp chondrocytes is unknown. Recent studies have reported that stanniocalcin 1 (STC1) increases Pit-1 mRNA expression in osteoblasts (38) and STC1 and STC2 have been shown to regulate Pi-uptake in chicken chondrocytes (39). STC1 stimulates renal Pi uptake and increases Pit1 expression in osteoblasts (40), whereas STC2 inhibits the Pit1 expression and renal Pi uptake (38). Thus, STC1 and STC2 have an opposite action in the regulation of Pit-1 expression. Therefore, it is intriguing to examine whether STC1 or STC2 is involved in the Pit-1 expression in chondrocytes. In preliminary experiments, we determined the expression of Stc1 and Stc2 mRNA in WT and Hyp chondrocytes using RT-PCR and real-time PCR. The Stc2 mRNA was expressed in both WT and Hyp chondrocytes at the same level (data not shown). However, the expression of Stc1 mRNA was decreased in Hyp

ATP synthesis and chondrogenesis - A notable and novel finding obtained in this study is that 3-BrPA inhibits apoptosis and mineralization in growth plate hypertrophic cartilage in vivo and primary chondrocytes in vitro. 3-BrPA is an alkylating agent that decreases cellular ATP via inhibition of hexokinase in glycolysis and is shown to promote cancer cell death through activation of mitochondrial pathway of apoptosis or necrosis (33). Of note, ATP-depleting effect of 3-BrPA is prominent only in tumor cells but not apparent in non-transformed cells (34). Hence, it has been proposed that 3-BrPA could be an anti-cancer agent for varieties of cancers. In addition to these effects on cancers, our results show that 3-BrPA inhibits the differentiation of cartilage, suggesting that ATP generation is also necessary for non-transformed chondrocytes to differentiate and that chondrogenesis is thus an energy-dependent biological event.

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
chondrocytes compared with WT chondrocytes (data not shown). These results suggest that STC1 but not STC2 regulates Pit-1 expression in chondrocytes.

Involvement of fibroblast growth factor23 - Fibroblast growth factor 23 (FGF23) is a hormone that regulates serum Pi levels (41). FGF23 requires Klotho for its signaling as the co-receptors in addition to the canonical FGFR1(IIIc) (42, 43). Mice transgenic for FGF23 displayed a reduction in Npt2a expression in the renal proximal tubules (44), indicating that FGF23 is a negative regulator of Npt2a expression, raising the possibility that Klotho-dependent FGF23 signaling regulates Pit-1 expression in chondrocytes as well. FGF23 expression was predominantly localized in osteoblasts, cementoblasts, and odontoblasts, with a sporadic expression in some chondrocytes, osteocytes and cementocytes (45). However, we were not able to demonstrate FGF23 expression in primary mouse chondrocytes by RT-PCR. Further studies are needed to elucidate the relationship between FGF23 signaling and Pit-1 expression in cartilage.

In conclusion, we have found in the present study that chondrogenesis is modulated by cellular Pi uptake via Pit-1 and cellular ATP synthesis and thus is a biological event that depends on mitochondrial energy generation. We believe that these findings should provide us with a novel concept and alternative approaches to study the cellular differentiation that occurs in physiological conditions and also to analyze the skeletal abnormalities seen in congenital hypophosphatemic disorders such as XLH.

FOOTNOTES

We thank Dr. Kenichi Miyamoto and Dr. Hiroko Segawa (University of Tokushima Graduate School, Tokushima, Japan) for the kind gift of mouse Pit-1 cDNA. This work was partly supported by the 21st Century COE program entitled “Origination of Frontier BioDentistry” at Osaka University Graduate School of Dentistry, supported by the Ministry of Education, Culture, Sports, Science and Technology and by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (T.Y. #A202290100).

The abbreviations used are: XLH, X-linked hypophosphatemia; Pi, phosphate; GAG, glycosaminoglycans; Npt2a, type Ila sodium-dependent Pi transporter; RT-PCR, Reverse transcription-polymerase chain reaction; PFA, phosphonoformic acid; ip, intraperitoneal injection; DAPI, 4',6-Diamidino-2-phenylindole; 3-BrPA, 3-Bromopyruvate; WT, wild-type; STC, Stanniocalcin; FGF23, fibroblast growth factor 23; ATPase, adenosine triphosphatase.

REFERENCES

1. Zuscik, M. J., Hilton, M. J., Zhang, X., Chen, D., and O'Keefe, R. J. (2008) J Clin Invest 118(2), 429-438
2. Winters, R. W., Graham, J. B., Williams, T. F., Mc, F. V., and Burnett, C. H. (1958) Medicine (Baltimore) 37(2), 97-142
3. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) Am J Hum Genet 60(4), 790-797
4. Eicher, E. M., Southard, J. L., Scriver, C. R., and Glorieux, F. H. (1976) Proc Natl Acad Sci U S A 73(12), 4667-4671
5. Miao, D., Bai, X., Panda, D. K., Karaplis, A. C., Goltzman, D., and McKee, M. D. (2004) Bone 34(4), 638-647.
6. Hayashibara, T., Hiraga, T., Sugita, A., Wang, L., Hata, K., Ooshima, T., and Yoneda, T. (2007) J Bone Miner Res 22(11), 1743-1751
7. Boyde, A., and Shapiro, I. M. (1980) Histochemistry 69(1), 85-94.
8. Kakuta, S., Golub, E. E., and Shapiro, I. M. (1985) Calcif Tissue Int 37(3), 293-299.
9. Mwale, F., Tchetina, E., Wu, C. W., and Poole, A. R. (2002) J Bone Miner Res 17(2), 275-283.
10. Shapiro, I. M., and Boyde, A. (1984) Metab Bone Dis Relat Res 5(6), 317-326.
11. Virkki, L. V., Biber, J., Murer, H., and Forster, I. C. (2007) Am J Physiol Renal Physiol 293(3), F643-654
12. Palmer, G., Zhao, J., Bonjour, J., Hofstetter, W., and Caverzasio, J. (1999) Bone 24(1), 1-7.
13. Mansfield, K., Teixeira, C. C., Adams, C. S., and Shapiro, I. M. (2001) Bone 28(1), 1-8.
14. Cecil, D. L., Rose, D. M., Terkeltaub, R., and Liu-Bryan, R. (2005) Arthritis Rheum 52(1), 144-154.
15. Fujita, T., Meguro, T., Izumo, N., Yasutomi, C., Fukuyama, R., Nakamuta, H., and Koida, M. (2001) *Jpn J Pharmacol* **85**(3), 278-281.
16. Guicheux, J., Palmer, G., Shukunami, C., Hiraki, Y., Bonjour, J. P., and Caverzasio, J. (2000) *Bone* **27**(1), 69-74.
17. Montessuit, C., Caverzasio, J., and Bonjour, J. P. (1991) *J Biol Chem* **266**(27), 17791-17797.
18. Wang, D., Canaff, L., Davidson, D., Corluka, A., Liu, H., Hendy, G. N., and Henderson, J. E. (2001) *J Biol Chem* **276**(36), 33995-34005.
19. Wu, L. N., Guo, Y., Genge, B. R., Ishikawa, Y., and Wuthier, R. E. (2002) *J Cell Biochem* **86**(3), 475-489.
20. Magne, D., Bluteau, G., Faucheux, C., Palmer, G., Vignes-Colombeix, C., Pilet, P., Rouillon, T., Caverzasio, J., Weiss, P., Daculsi, G., and Guicheux, J. (2003) *J Bone Miner Res* **18**(8), 1430-1442.
21. Shimomura, Y., Yoneda, T., and Suzuki, F. (1975) *Calcif Tissue Res* **19**(3), 179-187.
22. Rowe, P. S., Ong, A. C., Cockerill, F. J., Goulding, J. N., and Hewison, M. (1996) *Bone* **18**(2), 159-169.
23. Loghman-Adham, M. (1996) *Gen Pharmacol* **27**(2), 305-312.
24. Swenson, C. L., Weisbrode, S. E., Nagode, L. A., Hayes, K. A., Steinmeyer, C. L., Mathes, L. E. (1991) *Calcif Tissue Int* **48**(5), 353-361.
25. Geschwind, J. F., Ko, Y. H., Torbenson, M. S., Magee, C., and Pedersen, P. L. (2002) *Cancer Res* **62**(14), 3909-3913.
26. Jones, A. R., Gillan, L., and Milmlow, D. (1995) *Contraception* **52**(5), 317-320.
27. Ko, Y. H., Smith, B. L., Wang, Y., Pomper, M. G., Rini, D. A., Torbenson, M. S., Hullien, J., and Pedersen, P. L. (2004) *Biochem Biophys Res Commun* **324**(1), 269-275.
28. Gibson, G. (1998) *Microse Res Tech* **43**(2), 191-204.
29. Eguchi, Y., Srinivasan, A., Tomaselli, K. J., Shimizu, S., and Tsujimoto, Y. (1999) *Cancer Res* **59**(9), 2174-2181.
30. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91**(4), 479-489.
31. Rao, N. N., Gomez-Garcia, M. R., and Kornberg, A. (2009) *Annu Rev Biochem* **78**, 605-647.
32. Zalutskaya, A. A., Cox, M. K., and Demay, M. B. (2009) *J Cell Biochem* **108**(3), 668-674.
33. Pelicano, H., Martin, D. S., Xu, R. H., and Huang, P. (2006) *Oncogene* **25**(34), 4633-4646.
34. Xu, R. H., Pelicano, H., Zhou, Y., Carew, J. S., Feng, L., Bhalla, K. N., Keating, M. J., and Huang, P. (2005) *Cancer Res* **65**(2), 613-621.
35. Ecarot, B., Glorieux, F. H., Desbarats, M., Travers, R., and Labelle, L. (1992) *J Bone Miner Res* **7**(5), 523-530.
36. Tanaka, H., Seino, Y., Shima, M., Yamaoka, K., Yabuuchi, H., Yoshikawa, H., Masuhara, K., Takaoka, K., and Ono, K. (1988) *Bone Miner* **4**(3), 237-246.
37. Yoshikawa, H., Masuhara, K., Takaoka, K., Ono, K., Tanaka, H., and Seino, Y. (1985) *Bone* **6**(4), 235-239.
38. Yoshiko, Y., Candeliere, G. A., Maeda, N., and Aubin, J. E. (2007) *Mol Cell Biol* **27**(12), 4465-4474. Epub 2007 Apr 4416.
39. Wu, S., Yoshiko, Y., and De Luca, F. (2006) *J Biol Chem* **281**(8), 5120-5127. Epub 2005 Dec 5123.
40. Ishibashi, K., and Imai, M. (2002) *Am J Physiol Renal Physiol* **282**(3), F367-375.
41. Fukumoto, S., and Yamashita, T. (2007) *Bone* **40**(5), 1190-1195.
42. Kurosu, H., Ogawa, Y., Miyoshi, M., Yamamoto, M., Nandi, A., Rosenblatt, K. P., Baum, M. G, Schiavi, S., Hu, M. C., Moe, O. W., and Kuro-o, M. (2006) *J Biol Chem* **281**(10), 6120-6123. Epub 2006 Jan 6125.
43. Urakawa, I., Yamazaki, Y., Shimada, T., Iijima, K., Hasegawa, H., Okawa, K., Fujita, T., Fukumoto, S., and Yamashita, T. (2006) *Nature* **444**(7120), 770-774.
44. Shimada, T., Urakawa, I., Yamazaki, Y., Hasegawa, H., Hino, R., Yoneya, T., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2004) *Biochem Biophys Res Commun* **314**(2), 409-414.
45. Yoshiko, Y., Wang, H., Minamizaki, T., Ijuin, C., Yamamoto, R., Suemune, S., Kozai, K., Tanne, K., Aubin, J. E., and Maeda, N. (2007) *Bone* **40**(6), 1565-1573.

**FIGURE LEGENDS**
FIGURE 1. Apoptosis and related events in Hyp chondrocytes. A, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed using tibiae of 4-week-old WT and Hyp mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. Representative pictures obtained out of numerous sections of four mice from each group are shown. B, Number of TUNEL-positive cells in tibial growth plate of WT and Hyp mice. C, Quantitative determination of chondrocyte apoptosis. Cells were cultured in the differentiation medium in 96-well plates for 7 days. The determination was conducted using the Cell Death Detection ELISA PLUS after differentiation. Data are shown as apoptotic activity. D, Histochemical staining of WT and Hyp chondrocytes. Cells were cultured for 7 days in the differentiation medium and stained with alcian blue for GAG synthesis (top) and with alizarin red-S for mineralization (bottom). E, Quantification of alcian blue staining. F, Quantification of alizarin red staining. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from WT chondrocytes (p<0.05).

FIGURE 2. Activity of apoptotic signaling pathways. A, Caspase-9 activity in WT and Hyp chondrocytes. B, Caspase-3 activity in WT and Hyp chondrocytes. Activity was measured using the Caspase-Glo 9 and Caspase-Glo 3/7 assay kit after differentiation. C, Intracellular ATP levels in WT and Hyp chondrocytes. Cells were cultured at a density of 1 x 10^4 cells/well in 96-well plates for 24 hours. ATP levels were measured using the ATP assay kit.

FIGURE 3. Characterization of Hyp chondrocytes. A, Time-course of Pi uptake in WT (open circle) and Hyp (solid circle) chondrocytes. Cells were cultured for 7 days in the differentiation medium and Pi uptake was determined as described in Experimental Procedures. B, Expressions of Npt1, Npt2a and Pit-1 mRNA in WT and Hyp chondrocytes. Total RNA isolated from chondrocytes cultured for 24 hours was used for RT-PCR analysis using the primer pairs. β-actin was amplified as control. C, Time-dependent expression of Pit-1 mRNA by real-time PCR. D, Time-dependent expression of Npt2a mRNA by real-time PCR. The amount of Npt2a and Pit-1 of WT chondrocytes at day 0 was designated as 1.0 and normalized to GAPDH. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from WT chondrocytes (p<0.05).

FIGURE 4. Effects of PFA on chondrocyte differentiation. A, Intracellular ATP levels. Cells were cultured in the presence of 10^{-5}M PFA. ATP levels were measured using the ATP assay kit. B, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed on tibial sections from 31-day-old control and PFA-treated mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. Representative pictures obtained out of numerous sections of four mice from each group are shown. C, Number of TUNEL positive cells in tibial growth plate of control and PFA-treated mice. D, Histochemical staining of chondrocytes. Cells were cultured in the presence of 10^{-5} M PFA and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). E, Quantification of alizarin red staining. F, Caspase-9 activity. G, Caspase-3 activity. Cells were cultured in the presence of 10^{-5}M PFA. Activity was measured using the Caspase-Glo 9 and Caspase-Glo 3/7 assay. H, Serum Pi levels. Results are expressed as mean +/- SEM of four separate determinations. *Significantly different from control (p<0.05).

FIGURE 5. Npt2a and Pit-1 knockdown by siRNA in chondrocytes. A, siRNA for siNEGATIVE (Control), siNPT2a or siPIT-1 was transfected in chondrocytes and the expression of NPT2a, Pit-1 or GAPDH was analyzed by RT-PCR. B, Pi-uptake in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes was determined in the presence of 3 μCi/mL of KH2^{32}PO4. C, Intracellular ATP levels in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. D, Caspase-9 activity in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. E, Caspase-3 activity in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. F, Quantitative determination of chondrocyte apoptosis in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. G, Histochemical staining of siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. Cells were cultured and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). H, Quantification of alizarin red staining. We repeated the experiments twice using different preparation of primary chondrocytes and obtained the identical results. Results are expressed as mean +/- SEM of two separate determinations. *Significantly different from control (p<0.05).
FIGURE 6. Effects of Pit-1 overexpression in chondrocytes. A, Empty vector (Control) or Pit-1 was transfected in WT and Hyp chondrocytes. Pi-uptake was determined in the presence of 3 μCi/mL of KH$_2^{32}$PO$_4$. B, Intracellular ATP levels in Control or Pit-1-transfected WT and Hyp chondrocytes. C, Caspase-9 activity in control and Pit-1-transfected WT and Hyp chondrocytes. D, Caspase-3 activity in control and Pit-1-transfected WT and Hyp chondrocytes. E, Quantitative determination of apoptosis in control and Pit-1-transfected WT and Hyp chondrocytes. F, Histochemical staining of control and Pit-1-transfected WT and Hyp chondrocytes. Cells were cultured and stained with alizarin red-S for mineralization. Pit-1 expression was confirmed by RT-PCR. G, Quantification of alizarin red staining. We repeated the experiments twice using different preparation of primary chondrocytes and obtained the identical results. Results are expressed as mean +/- SEM of two separate determinations. *Significantly different from WT control ($p<0.05$). †Significantly different from Hyp control ($p<0.05$).

FIGURE 7. Effects of 3-BrPA on chondrocyte apoptosis and calcification. A, Effects of 3-BrPA on caspase-9 activity. B, Effects of 3-BrPA on caspase-3 activity. Cells were treated with $10^{-6}$ M 3-BrPA for 7 days and measured for caspase activity. C, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed on tibial sections from 31-day-old control and 3-BrPA-treated mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. D, Number of TUNEL-positive cells in tibial growth plate of control and 3-BrPA-treated mice. E, Histochemical staining of chondrocytes. Cells were cultured for 7 days in the presence of $10^{-6}$ and $10^{-5}$ M 3-BrPA and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). G, Quantification of alizarin red staining. H, Serum Pi levels. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from control ($p<0.05$).
Figure 3

A

[Graph showing nmol Pi/mg protein 5min against Days in culture with WT and Hyp lines.

B

[Western blot images of Npt1, Npt2a, Pit-1, and β-actin for WT and Hyp conditions.

C

[Graph showing Pit-1 mRNA expression levels against Days in culture with WT and Hyp lines.

D

[Graph showing Npt2a mRNA expression levels against Days in culture with WT and Hyp lines.]
Figure 4

A. ATP content (% of control)

B. Control vs. PFA images with TUNEL staining.

C. Number of TUNEL positive cells.

D. PFA (M) concentrations and Alizarin red positive area.

E. Caspase-9 activity (RLU).

F. Caspase-9 activity at different PFA concentrations.
Figure 4

G: Caspase-3 activity (RLU)
- Control: 25
- PFA: 15

H: Serum Pi (mg/dl)
- Control: 12
- 500: 12
- 1000: 8

PFA (mg/kg/day)
Figure 5

Graph H shows the Alizarin red positive area (mm²) for Control, siNpt2a, and siPit-1 treatments. The graph indicates a significant decrease in the Alizarin red positive area with siPit-1 treatment compared to Control and siNpt2a.
