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Premature chondrocyte apoptosis and compensatory upregulation of chondroregulatory protein expression in the growth plate of Goto–Kakizaki diabetic rats

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is much more detrimental to bone than previously thought. Specifically, it is associated with aberrant bone remodeling, defective bone microstructure, poor bone quality, and growth retardation. The T2DM-associated impairment of bone elongation may result from a decrease in growth plate function, but the detailed mechanism has been unknown. The present study, therefore, aimed to test hypothesis that T2DM led to premature apoptosis of growth plate chondrocytes in Goto–Kakizaki (GK) type 2 diabetic rats, and thus triggered the compensatory responses to overcome this premature apoptosis, such as overexpression of *Runt*-related transcription factor (Runx)-2 and vascular endothelial growth factor (VEGF), the essential mediators for bone elongation. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of epiphyseal sections successfully revealed increases in chondrocyte apoptosis in the hypertrophic zone (HZ) and chondro-osseous junction of GK rats. Quantitative immunohistochemical analysis further confirmed the overexpression of parathyroid hormone-related protein (PTHrP), Runx2 and VEGF, but not Indian hedgehog (Ihh) in the HZ. Analysis of blood chemistry indicated suppression of bone remodeling with a marked decrease in parathyroid hormone level. In conclusion, GK rats manifested a premature increase in chondrocyte apoptosis in the HZ of growth plate, and a compensatory overexpression of chondroregulatory proteins, such as PTHrP, Runx2, and VEGF. Our results, therefore, help explain how T2DM leads to impaired bone elongation and growth retardation.

1. Introduction

Diabetes mellitus (DM) is a global metabolic problem causing dysfunction of various tissues and organs, such as blood vessels (microangiopathy), kidney (diabetic nephropathy), and peripheral nerves (diabetic neuropathy) [1,2]. Several investigations have found that DM is also associated with abnormal bone metabolism, decreased bone mass, and/or poor bone quality, which are the primary causes of increased fracture risk especially in the hip and spine [1]. Furthermore, DM not only alters bone structure but also impairs longitudinal bone growth—another well-recognized consequence of DM—thereby leading to growth retardation in young humans and rodents [3–5]. This defective bone growth is of special concern at present because of subtypes of DM in young patients, such as maturity onset diabetes of the young (MODY), a monogenic familial disorder that results in a young-onset (age < 25) non-insulin dependent form of DM [6]. It is possible that the impaired bone elongation results from aberrant growth plate function, but the detailed mechanism is unknown.

The growth plate cartilage consisting of chondrocytes and extracellular matrix made of collagen and proteoglycans is located in the epiphyses of long bone [7,8]. It acts as the main region controlling longitudinal (endochondral) bone growth [8]. The growth plate chondrocytes are histologically divided into 3 zones, i.e., reserve zone (RZ), proliferative zone (PZ) and hypertrophic zone (HZ). The RZ contains small and low mitotic activity progenitor cells embedded in extracellular matrix, which gradually migrate to the PZ where they proliferate and become organized into

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parallel vertical columns [7,8]. In the HZ, mature hypertrophic chondrocytes finally undergo programmed cell death (apoptosis) and are replaced by capillaries (vascular invasion) and osteoblasts, which, in turn, are responsible for bone formation and bone elongation [7,8]. On the other hand, premature apoptosis of chondrocytes in the HZ could impair bone elongation [9]. We therefore hypothesized that premature chondrocyte apoptosis may be exaggerated in the growth plate of diabetic rats.

Since chondrocyte maturation and turnover are tightly controlled by chondroregulatory factors and their feedback loops, it is possible that premature chondrocyte apoptosis is accompanied by a compensatory increase in the production of chondroregulatory factors. In general, normal turnover of growth plate chondrocytes requires complex interactions among systemic and local factors. Insulin-like growth factor (IGF)-1 is considered the major systemic growth factor essential for stimulation of longitudinal bone growth [8]. Local chondrocyte-derived factors, particularly Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), and vascular endothelial growth factor (VEGF), orchestrate chondrocyte differentiation, vascular invasion, and subsequent bone formation (for reviews [8,10,11]). Besides humoral factors, chondrocyte differentiation is also controlled by transcription factors, such as *Runt*-related transcription factor (Runx)-2 that is the major transcriptional activator of chondrogenesis and osteogenesis [8].

Therefore, the objectives of the present study were to demonstrate premature chondrocyte apoptosis and the compensatory overexpression of various chondroregulatory factors, e.g., Runx2, PTHrP, and VEGF, in the growth plate of Goto–Kakizaki (GK) rats with type 2 diabetic mellitus (T2DM). Changes in the serum levels of bone turnover markers as well as calciotropic hormone levels were also determined.

2. Materials and methods

2.1. Animals

GK rats are non-obese T2DM substrain of Wistar rats with stable fasting hyperglycemia (~130–140 mg/dL) and insulin resistance [12–15]. Four-week-old female GK rats and age-matched wild-type (WT) Wistar rats were purchased from the Center for Laboratory Experimental Animals (CLEA), Japan, and housed in accordance with the Mahidol University policy for the care and use of animals for scientific purposes. This study has been approved by the ethics committee of the National Laboratory Animal Center (NLAC), Mahidol University. Rats were kept at NLAC in stainless steel cages under 12:12 h light–dark cycle. Room temperature was ~22–24 °C with relative humidity of ~50–60%. They were fed standard chow (Perfect Companion Co., Ltd., Thailand) and reverse osmosis water ad libitum. At the end of experiment, all rats (16 weeks old) were euthanized by 70 mg/kg sodium pentobarbitone i.p.

2.2. Analyses of bone turnover biomarker and hormone levels

Blood sample was collected by cardiac puncture for fasting blood glucose, hormone levels [i.e., insulin (Catalog No. EZRMI-13K; Millipore, St. Charles, Missouri, USA), intact PTH (iPTH; Catalog No. 3KG024, Scantibodies Laboratory, Santee, CA, USA), $25(OH)D_3$ (Catalog No. KAP1971, DIAsource ImmunoAssay, Belgium), $1,25(OH)_2D_3$ (Catalog No. MBS160920, MyBioSource, San Diego, CA, USA) and IGF-1 (Catalog No. MG100, R&D Systems, Minneapolis, MN, USA)], and bone turnover marker levels [i.e., C-terminal telopeptides of type I collagen (CTX-1; Catalog No. AC-06F1, Immunodiagnostic Systems, AZ, USA), N-terminal propeptide of type I procollagen (P1NP; Catalog No. AC-33F1, Immunodiagnostic Systems), and osteocalcin (Catalog No. 601505, Immutopics, San Clemente, CA, USA)].

2.3. Bone preparation for immunohistochemistry

Tibiae were collected for determining the expression levels of chondroregulatory factors (i.e., Runx2, PTHrP, Ihh, and VEGF) by quantitative immunohistochemistry. After being removed, bone was cleaned off the adhering tissue and immediately fixed at 4 °C in 0.1 M phosphate-buffered saline (PBS) containing 4% w/v paraformaldehyde. Thereafter, they were decalcified in 15% w/v ethylenediaminetetraacetic acid (EDTA; Sigma) for 3 weeks, dehydrated and then cleared in graded ethanol and xylene, respectively. After being embedded in paraffin, a bone specimen was longitudinally cut into 5 μm sections with a microtome (model RM2255; Leica, Nussloch, Germany).

2.4. Quantitative immunohistochemical analysis of Runx2, PTHrP, Ihh and VEGF

Bone sections were deparaffinized, rehydrated, and later incubated at 37 °C for 30 min in antigen retrieval solution (0.01 mg/mL proteinase K, 50 mM Tris–HCl pH 8.0 and 5 mM EDTA). They were then incubated with 3% H_2O_2 to inhibit endogenous peroxidase activity. Non-specific binding was blocked by 4–8% bovine serum albumin, 5–10% normal goat serum, and 0.1% Tween-20 in PBS. Thereafter, the sections were incubated at 4 °C overnight with rabbit polyclonal primary antibody against Ihh (Catalog No. sc-13088), Runx2 (Catalog No. sc-10758) or PTHrP (Catalog No. sc-20728), or mouse monoclonal primary antibody against VEGF (Catalog No. sc-53462; all purchased from Santa Cruz Biotechnology, Dallas, TX, USA). After incubation with primary antibody, the sections were washed and incubated with biotinylated anti-IgG, followed by incubation with streptavidin-conjugated horseradish peroxidase solution (Invitrogen, Carlsbad, CA, USA) and ImmPACT AMEC red substrate, 3-amino-9-ethylcarbazole (Vector, Burlingame, CA, USA). As for the negative controls, bone sections were incubated in blocking solution without primary antibody. Finally, all sections were counterstained with hematoxylin and examined under a light microscope using Image Pro Plus 5 (Media Cybernetics, Bethesda, MD, USA). Immunohistochemical signals were quantified as described previously [16,17].

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis

Chondrocyte apoptosis was determined by in situ cell death detection kit (Catalog No. 11684795910, Roche, Indianapolis, IN, USA). Bone sections were deparaffinized, rehydrated, and then incubated in antigen retrieval solution (0.01 mg/mL proteinase K, 10 mM Tris–HCl pH 8.0 and 5 mM EDTA). They were later permeabilized in 0.1% v/v Triton X-100 in 1× PBS before incubating with TUNEL reaction mixture at 37 °C for 60 min in the dark and moist condition. All slides were mounted with 85% glycerol and visualized under Zeiss Axio Observer.Z1 microscope (Carl Zeiss, Germany) operated with AxioVision 4. The number of apoptotic chondrocytes was counted in four fields per each section. Two sections from each animal were analyzed. The regions of interest included the RZ, PZ, and HZ as previously described by Yakar et al. [18] as well as the tibial metaphyseal chondro-osseous (CO) junction [19,20]. The TUNEL-positive signals were expressed as the number of TUNEL-positive cells per field.

2.6. Statistical analysis

The results are expressed as mean ± SE. Two sets of data were compared by unpaired Student's *t*-test. The level of significance was $P < 0.05$. All data were analyzed by GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. Results

Sixteen-week-old GK rats were found to have higher fasting blood glucose than the age-matched WT rats (93.08 ± 2.90 mg/dL and 131.9 ± 6.78 mg/dL in WT and GK groups, respectively; $P < 0.001$). In GK rats, the numbers of apoptotic chondrocytes in the HZ and CO junction, but not RZ and PZ, were significantly greater than those in the WT rats (Fig. 1A–D). Histological examination revealed that the zone of chondrocyte apoptosis moved toward early hypertrophic zone (data not shown), suggesting the presence of premature chondrocyte apoptosis. Interestingly, Runx2 expression in the HZ of GK rats was increased by 2-fold compared to that of the WT rats, while having no change in the RZ and PZ (Fig. 1E–H), consistent with the hypothesis that there was a compensatory response to overcome excessive loss of hypertrophic chondrocytes. Such the compensatory response might also occur at the systemic level since serum IGF-1—a potent stimulator of longitudinal bone growth [8]—was significantly elevated by ~130% in the GK rats (Fig. 1I).

Immunohistochemistry further revealed that PTHrP was abundant in the extracellular matrix of HZ and also present in the proliferative and hypertrophic chondrocytes (Fig. 2A). Quantitative immunohistochemical analyses showed the upregulation of PTHrP expression in the RZ (~4-fold) and HZ (~3-fold), whereas there was no change in Ihh expression in all three zones of growth plate in GK rats (Fig. 2B, C). VEGF was abundantly localized in the

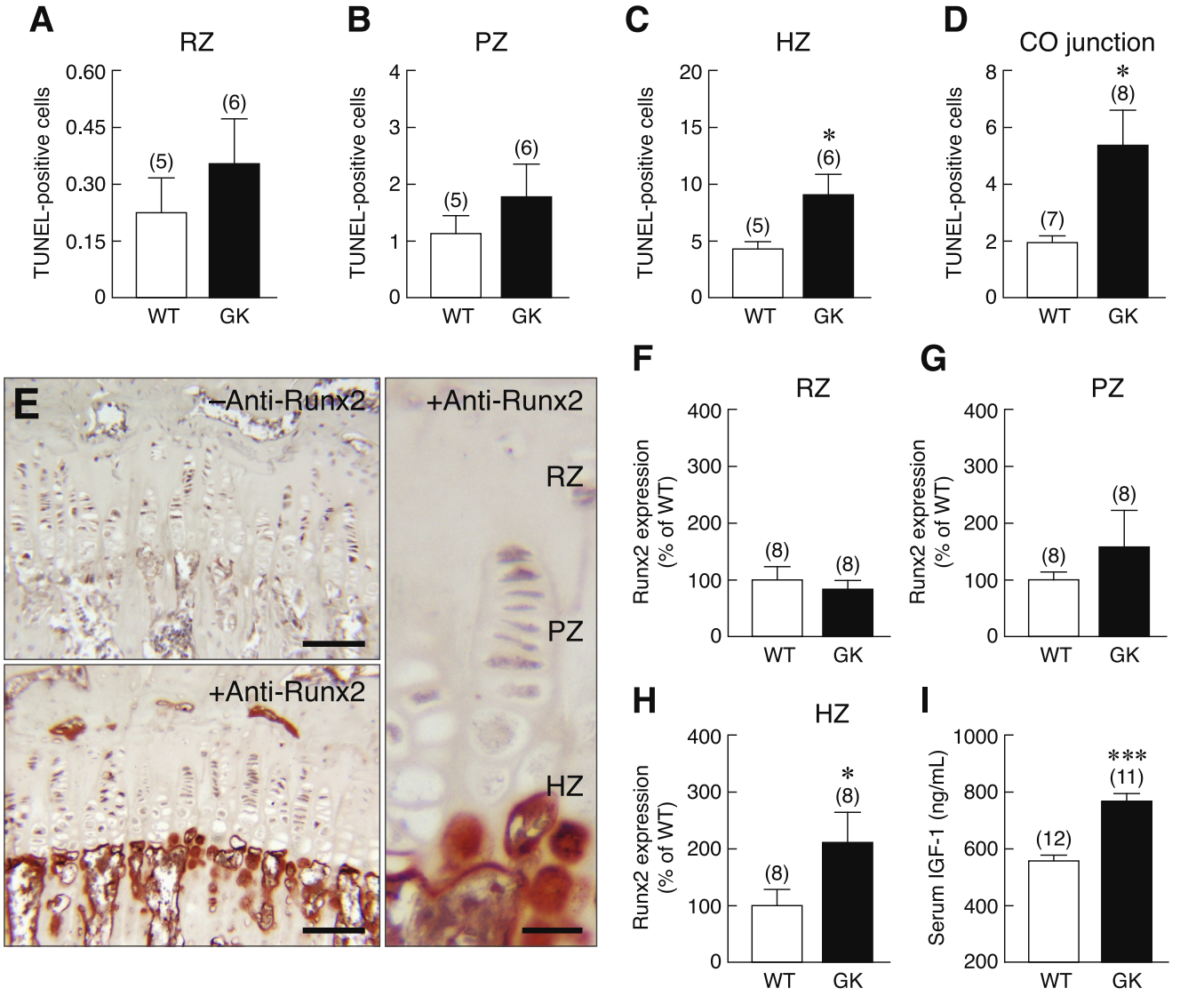

Fig. 1. (A–D) Numbers of apoptotic chondrocytes in the growth plate of wild-type (WT) and GK diabetic rats as determined by TUNEL assay. The regions of interest included the reserve zone (RZ), proliferative zone (PZ), hypertrophic zone (HZ), and chondro-osseous (CO) junction. (E) A representative immunohistochemical photomicrograph shows Runx2 protein in the growth plate of GK rats (200× magnification; scale bar, 100 μm). The paraffin-embedded decalcified tibial section was incubated with primary antibody against Runx2 (+Anti-Runx2), whereas the corresponding negative section was incubated in an absence of anti-Runx2 antibody (−Anti-Runx2). The growth plate area of a GK rat was magnified in the right panel. Scale bar, 25 μm. (F–H) Expression of Runx2 proteins in the RZ, PZ and HZ of WT and GK rats as determined by quantitative immunohistochemistry. (I) Serum IGF-1 levels in WT and GK rats. Numbers in parentheses are the numbers of animals in each group. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with WT.

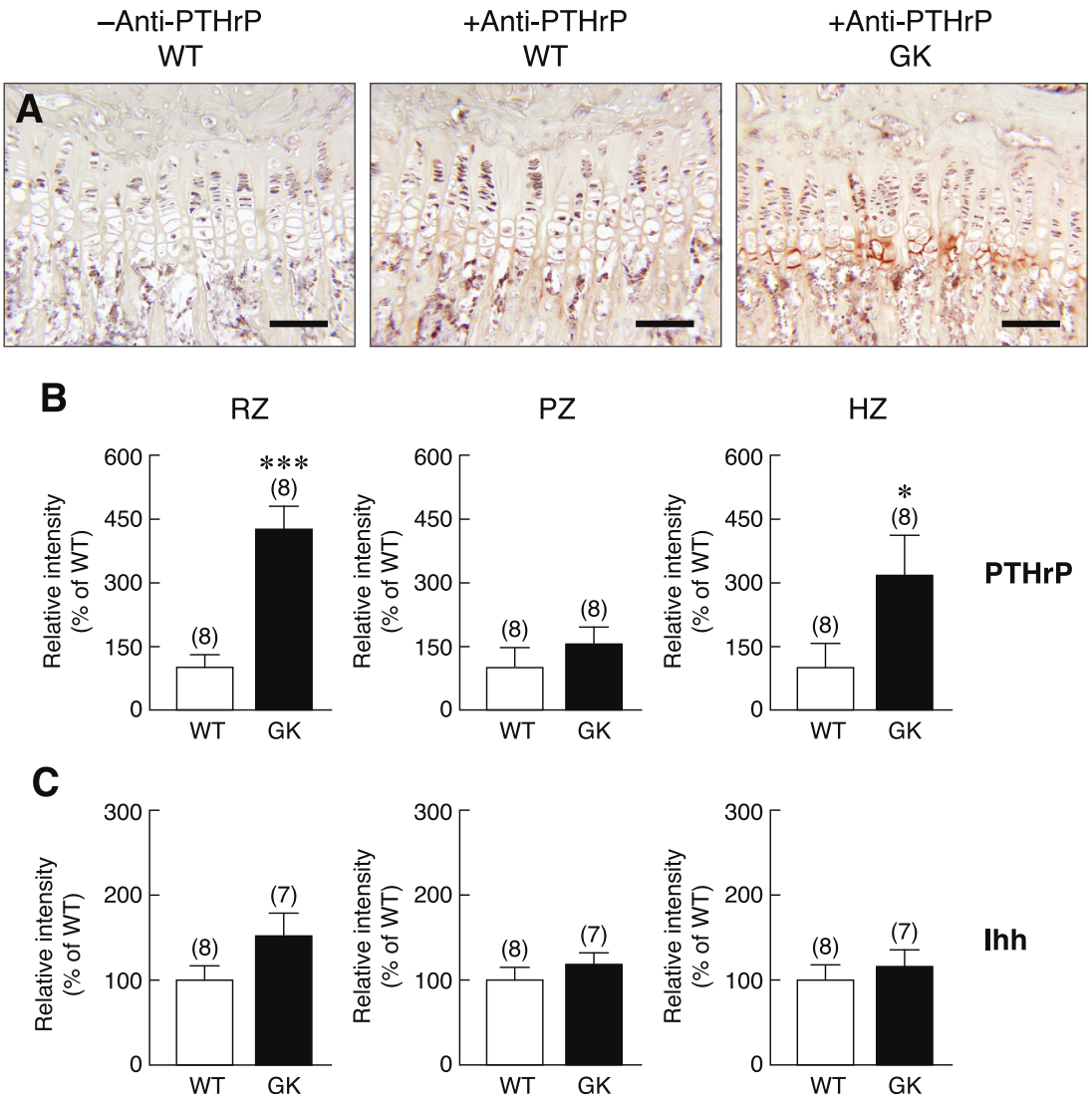

Fig. 2. (A) A representative immunohistochemical photomicrograph of PTHrP expression in the proximal tibial growth plate of WT and GK rats (200× magnification; scale bar, 100 μm). The tibial section was incubated with primary antibody against PTHrP (+Anti-PTHrP), whereas the corresponding negative control section was incubated in an absence of anti-PTHrP antibody (–Anti-PTHrP). (B–C) Relative immunohistochemical signals of PTHrP and Ihh proteins in the RZ, PZ, and HZ of WT and GK rats. Numbers in parentheses are the numbers of animals in each group. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with WT.

hypertrophic chondrocytes as well as in the endothelial cells lining the primary spongiosa (Fig. 3A). VEGF expression in the GK rats was dramatically increased in the RZ and HZ by 3.3- and 7-fold, respectively (Fig. 3B), suggesting an attempt to increase vascular invasion into both ends of the growth plate cartilage in GK diabetic rats.

Regarding the circulating levels of calciotropic hormones and bone turnover markers, the GK rats exhibited lower levels of iPTH and storage form of vitamin D [25(OH)D_3] compared to WT rats, while the levels of 1,25$(OH)_2D_3$, the active form of vitamin D, were higher (Fig. 4A–C). The overall bone turnover was reduced under diabetic condition since the levels of both bone resorption marker (i.e., CTX-1) and bone formation marker (i.e., osteocalcin) appeared lower in the GK rats than WT rats (Fig. 4D and E). However, the P1NP levels in GK and WT rats were similar (Fig. 4F).

4. Discussion

It has long been postulated that DM could cause osteopenia by disrupting bone remodeling [5,21,22], and impeded longitudinal bone growth by inducing abnormal differentiation of growth plate chondrocytes [9]. Here we provided corroborative evidence that further explained the aberrant growth plate function in GK diabetic rats. Specifically, the TUNEL assay revealed increases in apoptotic chondrocytes in HZ and CO junction in GK rats when compared to WT rats. Although normal hypertrophic chondrocytes are programmed to undergo apoptosis prior to vascular invasion and osteoblast-mediated bone formation [7,8,23], the DM-induced premature chondrocyte apoptosis might impair bone elongation, similar to that reported previously in diabetic mice [9,24]. Premature chondrocyte apoptosis might have resulted from several factors, e.g., glucose, insulin, and advance glycation end products (AGEs). Kayal and colleagues [24] demonstrated that chondrocyte apoptosis was significantly higher in diabetic mice, which could be blocked by insulin. Such increases in apoptotic chondrocytes were accompanied by the increased mRNA levels of osteoclastogenic genes and matrix degradation-related genes (e.g., RANKL, TNF-α and ADAMTS-4) [24]. On the other hand, AGEs formed by non-enzymatic reaction of reducing sugar and proteins, which can accumulate and disturb tissue functions [25], have been reported

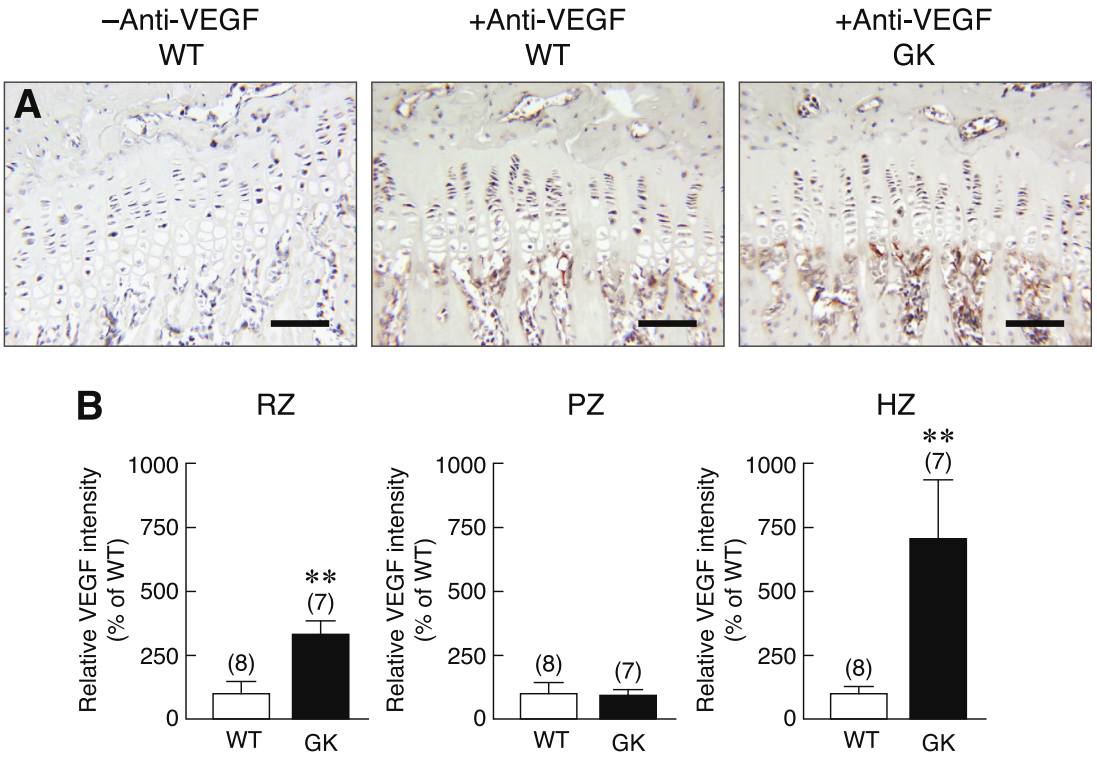

Fig. 3. (A) A representative immunohistochemical photomicrograph of VEGF expression in the proximal tibial growth plate of WT and GK rats (200× magnification; scale bar, 100 μm). The tibial section was incubated with primary antibody against VEGF (+Anti-VEGF), whereas the corresponding negative control section was incubated in an absence of anti-VEGF antibody (−Anti-VEGF). (B) Relative immunohistochemical signals (arbitrary unit) of VEGF protein in the RZ, PZ, and HZ of WT and GK rats. Numbers in parentheses are the numbers of animals in each group. $^{**}P < 0.01$ compared with WT.

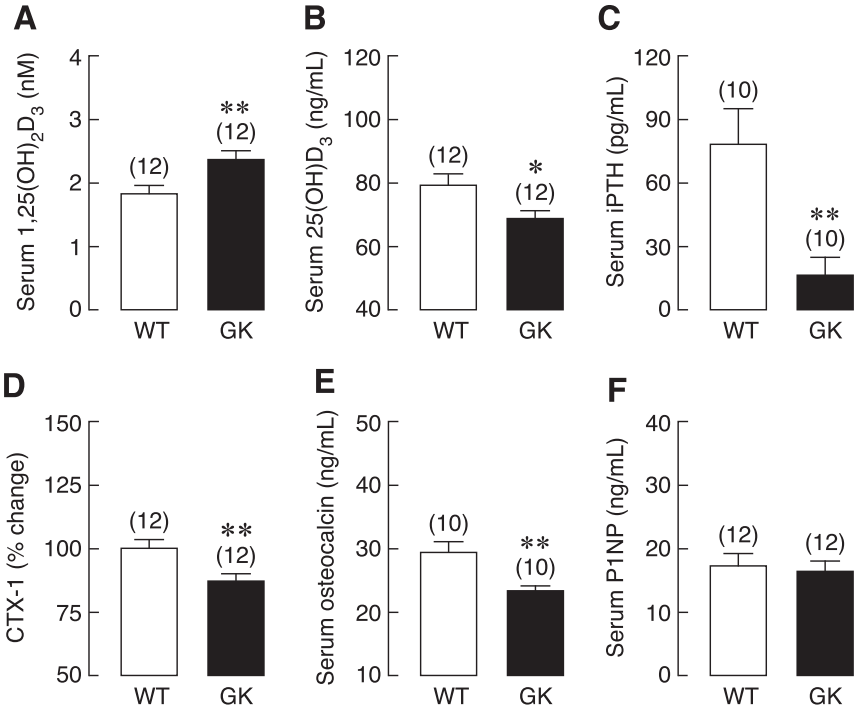

Fig. 4. Serum levels of calciotropic hormones, i.e., (A) $1,25(OH)_2D_3$, (B) $25(OH)D_3$, and (C) iPTH. Serum levels of bone turnover biomarkers, i.e., (D) CTX-1, (E) osteocalcin, and (F) P1NP in WT and GK diabetic rats. Numbers in parentheses are the numbers of animals in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT.

to induce endoplasmic reticulum stress and finally apoptosis in mouse chondrocytes [26].

In the present study, premature apoptosis occurred concurrently with the elevation of systemic growth factor level and the expression of transcription factor essential for promoting longitudinal bone growth, i.e., serum IGF-1 and Runx2 protein in the HZ, respectively. This finding could be a compensatory response to mitigate excessive loss of chondrocytes due to premature

apoptosis. The circulating IGF-1 and local Runx2 expression in the growth plate chondrocytes are known to promote chondrocyte differentiation into mature state, thereby inducing longitudinal bone growth [8,27]. Furthermore, the upregulation of PTHrP expression in the RZ and HZ also represented an effort to increase chondrocyte proliferation in order to maintain cell number in the growth plate of GK rats [8,10,11].

Strikingly, VEGF—a growth factor that couples angiogenesis to osteogenesis (for review [28])—was upregulated in the growth plate chondrocytes as well as in the endothelial cells (EC) of primary spongiosa of GK rats. VEGF from the hypertrophic chondrocytes normally stimulates blood vessel invasion and bone cell development near the CO junction. Recent investigations further revealed that VEGF was also secreted by osteoblasts and type H-EC, the latter of which was a specialized EC that induced angiogenesis and helped sustain chondrocyte and osteoblast functions to promote bone formation [29,30]. Proliferation of type H-ECs was positively regulated by hypoxia-inducible factor (HIF)-1 that was produced in greater amount under stress condition, e.g., hypoxia and hyperglycemia as in DM [31,32]. It was, therefore, possible that the upregulation of VEGF expression in the primary spongiosa of GK rats could be one of compensatory responses of hypertrophic chondrocytes, type H-EC, and/or osteoblasts to maintain bone formation in the metaphyseal region to palliate diabetic-induced bone loss and impairment of bone elongation [28,29,31,32].

DM not only suppressed longitudinal bone growth, but also suppressed whole body bone remodeling as indicated by decreases in the levels of bone turnover markers. Normally, bone remodeling is a coupled process of bone resorption and formation, and requires coordination of all three types of bone cells, namely osteocytes, osteoblasts, and osteoclasts [1,33,34]. Under normal conditions, change of mechanical stress is detected by osteocytes, which, in turn, transmit signals to the osteoblasts. Osteoblasts then stimulate osteoclast differentiation and subsequent bone resorption [1,33,34]. Once bone resorption ends, osteoblast-mediated bone formation takes place at the same site to fill up the resorption pit [1,33,34]. Therefore, the uncoupling of osteoblast and osteoclast functions, as evidenced in GK diabetic rats (Charoenphandhu and Krishnamra, unpublished observation), could lead to impairment of bone microstructure and remodeling process. In the present study, the decreased levels of bone formation marker (osteocalcin) and bone resorption marker (CTX-1) strongly suggested the T2DM-induced suppression of whole body bone remodeling, consistent with the previous reports that showed low bone formation in diabetic patients and rodents [5,35,36].

Furthermore, impaired calcium and vitamin D metabolisms in GK rats may also contribute to decreases in longitudinal bone growth and bone turnover. A marked decrease in serum PTH level as observed in GK rats was common in DM and contributed to fracture risk [35,37]. The underlying mechanism by which DM-induced decrease in PTH level has been unknown, but it could be explained, in part, by the elevated levels of $1,25(OH)_2D_3$, which was reported to directly suppress PTH secretion independent of plasma ionized calcium [38]. Excessive production of $1,25(OH)_2D_3$ thus diminished the vitamin D storage pool [39], thereby reducing the circulating level of $25(OH)D_3$ as observed in GK rats.

In conclusion, we elaborated the presence of premature chondrocyte apoptosis in the growth plate of GK rats, particularly in the HZ and CO junction, which could explain how impaired longitudinal bone growth occurred in T2DM. A massive loss of growth plate chondrocytes, which formed template for endochondral bone formation, was accompanied by a number of compensatory responses, such as increased serum IGF-1 level and overexpression of PTHrP, Runx2, and VEGF in the growth plate, presumably to help palliate the DM-associated suppression of bone elongation. Besides aberrant growth plate function, analysis of blood chemistry further revealed that bone turnover—a coupled process of osteoclast-mediated bone resorption and osteoblast-mediated bone formation—was reduced as indicated by low serum levels of osteocalcin and CTX-1, both of which might result from the deranged metabolism of calciotropic hormones (PTH and vitamin D). Our results have, therefore, confirmed that T2DM is detrimental to the chondroregulatory function and whole body bone metabolism.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

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