ORIGINAL ARTICLE

Mice Deficient in NF- κ B p50 and p52 or RANK Have Defective Growth Plate Formation and Post-natal Dwarfism

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NF- κ Bp50/p52 double knockout (dKO) and RANK KO mice have no osteoclasts and develop severe osteopetrosis associated with dwarfism. In contrast, Op/Op mice, which form few osteoclasts, and Src KO mice, which have osteoclasts with defective resorptive function, are osteopetrotic, but they are not dwarfed. Here, we compared the morphologic features of long bones from p50/p52 dKO, RANK KO, Op/Op and Src KO mice to attempt to explain the differences in their long bone lengths. We found that growth plates in p50/p52 dKO and RANK KO mice are significantly thicker than those in WT mice due to a 2-3-fold increase in the hypertrophic chondrocyte zone associated with normal a proliferative chondrocyte zone. This growth plate abnormality disappears when animals become older, but their dwarfism persists. Op/Op or Src KO mice have relatively normal growth plate morphology. In-situ hybridization study of long bones from p50/p52 dKO mice showed marked thickening of the growth plate region containing type 10 collagen-expressing chondrocytes. Treatment of micro-mass chondrocyte cultures with RANKL did not affect expression levels of type 2 collagen and Sox9, markers for proliferative chondrocytes, but RANKL reduced the number of type 10 collagen-expressing hypertrophic chondrocytes. Thus, RANK/NF- κ B signaling plays a regulatory role in post-natal endochondral ossification that maintains hypertrophic conversion and prevents dwarfism in normal mice.

Keywords: NF- κ B; RANK; growth plate; chondrocytes; dwarfism

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Introduction

Long bones are formed during fetal development through mesenchymal cell differentiation into chondrocytes, which form cartilaginous moulds of the bones. These moulds are invaded by blood vessels typically in their mid-regions where chondrocytes become hypertrophic and the matrix around them calcifies. Calcified cartilage is then resorbed by cells in the myeloid lineage

to facilitate formation of a marrow cavity, which is partly filled with cancellous (trabecular) bone, and of growth plates at each end of bones in a process called endochondral ossification (1-2). Calcified cartilage matrix at the chondroosseous junction of growth plates is removed continuously post-natally in mice during skeletal development, making room for vascular channels and new bone, and allowing longitudinal bone growth and expansion of the marrow cavity. Dysfunction of factors or genes that control any components of endochondral ossification could lead to dwarfism (3-4).

The precise cell types that are responsible for removing calcified cartilage have not been well characterized. In 1962, Dr. Takuma, for the first time, described a type of

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cell that resorbed cartilage, and named them as chondroclasts. This type of cell was also later called mineraloclasts and collagenoclasts (5-6). Using electronic microscopy, Nordahl *et al.* demonstrated that chondroclasts do not form ruffled borders and a clear zone, which are seen typically in activated osteoclasts, but they are stained positively for tartrate-resistant acid phosphatase (TRAP) (7). However, there is no functional assay or specific marker available to distinguish chondroclasts from osteoclasts, and it is still unclear, currently, if chondroclasts actually exist 50 years after they were first described (5). Studies from many types of osteoclast-deficient mice, such as RANKL^{-/-}, RANK^{-/-}, and NF- κ B p50/p52 knockout mice, clearly indicate that fetal endochondral ossification and marrow cavity formation can proceed in the absence of TRAP-positive osteoclasts (8-9).

Interestingly, one of the common phenotypes of these osteoclast-deficient mice is dwarfism, but the mechanisms responsible for dwarfism have not been elucidated. RANKL/RANK/NF- κ B signaling plays an essential role in osteoclast formation, but little is known about its role in chondrocyte biology (10). Recent studies indicate that chondrocytes, especially hypertrophic chondrocytes, regulate osteoclastogenesis by producing RANKL in response to Vitamin D (11) or BMP2 (12), and deletion of RANKL in hypertrophic chondrocytes causes severe osteopetrosis (13). β -catenin controls RANKL production by hypertrophic chondrocytes, thereby affecting bone mass (14). However, it remains unknown if osteoclasts or factors essential for osteoclastogenesis affect chondrocytes or their functions. In this study, we demonstrate that mice lacking NF- κ B p50 and p52 or RANK have thicker growth plates mainly due to an expanded hypertrophic chondrocyte zone. At the cellular level, RANKL treatment does not affect chondrocyte nodule formation in short-term micro-mass chondrocyte cultures, or the expression levels of the early chondrogenic markers, Sox9 and type-2 collagen. In contrast, RANKL significantly delays BMP2-induced type-10 collagen expression in long-term micro-mass chondrocyte cultures. Thus, RANK/NF- κ B signaling plays a regulatory role in post-natal endochondral ossification by maintaining the hypertrophic chondrocyte zone and preventing dwarfism in normal mice.

Materials and Methods

Animals

Generation of NF- κ B p50/p52 dKO mice (C57Bl/6x129), RANK KO (C57Bl/6), and Src KO (C57Bl/6) mice has been described previously (15-17). All mice were used when

they were 2-6 weeks old. Littermates of these KO mice have normal teeth eruption and skeletal development and were used as wild-type (WT) controls. Op/Op mice (C57Bl/6) and WT littermates were purchased from Jackson labs. Timed-pregnant CD1 mice were purchased from Charles River. The Institutional Animal Care and Use Committee of University Rochester Medical Center approved all animal studies.

Isolation and culture of mesenchymal cells from mouse limb buds

Limb bud mesenchymal cells were isolated from 11.5 day pregnant female CD1 mice, as described previously (18-19). Briefly, mice were euthanized by CO₂. Embryos were separated and the distal quarter for subridge distal tip of the limb was pooled and digested with Dispase (1 U·mL⁻¹) for 3 h at 37 °C. Cells were resuspended at a density of 1×10⁷ cells per mL in medium containing 40% DMEM and 60% F12. A total of 1×10⁵ cells in 10 μ L of media were placed in micro-mass and 1 mL of culture medium was added. Medium was changed every other day thereafter. For long-term cultures, ascorbic acid (50 μ g·mL⁻¹) and β -glycerol phosphate (10 mmol·L⁻¹) were added into the medium beginning on day 7. For histologic analysis, pellet cultures were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned and stained with Safranin-O and Fast-Green. BMP2 (40 μ g·mL⁻¹) or RANKL (200 μ g·mL⁻¹) was added into the culture every other day for the indicated time periods.

In situ hybridization

S³⁵-labeled sense and antisense UTP riboprobes were synthesized from plasmids (kindly provided by Dr. Jill Helms) with insertions of mouse *osteocalcin*, *type II collagen*, and *type X collagen*. The sections were incubated in standard hybridization buffer and hybridization was performed at 55 °C overnight, as previously described (18-19). Non-specifically-bound probes were hydrolyzed with RNase A (20 μ g μ L⁻¹), and final washes were carried out at high stringency at 55 °C with 2×SSC/50% formamide. Emulsion-dipped slides were exposed for various times.

Real-time quantitative RT-PCR

Total RNA was extracted by RNeasy Kit (Qiagen). cDNA was synthesized from 1 μ g of total RNA. The PCR reaction mix contained a final concentration of 1×SYBR Green PCR master mix (Applied Biosystems), and 10 pmol·L⁻¹ specific primers and 2.5 ng of cDNA. The PCR reactions were performed on a Rotogene 2000, and the following cycles were used for all amplification: 30 s at

94 °C, 30 s at 60 °C, and 30 s at 69 °C for a total of 40 cycles. The relative levels of mRNA of a specific gene were calculated using the standard curves generated from cDNA dilutions and normalized to *actin* as an internal control. The primer sequences include: Col2a, forward ACTGGTAAGTGGGGCAAGAC and reverse CCACACC-AAATTCCTGTCA; Sox9, forward TTCATGTCCGACCTGT-AACC and reverse TTGACAGCGTTCGAGGAGAG; and *actin*, forward AGATGTGGATCAGCAAGCAG and reverse GCGCAAGTAGGTTTTGTCA.

Statistics

Data are presented as means $\pm$ standard deviation, and all experiments were performed at least twice with similar results. Statistical analyses were performed with Stat view statistical software. Differences between two groups were compared using un-paired Student *t*-test and more than two groups were compared using one-way ANOVA, followed by a Bonferroni/Dunnet test. *P* values less than 0.05 are considered to be statistically significant.

Results

NF- κ Bp50/p52 dKO or RANK KO mice have thickened growth plates

We reported previously that NF- κ Bp50/p52 dKO mice develop osteopetrosis because they do not form osteoclasts, indicating that the expression of NF- κ B p50 and p52 proteins are essential for osteoclast formation during development (16). While characterizing the bones of p50/p52 dKO mice, we noticed that they have thickened growth plates (Figure 1A). The organization of growth plates from 2-week-old p50/p52 dKO mice into distinct proliferative, pre-hypertrophic and hypertrophic chondrocyte zones is not altered, but the hypertrophic zone thickness is increased 2-3 fold (Figure 1A).

NF- κ B activation is triggered through the RANKL/RANK signaling in osteoclasts. We examined the growth plates of 2-week-old RANK KO mice and found that, like the NF- κ Bp50/p52 dKO mice, the hypertrophic chondrocyte zone is thicker than normal (Figure 1B). Since both RANK KO and NF- κ B dKO mice have severe osteopetrosis due to an absence of osteoclasts, we next sought to determine if this growth plate phenotype is observed also in mice with defective osteoclasts. To do this, we examined bones from 2-week-old Op/Op mice, which form very few osteoclasts, and 2-week-old Src KO mice, which form increased numbers of osteoclasts, which cannot resorb bone effectively. No difference in the thickness of the growth plates (Figure 1C and 1D) was detected.

Increased type 10 collagen-expressing cells in hypertrophic chondrocyte zone of NF- κ Bp50/p52 dKO mouse growth plates

To determine if NF- κ B signaling affects chondrocyte function, we examined the expression pattern of chondrocyte marker genes by in-situ hybridization in long bone sections of p50/p52 dKO mice (Figure 2). Type-2 collagen is a marker of proliferating chondrocytes and type-10 collagen is a marker of hypertrophic chondrocytes. We found no difference in the expression of type-2 collagen between p50/p52 dKO and control mice. However, the expression of type-10 collagen is markedly extended in the growth plates of dKO mice (Figure 2A). Collagenase-3 (MMP13) is expressed in the skeleton during embryonic development and *Mmp13*^{-/-} mice show profound defects in growth plate cartilage with markedly increased hypertrophic zones, as well as a delay in endochondral ossification (20). We found that WT hypertrophic chondrocytes express high levels of MMP13, while in dKO mice MMP13-expressing cells were present within the calcified bone matrix (Figure 2A). To determine if there is a calcification defect in dKO mice, we performed Von Kossa staining and found that the increased bone matrix in mutant mice was calcified normally (Figure 2B).

The abnormal growth plate phenotype in RANK KO mice recovers as the mice age

NF- κ B p50/p52 dKO mice typically die around 2-3-week of age, which limits investigation of the role NF- κ B in chondrocytes of older mice, while RANK KO mice can survive to adulthood if they are fed soft food. To determine if the growth plate phenotype persists beyond 2 weeks of age, we performed histomorphometric analysis of long bones from 1-, 2-, 3-, and 6-week-old RANK KO mice (Figure 3). We found that the significant increase in the length of hypertrophic chondrocyte zone in RANK KO mice compared to WT littermates is confined to the first 2 weeks after birth. In RANK KO mice older than 3 weeks, the growth plate is of normal thickness. However, the growth retardation in 3-week-old RANK KO mice was still present when the mice are 6 weeks old. Accordingly, the length of the hypertrophic chondrocyte zone relative to the tibial length is still less in RANK KO mice at older ages than in WT littermates (Figure 3A). Thus, this early transient defect in endochondral ossification results in a dwarf phenotype in adulthood.

RANKL does not have a role in the early phase of chondrogenesis

Endochondral bone formation consists of two phases: an early phase, in which undifferentiated mesenchymal

cells become committed to the chondrocyte lineage, and a late phase, in which chondrocytes undergo proliferation, hypertrophic conversion, and calcification. We studied the potential role of RANK signaling in these 2 major phases in endochondral bone formation using 2 approaches. To determine if RANKL affects the commitment of mesenchymal cells to the chondrocyte lineage, we used a well-established micro-mass culture system, in which limb bud mesenchymal cells from E11.5 mouse embryos are cultured *in vitro* to form cartilaginous nodules (18-19). This short-term micro-mass culture system is used

to examine changes in the early phase of chondrogenesis. Previous studies reported that BMP-2 increases the size of chondrocyte nodules in micro-mass cultures (19). We confirmed that BMP-2 increased the expression of Sox9 and type-2 collagen genes, which are involved in early phase chondrogenesis (at 24 hours) and increased chondrocyte nodule area after 8 days of treatment. In contrast, RANKL had no effect on the expression levels of Sox9 and type 2 collagen mRNA (Figure 4A) nor on the basal and BMP2-induced nodule formation in the same culture conditions (Figure 4B).

Figure 1 Growth plate morphology of various osteopetrotic mice. Sections of tibial growth plates from 2-week-old NF- κ B p50/p52 dKO, RANK KO, Op/Op and Src KO mice and their littermate control mice stained with Safranin-O-Fast-Green (A), and H&E/alcian blue (B-D). Magnification $\times 4$ and $\times 10$ in left and right panels, respectively. The proliferative chondrocyte zone is indicated by * and the hypertrophic chondrocyte zone is indicated by #.

Figure 2 Expression of chondrocyte marker genes in tibial growth plates from 2-week-old NF- κ B p50/p52 dKO and littermate control mice. (A) In-situ hybridization showing the distribution of type-2 and -10 collagen (Col), and MMP13 mRNA. The proliferative chondrocyte zone is indicated by * and the hypertrophic chondrocyte zone is indicated by #. (B) Safranin O/von Kossa-stained plastic-embedded sections showing a thickened hypertrophic chondrocyte zone and increased volume of mineralized bone matrix in p50/p52 dKO mice.

RANKL inhibits chondrocyte hypertrophy

The late phase chondrogenesis features the conversion of proliferative chondrocytes to hypertrophic chondrocytes associated with decreased expression of type-

2 collagen and increased type-10 collagen and osteocalcin expression (18). To investigate if RANKL affects this late phase, we treated micro-mass cultures with RANKL for 8, 16 and 24 days and performed in-situ hybridization

to examine the expression patterns of type-2 and type-10 collagen. We found that BMP2 decreased type-2 and increased type-10 collagen expression progressively during this culture period, and this was most obvious at day 24 (Figure 5A). In contrast, RANKL alone had no effect on basal expression levels of type-2 or type-10 collagen at all time-points examined. However, RANKL

inhibited BMP2-induced cell hypertrophy and the cells remained predominantly at the proliferating stage, expressing type-2, but not type-10 collagen (Figure 5A) associated with few hypertrophic chondrocytes, which expressed less type-10 collagen than those in the control cultures (Figure 5B).

Figure 3 Histomorphometric analysis of growth plates from RANK KO mice. Histomorphometric analysis was carried out on H&E/Alcian blue-stained sections of tibiae from 1, 2, 3 and 6-week-old RANK KO and their littermate controls. Values are means $\pm$ SD of 3 mice per group. * P <0.05 vs control mice of the same age.

Figure 4 Effects of short-term RANKL treatment on micro-mass cultures from limb buds of WT mice. (A) Cultures were treated with RANKL, BMP2 or RANKL+BMP2 for 24 hrs and the expression levels of Sox9 and type 2 collagen mRNA were determined by qPCR. Fold changes of gene expression levels were calculated using values from PBS-treated cultures as 1. (B) Cells were cultured in chondrocyte-inducing medium and treated with RANKL, BMP2 or RANKL+BMP2 for 8 days. Values are the mean $\pm$ SD of 3 wells. * P <0.05 vs PBS-treated group.

Discussion

In this study, we demonstrated that mice deficient in NF- κ B p50 and p52 or RANK protein expression have thickened growth plates with a normal size proliferating zone and type-2 collagen expressing region, but a significantly thickened type-10 collagen expressing hypertrophic chondrocyte zone. At the cellular level, RANKL treatment did not affect the expression levels of genes controlling early phase chondrogenesis, nor chondrocyte nodule formation in short-term cultures. In contrast,

RANKL significantly delayed expression of BMP2-induced genes involved in late-phase chondrogenesis and reduced chondrocyte hypertrophy in long-term micro-mass cultures. Thus, the RANKL/NF- κ B signaling pathway appears to play a regulatory role in post-natal endochondral ossification by promoting conversion of proliferative to hypertrophic chondrocytes. These findings suggest that factors that inhibit RANKL/NF- κ B signaling in endochondral ossification during embryonic and post-natal skeletal development may contribute to dwarfism.

Our findings raise several interesting questions. First,

Figure 5 Effects of long-term RANKL treatment on micro-mass chondrocyte cultures. Micro-mass pellets from limb buds of WT mice were cultured in chondrocyte-inducing medium and treated with BMP2±RANKL for 8-24 days, fixed, paraffin embedded, and sectioned. A) and B) H&E/alcian blue-stained sections and type-2 and type-10 collagen (Col) in-situ hybridization (magnification x2 and x20, respectively) at different days of micro-mass cultures. Proliferative chondrocytes are indicated by black arrowheads and hypertrophic chondrocytes are indicated by red arrows in B.

NF- κ B p50/p52 dKO or RANK KO mice never form osteoclasts throughout their lifespan. Nevertheless, these mice develop a marrow cavity, which becomes largely filled with un-remodeled bone, suggesting that cells other than osteoclasts are responsible for removing mineralized cartilage matrix at the chondro-osseous junction to create a bone marrow cavity. NF- κ B regulates the expres-

sion of matrix metalloproteinases (21-22), which have been implicated in the resorption of calcified cartilage (23). Therefore, we speculate that matrix metalloproteinases produced by hypertrophic chondrocytes (24) dissolve the matrix around them under the control of RANKL expressed by hypertrophic chondrocytes (12, 25). Failure of this mechanism to remove hypertrophic chon-

drocytes in the absence of NF- κ B signaling in our mice could explain the thickening of their hypertrophic zones. Alternatively, the matrix could be removed by chondroclasts, which remain poorly defined cells. A recent study suggests that chondroclasts are osteoclasts (26), and the findings show clearly that osteoclasts have the ability to resorb calcified cartilage. We have found that NF- κ B p50/p52 dKO and RANK KO mice have no TRAP⁺ mononuclear cells or osteoclasts (16, 27-28). Our findings demonstrate definitively that cells other than osteoclasts also possess the ability to resorb calcified cartilage and suggest that chondroclasts in our mice must be TRAP⁺ mononuclear cells, because we also did not find any TRAP⁺ multinucleated cells at growth plates. Thus, cartilage in the embryonic bones of these KO mice is removed by an as yet unexplained process.

Secondly, we found that NF- κ B p50/p52 dKO and RANK KO mice, but not Op/Op or Src KO mice, have clearly abnormal growth plates. c-Fos KO mice also have a thickened growth plate phenotype (29). Op/Op mice do not express M-CSF, which is required for osteoclast formation, and they have very few osteoclasts. Src signaling is activated in osteoclasts and their precursors by both RANKL and M-CSF (30), but this clearly is required for osteoclast activation (15, 31), and not for osteoclast formation, and has no essential function in growth plates. Because NF- κ B p50/p52 dKO, RANK KO, and c-Fos KO mice all have no osteoclasts, coupled with the fact that c-Fos is activated downstream of NF- κ B (32), our finding suggests that the thickened growth plates in NF- κ B dKO, RANK KO and c-Fos KO mice may be more related to the RANKL/NF- κ B/c-Fos signaling defect in chondrocytes, rather than simply being the result of the absence or paucity of osteoclasts.

NF- κ B signaling regulates the growth and differentiation of mesenchymal cells into chondrocytes (33), and Sox9, a master regulator of chondrocyte differentiation, induces transcription of type-2 collagen and aggrecan (34). TNF down-regulates Sox9 expression through NF- κ B activation and inhibits chondrocyte differentiation in a number of pathologic settings, in which its expression is increased (26). However, the role of RANKL in chondrocyte function has not been investigated previously. We found that short-term RANKL treatment of mesenchymal cells had no effect on their differentiation into chondrocytes or expression of Sox9 and type-2 collagen. In contrast, RANKL remarkably reduced hypertrophy of chondrocytes, suggesting that the absence of RANKL signaling may increase the conversion of proliferative to hypertrophic chondrocytes to explain the thicker hypertrophic chondrocyte zone in RANK KO mice. A recent

study reports that chondrocytes express RANK protein on their cell surfaces, and that OPG-Fc treatment reduces MMP13 production of chondrocytes (35), supporting a functional role of RANK signaling in chondrocytes.

Finally, we found that the thickening of the hypertrophic chondrocyte zone in RANK KO mice is transient and disappears between 2-3 weeks post-natally, which is similar to the phenotype in MMP9 KO mice (23). The thickened hypertrophic chondrocyte zone is not present in the occasional NF- κ B dKO mice that survive until between 5- and 6-weeks-old (data not show). Our studies to-date do not adequately explain the transient nature of the growth plate defect in the KO mice, but because MMP expression is regulated by NF- κ B in many cell types, the defect is presumably related to the absence of NF- κ B signaling in chondrocytes or other cell types, which appears to be compensated for by a mechanism that remains to be identified.

Our study indicates that the RANKL/NF- κ B signaling may play a regulatory role in endochondral ossification that maintains hypertrophic conversion and prevents dwarfism in normal mice. Further study will be required to determine the molecular mechanisms by which the RANKL/NF- κ B signaling regulates chondrocyte functions.

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