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Endochondral bone formation in toothless (osteopetrotic) rats: failures of chondrocyte patterning and type X collagen expression

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ABSTRACT The pacemaker of endochondral bone growth is cell division and hypertrophy of chondrocytes. The developmental stages of chondrocytes, characterized by the expression of collagen types II and X, are arranged in arrays across the growth zone. Mutations in collagen II and X genes as well as the absence of their gene products lead to different, altered patterns of chondrocyte stages which remain aligned across the growth plate (GP). Here we analyze GP of rats bearing the mutation toothless (tl) which, apart from bone defects, develop a progressive, severe chondrodystrophy during postnatal weeks 3 to 6. Mutant GP exhibited disorganized, non-aligned chondrocytes and mineralized metaphyseal bone but without cartilage mineralization or cartilaginous extensions into the metaphysis. Expression of mRNA coding for collagen types II (Col II) and X (Col X) was examined in the tibial GP by in situ hybridization. Mutant rats at 2 weeks exhibited Col II RNA expression and some hypertrophied chondrocytes (HC) but no Col X RNA was detected. By 3rd week, HC had largely disappeared from the central part of the mutant GP and Col II RNA expression was present but weak and in 2 separate bands. Peripherally the GP contained HC but without Col X RNA expression. This abnormal pattern was exacerbated by the fourth week. Bone mineralized but cartilage in the GP did not. These data suggest that the tl mutation involves a regulatory function for chondrocyte maturation, including Col X RNA synthesis and mineralization, and that the GP abnormalities are related to the Col X deficiency. The differences in patterning in the tl rat GP compared to direct Col X mutations may be explained by compensatory effects.

KEY WORDS: collagen type II, collagen type X, chondrocyte, chondrodystrophy, osteopetrosis

Introduction

The pacemaker of endochondral bone formation is cell division and hypertrophy of chondrocytes in growth plates (Farnum and Wilsman, 1989; Hunziker, 1994). It is the proliferation, vectorial extracellular matrix production and hypertrophy of chondrocytes together with mineralization of the longitudinally aligned matrix components that maintain the alignment and relative proportion of cell types during longitudinal bone growth (Vanky et al., 1998). Furthermore, the mineralized cartilage columns derived from growth plates provide the scaffolding onto which metaphyseal bone matrix is secreted and mineralized by osteoblasts (Marks, 1998). In growth plate cartilages collagen type II (Col II) is synthesized by proliferating chondrocytes. When these cells differentiate into hypertrophied chondrocytes deeper in the growth plate, type II collagen synthesis ceases and type X collagen (Col X) begins to be produced (Iyama et al., 1991; Schmid et al., 1991). Thus, Col II and X are exclusively expressed in proliferating and hypertrophic chondrocytes, respectively (Castagnola et al., 1986).

Osteopetrotic mutations are characterized by skeletal sclerosis resulting from interceptions of the development and/or function of bone-resorbing cells, osteoclasts (Popoff and Marks, 1995). In specific mutations a variety of other cells/tissues may be affected.

Abbreviations used in this paper: Col II, collagen type II; Col X, collagen type X; CSF-1, colony-stimulating factor-1; DIG, digoxygenin; GP, growth plate; HC, hypertrophied chondrocyte; RHT, ruthenium hexamine trichloride; tl, toothless mutation in the rat.
In the toothless (tl) mutation in the rat (Marks, 1977) a chondrodystrophy (Seifert 1994; 1996) develops by the third postnatal week. Injections of colony-stimulating factor-1 (CSF-1) improve the skeletal sclerosis (Marks et al., 1993, 1992) but are without any effect on the chondrodystrophy (Odgren et al., 1999). This chondrodystrophy in tl rats affects the development of cartilage and bone cells and endochondral ossification (Seifert, 1996). Specifically the zone of proliferating chondrocytes increases with age and the hypertrophic chondrocyte zone decreases, both effects beginning and being most obvious in the central regions.

Given these abnormalities in chondrocyte differentiation in growth plates of tl rats, we have compared postnatal gene expression for collagens type II and X, regional chondrocyte morphology and cartilage mineralization in tibial growth plates from mutant and normal rats. These data are expected to clarify the interdependent contributions of cartilage and bone cells during skeletal development.

**Results**

Figure 1 demonstrates the differences in organization and composition of the proximal tibial epiphyseal plates and metaphyses in normal rats and osteopetrotic littermates one month after birth. The growth plate in normal rats (Fig. 1A) is well organized with linear progressions of proliferating (p) and hypertrophied (h) chondrocytes and a matrix that stains metachromatically.

**Figs. 2 and 3.** Representative photomicrographs of the proximal tibia from a 2-week-old normal rat (N-Fig. 2) and its toothless (tl-Fig. 3) littermate stained with toluidine blue (A) and illustrating gene expression by *in situ* hybridization for collagens type II (B) and X (C) in adjacent sections. Notice that there is no mRNA expression for collagen type X in tl rats (Fig. 3C). X40.
The differential expression of Col II and X in normal and mutant growth plates is shown at higher magnification in Figures 4 and 5, respectively. Here one can see that in normal rats Col II RNA is highly expressed by a band of young proliferating chondrocytes (Fig. 4A,B). Near the zone of hypertrophy expression for this gene is reduced, and it is not detected in hypertrophied chondrocytes (Fig. 4B). In contrast, Col X RNA in normal rats is expressed exclusively by hypertrophied chondrocytes (Fig. 4A,C), and this expression ends abruptly at the chondro-osseous junction where the growth plate abuts the proximal tibial metaphysis. In 2-week-old tl rats (Fig. 5) Col II RNA is expressed by proliferating chondrocytes (Fig. 5A,B) but lacks the intense band of expression seen in the young proliferating cells in normal littermates (Fig. 4B). There is no expression of Col X RNA by mutant chondrocytes (Fig. 4C) even though this section contains hypertrophied chondrocytes (Fig. 4A).

Next we evaluated gene expression for these two collagens in the proximal tibial growth plates of 3-week-old animals. Expression of Col X RNA was not detectable in mutant growth plates (data not shown). Thus, the results at this age for Col X were identical to what we found in 2-week-old animals (Figs. 4C and 5C). Col II RNA expression in normal 3-week-old rats (Fig. 6) was compact, confined to the zone of proliferating chondrocytes (Fig. 6A,B) and was most intense in the younger proliferating cell population. In mutants (Figs. 7 and 8), Col II expression differed in the lateral and central regions of the proximal tibial growth plate. In the lateral growth plate Col II expression was linear and confined to proliferating cells (Fig. 7A,B). Centrally, Col II expression spread throughout the growth plate and was much less intense than its expression laterally in mutants. At higher magnification (Fig. 8) one can clearly see the lack of uniform expression of Col II in the central growth plate (Fig. 8B) where two bands of higher expression are separated by a region of low expression (rt. side of Fig. 8B). Thus, instead of the suppressed expression of Col II during the differentiation of proliferating normal chondrocytes (Fig. 6B), mutant chondrocytes exhibit cyclical expression for Col II in a thickened growth plate with few hypertrophic cells (right sides of Fig. 8A and B).

Based on the reported relationship between Col X and mineralization in cartilage (Iyama et al., 1991; Kirsch and Von der Mark, 1991; Schmid et al., 1991), we examined the extent of mineralization...
In summary, these data demonstrate that in tl rats: 1) type X collagen RNA is not expressed in the growth plate, even though hypertrophic chondrocytes are present, albeit in reduced number and uneven distribution, 2) type II collagen expression in growth plates is present but irregular; compact in the lateral part of the growth plate and cyclical and diffuse centrally, 3) cartilage mineralization fails but bone does mineralize, 4) centrally, cartilage does not extend into the metaphysis (presumably because of the failure of chondrocyte hypertrophy) and bone trabeculae form here without a cartilage scaffold, 5) local areas of pyknotic chondrocyte clusters early in the proliferative zone are followed by regions of degenerating cells and restricted chondrocyte hypertrophy deeper in the growth plate.

Discussion

During long bone growth endochondral ossification proceeds through consecutive steps of concurrent chondrocyte proliferation and production of Col II followed by down-regulation of Col II, hypertrophy and production of Col X. Recent data indicate that 80% of the growth in murine bones is related to chondrocyte hypertrophy (Vanky et al., 1998). Thus, it is not surprising that in tl rats where few chondrocytes hypertrophy, long bone growth is retarded (Seifert, 1998; Odgren et al., 1999). Mineralization of cartilage normally begins in the high hypertrophic zone and is mediated by matrix vesicles from these cells. The regulation of chondrocyte maturation has recently been shown to result from the local interactions of Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) and its receptor (Kronenberg et al., 1997). The expression of Ihh in maturing chondrocytes upregulates PTHrP secretion from the perichondrium which, in turn, acts as a paracrine suppressor of chondrocyte hypertrophy through PTHrP receptors on prehypertrophic cells (Lee et al., 1996; Philbrick et al., 1996; Vortkamp et al., 1996, 1998; Zou et al., 1997; Philbrick 1998). Hypertrophic chondrocytes also slow the maturation of their own precursors in a negative-feedback loop and delay apoptosis by upregulating Bcl-2 expression (Amling et al., 1997). Recently, BMP-6 has been shown to be a positive regulator of avian chondrocyte differentiation (Grimsrud et al., 1999). These mechanisms produce a growth plate with uniformly thick zones of chondrocyte proliferation and hypertrophy (Fig. 1A). In the absence of PTHrP, growth plates are thin and mineralization accelerated (Amizuka et al., 1994; Karaplis et al., 1994; Weir et al., 1996). Maturation and death of hypertrophic chondrocytes occurs, at least in part, by signals from endothelial cells at the chondro-osseous junction (Bittner et al., 1998) and involves the secretion of enzymes that cross-link a variety of matrix molecules (Nurminskaya et al., 1998), by upregulating Bcl-2 expression (Amling et al., 1997). Recently, BMP-6 has been shown to be a positive regulator of avian chondrocyte differentiation (Grimsrud et al., 1999). These mechanisms produce a growth plate with uniformly thick zones of chondrocyte proliferation and hypertrophy (Fig. 1A). In the absence of PTHrP, growth plates are thin and mineralization accelerated (Amizuka et al., 1994; Karaplis et al., 1994; Weir et al., 1996). Maturation and death of hypertrophic chondrocytes occurs, at least in part, by signals from endothelial cells at the chondro-osseous junction (Bittner et al., 1998) and involves the secretion of enzymes that cross-link a variety of matrix molecules (Nurminskaya et al., 1998), stabilizing the mineralized cartilaginous scaffold onto which osteoblasts deposit metaphyseal bone. In tl rats, proliferation of some central chondrocytes is aborted, differentiation and hypertrophy of the remainder are delayed, expression of Col II is irregularly distributed and extended, and expression of Col X never occurs. Mineralization of cartilage fails, there is no cartilaginous scaffolding for bone formation in the central metaphysis and bone here forms without a cartilaginous core (Fig. 1B). Laterally, the growth plate...
distortions are not as spectacular presumably because of adjacent periosteal collar influences on chondrocyte differentiation (Bianco et al., 1998; Long and Linsenmayer, 1998). Nevertheless, Col X expression also fails in this region of the mutant growth plate. It has recently been shown (Zerega et al., 1999) that the addition of PTHrP to cultures of hypertrophied avian chondrocytes suppresses both expression of Col X and mineralization by these cells. Given the perichondrial location of PTHrP secreting cells in growing long bones (Zou et al., 1997), the failure of Col X expression and mineralization of cartilage by hypertrophied chondrocytes in the lateral areas of the growth plate of tl rats could be due to overexpression of PTHrP by adjacent perichondrial cells. Explaining the pathology of central areas of the tl growth plate (Fig. 1B) is more difficult and may involve abnormalities in the PTHrP-Ihh axis, BMP-6 and its receptor or Bcl-2 expression. Thus, expression of these genes needs to be evaluated systematically in the tl growth plate during development to understand the mechanisms for regional differences in chondrocyte maturation. Furthermore, a comprehensive analysis of the mechanisms of the premature cell death and loss of chondrocytes in the mutant growth plate is warranted.

Type X collagen is a short, non-fibrillar collagen synthesized by hypertrophic chondrocytes before mineralization and vascular invasion of the growth plate (Iyama et al., 1991; Kirsch and Von der Mark, 1991; Reichenberger et al., 1991; Schmid et al., 1991; Claassen and Kirsch, 1994). Its appearance only after chondrocyte hypertrophy, together with its expression by chondrocytes in sites of mineralization at tendon insertions (Niyibizi et al., 1996) and osteoarthritic transformation of articular cartilage (Hoyland et al., 1991), have suggested a role for type X collagen in mineralization. Type X collagen is found in two forms in the extracellular matrix (Schmid and Linsenmayer, 1990): as fine filaments adjacent to hypertrophic chondrocytes and in the interstitial matrix in association with type II collagen (Chen et al., 1992). Degradation of Col X occurs in the metaphysis by the combined actions of degradative enzymes present in osteoclasts (Sires et al., 1995; Vu et al., 1998).

Mutations in the Col X gene lead to metaphyseal chondrodysplasias characterized by thin growth plates and metaphyses (Jacenko et al., 1993; Olsen, 1995; Pokharel et al., 1995) quite opposite the skeletal manifestations in tl rats (Seifert, 1994, 1996). Cloning of the mouse gene (Apte et al., 1992; Elima et al., 1993) made Col X-deficient mice available for analysis. Early studies revealed (Rosati et al., 1994) no gross abnormalities in bone growth or development in these mice, but subsequent analyses (Kwan et al., 1997) showed reductions in resting chondrocytes and articular cartilage thickness and a shift of matrix vesicles and proteoglycans from the zone of hypertrophy to earlier stages in chondrocyte differentiation. Furthermore, Col X null mice show no abnormalities in chondrocyte morphology or differentiation patterns including premature apoptosis (Kwan et al., 1997). These subtle deficiencies in Col X deficient mice contrast with the manifestations in tl rats where failure to express Col X is associated with a failure of mineralization and irregular, thick growth plates.

The chondrodystrophy that is characteristic of the tl mutation (Seifert, 1994, 1996) appears to result from central regional derangements in chondrocyte patterning (proliferation and hypertrophy) and gene expression. Hypertrophic chondrocytes occur in reduced numbers and in skewed distributions in tibial growth plates, and fail to express Col X RNA. Col II RNA is present, not in well-aligned layers of cells, but rather concentrated to lateral parts of the tibia. These derangements could be caused by deficiencies in the cartilage matrix, which affect cartilage mineralization and interfere with the interaction of endothelial cells with cartilage matrix. This failure could interfere with the supply of nutrients and oxygen to chondrocytes and with the entrance of osteoblast precursors. The end result of these abnormalities in chondrocyte development is the retardation of long bone growth, presumably mediated by the failure of the growth plate to provide a mineralized cartilage surface for the orderly attraction and differentiation of bone cell precursors.
bone that produce the predictable, coordinated differentiation of cartilage and bone cells that mediate longitudinal bone growth. Given the abnormalities in cartilage (Seifert, 1994, 1996) and bone (Seifert et al., 1988; Shalhoub et al., 1991; Sundquist et al., 1995; Wisner-Lynch et al., 1995; Marks, 1997; Watanabe et al., 1997, 1998) cells and in the skeletal vasculature (Aharinejad et al., 1995), this mutation will be a valuable model for exploring the roles of these cells and tissues in the development and maintenance of the skeleton. How a single, autosomal recessive mutation in tl rats produces such a unique constellation of effects including compensatory adjustments remains to be established.

Materials and Methods

All the experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical Center (UMMMC). Animals were maintained and used at the UMMC according to the recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985).

Source of animals and tissue processing

Rats were obtained from our breeding colonies at the UMMC and mutants (tl/tl) were identified radiographically at birth (Schneider et al., 1988) by the failure of development of marrow cavities in long bones. Normal homozygotes (+/+ ) and heterozygotes (tl/+ ) are distinguishable only by breeding, and normal littermates included both these genotypes. Rats of normal and mutant phenotypes were killed by cervical dislocation at 2,3 and 4 weeks after birth. The proximal tibiae were quickly isolated, cleaned of muscles, bisected in the sagittal plane and immersion fixed in freshly prepared 4% paraformaldehyde in RNase-free phosphate-buffered saline, pH 7.0, at 4°C for 24 h. Bisected tibiae including the proximal growth plate were demineralized in 0.3 M EDTA, pH 7.0, at 4°C for 24 h. Bisected tibiae including the proximal growth plate were demineralized in 0.3 M EDTA, pH 7.0, at 4°C, dehydrated and vacuum-embedded in paraffin. Serial sections were cut at 7 µm, floated on RNase-free water and mounted on clean glass slides pretreated with aminopropyl triethoxysilane (Sigma, St. Louis, MO). Blocks and glass-mounted sections were stored at 4°C until used.

Tibiae were also removed and sectioned in the mid-sagittal plane. Under magnification, parallel 1-mm thick parasagittal slices containing the proximal growth plate cartilage and metaphysis were cut, fixed and processed undecalcified according to the method of Hunziker and Hermann (1982) (Hunziker, 1994) which optimally preserves chondrocytes in their fully expanded state. Briefly, slices were fixed for 2 h in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, containing 0.7% ruthenium hexamine trichloride (RHT; Polysciences, Inc., Warrington, PA, USA). Slices were then rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.7% RHT. The presence of RHT, a low molecular weight cationic dye of high positive charge density, in the fixatives causes precipitation of pericellular proteoglycans and maintains plasma membrane bonds with the surrounding extracellular matrix. This minimizes extraction of proteoglycans and the artifactual shrinkage of chondrocytes that accompany conventional aqueous fixation methods. Slices were dehydrated in ethanol and embedded in epoxy resin. Semi-thin sections (1 µm) were cut and stained for mineral by the von Kossa method (Seifert, 1996) and counterstained in 0.5% toluidine blue in 1% sodium borate.

Probes for hybridization

Clones containing rat Col II and X mRNA sequences were selected from a femur cDNA library as described, and inserts were sequenced (Wurtz et al., 1998). Aligned sequences were deposited under the accession numbers AJ224879 and AJ131848, respectively, in the EMBL database.

RNA probes were synthesized in the presence of digoxigenin (DIG)-modified UTP by an in vitro transcription kit (Boehringer Mannheim, Mannheim, Germany) as directed by the manufacturer. Controls including
sense probes and the insert of pSPT19-Neo (Boehringer Mannheim) were also transcribed. The oligonucleotides transcribed for Collagen α(I) (II) and Collagen α(X) (X) were specific and showed no crossreactivity (Wurtz et al., 1998; and unpublished data).

**In situ hybridization**

The sections obtained from sagitally sectioned tibiae were reacted with the DIG-labeled riboprobes for Col II, Col X or control probes in an *in situ* hybridization protocol developed at the Center for Oral Biology specifically for analyzing gene expression in mineralized tissues. This method has been described (Liao et al., 1998; Wurtz et al., 1998; Marks et al., 1999). Briefly, deparaftinized sections were exposed to 0.2 M HCl, proteinase K and glycine, post fixed in 4% paraformaldehyde, reacted with triethanolamine and hybridized overnight at 42°C in a sealed humid chamber while exposed to 0.5 ng DIG-UTP-labeled riboprobe/μL. High stringency washes and RNase treatment preceded identification of specifically bound DIG-labeled riboprobes by reacting with an alkaline phosphatase-coupled DIG antibody (Boehringer Mannheim). Exposure times for development ranged from 100 to 180 min depending upon the probe and the age of the animal. Gene expression for Col II and X in the tibial growth plate was evaluated in adjacent sections of the same block for each probe and every fifth section was stained with toluidine blue to identify more precisely the cell populations expressing each gene. Sections from normal and mutant rats of the same age and genotype were analyzed to determine the sites, times, intensity and patterns of gene expression for Col II and X in the proximal epiphyseal plate. All control probes were negative.

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