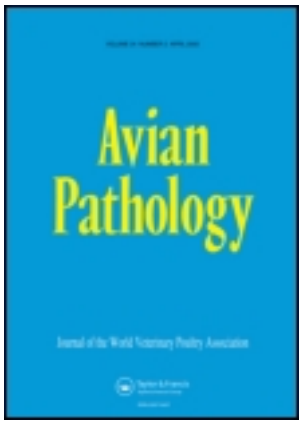

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Expression of type X collagen, Indian hedgehog and parathyroid hormone related-protein in normal and tibial dyschondroplastic chick growth plates

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Tibial dyschondroplasia (TD) is a form of aberrant endochondral ossification in chickens, in that a plug of avascular cartilage (TD lesion) is formed within the growth plate. Histologically, the lesion is filled with apparently transitional chondrocytes that have been unable to differentiate to hypertrophic chondrocytes. We have examined the spatial expression of mRNAs for type X collagen, Indian hedgehog (Ihh) and Parathyroid Hormone-related protein (PTHrP) in the TD growth plate by *in situ* hybridization in order to ascertain at which stage chondrocyte differentiation is arrested in TD. In the normal growth plate, type X collagen mRNA was expressed by both prehypertrophic and hypertrophic chondrocytes. Indian Hedgehog mRNA was detected in a band of prehypertrophic chondrocytes and PTHrP expression was localized to a narrow band of prehypertrophic chondrocytes and in osteoblasts within the diaphysis. In TD sections, collagen X expression was seen within differentiating cells, within a small number of lesion cells, and within hypertrophic chondrocytes on the diaphyseal side of the lesion. Ihh expression was also seen within the differentiating cells and throughout the lesion. These data indicate that chondrocyte differentiation is arrested at the transitional stage just prior to hypertrophy. Contrary to the previously reported PTHrP expression patterns in TD chicks by immunohistochemistry, PTHrP mRNA was not detected in the TD lesion. This observation probably reflects the cessation of PTHrP gene expression by chondrocytes in the more severe TD lesions. The results from the present study also imply that the arrest of cell differentiation in TD is independent of PTHrP and that endochondral ossification in the post-hatch avian growth plate may involve additional regulatory pathways.

Introduction

During vertebrate skeletal development, cartilage acts as a template for bone formation, and the chondrocytes are responsible for producing a calcified matrix upon which new bone will develop. This process is known as endochondral ossification, which mediates bone elongation in the epiphyseal growth plates of long bones (Hunziker, 1994). As a pre-requisite to bone deposition, chondrocytes within the epiphyseal growth plate undergo a programme of cellular proliferation and differentiation. Cell hypertrophy is followed by the mineralization of the hypertrophic cartilage matrix prior to the formation of osteoid by invading osteogenic cells (Poole, 1991).

Chondrocyte hypertrophy is a tightly regulated form of cellular maturation, characterized by increased activity of alkaline phosphatase (ALP) and synthesis of type X collagen which are well-known markers of chondrocyte hypertrophy (Cancedda *et al*., 1995; Kwan, 2000). The mechanisms controlling the rate at which growth plate chon-

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drocytes proliferate and subsequently differentiate are not currently understood. The secreted proteins Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) have recently been implicated in a negative feedback loop which controls long bone development by attenuating chondrocyte differentiation in the embryonic mouse limb (Vortkamp *et al*., 1996). Ihh is one of the three mammalian homologues of the *Drosophila* hedgehog gene (hh) that regulates segmentation and anterior–posterior patterning of the imaginal discs in the drosophila embryo, the cells of which give rise to adult epidermal structures e.g. the wings and the antennae (Kalderone, 2000). The mammalian hh genes, namely Sonic hedgehog (Shh), Desert hedgehog and Indian hedgehog (Ihh), are important in patterning of many tissues and structures. PTHrP was first identified as a pathogenic factor for malignancy-associated hypercalcaemia, a condition caused by tumours secreting a circulating bone-resorbing factor (Suva *et al*., 1987; Burtis *et al*., 1987). Both parathyroid hormone (PTH) and PTHrP share amino acid sequence homologies and bind to the same class I PTH/PTHrP receptor (Jüppner *et al*., 1991). However, the functions of PTHrP differs from that of PTH as being a regulator of cellular growth and differentiation (Philbrick *et al*., 1996).

In the model proposed by Vortkamp *et al.* (1996), Ihh is produced by chondrocytes within the pre-hypertrophic zone and it inhibits chondrocyte differentiation via stimulating the production of PTHrP by cells located within the periarticular perichondrium. Biological actions of PTHrP are mediated by the PTH/PTHrP receptor, which is expressed by pre-hypertrophic chondrocytes proximal to those expressing Ihh (Vortkamp *et al.*, 1996; Ben-Bassat *et al.*, 1999). Disruption of the PTHrP gene in mice causes accelerated chondrocyte hypertrophy (Karaplis *et al*., 1994; Amizuka *et al*., 1994) and PTHrP itself has been shown to be able to inhibit chondrocyte hypertrophy in organ culture (Vortkamp *et al*., 1996) and cell culture studies (Loveys *et al*., 1993).

Avian tibial dyschondroplasia (TD) is commonly seen in rapidly developing birds and particularly in meat-type (broiler) chickens. It is characterized grossly by an opaque plug of avascular and non-mineralized cartilage (the lesion) present within the proximal metaphysis of the chicken tibiotarsus, and also occasionally the tarsometatarsus (Leach & Nesheim, 1965). It occurs spontaneously, and may be similar to osteochondrosis in mammals (Poulos *et al.*, 1978). The disruption of growth plate histology indicates that TD is a disorder of endochondral ossification (Hargest *et al*., 1985). Uncalcified, pliable cartilage within the metaphysis of long bones can result in several skeletal problems within the bird: the generation of abnormal biomechanical loads through the lesion and reduced bone growth within the vicinity of the lesion may both contribute to angular deformities such as tibial bowing and ultimately lameness (Lynch *et al*., 1992).

Dyschondroplasia is generally recognized to be due to a failure of growth plate chondrocytes to differentiate fully to the hypertrophic phenotype (Farquharson *et al*., 1992; Hargest *et al*., 1985; Poulos *et al.*, 1978). Biochemical changes to the cartilage extracellular matrix of the TD lesion have also been observed that may contribute to the failure of chondrocytes to differentiate (Tselepis *et al*., 1996, 2000). In this study, we examined the expression of markers of the prehypertrophic and hypertrophic chondrocytes such as Ihh, PTHrP and type X collagen respectively in post-hatch chicken growth plates in order to define the differentiation stage of TD chondrocytes by *in situ* hybidization and to examine whether the Ihh/PTHrP regulatory pathway is implicated in the initiation and pathological development of avian tibial dyschondroplasia.

Materials and Methods

Preparation of normal and TD growth plate sections for in situ *hybridizations*

Experiments were conducted using groups of broiler chickens (Cobb strain) reared under two different dietary regimes in compartments of electrically heated tier brooders. Food and water were available *ad libitum* and diets, based on wheat and soya meal, were either a control diet, calculated to contain 12 g/kg Ca and 7.6 g/kg P, or a TD-inducing diet, calculated to contain 7.5 g/kg Ca and 7.6 g/kg P (Rennie *et al*., 1993). The birds were sacrificed at week 3 by cervical dislocation. The right and left proximal tibiotarsi were dissected and scored for the level of severity of TD (Thorp, 1988). Proximal tibiotarsi from 5 normal and 5 grade 6 TD lesions (i.e. large lesion occupying the entire metaphysis) based on the visual scoring system described by Thorp (1988) were fixed overnight in 4% paraformaldehyde, demineralized in EDTA (10%) and embedded in paraffin wax. Sections were cut at 8 μm on a LKB 2218 microtome and mounted onto 3-aminopropyltriethoxysilane (APES) coated slides. The latter were prepared by washing slides in 2% (w/v) SDS for 30 min and rinsed in running distilled water to remove all detergent. They were then dipped in acetone for 2 min and then acetone containing 2% (v/v) APES for 5 min. The slides were drained, rinsed in distilled water and dried overnight at 50°C. All solutions were prepared RNase free using 0.1% diethylpyrocarbonate (DEPC) treatment followed by autoclaving, or were purchased as molecular biology grade materials containing no DNase or RNase activities. All glassware was baked at >200°C overnight. Tissue sections were prepared as described earlier and mounted onto APES coated slides using warmed DEPC treated water. Normal and TD samples were run side by side. Slides were dried overnight at 45°C and stored at room temperature in an airtight container.

Riboprobe preparation

Riboprobes for chick collagen type X, Ihh and PTHrP mRNAs were prepared by transcribing cDNAs of the appropriate genes inserted into pGEM-T vectors (Promega, Southampton, UK) using the following protocols: cDNA probes were prepared using RT-PCR with oligonucleotide primers designed to amplify specific regions of genes of interest of 250 to 350 nucleotides in length. PCR primers for chick type X collagen, Ihh and PTHrP cDNAs were designed using Bio/Oligo software (Bio/Gene Ltd, Cambridgeshire, UK) and are shown in

Table 1. *Oligonucleotide primer sequences for chicken PTHrP, type X collagen and Indian hedgehog used in the preparation of riboprobes for* in situ *hybridization.*

Sequence (5′–3′)	Use
GTA TGG AAG CAA TGT AAC	Forward chick PTHrP primer
TGT AAT CAG AAA GGA AGC	Reverse chick PTHrP primer
AGC AGG AGC AAA TCA AGC	Forward chick type X collagen primer
CAG AGG AAT AGA GAC CAT	Reverse chick type X collagen primer
CAT CAT CTT CAA GGA CGA GGA GAA C	Forward chick Indian hedgehog primer
TAC TTG TTG CGG TCC CTG TCT GAC	Reverse chick Indian hedgehog primer

Table 1. RNA used for the RT-PCR was isolated from fifty 17-day-old embryonic chick sterna. Cells were released and lysed by homogenization with a Polytron (IKA Labortechnik, Germany) in 5 ml Tri Reagent (Sigma, Poole, UK). The manufacturer's protocol for Tri Reagent RNA isolation was followed and the resulting pellet was dissolved in RNase and DNase free distilled water. For RT-PCR the isolated chick RNA was treated with RQ1 RNase free DNase (Promega) to remove contaminating DNA at 37°C for 15 to 30 min. The enzyme was denatured at 95°C for 10 min and removed by phenol:chloroform extraction. RNA was purified from the aqueous phase of the phenol/chloroform extract by ethanol precipitation. The RNA was then dissolved in an appropriate volume of DEPC treated water and reverse transcribed to cDNA using murine Moloney leukaemia virus reverse transcriptase and random primers (Promega) at 37°C for 60 min before PCR. The PCR products were cloned into pGEM-T vectors, amplified in JM109 *E. coli* and the plasmid DNA isolated by acid phenol:chloroform purification. The DNA was sequenced using M13 primers to confirm cloning and the orientation of the insert. Sequences used for probe synthesis showed no homology to known genes other than those intended.

Digoxigenin (DIG) (Roche Diagnostics, East Sussex, UK) labelled riboprobes (both sense and antisense) were synthesized from the cloned PCR products. DIG-labelled RNA was transcribed from the SP6 or T7 RNA polymerase promoter regions as per the manufacturer's instructions. The transcription of the cloned sequence within the pGEM-T vector separately with T7 and SP6 RNA polymerases produces either the sense or antisense probes depending on the orientation of the cloned DNA insert. The template DNA was removed by DNase digestion and the riboprobe was precipitated in 4M LiCl and ethanol. Riboprobes were electrophoresed on RNase free 1% (w/v) agarose/0.01 M EDTA in 0.445M Tris borate, pH 8.3 (TBE) gels and visualized with 1μg/ml ethidium bromide. DIG labelling of the riboprobe was assessed by blotting 1μl of 1:10, 1:100 and 1:1000 (v/v) dilutions onto Hybond-N nylon membrane (Amersham, UK) and visualizing with an alkaline phosphatase conjugated DIG antibody and nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics). The level of DIG labelling of antisense and sense riboprobes was compared, and the lowest concentration of each that gave a reasonable signal was used at the hybridization step.

In situ *hybridization*

In each hybridization experiment, two sets of sections from a single tissue block were hybridized separately with the antisense and sense (controls) riboprobes under identical experimental conditions as described later. Sections were cleared in xylene twice for 5 min and rehydrated through 100%, 95% and 70% (v/v) ethanols followed by DEPC treated distilled water. The fixed tissue was permeabilized with 10 μg/ml proteinase K in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) at 37°C for 30 min and post-fixed in 4% (v/v) paraformaldehyde/10 mM KH_2PO_4/K_2HPO_4, 137mM NaCl and 2.7mM KCl, pH7.4 for 5 min. After rinsing twice in water the sections were acetylated in 0.1 M triethanolamine/0.25% (v/v) acetic anhydride for 5 min twice to reduce non-specific binding to proteins, and then washed in 5 × SSC (0.75M NaCl, 75mM sodium citrate, pH 7.2) for 15 min. The riboprobe was denatured at 80°C for 2 min, cooled and then mixed with prewarmed hybridization mix containing 50% (v/v) formamide/10% (v/v) dextran sulfate/5 × SSC/40 μg/ml sheared herring sperm DNA to a final dilution of typically 1:100. Each section was incubated with 20μl probe/hybridization mix under a silanized, plastic coverslip (Hybrislip, Sigma) at 45°C overnight in an atmosphere saturated with 5 × SSC/50% (v/v) formamide. In addition to the sense controls, extra negative control sections were prepared by incubating with only the hybridization mix.

Stringency washing and visualization of signal

Post hybridization, the coverslips were floated off in 2 × SSC at room temperature for 1 h. Two sections which had been hybridised with the anti-sense probe and two sections hybridised with sense probe were given a 1 h stringency wash at 55°C of 2 × SSC to remove non-specifically bound probe. A replicate set of four sections was washed in 2 × SSC and 1 × SSC consecutively, each wash for 1 h at 55°C. A third replicate set of four sections was washed in 2 × SSC, then in 1 × SSC followed by 0.5 SSC, each wash for 1 h at 55°C. All washes used Hybaid Omnislide wash modules. Sections were equilibrated in 100 mM Tris–HCl/150 mM NaCl, pH 7.5, and 200μl of a 1:500 dilution of alkaline phosphatase labelled DIG antibody diluted in 100 mM Tris–HCl/NaCl, pH 7.5, 1% (w/v) blocking reagent (Roche Diagnostics), was added to each and incubated at room temperature for 1 h. Antibody was washed off with two washes of Tris/NaCl (pH 7.5) and the tissue further equilibrated in 100 mM Tris–HCl/100 mM NaCl/50 mM MgCl, pH 9.5 for 10 min. The colour reaction used 500 μl Tris/NaCl/MgCl, pH 9.5, 2.25μl NBT (75 mg/ml), 1.75μl BCIP (50 mg/ml), and 2.5μl levamisole (1M) per section and was left in the dark for typically 2 to 3 hs. The reaction was stopped in TE buffer (pH 8.0) and slides were rinsed in running tap water before being mounted under Aquamount (BDH Laboratory Supplies, UK).

Results

All the lesions in the tibiotarsi examined in the present report were of grade 6 severity. At least 30 sections from each tissue block were processed for *in situ* hybridations. All 5 TD and 2 normal growth plate blocks were examined. The results reported here were typical observations which were consistently observed within all the normal and TD tibia examined and the results showed very little variations within the two groups. Histological features of the longitudinal sections of the proximal end of normal and tibial dyschondroplastic three-week-old chick growth plates can be clearly seen and compared in Figure 1 and Figure 2. The growth plate of a TD chick of the same age is three times the size/width of the normal growth plate. Epiphyseal and metaphyseal blood vessels are prominent within the normal growth plate whereas the TD growth plate appears to be relatively avascular with drastically reduced metaphyseal blood supply. The proliferative zones of normal and TD growth

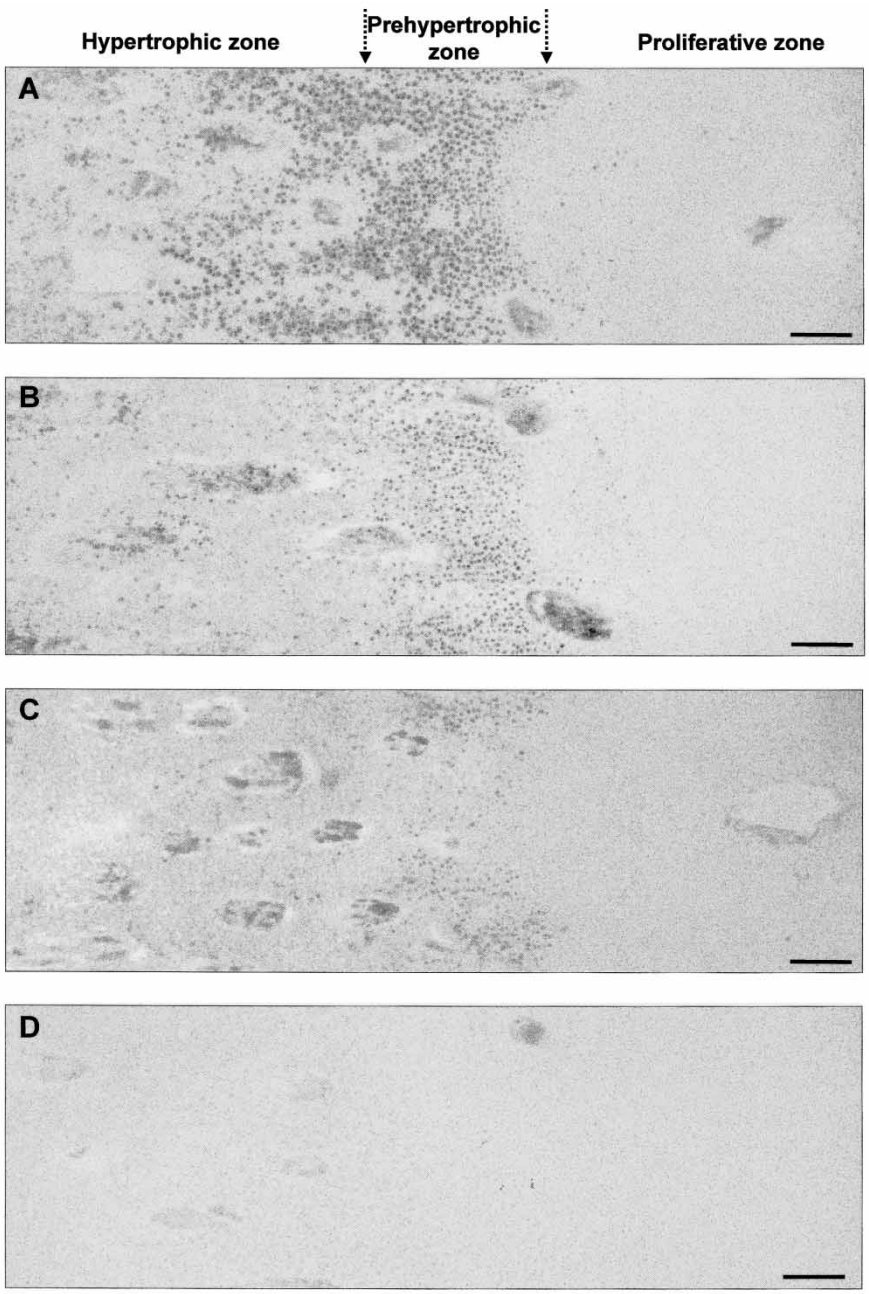

Figure 1. In situ *hybridization of longitudinal sections of normal 3-week-old chick growth plate using DIG-labelled antisense riboprobe specific for mRNAs of chick (A) type X collagen; (B) Indian hedgehog (Ihh); and (C) Parathyroid Hormone Related-protein (PTHrP). The probe was visualized with an alkaline phosphatase antibody for DIG and the NBT/BCIP colour reaction. (A) Specific positive staining can be seen in the prehypertophic zone and part of the hypertrophic zone in the healthy growth plate. Staining intensity reduces as chondrocytes mature further within the region of calcification. (B) Positive staining is evident within the prehypertrophic zone of the normal 3-week-old chick growth plate. A few cells either side of this region and on the proximal edge of blood vessels in the hypertrophic zone also stain. (C) The expression domain of PTHrP mRNA within the normal chick growth plate is restricted to a band within the prehypertrophic zone. Proliferative and hypertrophic zones do not show expression of the PTHrP mRNA. Background darkening of the matrix in the hypertrophic zone is artefactual. (D) Example of a negative control section hybridized with a sense probe to chick Indian hedgehog. All other sense probes yielded no observable staining. Scale bar = 100μm.*

plates are similar in appearance consisting of columns of flattened chondrocytes of around 25 cells each (Figure 3a and e). The columns of prehypertrophic chondrocytes in TD are longer than those seen in the normal growth plate. The chondrocytes within the lesion are of similar size to those in the prehypertrophic zone (Figure 3b,f and g). Many empty lacunae are also seen in the middle and distal parts of the lesion. On the diaphyseal side of the lesion, large chondrocytes, comparable in size and morphology to the hypertrophic chondrocytes in the normal growth plate (Figure 3c), are present between large blood vessels and bone marrow spaces. The extracellular matrix here

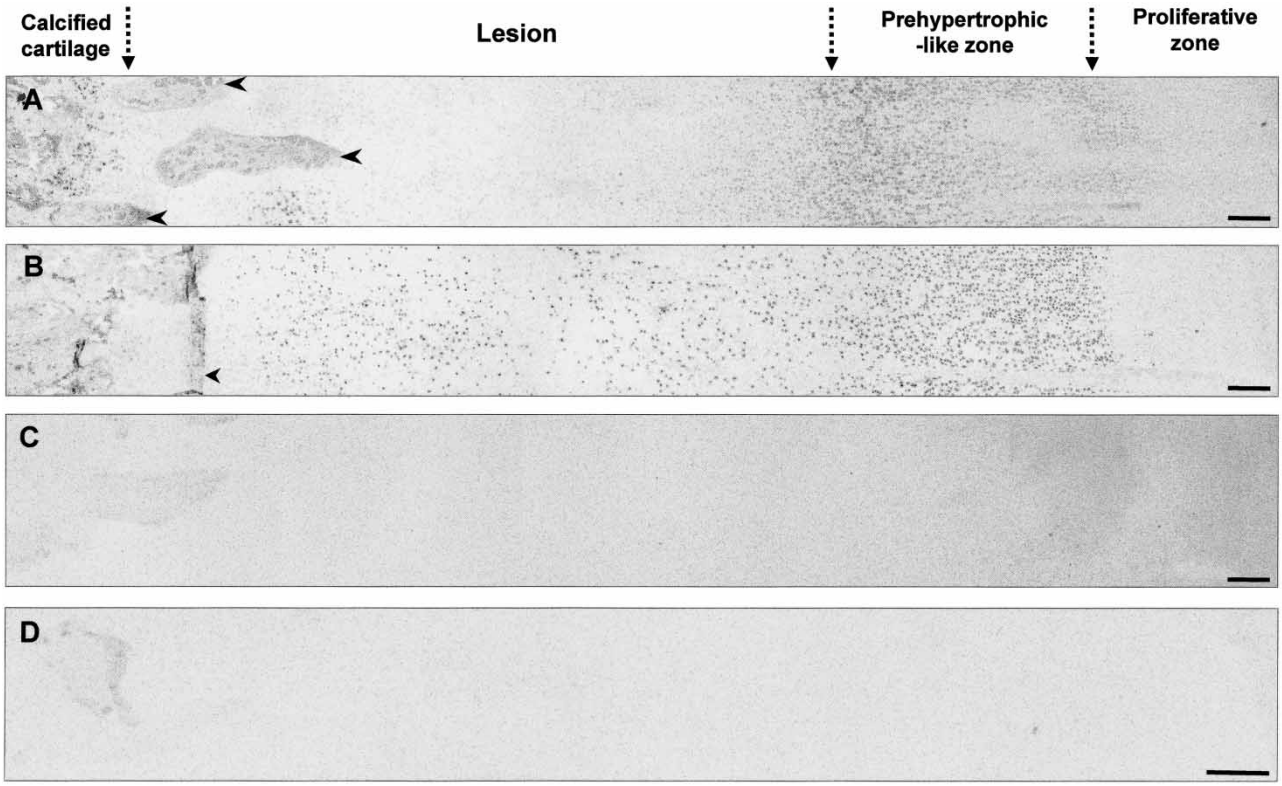

Figure 2. *Photomicrograph montages of longitudinal sections of 3-week-old chick dyschondroplastic tibial (TD) chick growth plate using DIG-labelled antisense riboprobe specific for mRNAs of chick (A) type X collagen; (B) Indian hedgehog; (C) PTHrP TD growth plate. The probe was visualized with an alkaline phosphatase antibody for DIG and the NBT/BCIP colour reaction. (A) In the TD afflicted growth plate positive staining is initially present in prehypertrophic-like chondrocytes, and staining is present in differentiated cells. The lesion shows few positive cells in its proximal part and only pale background staining in the central and distal regions. Groups of large chondrocytes on the diaphyseal side of the lesion near large blood vessels (arrowheads) also show positive staining. (B) Prehypertrophic-like chondrocytes in the TD growth plate show specific expression of Ihh mRNA. This expression is continuous with cells within the lesion, although the number of positive cells is reduced, probably because most cells are dead. Outside the lesion, at the proximal end of the diaphysis, no staining is seen. (The structures highlighted by arrowheads are artefactual folds.) (C) There is no specific staining of the TD growth plate. (D) Negative control section of the TD growth plate hybridized with a sense probe to chick Indian hedgehog. All other sense probes yielded no observable staining. Scale bar = 100μm.*

is darkly stained, and appears similar to that of the zone of calcification seen in the normal growth plate (Figure 3h).

In situ *hybridization for type X collagen mRNA*

The expression of the type X collagen mRNA by differentiating chondrocytes in the normal growth plate is clearly localized to a very distinct band across its width (Figure 1a). No expression of type X collagen mRNA by proliferative chondrocytes was observed. Type X collagen mRNA expression begins in the prehypertrophic zone and higher levels of expression are associated with the hypertrophic cells adjacent to the prehypertrophic zone. The number of positive cells appeared to be decreased distally and in the region of calcified cartilage few hypertrophic chondrocytes showed any expression (Figure 3a to c).

In the TD growth plate, collagen X mRNA was also expressed by prehypertrophic differentiating chondrocytes (Figure 2a) and not by proliferative cells. The level of expression in prehypertrophic chondrocytes appears to became more pronounced distally, up to the boundary with the lesion (Figure 2a and Figure 3f). The apparent increase in staining intensity in the distal region of the prehypertrophic zone was not as clear as in the normal growth plate. No significant level of type X expression was observed within the TD lesion (Figure 2a and Figure 3g).

Groups of large chondrocytes directly distal to the TD lesion, also showed type X collagen mRNA. These cells appeared morphologically to be hypertrophic chondrocytes and were interspersed between the large metaphyseal blood vessels and the blood vessels and marrow spaces of the trabecular bone (Figure 3h). Sense riboprobe negative controls in normal and TD tissues did not show any hybridization of cells within the growth plate, articular cartilage or bone.

In situ *hybridization of Indian hedgehog mRNA*

Expression of Indian hedgehog mRNA in the normal, three-week-old chick growth plate was restricted to the early differentiating chondrocytes of the prehypertrophic zone (Figure 1b). No Ihh mRNA was observed in the proliferative zone (Figure 4a to c) and there was a marked boundary

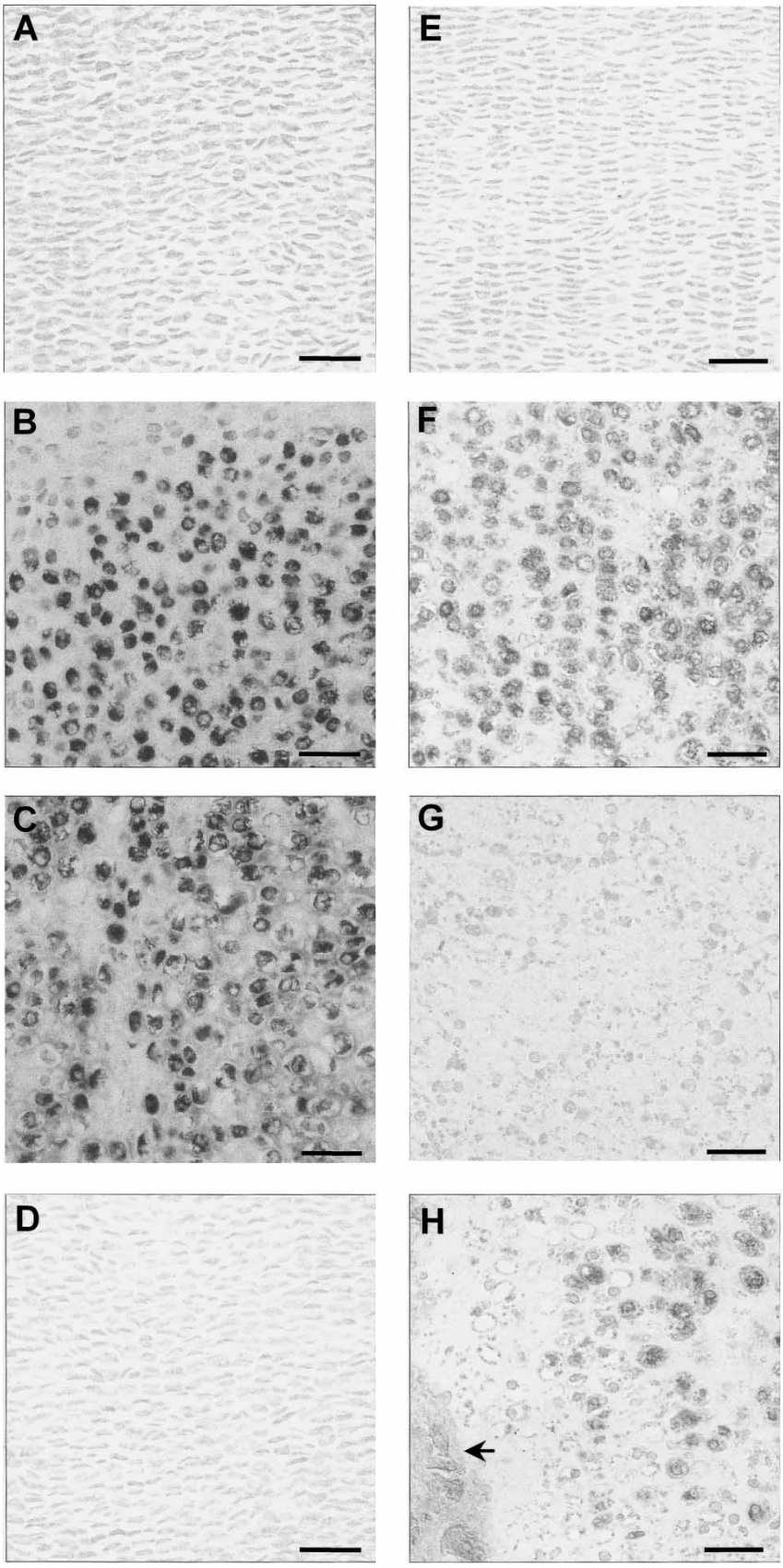

Figure 3. *Higher magnification of* in situ *hybridization using a riboprobe specific for type X collagen mRNA in the proximal tibial growth plate of normal (A–D) and TD (E–H) 3 week old chicks. DIG labelled probe, visualized with a DIG-alkaline phosphatase conjugated antibody and NBT/BCIP. In the growth plate proliferative chondrocytes do not label positive for type X collagen expression (A), but prehypertrophic (B) and hypertrophic chondrocytes (C) show staining specific to the cytoplasm. (D) Sense probe negative control, normal growth plate. In the TD growth plate proliferative chondrocytes (E) are negative, whereas differentiating cells (F) show cytoplasmic staining. The lesion (G) does not contain any positive cells, but outside the lesion at the proximal end of the diaphysis (H) many large, apparently hypertrophic chondrocytes exhibit type X collagen expression. The arrowed structure in "H" is a large blood vessel. Scale bars = 50μm.*

between these negative and positive regions. The more fully differentiated chondrocytes within the hypertrophic zone were negative (Figure 4c).

Ihh mRNA in the TD growth plate was also localized to the prehypertrophic-like zone (Figure 2b and Figure 4g) with chondrocytes in the proliferative zone showing no Ihh mRNA (Figure 4f). The point at which positive staining began defined the proximal end of the prehypertrophic zone, and the start of chondrocyte differentiation. Expression was detected throughout the TD lesion, with the majority of cells staining intensely (Figure 4g and Figure 4h) although some chondrocyte lacunae appeared empty. The positive cells were separated by a large volume of interterritorial matrix and were spaced further apart in the more distal region of the lesion. The hypertrophic chondrocytes distal to the lesion and at the end of the diaphysis were generally negative for Ihh expression although faint staining was observed in a small number of cells (Figure 2b and Figure 4i). Negative controls using sense riboprobes gave no staining in normal (Figure 4d and e) or TD growth plate sections (Figure 4j).

In situ *hybridisation for parathyroid hormone-related protein mRNA*

In the normal chick growth plate PTHrP mRNA was localized to a specific band of prehypertrophic chondrocytes (Figure 1c). The positive band of cells was narrower than that of the Indian hedgehog mRNA domain, and limited to the smaller of the round, differentiating cells (Figure 5b). Proliferative and hypertrophic chondrocytes did not show any expression (Figure 1c and Figure 5a and c). Osteoblasts upon the surface of newly forming spicules of trabecular bone in both normal and TD birds were densely positive for PTHrP (Figure 5d and e). No specific labelling of PTHrP mRNA was observed at the perichondrium or periarticular surfaces (not shown). *In situ* hybridization using the PTHrP probe in the dyschondroplastic sections gave no labelling of chondrocytes in any region of the growth plate (Figure 2c).

Discussion

During bone elongation, terminal differentiation of growth plate chondrocytes is an important prerequisite to the normal development and repair of the skeleton. Many transcription and soluble growth factors, including Insulin-like Growth Factor-1 (Lazowski *et al*., 1994; Ren *et al*., 1997), Transforming Growth Factor β (Thorp *et al*., 1992), Fibroblast Growth Factors (Deng *et al*., 1996; Peters *et al*., 1993), Ihh (Vortkamp *et al*., 1996), PTHrP (Karaplis *et al*., 1994; Amizuka *et al*., 1994; Vortkamp *et al*., 1996; Farquharson *et al*., 1999) are involved in chondrocyte differentiation (Cancedda *et al*., 1995). Such diversity of controlling molecules suggests that chondrocyte proliferation and differentiation are likely to be mediated through a number of interacting control loops.

Tibial dyschondroplasia (TD) is a consequence of impaired growth plate chondrocyte differentiation, which leads to defective ossification and bone morphogenesis (Hargest *et al*., 1985; Farquharson *et al*., 1992; Loveridge *et al*., 1993; Thorp *et al*., 1993; Farquharson & Jefferies, 2000). Based on the current perceptions of the regulation of cell differentiation in the growth plate, the pathology of TD may be related to the disruption of the Ihh/PTHrP control loop as reviewed in the introduction (Vortkamp *et al*., 1996). Also, chondrocyte maturation within the TD lesion appears to be arrested at the prehypertrophic stage when studied morphologically and biochemically (Hargest *et al*., 1985; Farquharson *et al*., 1992).

The data presented here have shown that collagen X mRNA expression in 3-week chicks is initiated at the prehypertrophic stage, and expression is maintained throughout the hypertrophic zone which is consistent with previously observed expression patterns of type X collagen in the growth plate by immunohistochemistry (for review see Kwan, 2000). In the TD growth plate, type X collagen mRNA is present in a band of cells of the proximal border of the TD lesion. Their cellular morphologies, anatomical positions and the presence of collagen X mRNA suggest these cells have a pre-hypertrophic chondrocyte phenotype. This observation, together with the relatively elongated prehypertrophic zone, clearly indicates a delay in the maturation of cells to hypertrophic chondrocytes in TD, the newly synthesized collagen X molecules accumulate intracellularly and are not deposited within the matrix of the TD lesion (Farquharson *et al*., 1995; Tselepis *et al*., 1996; Reginato *et al*., 1998). We have also observed that interactions between type X collagen and the $\alpha_2\beta_1$ integrin of chondrocytes can upregulate collagen X gene expression implying type X collagen deposi-

Figure 4. In situ *hybridization of a DIG labelled riboprobe specific for Indian hedgehog gene expression in the normal (A–E) and TD (F–J) chick growth plates. (A) In the normal growth plate proliferative chondrocytes do not label. (B) Cells within the prehypertrophic zone stain, demonstrating expression of Ihh mRNA. (C) Hypertrophic chondrocytes do not stain positively. (D) Sense control, proliferative zone, normal growth plate. (E) Sense control, prehypertrophic zone, normal growth plate. In the TD growth plate (F) proliferative chondrocytes do not show Ihh expression. (G) Prehypertrophic-like chondrocytes stain darkly. (H) Ihh mRNA is present in cells within the lesion. (I) At the proximal end of the diaphysis cells are predominantly unlabelled, although staining is commonly seen in a small number of cells. The arrowed structures in "C" and "I" are blood vessels and haematopoietic tissue extending from the bone. (J) Sense control, prehypertrophic zone, TD growth plate. Scale bars = 50μm.*

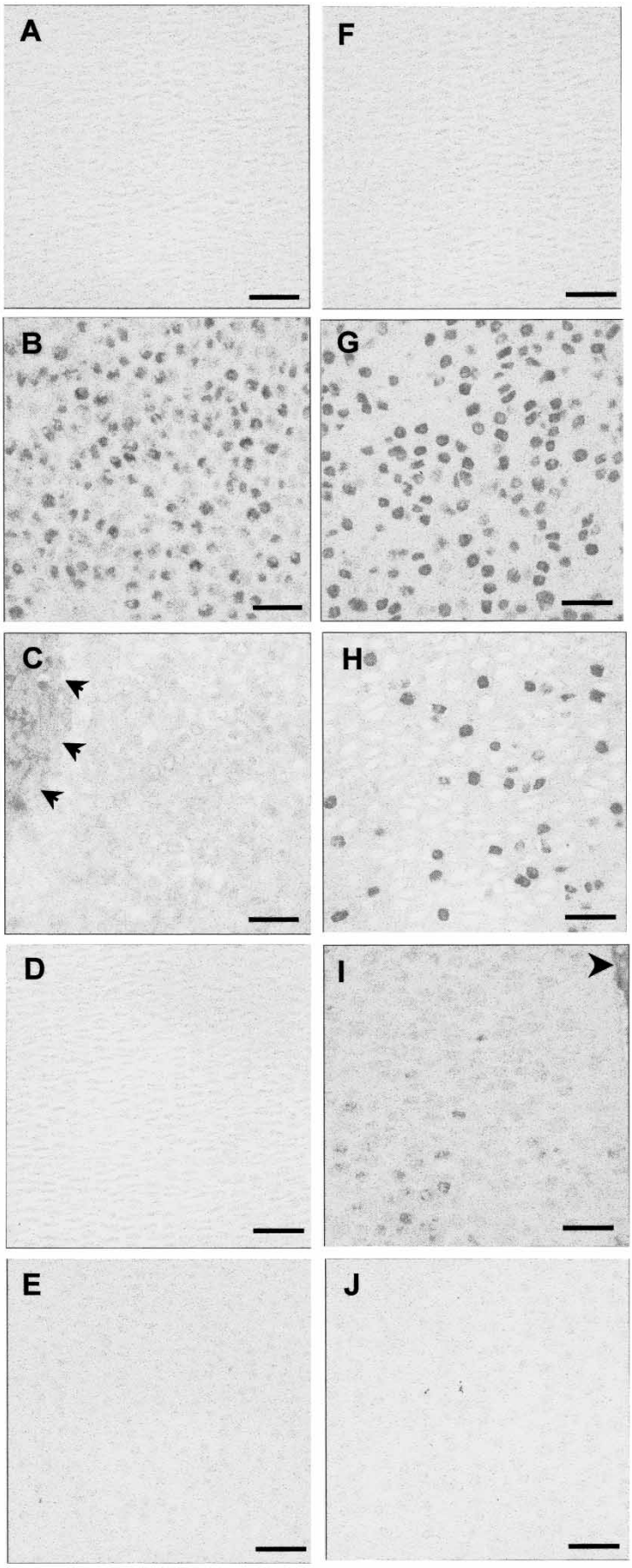

Figure 4

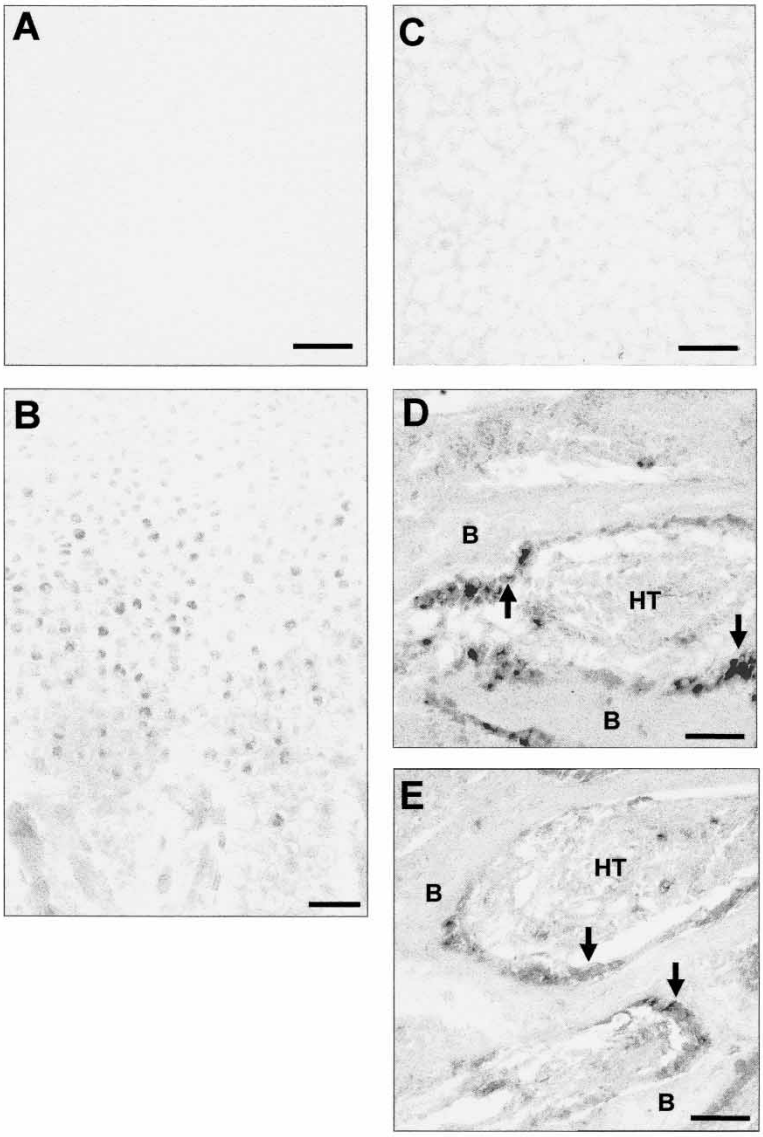

Figure 5. In situ *hybridization of a probe specific for PTHrP mRNA in the normal (A–D) and TD (E) chick growth plate. (A) Proliferative and (C) hypertrophic chondrocytes do not show expression of PTHrP but a thin band of labelling is present within the prehypertrophic zone (B). This band is thinner than the band of Indian hedgehog expression also seen in this study. Chondrocytes positive for PTHrP are small transitional cells. Larger transitional cells are negative. (D) Osteocytes within the developing bone do not show expression of PTHrP, but many osteoblasts lining the bony spicules are labelled. The staining does appear to be cytoplasmic and clear spaces can be seen at the sites of nuclei (arrow). (E) Osteoblasts lining the bony spicules in the metaphysis of TD chicks also showed labelling of PTHrP mRNA. HT – medullary cavity and haematopoetic tissue. B – spicules of trabecular bone developing from calcified cartilage. Scale bars = 50μm.*

tion may have a positive feedback control over its biosynthesis (Rees & Kwan, unpublished data). In this context, the deposition of type X collagen may be important in the full maturation of the prehypertrophic chondrocytes by stimulating collagen X deposition and cell–collagen interactions. In TD, the absence of type X collagen deposition in the extracellular matrix may therefore lead to the reduction and eventual cessation of type X collagen gene expression in the lesion observed by *in situ* hybridization in this study. The presence of a small number of hypertrophic chondrocytes expressing collagen X at the diaphyseal end of the growth plate suggests that these cell are undergoing differentiation which may lead to renewed bone growth. It is generally accepted that the appearance of 'normal' chondrocytes distal to the TD lesion are signs of repair where many hypertrophic chondrocyte markers are expressed.

The expression pattern of Indian hedgehog mRNA observed in the normal growth plate in the present study was similar to the pattern seen during embryonic mouse bone formation (Iwasaki *et al*., 1997; Vortkamp *et al*., 1996). We have shown here that Ihh mRNA is expressed by prehypertrophic chondrocytes in the growth plate of 3-week-old chickens, and that it remains a good marker for prehypertrophic chondrocytes in post-hatch tissue. This observation also confirms that collagen X mRNA expression overlaps with the

proximal Ihh mRNA expression domain in the growth plate (Vortkamp *et al*., 1996). The observed expression of Ihh mRNA by viable TD lesion cells suggests that these cells do not cease Ihh expression in the normal manner that has been associated with further differentiation, and that cells within the TD lesions are not undergoing degeneration but are still biologically viable. The TD growth plate chondrocytes are therefore developmentally arrested at the prehypertrophic stage, and are unable to progress and become hypertrophic. In the TD growth plate, the extended zone of Ihh mRNA expression may lead to high extracellular matrix levels of Ihh protein. It has been suggested that high local concentrations of the amino-terminal fragment of Ihh can function directly as a morphogen to promote chondrocyte differentiation (Karaplis & Vautour, 1997; Akiyama *et al*., 1997; Stott & Chuong, 1997). Our observations provide no evidence to support such a role for Ihh in chondrocyte differentiation. However, further immunolocalization studies are required to ascertain the levels of Ihh protein in the TD lesion.

A number of previous studies on PTHrP expression have reported contrasting observations. PTHrP protein has been immunolocalized to the proliferating chondrocytes, transitional chondrocytes and the perichondrium of embryonic mouse growth plate (Lee *et al*., 1996). In the juvenile chick growth plate, intracellular localization of PTHrP protein was observed throughout all zones by Farquharson *et al*. (2001a) and in the pre-hypertrophic and hypertrophic chondrocytes by Medill *et al*. (2001). Our *in situ* hybridization experiments clearly localized PTHrP mRNA only to a narrow band of immature prehypertrophic chondrocytes and therefore complements the observation by Medill *et al*. (2001) that PTHrP expression is limited to specific zones of chondrocyte maturation and supports the notion that PTHrP expression is integral to the growth plate chondrocytes in juvenile chicks. The underlying reason for the apparent discrepancies of these reported PTHrP expression patterns in the chick is at present not known. However, if PTHrP mRNA is transiently expressed in the prehypertrophic zone it is conceivable that PTHrP is retained by the maturing cells and therefore detectable by immunohistochemistry after differentiating into hypertrophic cells. Indeed, PTHrP has not been localized within the matrix (Farquharson *et al*., 2001a,b; Medill *et al*., 2001).

The absence of periarticular and perichondrial expression of PTHrP mRNA raises the possibility that the Ihh/PTHrP mechanism that regulates chondrocyte differentiation in the mouse embryo is not equivalent to that in the post-hatch avian growth plate. According to the current theory, an increase in Ihh expression would lead to increased expression of PTHrP in the TD lesion. On the contrary, PTHrP mRNA was not detected in the TD growth plates we examined even though the tissue was prepared similarly, the same probe was used and the experiments were run in parallel to the normal growth plate sections under identical conditions. The present observation suggests that the arrest of cell differentiation in TD is independent of PTHrP. However, reduced levels of PTHrP expression have been reported by Farquharson *et al*. (2001a,b). The apparent absence of PTHrP mRNA in our TD lesions probably indicates the cessation of PTHrP expression in a more severe form of TD, assuming that there is a corresponding decrease in PTHrP expression with TD lesion development. The absence of PTHrP mRNA in our *in situ* hybridization studies is unlikely to be due to the lack of sensitivity of the methods employed since osteoblasts from the both normal and TD growth plates were shown to strongly express PTHrP mRNA.

In summary, we have used genetic markers of chondrocytic phenotypes to show that chondrocytes in the TD growth plate are developmentally arrested in a prehypertrophic state. We have shown that type X collagen mRNA is a marker for both prehypertrophic and hypertrophic chondrocytes. This study therefore ascertained that the TD growth plate is a good model of disrupted endochondral ossification. Our observations also suggest that the Ihh/PTHrP mechanism by which chondrocyte differentiation is regulated in embryonic mice does not act in the same manner in the post-hatch avian growth plate and therefore it is conceivable that the Ihh/PTHrP feedback control is not the only mechanism in mediating chondrocyte differentiation in neonatal bone growth in chick and other animal systems. Our data suggest that the arrest of hypertrophy is independent of PTHrP, as we detected none in the TD growth plate. It is probable that other regulatory mechanisms are involved in the arrest of chondrocyte differentiation in TD. Delta/Notch, the cell fate signalling system that has been implicated in halting chondrocyte differentiation in embryonic chicks (Crowe *et al*., 1999), may have a role in the development of TD. The present study, therefore, provides a basis for further investigations of the molecular mechanisms of the development of avian TD involving these other known regulatory factors.

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References

Akiyama, H., Shigeno, C., Hiraki, Y., Shukunami, C., Kohno, H., Akagi, M., Konishi, J. & Nakamura, T. (1997). Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5. *Biochemical & Biophysical Research Communications*, *235*, 142–147.

Amizuka, N., Warshawsky, H., Henderson, J.E., Goltzman, D. & Karaplis, A.C. (1994). Parathyroid hormone-related peptide-depleted mice show altered endochondral bone-formation. *Journal Cell Biology*, *126*, 1611–1623.

Ben-Bassat, S., Genina, O., Lavelin, I., Leach, R.M. & Pines, M. (1999). Parathyroid receptor gene expression by epiphyseal growth plates in rickets and tibial dyschondroplasia. *Molecular & Cellular Endocrinology*, *149*, 185–195.

Burtis, W.J., Wu, T., Bunch, C., Wysolmerski, J.J., Isogna, K.L., Weir, E.C., Broadus, A.E. & Stewart, A.F. (1987). Identification of a novel 17,000 dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. *Journal of Biological Chemistry*, *161*, 7151–7156.

Cancedda, R., Cancedda, F.P. & Castagnola, P. (1995). Chondrocyte differentiation. *International Review of Cytology*, *159*, 265–358.

Crowe, R., Zikherman, J. & Niswander, L. (1999). Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation. *Development*, *126*, 987–998.

Deng, C.X., Wynshaw-Boris, A., Zhou, F., Kuo, A. & Leder, P. (1996). Fibroblast growth receptor 3 is a negative regulator of bone growth. *Cell*, *84*, 911–921.

Farquharson, C., Whitehead, C.C., Rennie, J.S., Thorp, B.H. & Loveridge, N. (1992). Cell proliferation and enzyme activities associated with the development of avian tibial dyschondroplasia: An in situ biochemical study. *Bone*, *13*, 59–67.

Farquharson, C., Berry, J.L., Mawer, E.B., Seawright, E. & Whitehead, C.C. (1995). Regulators of chondrocyte differentiation in tibial dyschondroplasia: an in vivo and in vitro study. *Bone*, *17*, 279–286.

Farquharson, C., Houston, B., Seawright, E. & Jefferies, D. (1999). PTHrP and PTH/PTHrP receptor expression within maturationally distinct chondrocyte populations of the growth plate. *Journal of Bone and Mineral Research*, *14*(Suppl 1), S292.

Farquharson, C. & Jefferies, D. (2000). Chondrocytes and longitudinal bone growth: The development of tibial dyschondroplasia. *Poultry Science*, *79*, 994–1004.

Farquharson, C., Seawright, E. & Jefferies, D. (2001). Parathyroid hormone-related peptide expression in tibial dyschondroplasia. *Avian Pathology*, *30*, 327–335.

Farquharson, C., Jefferies, D., Seawright, E. & Houston, B. (2001). Regulation of chondrocyte terminal differentiation in the postembryonic growth plate: The role of the PTHrP–Indian Hedgehog axis. *Endocrinology*, *142*, 4131–4140.

Hargest, T.E., Leach, R.M. & Gay, C.V. (1985). Avian tibial dyschondroplasia. I. Ultrastructure. *American Journal of Pathology*, *119*, 175–190.

Hunziker, E.B. (1994). Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microscopy Research and Technique*, *28*, 505–519.

Iwasaki, M., Le, A.X. & Helms, J.A. (1997). Expression of Indian hedgehog, bone morphogenetic protein 6 and gli during skeletal morphogenesis. *Mechanism of Development*, *69*, 197–202.

Jüppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X.F., Schipani, E., Richards, J., Kolakowski Jr., L.F., Hock, J., Potts Jr., J.T., Kronenberg, H.M. & Segre, G.V. (1991). A G protein-linked receptor for parathyroid hormone-related peptide. *Science*, *254*, 1024–1026.

Kalderone, D. (2000). Transducing the Hedgehog signal. *Cell*, *103*, 371–374.

Karaplis, A.C., Luz, A., Glowacki, J., Bronson, R.T., Tybulewicz, V.L.J., Kronenberg, H.M. & Mulligan, R.C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes and Development*, *8*, 277–289.

Karaplis, A.C. & Vautour, L. (1997). Parathyroid hormone-related peptide and the parathyroid hormone/parathyroid hormone-related peptide receptor in skeletal development. *Current Opinion in Nephrology and Hypertension*, *6*, 308–313.

Kwan, A.P.L. (2000). Type X Collagen. In B. Caterson, C.W. Archer, J.R. Ralphs & M. Benjamin (Eds.), *Biology of the Synovial Joint*. Amsterdam, The Netherlands: Harwood Academic Publishers.

Lazowski, D.A., Fraher, L.J., Hodsman, A., Steer, B., Modrowski, D. & Han, V.K. (1994). Regional variation of insulin-like growth factor-I gene expression in mature rat bone and cartilage. *Bone*, *15*, 563–576.

Leach, R.M. & Nesheim, M.C. (1965). Nutritional, genetic and morphological studies of an abnormal cartilage formation in young chicks. *Journal of Nutrition*, *86*, 236–244.

Lee, K., Lanske, B., Karaplis, A.C., Deeds, J.D., Kohno, H., Nissenson, R.A., Kronenberg, H.M. & Segre, G.V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology*, *137*, 5109–5118.

Loveridge, N., Farquharson, C., Hesketh, J.E., Jakowlew, S.B., Whitehead, C.C. & Thorp, B.H. (1993). The control of chondrocyte differentiation during endochondral bone growth in vivo: changes in TGF-β and the proto-oncogene c-*myc*. *Journal of Cell Science*, *105*, 949–956.

Loveys, L.S., Gelb, D., Hurwitz, S.R., Puzas, J.E. & Rosier, R.N. (1993). Effects of parathyroid hormone-related peptide on chick growth plate chondrocytes. *Journal of Orthopaedic Research*, *11*, 884–891.

Lynch, M., Thorp, B.H. & Whitehead, C.C. (1992). Avian tibial dyschondroplasia as a cause of bone deformity. *Avian Pathology*, *21*, 275–285.

Medill, N.J., Praul, C.A., Ford, B.C. & Leach, R.M. (2001). Parathyroid hormone-related peptide expression in the epiphyseal growth plate of the juvenile chicken: evidence for the origin of the parathyroid hormone-related peptide found in the epiphyseal growth plate. *Journal of Cellular Biochemistry*, *80*, 504–511.

Peters, K., Ornitz, D., Werner, S. & Williams, L. (1993). Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Developmental Biology*, *155*, 423–430.

Philbrick, W.M., Wysolmerski, J.J., Galbraith, S., Holt, E., Orloff, J.J., Yang, K.H., Vasavada, R.C., Weir, E.C., Broadhus, A.E. & Stewart, A.F. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiological Reviews*, *76*, 127–173.

Poole, A.R. (1991). The growth plate: cellular physiology, cartilage assembly and mineralization. In B.K. Hall (Ed.), *Cartilage Molecular Aspects*. Ann Arbor: CRC Press.

Poulos, P.W., Reiland, S., Elwinger, K. & Olsson, S.E. (1978). Skeletal lesions in the broiler, with special reference to dyschondroplasia (osteochondrosis). Pathology, frequency, and clinical significance in two strains of birds on high and low energy feed. *Radiology Supplement*, *358*, 229–276.

Reginato, A.M., Bashey, R.I., Rosselot, G., Leach, R.M., Gay, C.V. & Jimenez, S.A. (1998). Type X collagen biosynthesis and expression in avian tibial dyschondroplasia. *Osteoarthritis and Cartilage*, *6*, 125–136.

Ren, P., Rowland 3rd, G.N. & Halper, J. (1997). Expression of growth factors in chicken growth plate with special reference to tibial dyschondroplasia. *Journal of Comparative Pathology*, *116*, 303–320.

Rennie, J.S., Whitehead, C.C. & Thorp, B.H. (1993). The effect of dietary 1,25-dihydroxycholecalciferol in preveting tibial dyschondroplasia in broilers fed diets imbalanced in calcium and phosphorus. *British Journal of Nutrition*, *69*, 809–816.

Stott, N.S. & Chuong, C.M. (1997). Dual action of sonic hedgehog on chondrocyte hypertrophy: retrovirus mediated ectopic sonic hedgehog expression in limb bud micromass culture induces novel cartilage nodules that are positive for alkaline phosphatase and type X collagen. *Journal of Cell Science*, *110*, 2691–2701.

Suva, L.J., Winslow, G.A., Wettenhall, E.H., Hammonds, R.G., Moseley, J.M., Diefenbach-Jaggar, H., Rodda, C.P., Kemp, B.E., Rodriguez, H., Chen, E.Y., et al. (1987). A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science*, *237*, 893–896.

Thorp, B.H. (1988). Relationship between the rate of longitudinal bone growth and physeal thickness in the growing fowl. *Research in Veterinary Science*, *45*, 83–85.

Thorp, B.H., Anderson, I. & Jakowlew, S. (1992). Transforming growth factor-b1, -b2 and -b3 in cartilage and bone cells during endochondral ossification in the chick. *Development*, *114*, 907–911.

Thorp, B.H., Farquharson, C., Kwan, A.P.L. & Loveridge, N. (1993). Osteochondrosis/dyschondroplasia: a failure of chondrocyte differentiation. *Equine Veterinary Journal Supplement*, *16*, 13–18.

Tselepis, C., Hoyland, J.A., Barber, R.E., Thorp, B.H. & Kwan, A.P.L. (1996). Expression and distribution of cartilage matrix macromolecules in avian tibial dyschondroplasia. *Avian Pathology*, *25*, 305–324.

Tselepis, C., Kwan, A.P.L., Thornton, D. & Sheehan (2000) The biochemical characterization of aggrecan from normal and tibial-dyschondroplastic chicken growth-plate cartilage. *Biochemcal Journal*, *351*, 517–525.

Vortkamp, A., Kaechoong, L., Lanske, B., Segre, G.V., Kronenberg, H.M. & Tabin, C.J. (1996). Regulation of rate of cartilage differentiation by Indian Hedgehog and PTH-related protein. *Science*, *273*, 613–622.

ZUSAMMENFASSUNG

Expression von Typ X-Kollagen, Indian Hedgehog und Parathyroidhormon-verwandtem Protein in normalen und dyschondroplastischen Wachstumsplatten in der Tibia beim Huhn

Die tibiale Dyschondroplasie (TD) ist eine Form von abweichender endochondraler Ossifikation beim Huhn, bei der sich in der Wachstumsplatte ein gefäßloser Knorpelpfropf (TD Läsion) bildet. Histologisch betrachtet ist diese Stelle gefüllt mit vermeintlichen Übergangschondrozyten, die nicht in der Lage waren, sich zu hypertrophen Chondrozyten zu differenzieren. Wir haben die räumliche Verteilung der mRNS von Typ X-Kollagen, Indian Hegdehog (Ihh) und dem Parathyroidhormon-verwandten Protein (PTHrP) in der TD-Wachstumsplatte durch in situ Hybridisation untersucht, um festzustellen, in welchem Stadium die Chondrozytendifferenzierung in der TD stehen geblieben ist. In der normalen Wachstumsplatte wurde Typ X-Kollagen-mRNS sowohl von prähypertrophen als auch von hypertrophen Chondrozyten gebildet. Indian Ihh-mRNS wurde in einer Bande von prähypertrophen Chondrozyten nachgewiesen und PTHrP-Expression wurde in einer schmalen Bande von prähypertrophen Chondrozyten und in Osteoblasten in der Diaphyse lokalisiert. In TD-Schnitten wurde Kollagen X-Expression beobachtet in sich differenzierenden Zellen, in einer geringen Anzahl von Läsionszellen und in hypertrophischen Chondrozyten auf der diaphysalen Seite der Läsion. Ihh-Expression wurde ebenfalls in den sich differenzierenden Zellen und überall in der Läsion beobachtet. Diese Daten zeigen, dass die Chondrozytendifferenzierung in einem Übergangsstadium unmittelbar vor der Hypertrophie angehalten wird. Im Gegensatz zu den kürzlich beschriebenen mittels Immunhistochemie ermittelten PTHrP-Expressionsmustern in TD-Hühnern war in unseren Untersuchungen die PTHrP-mRNS nicht in der TD-Läsion zu finden. Diese Beobachtung kann den Stillstand der PTHrP-Genexpression von Chondrozyten in hochgradigen TD-Veränderungen erklären. Die Ergebnisse dieser Studie implizieren auch, dass der Stillstand in der Zelldifferenzierung bei der TD unabhängig von PTHrP ist und dass in die enchondrale Ossifikation in der aviären Wachstumsplatte nach dem Schlupf wahrscheinlich zusätzliche Regulationswege involviert sind.

RESUMEN

Expresión de colágeno tipo X, Indian Hedgehog y proteína relacionada con la hormona paratiroidea en placas de crecimiento de pollos normales y con discondroplasia tibial

La discondroplasia tibial (TD) es una forma de osificación endocondral aberrante en pollos, en la cual se forma un tapón de cartílago avascular en la placa de crecimiento (lesión de TD). Histológicamente, esta lesión está aparentemente formada por condrocitos transicionales que no han podido diferenciarse a condrocitos hipertróficos. En este estudio, se ha examinado la presencia de expresión espacial de mARNs para el colágeno X, el *Indian Hedgehog* (Ihh) y la proteína relacionada con la hormona paratiroidea (PTHrP) en la placa de crecimiento con TD mediante hibridación *in situ,* con el objetivo de determinar en que estadio de diferenciación queda detenido el condrocito en la TD. En la placa de crecimiento normal, el mARN de colágeno tipo X se expresa tanto en condrocitos prehipertróficos como hipertróficos. EL *Indian Hedgehog* mARN se detecta en una banda de condrocitos prehipertróficos y la expresión de PTHrP se localiza en una estrecha banda de condrocitos prehipertróficos y en una banda de osteoblastos en la diáfisis. En las secciones de TD, la expresión de colágeno X se observó en células en diferenciación, en un pequeño número de células en la lesión y en condrocitos hipertróficos en la banda de la diáfisis de la lesión. La expresión de la Ihh se observó también en células en diferenciación y por toda la lesión. Estos datos indican que la diferenciación de los condrocitos queda detenida en un estadio de transición justo antes de la hipertrofia. Contrariamente a los datos publicados previamente sobre los patrones de expresión de la PTHrP en pollos con TD mediante inmunocitoquímica, el mARN de la PTHrP no se observó en la lesión de TD. Esta observación puede reflejar el cese de la expresión del gen de la PTHrP por parte de los condrocitos en las lesiones de TD más severas. Los resultados de este estudio también demuestran que el arresto de la diferenciación celular en la TD es independiente de la PTHrP y que la osificación endocondral en la placa de crecimiento una vez el pollo ha nacido podría involucrar otras rutas de regulación adicionales.