Chondrocytes and Longitudinal Bone Growth: The Development of Tibial Dyschondroplasia

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ABSTRACT Growth plate cartilage is central to the process of bone elongation. Chondrocytes originating within the resting zone of the growth plate proceed through a series of intermediate phenotypes: proliferating, prehypertrophic and hypertrophic, before reaching a terminally differentiated state. Disruption of this chondrocyte maturational sequence causes many skeletal abnormalities in poultry such as tibial dyschondroplasia (TD), which is a common cause of deformity and lameness in the broiler chicken. Cell and matrix components of the growth plate have been studied in order to determine the cause(s) of the premature arrest of chondrocyte differentiation and retention of prehypertrophic chondrocytes observed in TD. Chondrocyte proliferation proceeds normally in TD, but markers of the differentiated phenotype, local growth factors, and the vitamin D receptor are abnormally expressed within the prehypertrophic chondrocytes above, and within, the lesion. Tibial dyschondroplasia is also associated with a reduced incidence of apoptosis, suggesting that the lesion contains an accumulation of immature cells that have outlived their normal life span. Immunolocalization studies of matrix components suggest an abnormal distribution within the TD growth plate that is consistent with a failure of the chondrocytes to fully hypertrophy. In addition, the collagen matrix of the TD lesion is highly crosslinked, which may make the formed lesion more impervious to vascular invasion and osteoclastic resorption. Recent studies have applied the techniques of differential display and semiquantitative reverse transcriptase-polymerase chain reaction to RNA obtained from discrete populations of growth plate chondrocytes of different maturational phenotypes. This strategy has allowed us to compare phenotypically identical cell fractions from normal and TD growth plates in an attempt to identify possible candidate genes for TD.

(*Key words*: growth plate, chondrocyte, endochondral, bone growth, tibial dyschondroplasia)

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INTRODUCTION

Normal Bone Growth

Bone growth occurs at regions of specialized cartilage—the growth plates—situated at both ends of all long bones. The growth plate comprises chondrocytes and their extracellular matrix where proteoglycans and collagen type II predominate. A characteristic of endochondral bone growth is the precise temporal and spatial organization of chondrocytes within the growth plate where they exhibit a series of well-defined maturational stages. The chondrocytes are distinguished by changes in their rate of proliferation and morphology and by the synthesis of the proteins that form the extracellular matrix. These events lead to a differentiated phenotype and the synthesis by the hypertrophic chondrocytes of a matrix that mineralizes and is eventually replaced by bone through the actions of osteoclasts and osteoblasts (Figure 1).

The rate of longitudinal bone growth is controlled by biomechanical factors and numerous systemic and local growth mediators that interact to regulate the activities of the growth plate chondrocytes. Chondrocyte proliferation, matrix turnover, and changes in chondrocyte shape and size are three essential activities that are finely coordinated to achieve the rapid and characteristic alterations in both cells and matrix during bone elongation.

Proliferative chondrocytes of avian growth plates are arranged into columns, but, unlike mammals, they are not easily distinguishable due a much higher density and less extracellular matrix (Howlett, 1979). In the proliferative zone, the cells have a flattened, oblate spheroid shape, and the percentage of dividing chondrocytes within the chick growth plate has been estimated at 24% (Farquharson et al., 1992a). Chondrocyte differentiation starts immediately after cessation of division (Breur et al., 1994)

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Abbreviation Key: 1,25-D = 1,25-dihydroxyvitamin D3; 25-D = 25-hydroxyvitamin D3; ALP = alkaline phosphatase; FGF = fibroblast growth factor; IGF = insulin-like growth factor; KS = keratan sulfate; PCNA = proliferating cell nuclear antigen; PTHrP = parathyroid hormone-related peptide; TD = tibial dyschondroplasia; TRAP = tartrate-resistant acid phosphatase; VDR = vitamin D receptor.

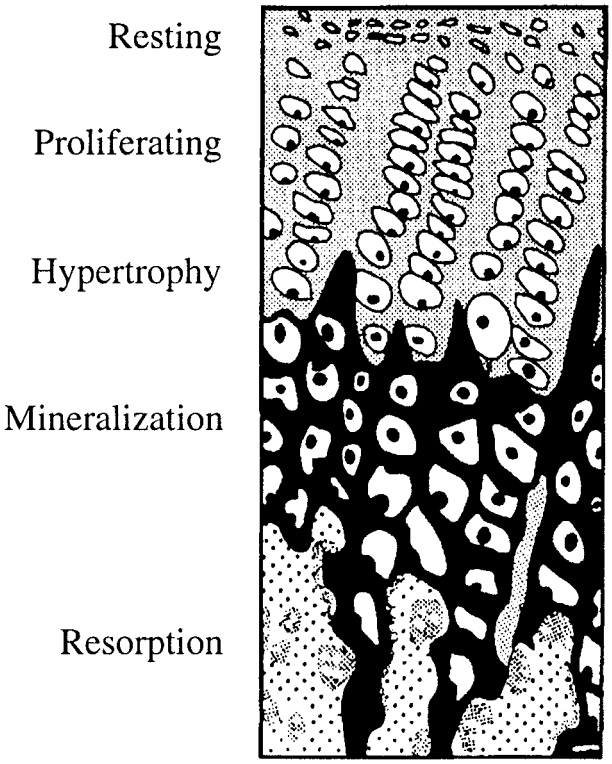

FIGURE 1. Spatial and temporal organization of chondrocytes within the growth plate.

and is characterized by the chondrocytes becoming more voluminous and spherical with increases in rough endoplasmic reticulum and Golgi apparatus, reflecting increased matrix production. Associated with this hypertrophic phenotype are increased activity of plasma membrane alkaline phosphatase (ALP), synthesis of collagen type X and downregulation of collagen type II, secretion of osteonectin and osteopontin, and expression of the vitamin D receptor (VDR).

Each chondrocyte of the growth plate moves through the entire spectrum of maturational stages while remaining in a spatially fixed location throughout its existence. The time taken for a chondrocyte to move from a proliferative to a terminally differentiated phenotype is species dependent and is approximately 21 h in the broiler chick (Thorp 1988). In contrast, the mean cycle time of proliferating cells within the growth plate of man and rat is 20 and 2 d, respectively (Kember and Sissons, 1976).

Many studies investigating the rate of longitudinal bone growth have concentrated on proliferation and volume changes in the hypertrophic chondrocytes. In mammals, a linear relationship exists between the vertical height of the hypertrophic chondrocyte in the direction of growth and the rate of bone growth. This variation in cell height accounts for the different growth rates between species and also the different growth rates that occur at the proximal and distal ends within the same long bone (Hunziker et al., 1987; Breur et al., 1991). This relationship, however, is not the case in birds, and it is the thickness of the avian growth plate that is related to the rate of longitudinal bone growth (Thorp, 1988). Further studies have indicated that variation in the size of the hypertrophic chondrocyte in different avian species is small, and the largest factor in determining the rate of growth is the size of the proliferative cell population within the growth plate (Kirkwood et al., 1989; Kember et al., 1990).

Alkaline phosphatase has long been associated with matrix mineralization (Robison, 1923), and inhibition of enzyme activity impairs the mineralization process. The exact role of ALP remains unclear, but the catalytic actions of ALP are thought to result in an enrichment of orthophosphate for incorporation into nascent $CaPO_4$ mineral (Anderson, 1995). Collagen type X is distributed almost exclusively in the matrix of hypertrophic cartilage, and a role for this collagen type in the mineralization process has been proposed. Recent data have indicated, however, that type X-null mice have no gross defects in long bone development (Rosati et al., 1994), and, therefore, collagen type X may have a supportive role rather than being involved in cartilage mineralization.

Tibial Dyschondroplasia

Disruption of normal growth plate chondrocyte maturation leads to tibial dyschondroplasia (TD) in broilers, layers, turkeys, and ducks. It is characterized by a plug of noncalcified avascular opaque cartilage situated below the growth plate and extending into the metaphysis (Figure 2). It is most commonly found in the proximal tibiotarsus, but has been reported to occur in most long bones. Dyschondroplasia was first described by Leach and Nesheim (1965) as a spontaneously occurring cartilage abnormality, but it is now known that both nutrition and selective breeding can affect the incidence of TD. Some of the

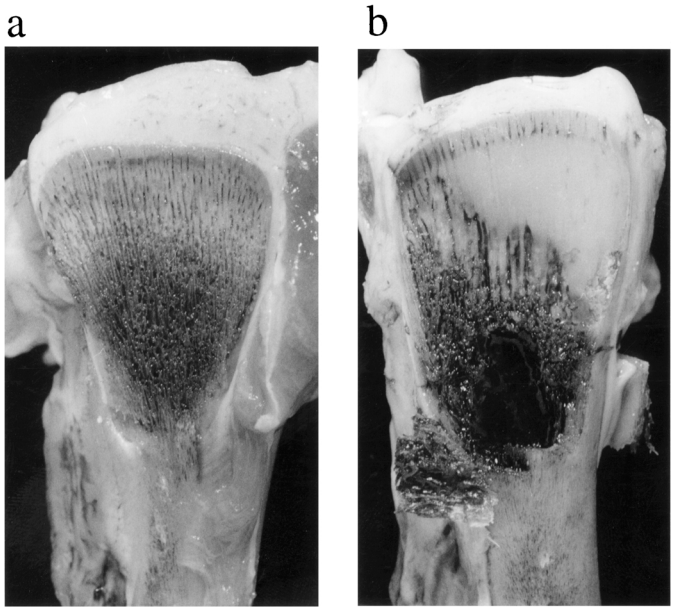

FIGURE 2. Comparison of normal (a) and dyschondroplastic (b) growth plates. The tibial dyschondroplasia lesion is opaque and avascular and occupies a large area of the metaphysis under the growth plate.

dietary supplements or modifications that are known to increase the incidence are altered Ca:P ratio, cysteine, homocysteine, salicylic acid, or copper content, as well as the inclusion of mycotoxins (Fusarium species) and commercial fungicides (thiuram and disulfiram). In contrast, vitamin D metabolites, and in particular 1,25-dihydroxycholecalciferol, are the only dietary factors known to prevent TD (for review see Whitehead, 1997). Due to the number of diverse causes of TD and to the absence of a common factor that links all causative agents, it is possible that several mechanisms may exist that result in the accumulation of chondrocytes and the formation of the cartilaginous plug that characterize dyschondroplasia.

The size of the TD lesion is variable. It can exist as a small cartilaginous mass within a discrete area under the growth plate, or it can occupy the metaphysis under the entire width of the growth plate. Interestingly, dyschondroplasia within the proximal tibiotarsi appears to be bilateral, and the incidence and severity of the TD lesion is similar in both legs. The small lesions are likely to be subclinical, whereas the larger lesions have been shown to lead to significant bone deformities and lameness in broilers. Abnormal, nonuniform bone growth within the area of the lesion leads to increased tibial plateau angle and tibial bowing (Lynch et al., 1992), which can cause angular and rotational deformities within the bone. These deformities may, through altered biomechanical forces, lead to other bone deformities and gait problems.

The precise underlying cellular defect that occurs in TD remains unclear. There have been many hypotheses that include a defect in the metaphyseal vasculature such that invasion of the hypertrophic cartilage is impaired (Riddell, 1975), abnormal matrix structure leading to an inhibition of vascularization and mineralization (Poulos, 1978), and a failure in osteoclastic cartilage resorption (Lawler et al., 1988). It is now generally accepted, however, that TD is a consequence of an inability of the maturing chondrocytes to undergo terminal differentiation, which normally leads to vascularization and mineralization. This series of events would also explain the accumulation of prehypertrophic chondrocytes that are evident in histological sections of TD lesions. This characteristic of TD distinguishes it from the thickened growth plates noted in hypocalcemic rickets, which is due to an accumulation of randomly orientated proliferating chondrocytes, and also from hypophosphatemic rickets, in which hypertrophic chondrocytes accumulate.

The regulation and coordination of the mechanisms involved in the transition from a proliferative to a terminally differentiated phenotype is central to the development of TD, but, to date, this process is not fully understood. In this article, we describe a series of cellular- and molecular-based studies to obtain a better understanding of the etiology of TD.

GROWTH PLATE MATRIX

Components

Collagen type II is the principal structural protein and predominant collagen of the growth plate matrix. It interacts with collagen types IX and XI to form heterotypic fibrils that are distributed throughout the cartilage matrix. During chondrocyte maturation, collagen type II expression decreases, and the hypertrophic chondrocytes initiate the synthesis of collagen type X. This collagen type is a nonfibrillar, short-chain collagen, and although it is known to associate with collagen type II fibrils, its exact function is unknown. Together with the various collagen types there are a number of proteoglycans, of which aggrecan predominates, as well as noncollagenous proteins such as osteopontin and osteonectin within the growth plate matrix. As chondrocytes differentiate and hypertrophy, concomitant changes also occur within the extracellular matrix, and evidence suggests that chondrocytes must provide the correct extracellular network and establish cell-matrix interactions to allow progressive differentiation. Although the structure-function relationship is not fully understood it is possible that abnormal matrix synthesis and interactions may contribute to the development of TD.

Collagen type X, due to its synthesis by exclusively hypertrophic chondrocytes in the growth plate, has been extensively studied. Immunohistochemistry has indicated sparse matrix staining within the TD lesion and, where present, was restricted to the proximal part of the lesion and associated, in some cases, with sites of vascular invasion (Farquharson et al., 1995). Collagen type X intracellular staining was also noted within TD chondrocytes but only in those without positive extracellular matrix staining, suggesting a defect in its secretion or incorporation into the matrix (Farquharson et al., 1995; Tselepis et al., 1996; Reginato et al., 1998). Increased collagen type X within the TD lesion has been reported (Wardale and Duance, 1996; Reginato et al., 1998); however, this result is misleading and is likely to be distorted due the large proportion of proliferating chondrocytes present in normal tissue. A more meaningful comparison was done by Bashey et al. (1989) who reported collagen type X production within the TD lesion to be less than 50% of the amount found in pure hypertrophic cartilage.

Collagen type X mRNA, although not present in the main body of the lesion, has been noted in the cells proximal and distal to the lesion (Chen et al., 1993; Knopov et al., 1997; Pines et al., 1998). The presence of mRNA within the proximal lesion bordering the junction with the normal growth plate is similar to the pattern of distribution achieved by collagen type X immunohistochemistry (Farquharson et al., 1995). This relationship strongly suggests that prior to chondrocyte developmental arrest and formation of the lesion, the chondrocytes had initiated differentiation, at least in part, to the hypertrophic stage. Less controversy exists about the distribution of collagen type II, which is present throughout the normal and TD growth plate, with the matrix of the TD lesion staining strongly (Chen et al., 1993; Farquharson et al., 1995; Tselepis et al., 1996). Collagen type II mRNA has, however, been reported to be present (Tselepis et al., 1996; Knopov et al., 1997) or absent (Chen et al., 1993) within the lesion.

Immunostaining of noncollagenous proteins, osteopontin and osteonectin, in TD tissue indicates their presence in the matrix distal to the lesion but their absence within the lesion itself (Wu et al., 1996; Pines et al. 1998). This distribution is similar to that of collagen types II and X, and the presence of matrix gene expression distal to the lesion suggests a process of repair and the resumption of normal development.

In general, dyschondroplastic cartilage has a similar concentration of proteoglycans to the normal growth plate (Lowther et al., 1974; Freedman et al. 1985). Aggrecan, the large aggregating proteoglycan of growth plate cartilage, has been detected by using antibodies to potential glycosaminoglycan epitopes of aggrecan, namely chondroitin 4- and 6-sulfate and keratan sulfate (KS). Staining for C4 and chondroitin 6-sulfate within normal and TD tissue was of similar intensity, and distribution and was localized to the matrix throughout the growth plate (Farquharson et al., 1994). Little KS staining was observed in the hypertrophic zone of the normal growth plate, whereas in the dyschondroplastic lesion intense matrix staining was present (Farquharson et al., 1994). This observation is consistent with a change from aggrecan to decorin and biglycan synthesis during normal chondrocyte maturation (Bianco et al., 1990) and the lack of attainment of full chondrocyte hypertrophy in TD. In a similar study, little detectable staining for chondroitin 4-sulfate, chondroitin 6-sulfate, and KS was noted throughout the lesion, and staining, where found, was limited to an intracellular location (Tselepis et al., 1996). These authors also showed by in situ hybridization that aggrecan gene expression in the TD lesion was less than in normal hypertrophic chondrocytes. Biglycan distribution and expression was also found to be abnormal within the lesion, whereas decorin, which was less abundant than aggrecan or biglycan, was normally distributed within the TD growth plate. Altered proteoglycan distribution in TD and in birds with a high genetic predisposition to TD, but without the presence of a lesion (Ling et al., 1996), may have implications in the mineralization process. A decrease in proteoglycan concentration of the maturing chondrocyte matrix is a prerequisite for the growth of hydroxyapatite crystals.

Structure and Integrity

Collagen undergoes a number of posttranslational modifications, one of which is the action of lysyl oxidase to initiate the formation of the mature nonreducible intermolecular crosslinks (pyridinoline and deoxypyridinoline) that confer strength and stability to the growth plate cartilage. The integrity of the collagen network influences biomechanical properties of the growth plate cartilage and may alter its resistance to osteoclastic resorption. Collagen crosslinking in dyschondroplasia has recently been investigated, and although a decrease has been reported (Chen et al., 1993), the consensus of quantitative studies (Orth et al., 1991, 1993, 1994; Farquharson et al., 1996, Wardale and Duance, 1996) indicates increased pyridinium crosslinks within the lesion. In normal and TD tissue, the pyridinoline concentration was found to be 10-fold higher in the proliferating zone than in the mature, collagen type X-containing tissue (Farquharson et al., 1996). This increase in concentration may be an essential adaptation (via increased collagenase activity and collagen turnover) of the matrix for vascular invasion and cartilage resorption to occur. Deoxpyridinoline was only observed in the more differentiated zones of the growth plate, and a progressive increase in the concentration of both crosslinks was noted from the proximal to the distal parts of the lesion (Orth et al., 1994; Farquharson et al., 1996) (Figure 3). Normal amounts of pyridinium crosslinks above the lesion (Farquharson et al., 1996) suggest that the high concentrations noted in the distal lesion may reflect a tissue that is failing to turn over due to reduced matrix metalloproteinase activity (Rath et al., 1997). This failure may exacerbate the condition but is unlikely to be the primary cause of TD.

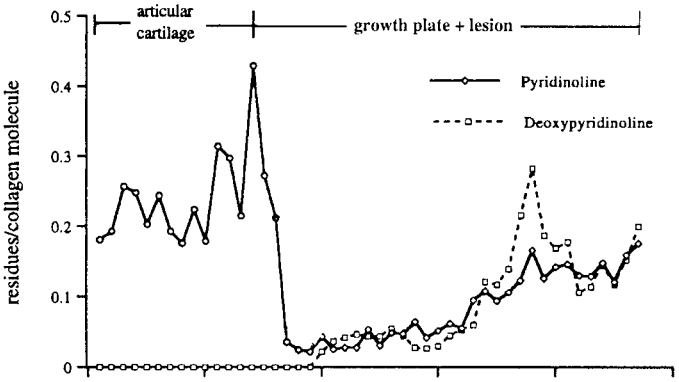

FIGURE 3. Distribution of pyridinium collagen crosslinks within the dyschondroplastic growth plate. Pyridinoline concentrations are highest in the proliferating zone and decrease with increasing chondrocyte differentiation. Deoxypyridinoline is first detected in the prehypertrophic zone and higher concentrations of both collagen crosslinks are found in the distal lesion. Figure adapted from original (Farquharson et al., 1996).

Programed Cell Death

The fate of the terminally differentiated hypertrophic chondrocytes is unclear. It is accepted however, that the differentiated chondrocyte must be removed to maintain the steady state thickness of the growth plate. It was long considered that the terminally differentiated chondrocyte died through necrosis, but this is now considered not to be the case. Authorities now contend that terminally differentiated chondrocytes either redifferentiate into bone cells (Cancedda et al., 1995), proliferate with one daughter chondrocyte dying and the other becoming an osteoblast (Roach et al., 1995), or undergo programed cell death (apoptosis) (Farnum and Wilsman, 1987; Gibson et al., 1995; Hatori et al., 1995). If chondrocyte removal were impaired, the retained tissue would interfere with the formation of endochondral bone. A number of recent studies have investigated chondrocyte apoptosis in TD in order to determine if disruption to this process can

explain the accumulation of cartilage in the metaphysis. Apoptosis in chondrocytes has been identified by a number of standard techniques including nucleosomal laddering, morphology, and DNA end-labeling procedures (Ohyama et al., 1997). Small TD lesions have been found to contain few, if any, apoptotic chondrocytes, whereas in severe lesions numerous apoptotic cells were present (Praul et al., 1997; Rath et al., 1998). Praul and colleagues (1997) suggested that these results imply that apoptosis was not a primary cause of TD but was secondary to the formation of the cartilaginous plug. Interestingly, in these studies (Praul et al., 1997; Rath et al., 1998), apoptosis was not observed in the hypertrophic chondrocytes of normal growth plates, although apoptotic hypertrophic cells have been reported in the chick (Hatori et al., 1995; Roach et al., 1995; Ohyama et al., 1997). Alternatively, because of the poor accessibility of cartilage to phagocytes, which are responsible for the removal of apoptotic cells, the inactive and avascular TD cartilage may persist and contribute to the pathology of the disorder (Rath et al., 1998). In contrast, a further study has shown that the TD lesion is associated with an impairment of apoptosis, which suggests that the accumulated prehypertrophic chondrocytes of the lesion have outlived their normal life span (Ohyama et al., 1997). This lack of apoptosis may be linked to the reduced expression of c-myc noted in the TD growth plate (Loveridge et al., 1993) and, in part, be responsible for the retention of the chondrocytes and extracellular matrix observed in TD. 1,25-dihydroxyvitamin D_3 (1,25-D) is known to promote apoptosis in many cell types, and it would therefore be of interest to determine if the prevention of TD by dietary 1,25-D involves the chondrocyte apoptotic pathway.

CHONDROCYTE ACTIVITIES

Proliferation

Evidence that the TD lesion emanates from the growth plate and is due to the accumulation of growth plate chondrocytes has been shown clearly in tritiated thymidine experiments (Gay and Leach, 1985). Four days after radioisotope injection, labeled cells that originated within the proliferating zone were found deep in the lesion. In similar and extended studies (Farquharson et al., 1992a), marked differences in chondrocyte proliferation were not found between normal growth plates and those with moderate lesions, although in severe lesions, proliferation was slightly reduced (Figure 4a). These results rule out an increased rate of proliferation as a possible mechanism for the accumulation of cartilage observed in TD. In a further study of chondrocyte proliferation, staining for proliferating cell nuclear antigen (PCNA) has been detected within the lesion (Pines et al., 1998). This result is in contrast to other results in which dividing cells were not found within the lesion (Gay and Leach, 1985; Farquharson et al., 1992a), but the authors (Pines et al., 1998) suggest that the presence of proliferating chondrocytes within the lesion is a primary event in the etiology of TD.

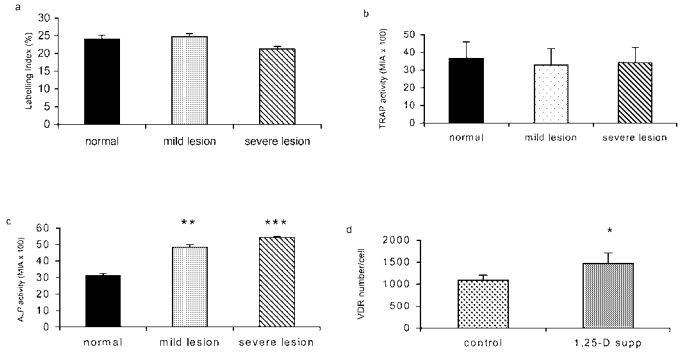

FIGURE 4. Quantification of chondrocyte labeling index (a), osteoclastic tartrate-resistant acid phosphatase (TRAP) activity (b), and chondrocyte alkaline phosphatase (ALP) activity (c) in normal birds and those with mild or severe tibial dyschondroplasia (TD) lesions. The labeling index and TRAP activity were unaffected in dyschondroplasia, whereas ALP activity in the prehypertrophic chondrocytes increased with lesion severity. Chondrocyte vitamin D receptor (VDR) number was increased in birds fed a diet supplemented with 1,25-dihydroxyvitamin D (d). Significantly different from normal birds at $^{*}P < 0.05$; $^{**}P < 0.01$; or $^{***}P < 0.001$. Figure adapted from originals (Farquharson et al., 1993; Berry et al., 1996).

This presence of proliferating chondrocytes could explain the lack of markers of chondrocyte hypertrophy such as ALP activity, collagen type X, and osteopontin. However, PCNA may also be expressed in association with DNA repair and also, possibly due to its long half-life, in nondividing cells as evidenced by PCNA staining in hypertrophic chondrocytes of normal rabbit growth plates (Aizawa et al., 1997). This lack of specificity suggests that PCNA cannot be regarded as a definitive marker for proliferating cells (Yu and Filipe, 1993).

Enzyme Activities

Glucose 6-phosphate dehydrogenase activity has been shown to be closely correlated to chondrocyte proliferation within the epiphyseal growth plate (Farquharson and Loveridge, 1990), presumably through its role in ribose sugar synthesis via the pentose phosphate pathway. Enzyme activity was not found to be raised in proliferating chondrocytes of severely affected chicks (Farquharson et al., 1992a). This lack of activity is in agreement with cell proliferation studies (Gay and Leach, 1985; Farquharson et al., 1992a) and enzyme studies of Zhang et al. (1997), who noted no elevation in triose-phosphate isomerase activity, an enzyme implicated in ribose sugar synthesis. These studies further support the hypothesis that TD is not due to excessive chondrocyte proliferation.

Walser et al. (1982) have proposed that a possible cause of TD is a failure of growth plate cartilage resorption. Increased osteoclastic resorption can occur either through higher individual osteoclastic activity or an increase in their number. Tartrate-resistant acid phosphatase (TRAP) activity has been used as a marker of osteoclastic activity and cartilage resorption (Lawler et al., 1988; Farquharson et al., 1992a). Individual osteoclastic TRAP activity was not correlated with lesion severity in 3-wk-old chicks fed on a diet with calcium and phosphorus imbalance (Far-

quharson et al., 1992a) (Figure 4b). This result is in broad agreement with the semiquantitative study of Lawler et al. (1988), which found inconsistent reductions in TRAP activity in osteoclasts of *Fusarium equesti*-induced TD. Osteoclast numbers under the lesion were found to be decreased in bones with severe lesions (Walser et al., 1982; Farquharson et al., 1992a), but this result was only found to be significant in the study of Walser and colleagues (1982).

Alkaline phosphatase activity has long been recognized as a marker of chondrocyte differentiation and may be involved in cartilage mineralization (Anderson, 1985). Decreased ALP activity in the chondrocytes of the lesion (Gay et al., 1985; Farquharson et al., 1992a,) is possibly secondary to chondrocyte necrosis that is known to occur (Gay et al., 1985; Hargest et al., 1985a). Enzyme activity at the distal edge of the lesion may represent the presence of a repair mechanism (as observed with the matrix proteins) and the initiation of mineralization and is in accord with ultrastructural studies and elemental analysis in which mineralization has been observed in this area of the lesion (Hargest et al., 1985a,b). Quantification of ALP activity by in situ microdensitometry has indicated that enzyme activity of the prehypertrophic chondrocytes situated proximal to the lesion is higher with increasing severity of the lesion (Farquharson et al., 1992a; Loveridge and Farquharson 1993) (Figure 4c). Given the role of ALP activity in mineralization, it might have been predicted that ALP activity would have been decreased throughout the TD growth plate. The activity of ALP may be related to the process of differentiation of the chondrocyte phenotype, which, in turn, is regulated by a variety of autocrine and paracrine growth factors. Taken together, these and other results (Gay et al., 1985) suggest that the impaired mineralization noted in TD is not a consequence of a lack of ALP activity.

Other enzyme activities, including lactate dehydrogenase (Farquharson et al., 1992a), carbonic anhydrase (Gay et al., 1985), esterase, malate dehydrogenase, and peroxidase (Zhang et al., 1997), have all been studied in TD. Activity of lactate dehydrogenase and carbonic anhydrase within the prehypertrophic chondrocytes of normal and TD growth plates were similar, but activity within the lesion was lower and greatly diminished in the chondrocytes of the distal lesion. This lack of activity within the lesion is probably due to cell death and necrosis and suggests that these two enzymes are neither limiting nor primary to the etiology of TD.

REGULATORS OF CHONDROCYTE DIFFERENTIATION

Growth Factors

Peptide growth factors are known to be central to chondrocyte differentiation, and recent studies have indicated that the pathogenesis of dyschondroplasia may be linked to abnormal expression of a number of these autocrine/paracrine factors. Transforming growth factor-β (TGF-β) regulates chondrocyte differentiation, and TGF-β3 has been localized to the prehypertrophic and hypertrophic chondrocytes of the chick growth plate (Thorp et al., 1995). In TD there was a marked decrease in TGF-β3 within the prehypertrophic chondrocytes above the lesion (Loveridge et al., 1993; Thorp et al., 1995). In areas of repair at the periphery of the TD lesion, chondrocytes adjacent to the invading metaphyseal vessels stain positively for TGF-β3, supporting the concept that TGF-β is important for the induction of angiogenesis. Further studies have indicated that the reduction in immunoreactive TGF-β3 in TD growth plates is a consequence of reduced steady-state levels of TGF-β3 mRNA (Law et al., 1996). In a separate study, cytoplasmic and extracellular staining with antibodies to TGF-β1 and -β2 isoforms was normal in the cells of the lesion and in those proximal to it (Ren et al., 1997). This study implies that changes in TGF-β expression in TD may be limited to the β3 isoform. Other major regulators of endochondral ossification, basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), epidermal growth factor, and transforming growth factor-α (TGF-α), are found predominantly within the more mature chondrocytes of the growth plate. In TD, the intensity of immunocytochemical staining for bFGF, IGF-I, epidermal growth factor, and tumor necrosis factor-α was greatly reduced (Thorp et al., 1995; Twal et al., 1996; Ren et al., 1997) as was the number of positively IGF and FGF stained chondrocytes (Thorp et al., 1995; Twal et al., 1996). Increased staining of growth factors was noted in areas of repair at the periphery of the lesion, and overall the changes in the staining pattern noted in TD appeared very similar to those noted with TGF-β (Loveridge et al., 1993).

Parathyroid hormone-related peptide (PTHrP) acts as a negative regulator of growth plate chondrocyte differentiation, thereby mediating the effects of Indian hedgehog (Ihh) on bone growth (Vortkamp et al., 1996). PTHrP and its receptor (PTHR) have been found, by in situ hybridization studies, to be expressed at discrete locations in the growth plate. Recent studies on the TD growth plate have indicated that normal expression of the PTHR gene was observed in the lower proliferating chondrocytes situated proximal to the lesion. No expression was observed within the lesion. Interestingly, PTHR expression was completely absent within rachitic growth plates of vitamin D-deficient chicks. This observation was possibly due to elevated parathyroid hormone levels and suggests that parathyroid hormone is not involved in the etiology of TD (Bassat et al., 1999).

Vitamin D Metabolites

Vitamin D metabolites play a major role in chondrocyte metabolism and appear to be essential for normal bone formation. The actions of this hormone are mediated through its interaction with its nuclear receptor (VDR) and subsequent regulation of gene expression. Receptors for 1,25-D have been located on growth plate chondrocytes, suggesting a direct role for this metabolite in chon-

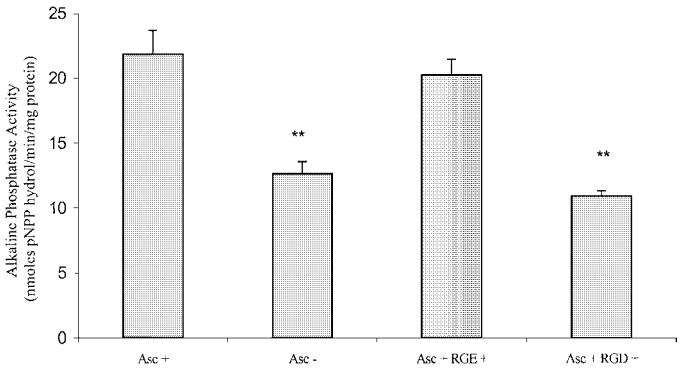

FIGURE 5. In vitro effect of RGD-containing peptides or control peptides (RGE) on chondrocyte alkaline phosphatase (ALP) activity. Addition of RGD peptides inhibited enzyme activity. Significantly different from the +AA treatment at $^{**}P < 0.01$; $^{***}P < 0.001$. Figure adapted from original (Farquharson et al., 1998).

drocyte metabolism (Berry et al., 1996). Although results from in vitro chondrocyte culture experiments have been inconsistent, a stimulation of chondrocyte differentiation by 1,25-D has been shown in vivo (Farquharson et al., 1993). This capacity for 1,25-D to promote chondrocyte differentiation may be responsible for its ability to normalize growth plate development and to prevent TD when given as a dietary supplement (Rennie et al., 1993). If bone growth normalization is indeed the case, it suggests that the rate of endogenous synthesis of 1,25-D from its precursor 25-D in the kidney may be deficient in the fast-growing young broiler. Strains with a greater predisposition toward TD have lower concentrations of plasma 1,25-D (Parkinson et al., 1996), but the circulating concentrations of 1,25-D in TD chicks are normal (Rennie et al., 1993). Although a change in the local concentration of the metabolite is possible, the fact that chondrocytes within the lesion have a reduced number and affinity of VDR (Berry et al., 1996) indicates that their ability to respond to 1,25-D may be reduced, and higher concentrations of 1,25-D may be required to achieve normal effects. The finding that dietary 1,25-D increases chondrocyte VDR number lends support to the hypothesis that 1,25-D may prevent TD by an effect on VDR synthesis (Figure 4d).

Ascorbic Acid

In vitro studies have shown consistently that ascorbic acid promotes ALP activity in cultured chondrocytes. The promotion of differentiation by ascorbic acid may be related to its requirement as a cofactor for prolyl and lysyl hydroxylase in the synthesis and secretion of stable triple-helical collagen. The formation of a collagen-rich matrix promotes cell-matrix interactions, via integrins, and provides a permissive environment for subsequent differentiation and gene expression (Habuchi et al., 1985). In chondrocyte culture experiments, addition of exogenous peptides containing the integrin recognition motif RGD has been shown to inhibit ascorbic acid-induced chondrocyte ALP activity (Figure 5). These peptides effectively block the cell-matrix interaction and lead to impairment of normal cell-matrix interactions (Farquharson et al., 1998). In addition, chicks supplemented with dietary ascorbic acid (1 g/kg) had higher concentrations of collagen and proteoglycan within their growth plates (Farquharson et al., 1998). Ascorbic acid may also enhance other hydroxylation reactions, including a stimulatory effect of the 1α-hydroxlase enzyme that converts 25-D to 1,25-D. It is possible that ascorbic acid exerts a synergistic action, with 1,25-D, on TD (Whitehead, 1997), by modulating the composition of the growth plate matrix, which complements the chondrocyte-differentiating actions (Farquharson et al., 1993) of dietary- or ascorbic acid-induced 1,25-D synthesis.

C-Myc Protein

Strong evidence exists for the involvement of the c-myc proto-oncogene in the regulation of cell proliferation. However, several groups have reported that the regulation of c-myc expression and DNA synthesis are uncoupled and that the differentiation of some cell lines is not accompanied by a simple decrease in c-myc expression. Furthermore, elevated levels of c-myc protein have been noted in a number of differentiating cells in vivo, such as colon crypt cells (Stewart et al., 1986), lens epithelia (Nath et al., 1987), and, recently, growth plate chondrocytes (Farquharson et al., 1992b). Differentiating chick chondrocytes contain significantly more c-myc protein than proliferating chondrocytes, suggesting a role in the differentiation process. In TD, the c-myc content of the prehypertrophic chondrocytes is significantly reduced in comparison to similar cells within normal growth plates. In contrast, there is no significant difference in the c-myc protein content of proliferative chondrocytes of normal and TD birds (Loveridge et al., 1993). This finding further suggests that the initial steps in chondrocyte differentiation are characterized by increased c-myc protein, and a possible link between c-myc, TGF-β, and 1,25-D in the chondrocyte differentiation process has been proposed (Loveridge et al., 1992). 1,25-D is known to regulate chondrocyte differentiation and modulates TGF-β and c-myc, whereas c-myc alters the expression of the VDR. This relationship suggests that c-myc and TGFβ are essential components of the signal cascade regulated by 1,25-D in growth plate chondrocytes. The lack of mineralization in TD may also be related to lower levels of c-myc protein. Mineralization of the growth plate matrix is mediated by the actions of matrix vesicles (Anderson, 1995) that are formed by a number of processes, which may include apoptosis (Akiska and Gay, 1985). C-myc protein is a potent inducer of apoptosis, and a decrease in c-myc protein concentration may lead to decreased matrix vesicle synthesis and a failure of mineralization. Supporting evidence for this hypothesis has recently been provided by Nie et al. (1995) who demonstrated that in TD there was a defect in the formation of matrix vesicles.

IN VITRO CELL CULTURE STUDIES

Cell culture has been widely used to determine a variety of chondrocyte characteristics, but few studies have been published in which the metabolic activities of the dyschondroplastic chondrocyte were investigated. In one early study, chondrocytes isolated from large TD lesions were found to have abnormal morphology and reduced mitogenic activity and proteoglycan synthesis (Rosselot et al., 1994). This diminished metabolic activity may reflect the situation found in explant cultures of TD tissue (Freedman et al., 1985; Lilburn and Leach, 1980) or simply be a consequence of reduced cell viability within the lesion (Hargest et al., 1985a). In addition, type X collagen synthesis by TD chondrocytes was reduced, but mRNA levels were substantially higher than in normal chondrocytes (Wardale and Duance, 1996; Reginato et al., 1998). This discrepancy may be a result of a block in translation or posttranslational modification of the protein or, alternatively, may reflect a defect in the secretion or incorporation of this collagen type into the extracellular matrix. Collagen type X and TGF-β production, high levels of ALP activity, and matrix mineralization were all reported in lesion chondrocytes grown in a high cell density culture system (Farquharson et al., 1995). Although the amount of collagen type X synthesized may be impaired, these qualitative results indicate that, in culture, lesion chondrocytes have the ability to terminally differentiate and mineralize (Farquharson et al., 1995). These results suggest that the primary abnormality in TD is related to a developmental fault that is only operative in vivo.

IDENTIFICATION OF CANDIDATE GENES

Dyschondroplasia has a strong genetic component that has led to the generation of experimental lines of broilers selected for a high and low incidence of TD (Sheridan et al., 1978; Thorp et al., 1993). These lines serve as valuable models with which to identify genes involved in the development of dyschondroplasia and to map candidate genes, once their role in initiating the disorder has been established.

The characterization of candidate genes for TD has been concentrated on genes known to be associated with chondrocyte differentiation and hypertrophy. A number of these are markers of hypertrophy, which are downregulated in the TD lesion, such as collagen type X (Farquharson et al., 1995; Tselepis et al., 1996), the proteoglycan aggrecan (Farquharson et al., 1994; Tselepis et al., 1996), and osteopontin and osteonectin (Wu et al., 1996; Pines et al., 1998). However, we have recently identified two genes expressed in chondrocytes by differential display (Jefferies et al., 1998a), Ex-FABP and B-cadherin, that are upregulated in the hypertrophic zone of the normal growth plate but also show elevated expression in the TD lesion (Jefferies et al., 2000). The reason for this upregulation is unclear, but it could indicate that the TD phenotype is more complex than previously thought.

TABLE 1. Candidate genes for tibial dyschondroplasia screened for expression levels in Percoll-fractionated chondrocytes

1.	Collagen types II and X
2.	Transforming growth factor β 1, β 2, and β 3
3.	Bone morphogenic protein (BMP) 2, 4, 5, 6, 7
4.	BMP receptor kinase 1, 2 and 3
5.	Vitamin D receptor
6.	Parathyroid hormone-related peptide (PTHrP)
7.	PTH-PTHrP receptor
8.	Ex-FABP
9.	HT7
10.	B-cadherin

Although many studies have identified differential expression of genes or gene products in TD, which have given valuable insights into the nature of the condition, many have been associated with the gross abnormalities of the lesion. Because TD involves a failure of chondrocyte differentiation, it can be misleading to compare patterns of gene expression in cells isolated from TD lesion and nonlesion tissues directly. Some chondrocyte cell phenotypes will be absent from the lesion, whereas others will be altered. This lack of specificity will give rise to predominantly secondary changes associated with the pathology rather than the etiology of dyschondroplasia. In order to circumvent this problem, we have made use of a strategy that utilizes Percoll density gradient centrifugation to separate normal growth plates into cell fractions of differing maturational development (Houston et al., 1999). This procedure allows the comparison of TD and non-TD cell fractions containing cells proximal to, and outwith, the lesion. These cells would normally progress to the fully hypertrophic phenotype in the normal growth plate but, in TD, fail to hypertrophy and give rise to the lesion. Any misexpression of a gene would strongly implicate that gene in the etiology of dyschondroplasia. By using this approach, we have recently screened a number of candidate genes for TD, using semiquantitative reverse transcriptase-polymerase chain reaction (Table 1) (Jefferies et al., 1998b). None of these genes showed any difference in levels of expression between TD and normal growth plate cell fractions, which may suggest that at least quantitatively, at the mRNA level, none of these genes appear to be implicated in initiating dyschondroplasia. These results cast doubt on the involvement of genes such as TGF-β3 (Thorp et al., 1995) and the VDR (Berry et al., 1996) and highlight the importance of studying gene expression in cells proximal to the lesion. It is in these cells that the peturbation of differentiation that gives rise to TD must first occur.

CONCLUSIONS AND FUTURE DIRECTIONS

Despite extensive research, the etiology of dyschondroplasia remains unclear. This lack of clarity is in part due to a number of apparently disparate factors causing TD, possibly by several distinct mechanisms, but each resulting in the occurrence of a histologically similar lesion. However, the observation that this disorder develops in the maturing prehypertrophic chondrocytes, which

are then unable to differentiate into fully hypertrophic chondrocytes, may be fundamental to its progression. Such chondrocytes would fail to elicit the biochemical changes associated with hypertrophy that are a prerequisite for cartilage vascularization, mineralization, and resorption by osteoclasts. Many morphological, biochemical, and molecular changes occurring in TD have been reported, but interpretation of these findings is often difficult because it is unknown whether any observed change inhibits the differentiation process or is a result of, and secondary to, the impaired differentiation. If the primary cellular developmental fault is to be identified, it is imperative that significance should be attached to changes that occur in the prehypertrophic cells proximal to the lesion and not within the lesion itself. It is the prehypertrophic chondrocytes that will eventually form the lesion and that are more likely to be free of secondary pathologies.

The identification of the genes involved in the control of growth plate chondrocyte development, together with a knowledge of how they influence signaling processes, matrix synthesis, and cellular events such as hypertrophy and cell death, are essential for a more complete understanding of endochondral bone growth. This information is now being obtained by various approaches, and careful interpretation of the accumulating data will enable us to obtain a more comprehensive understanding of chondrocyte development within the growth plate. Such fundamental information will be critical to our understanding of the primary developmental fault that occurs in dyschondroplasia. Identification of the genes involved in the perturbation of chondrocyte differentiation in TD may open the way to elimination of the disorder by genetic selection.

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