Original Article

Chondrocytes of the tibial dyschondroplastic lesion are apoptotic

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ABSTRACT Tibial dyschondroplasia (TD) is a disease characterized by the formation of an avascular, non-mineralized lesion along the mature face of the epiphyseal growth plate in rapidly growing chickens. In the normal growth plate, cells progress from a proliferative phase to hypertrophy where the tissue is vascularized and replaced by trabecular bone. In TD, cells apparently cease their development early in the transition to hypertrophy. These diseased cells are not removed by vascularization nor does mineralization occur. The resulting lesion increases in size as proliferative cells continue to divide in the absence of removal and replacement of cartilage by bone. This laboratory has previously reported that cells of the TD lesion have the morphological appearance of necrotic cells or in some cases apoptotic cells. In this study we examine in more detail the status of cells comprising the TD lesion using molecular techniques. Genomic DNA isolated from cells of severe TD lesions show the nucleosomal laddering indicative of apoptosis, while DNA isolated from proliferative and hypertrophic cells does not. This result was confirmed by the use of the Cell Death Detection ELISA™ which shows quantitatively that cells from severe TD lesions contain nearly twice as many nucleosomal fragments as cells from the hypertrophic zone while proliferative chondrocytes do not have significant fragmentation. *In situ* examination of the epiphyseal growth plate with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) clearly shows that the cells of the severe TD lesion are apoptotic. Cells from smaller lesions are stained to a lesser extent or not at all by TUNEL. We believe that the apoptosis seen in TD is a secondary effect of the disease and not its primary cause.

KEY WORDS: *apoptosis, tibial dyschondroplasia, skeletal growth, epiphyseal growth plate, cartilage*

Introduction

Tibial Dyschondroplasia (TD) is a cartilage abnormality which has a particularly high incidence among young rapidly growing chickens. This disease is similar to Jansen's metaphyseal dysplasia in humans (Juppner, 1996) and osteochondrosis in other mammals (Glade and Belling, 1984; Hill *et al.*, 1984; Corbellini *et al.*, 1991). First described by Leach and Nesheim (1965), TD is characterized by the presence of an avascular non-mineralized lesion in the proximal tibiotarsus and tarsometatarsus.

In the normal growth plate, chondrocytes progress from a rapidly dividing proliferative phase to a non-dividing hypertrophic phase. This progression from the proliferative phase to hypertrophy usually occurs in 24-48 hours (Gay and Leach, 1985). Recent studies have shown that parathyroid hormone related peptide (PTHrP) and Indian hedgehog (Ihh) are key signals in controlling the progression of chondrocytes from proliferation to hypertrophy (Amizuka *et al.*, 1996; Lanske *et al.*, 1996; Lee *et al.*, 1996; Vortkamp *et al.*, 1996). Highlighting the importance of this negative feedback loop is the discovery that Jansen's metaphyseal dysplasia is caused by a mutation in the PTH/PTHrP receptor which results in the constitutive activation of this receptor (Schipani *et al.*, 1995). Once chondrocytes have progressed from the proliferative phase to hypertrophy the terminal hypertrophic chondrocytes at the chondro-osseous junction undergo apoptosis, are calcified, and are replaced by bone (Farnum and Wilsman, 1987, 1989; Gibson *et al.*, 1995; Hatori *et al.*, 1995). In TD the orderly development of the chondrocytes is disrupted. ^{3}H-thymidine incorporation and electron microscopy have revealed that chondrocytes in afflicted growth plates seem to become arrested in their development early in hypertrophy (Gay and Leach, 1985; Hargest *et al.*, 1985). The proliferative chondrocytes continue to divide and the cells begin to undergo hypertrophy, but the tissue is not calcified nor is it replaced. This leads to a progressive increase in the size of the lesion which can persist for several weeks. Cells of the lesion show morphological signs of necrosis and in some cases

Abbreviations used in this paper: TD, tibial dyschondroplasia; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; PTH, parathyroid hormone; PTHrP, parathyroid hormone related peptide; Ihh, Indian hedgehog.

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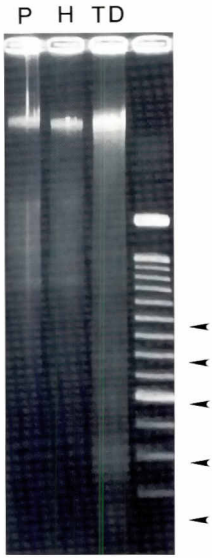

Fig. 1. Agarose gel electrophoresis of DNA isolated from growth plate chondrocytes. *Genomic DNA isolated from chondrocytes comprising severe TD lesions shows the nucleosomal laddering (indicated by arrows) characteristic of apoptosis while genomic DNA from proliferative (P) and hypertrophic (H) chondrocytes does not. M, 100bp DNA ladder.*

apoptosis (Hargest *et al.*, 1985). Biochemical analysis of cells comprising the TD lesion reveal that they contain less protein, DNA, and RNA than chondrocytes of the normal growth plate (Freedman *et al.*, 1985). The metabolic activity of chondrocytes from severe lesions, as measured by ^{3}H-thymidine and $^{35}SO_4$ incorporation, is greatly reduced when compared to cells of the normal growth plate (Rosselot *et al.*, 1994).

This laboratory and others have demonstrated that a plethora of gene products show diminished expression in the TD lesion. These proteins include osteonectin, bFGF, alkaline phosphatase, carbonic anhydrase, osteopontin, aggrecan, and biglycan (Gay *et al.*, 1985; Knopov *et al.*, 1995; Tselepis *et al.*, 1996; Twal *et al.*, 1996; Wu *et al.*, 1996). *In situ* hybridization analysis also indicates that collagen II, collagen X, and osteopontin expression is diminished in TD lesions (Chen *et al.*, 1993; Knopov *et al.*, 1995).

Programmed cell death is now recognized as an important part of development which counter balances cell proliferation. Apoptosis, the term used to describe this process (Wyllie *et al.*, 1980), is characterized morphologically by a reduction in cell volume, and dense condensation of chromatin followed by the disintegration of the cells into small vesicle-like (apoptotic) bodies. These apoptotic bodies are normally phagocytosed rapidly by surrounding cells without inducing inflammation. This process is distinguished from necrosis in which the cytoplasm swells, the membrane ruptures, and the release of cellular contents often induces the inflammatory response. On a molecular level apoptotic cells are characterized by the degradation of their DNA into nucleosomal or oligonucleosomal fragments (Wyllie, 1980). These fragments are absent in necrotic cells.

In the present study we have used a variety of molecular techniques to determine whether the chondrocytes comprising the TD lesion are apoptotic. We believe that our observation of widespread apoptosis in TD lesions explains the altered metabolism of these cells and leads us to suggest new, more fruitful avenues of research into this disease.

Results

Agarose gel electrophoresis of genomic DNA prepared from proliferative chondrocytes, hypertrophic chondrocytes, and chondrocytes isolated from TD lesions is shown in Figure 1. Nucleosomal laddering is present in DNA prepared from cells of the TD lesion but is not evident in proliferative or hypertrophic chondrocytes.

To obtain a more quantitative comparison of the amount of nucleosomal fragmentation in TD cells versus proliferative and hypertrophic cells, a commercial apoptosis detection ELISA was used. The Cell Death Detection ELISA™ utilizes a sandwich ELISA of anti-histone antibodies and peroxidase conjugated anti-DNA antibodies to quantitate nucleosomal fragmentation and thus the extent of apoptotic death in a cell population. The results in Figure 2 show that nucleosomal fragmentation in chondrocytes isolated from TD lesions is significantly greater than in hypertrophic chondrocytes, while the level of fragmentation in proliferative chondrocytes was not statistically significant from controls.

The normal growth plate was not labeled with TUNEL under our conditions (Fig. 3). Proliferative and prehypertrophic chondrocytes adjacent to TD lesions showed the same low level of staining seen in normal growth plates. Cells of the severe TD lesion are intensely labeled by TUNEL (Fig. 4). Small and medium TD lesions were examined and the results were variable. In some cases there was a progressive increase in the intensity of labeling from small lesions to severe lesions (Fig. 4C-E), while in other cases the small and

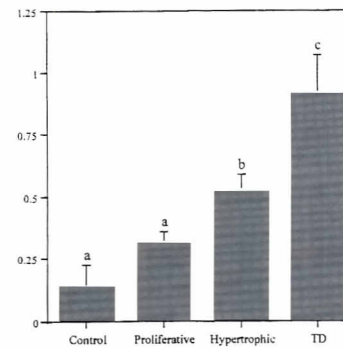

Fig. 2. Cell Death Detection ELISA of growth plate chondrocyte lysates. *The Cell Death Detection ELISA™ shows that chondrocytes isolated from severe TD lesions have significantly ($P<0.05$) more nucleosomal fragmentation than hypertrophic chondrocytes. Proliferative cells do not contain a statistically significant amount of nucleosomal fragmentation when compared to controls incubated without cell extract. Values plotted are mean±SEM.*

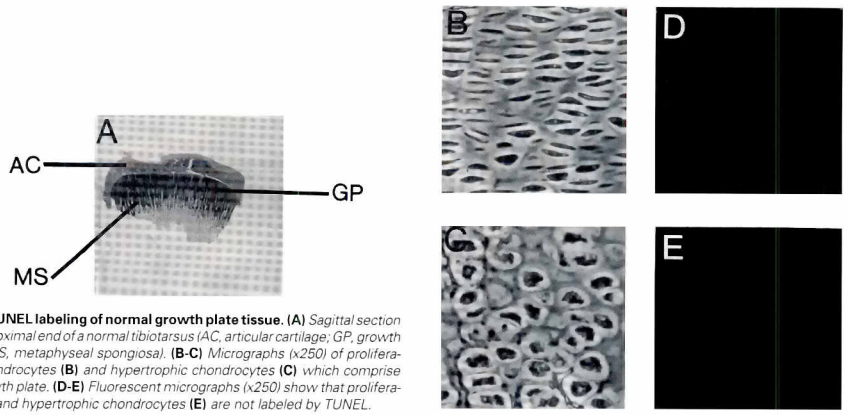

Fig. 3. TUNEL labeling of normal growth plate tissue. (A) *Sagittal section of the proximal end of a normal tibiotarsus (AC, articular cartilage; GP, growth plate; MS, metaphyseal spongiosa).* **(B-C)** *Micrographs (x250) of proliferative chondrocytes* **(B)** *and hypertrophic chondrocytes* **(C)** *which comprise the growth plate.* **(D-E)** *Fluorescent micrographs (x250) show that proliferative* **(D)** *and hypertrophic chondrocytes* **(E)** *are not labeled by TUNEL.*

medium lesions were unlabeled. Negative controls of severe TD lesions in which TdT was omitted from the labeling mixture were completely unlabeled (Fig. 4F).

Discussion

Farnum and Wilsman (1987, 1989) have observed that most hypertrophic cells of the porcine growth plate appear intact and viable. However, the terminal hypertrophic chondrocyte of the growth plate does have an apoptotic morphology immediately before metaphyseal vascular penetration and replacement by bone. Zenmyo *et al.* (1996) have reported that only the terminal hypertrophic chondrocytes of the porcine growth plate label with TUNEL and appear apoptotic when examined by electron microscopy. Gibson *et al.* (1995) have observed that in the chick sterna only those hypertrophic cells at the edge of the invading vasculature undergo apoptosis. These observations are at some variance with the observation of Hatori *et al.* (1995) who found that the vast majority of hypertrophic chondrocytes in the avian growth plate labeled with TUNEL. This result would appear to be misleading as their own cytometric analysis revealed that only 9% of hypertrophic chondrocytes and 2% of proliferative cells are apoptotic. The results of these four studies suggest that normally, hypertrophic chondrocytes undergo apoptosis immediately before vascular penetration while the majority of other hypertrophic chondrocytes not in close proximity to invading blood vessels are fully viable.

The study presented here has focused on examining chondrocytes of the tibial dyschondroplastic lesion. In this disease, cells appear to arrest early in hypertrophy and subsequently a non-mineralized, non-vascularized lesion forms. This lesion increases in size as proliferative cells continue to divide and begin to differentiate toward hypertrophy in the absence of chondrocyte replacement by bone. We observe extensive nucleosomal laddering, indicative of apoptosis, in DNA prepared from chondrocytes isolated from severe TD lesions while we do not observe nucleosomal laddering in DNA from hypertrophic or proliferative chondrocytes. The work of Farnum and Wilsman (1987, 1989), Gibson *et al.* (1995), Hatori *et al.* (1995) and Zenmyo *et al.* (1996) led us to expect nucleosomal laddering in DNA from hypertrophic cells; however our DNA preparations did not allow us to detect such low levels. Our observations of genomic DNA show that cells isolated from TD lesions have a much greater amount of nucleosomal laddering than hypertrophic chondrocytes.

To get a more quantitative assessment of the relative extent of apoptosis we measured nucleosomal fragmentation in normal proliferative and hypertrophic cells, as well as chondrocytes isolated from severe TD lesions using a commercially available ELISA. The results of these experiments show that TD cells contain nearly twice as many nucleosomal fragments as hypertrophic cells whereas proliferative chondrocytes did not contain statistically significant amounts. We believe that this is a conservative figure and may actually underestimate the fragmentation in TD cells. To assay equal numbers of each type of chondrocyte, cells were counted with a hemacytometer. It is possible that a significant percentage of what were counted as cells from severe TD lesions may in fact have been apoptotic bodies. If this were true then the DNA assayed from TD lesions would have come from fewer cells than was the case for proliferative and hypertrophic chondrocytes.

Severe TD lesions are labeled extensively by TUNEL, whereas little or no labeling of the normal growth plate was observed. This is in agreement with the reports of Rath *et al.* (1996) and Zenmyo *et al.* (1996). We believe the differences between our observations and those of Hatori *et al.* (1995), who observed extensive labeling of hypertrophic chondrocytes in the normal avian growth plate, are probably due to methodologies. The latter study does not explicitly indicate the optimized labeling conditions, so comparison to our study is impossible. All cells contain some levels of DNA damage and consequently can be labeled to some extent by TUNEL (Eastman and Barry, 1992). By altering either the treatment of

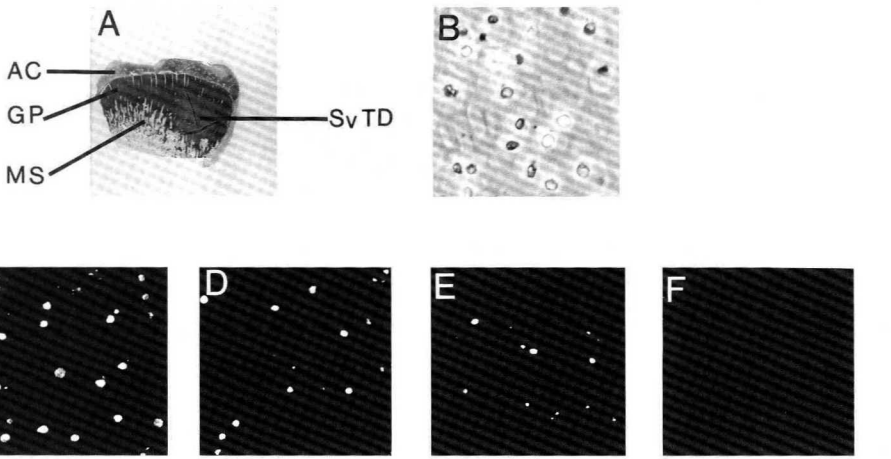

Fig. 4. TUNEL labeling of TD lesions of differing severity. (A) *Sagittal section of the proximal end of a tibiotarsus afflicted with TD (AC, articular cartilage; GP, growth plate; MS, metaphyseal spongiosa).* **(B)** *A micrograph (250x) of cells comprising the TD lesion.* **(C-E)** *Fluorescent micrographs show that severe, medium, and slight lesions (respectively) are progressively less labeled by TUNEL.* **(F)** *A severe TD lesion treated as in (C-E) but with terminal transferase omitted shows no labeling.*

tissue before labeling or the time of labeling, a varying intensity of staining may be obtained. Severe TD lesions clearly contain much more DNA fragmentation than any chondrocytes of the normal growth plate. Smaller lesions are labeled, but to a progressively lesser extent than severe lesions or not at all. Apoptosis is therefore most likely a secondary effect which manifests itself once the disease has caused the lesion to reach a critical size. The size of the lesions is thus an important consideration when studying TD. Some of the apparent discrepancies in the literature concerning TD may be the result of examining lesions of various sizes. The results of Farquharson *et al.* (1995), who obtained viable chondrocytes from TD lesions, is surprising in light of the observations presented here. These chondrocytes may have been isolated from less severe lesions that still had large numbers of viable cells. We have not been able to obtain a significant number of viable cells from a severe lesion.

A previous report from this laboratory focused on the proximal edge of the TD lesion and found that many of these chondrocytes appeared necrotic (Hargest *et al.*, 1985). The current study has focused on an examination of the center of severe lesions and shows by several methods that cells of the severe TD lesion are apoptotic. The amount and location of these apoptotic chondrocytes far exceeds what is observed in the normal growth plate. This observation is consistent with previous results showing that cells of the TD lesion when compared to cells of the normal growth plate have: 1) a decreased ^{3}H-thymidine and $^{35}SO_4$ incorporation (Rosselot *et al.*, 1994), 2) a decreased DNA and total protein content (Hargest, *et al.*, 1985), 3) decreased levels of alkaline phosphatase, carbonic anhydrase, bFGF, osteonectin, osteopontin, aggrecan, and biglycan (Gay *et al.*, 1985; Knopov *et al.*, 1995; Tselepis *et al.*, 1996; Twal *et al.*, 1996; Wu *et al.*, 1996), and 4) a reduction in the collagen II, collagen X, osteopontin, aggrecan, and biglycan mRNA levels (Chen *et al.*, 1993; Knopov *et al.*, 1995; Tselepis *et al.*, 1996). We propose that further study of gene expression in severe TD lesions is likely to be futile due to the apoptotic nature of these cells. More likely to be fruitful are the approaches of Farquharson *et al.* (1992), Loveridge *et al.* (1993) and Law *et al.* (1996) who have focused on the transcripts and proteins of the prehypertrophic zone, proximal to the lesion.

The recent studies on the control of chondrocyte hypertrophy by a PTHrP-Ihh negative feedback loop (Amizuka *et al.*, 1996; Lanske *et al.*, 1996; Lee *et al.*, 1996; Vortkamp *et al.*, 1996) and the discovery that Jansen's metaphyseal dysplasia is caused by a constitutively active PTHrP receptor (Schipani *et al.*, 1995) leads us to propose a hypothesis for the cause of TD. We suggest that TD is caused by a disruption of the PTH/PTHrP-Ihh negative feedback loop. Disruption in the level of PTH/PTHrP, Ihh, or their receptors could inhibit the normal progression of chondrocytes from proliferation to hypertrophy. These developmentally arrested chondrocytes would not produce proper levels of angiogenic signals, such as bFGF (Twal *et al.*, 1996), and thus the cartilage would not become vascularized. As the size of the lesion increases, chondrocytes in the center of the lesion would become increasingly cut off from factors normally present in the vascular supply which prevent premature apoptosis (Ishizaki *et al.*, 1994). This situation is analogous to experimentally induced ischaemia (Trueta and Amato, 1960; Riddell, 1975) and to metaphyseal injury in abused infants in which a lesion similar to TD has been

reported (Kleinman *et al.*, 1991). These apoptotic chondrocytes are not readily removed because many of the surrounding chondrocytes are also apoptotic, and the cells within the lesion are embedded in an extensive extracellular matrix which is resistant to vascularization. Future study in our laboratory will focus on the genes of the PTH/PTHrP-Ihh negative feedback loop and their expression in the prehypertrophic zone of avian growth plates afflicted with TD.

Materials and Methods

Source of tissue

Normal growth plate tissue and spontaneous (non-induced) TD tissue was obtained from commercial male chicks (Peterson x Arbor Acre, Metz Hatchery, Belleville, PA, USA) raised on a commercial broiler feed at the Pennsylvania State University Poultry Education and Research Center. At four weeks of age chickens were sacrificed by cervical dislocation and their tibiotarsi were removed.

Nucleosomal laddering

Chondrocytes were isolated from the proliferative and hypertrophic zones of normal growth plates as well as from TD lesions by the procedure of Rosselot *et al.* (1992). Genomic DNA from $1x10^7$ chondrocytes of each type was prepared by standard techniques (Ausubel *et al.*, 1989). Genomic DNA was electrophoresed on a 1.7% agarose gel, stained with ethidium bromide, and photographed.

Cell death detection ELISA™

A Cell Death Detection ELISA™ kit was obtained from Boehringer Mannheim (Indianapolis, IN, USA) and the manufacturers instructions were followed. Briefly, proliferative, hypertrophic, and TD chondrocytes were isolated as described above, counted with a hemacytometer and lysed. Following centrifugation the supernatant was incubated in microtiter plates previously coated with anti-histone antibodies. The bound nucleosomal fragments were then incubated with anti-DNA peroxidase linked antibody and ABTS (2,2'-azino-di[3-ethylbenzthiazoline sulfonate(6)]) substrate. Absorbance was measured with a Bio-Tek EL311 microtiter plate reader (BioTek Instruments Inc., Winooski, VT, USA) at 405 nm.

In situ apoptosis assay

Frozen sections from normal growth plates and from TD afflicted birds were prepared as described by Twal *et al.* (1994). Sections were fixed with 10% buffered formalin and incubated with proteinase K as described by Darfler and Karaszkiewicz (1995). Sections were then incubated for 15 min at 37°C with 25 µl of a solution containing: 250 U/ml terminal transferase, 50 nmol/ml digoxigenin-dUTP, 2.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 0.25 mg/ml BSA, and 25 mM Tris-HCl pH 6.6 (Boehringer Mannheim, Indianapolis, IN, USA). The sections were rinsed and then incubated with a 1:5 dilution of anti-digoxigenin antibody conjugated to fluorescein (Boehringer Mannheim, Indianapolis, IN, USA) for 15 min at room temperature. The sections were rinsed and mounted with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Fluorescence micrographs were taken with a Bio-Rad MRC 600 (Bio-Rad Laboratories, Hercules, CA, USA) confocal microscope.

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