The control of chondrocyte differentiation during endochondral bone growth in vivo: changes in TGF-β and the proto-oncogene c-myc

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SUMMARY

The expression of transforming growth factor-β and the c-myc proto-oncogene was studied in situ in the chondrocytes of the tibial growth plate of normal chicks and those with avian tibial dyschondroplasia in which the chondrocytes are developmentally arrested in the transitional phase between proliferation and differentiation. This results in an accumulation of unmineralised and avascular cartilage. Dyschondroplastic chicks showed reduced c-myc expression in the transitional chondrocytes but unaltered levels in the proliferating chondrocytes. Transforming growth factor-β expression was reduced in the transitional chondrocytes of dyschondroplastic chicks. In areas where the lesion was being repaired there was evidence of increased expression of both c-myc protein and transforming growth factor-β. Addition of 1,25-dihydroxyvitamin D to the diet, which is known to reduce the incidence of dyschondroplasia, resulted in an increase in c-myc production. These results suggest that both transforming growth factor-β and the proto-oncogene c-myc may be important elements of the cascade of events that lead to chondrocyte differentiation, hypertrophy and mineralisation.

Key words: transforming growth factor-β, proto-oncogene c-myc, growth plate, chondrocyte differentiation, endochondral ossification, chicken dyschondroplasia, osteoclasts, cartilage

INTRODUCTION

During longitudinal bone growth, chondrocytes within the epiphyseal plate pass through a series of well defined stages (see Brighton, 1978), characterised by changes in proliferation rate, shape and size, and synthesis and deposition of extracellular matrix components (Hunziker and Schenk, 1989). Consequently, the growth plate of the chick (Howlett, 1979) and other species can be divided into several distinct zones containing resting, proliferating, maturing/transitional and hypertrophic cells.

Controversy exists concerning the molecular control and co-ordination of the mechanisms involved in the transition of cells from a proliferative to a differentiating state, partly due to the fact that studies on isolated cells may not reflect the responses of cells in their normal microenvironment in vivo (Nathan and Sporn, 1991). The spatial and temporal organisation of the growth plate makes it ideal for the in situ biochemical study (Zanelli and Loveridge, 1991) of the regulation of these mechanisms during bone growth. The morphological changes associated with chondrocyte maturation are well defined and we and others have recently reported that chondrocytes undergoing differentiation and hypertrophy express high levels of transforming growth factor-β (TGF-β) (Thorpe et al., 1992; Jingushi et al., 1990) and the product of the proto-oncogene c-myc (Farquharson et al., 1992a) but the importance of these proteins in endochondral ossification has, thus far, not been defined. However, recent reports indicate that c-myc is essential in apoptosis (Evan et al., 1992; Shi et al., 1992) and it has been suggested that growth plate mineralisation involves this process (Kardos and Hubbard, 1982; Akisaka and Gay, 1985).

Avian tibial dyschondroplasia (ATD) results from a defect in endochondral ossification and is characterised by an accumulation of non-mineralised avascular cartilage extended distally from the proximal tibiotarsal epiphysis (Leach and Nesheim, 1965; Riddell et al., 1971; Poulos, 1978). It occurs spontaneously, and may be similar to osteochondrosis (Poulos, 1978), a generalised cartilage defect that has been reported in rapidly growing mammals (Rejno and Stromberg, 1978) and metaphyseal chondroplasia in humans (Rimoin et al., 1974). Although the incidence of ATD is affected by a number of factors such as nutrition
peroxidase procedure (Farquharson et al., 1992a). After fixation chilled immediately in -hexane at -70°C (Loveridge et al., 1991). After washing, sections were reacted with 3,3-diaminobenzidine and hydrogen peroxide. As control procedures, an appropriate dilution of normal mouse serum (1:3,000 to give a similar IgG concentration to the c-myc antibody) was substituted for the primary antibody in duplicate sections. The transitional (prehypertrophic) chondrocytes were identified on the basis of both their position relative to the epiphyseal vessels and their more rounded appearance than that of the ovoid proliferating cells as described by Duff (1984).

Quantification of c-myc protein content was done with a Vickers M85 microdensitometer (Vickers Ltd, York, UK) as described previously (Farquharson et al., 1992a). The following machine settings were used: wavelength, 470 nm; objective, ×40; mask A1 (6 μm diameter); 7 normal and 7 dyschondroplastic chicks (5 grade 3, 2 grade 4) were examined and a minimum of 10 readings were made from 2 serial sections of each growth plate. The results are presented as the mean integrated absorbance × 100 (MIA × 100) ± s.e.m. and statistical analysis was by Student’s t-test.

Immunolocalisation of TGF-β3

Rabbit polyclonal antibodies were generated against synthetic peptides corresponding to unique regions of chicken TGF-β3 as previously described (Jakowlew et al., 1991). Anti-P 50-60 (Jingushi et al., 1990), generated against a peptide corresponding to amino acids 50 to 60 of mature chicken TGF-β3, was used to detect TGF-β3.

The specificity of the antibodies for their corresponding TGF-β isoforms was confirmed by western blot analysis as previously demonstrated and it is reported that this antibody recognises the active form of TGF-β3 (Flanders et al., 1988, 1990, 1991). Sam-

**MATERIALS AND METHODS**

Broiler chicks (male: Ross I strain) were maintained from hatch ing to 3 weeks of age on a diet containing 7.5 g/kg calcium and 7.6 g/kg phosphorus and 25 μg/kg vitamin D3. Proximal tibiotarsi were dissected and examined for the presence of the dyschond roplastic lesion by cutting serial sagittal sections through the bone extremity. The severity of the dyschondroplastic lesion was visually scored ranging from 1 (mild) to 6 (very severe) as described previously (Farquharson et al., 1992c). Samples of bones with or without ATD were processed for histology, immunocytochemistry and enzyme cytochemistry as described below.

**Histology**

The chicks were injected with heparin before being killed by a barbiturate overdose. After aortic catheterisation the pelvic limbs were perfused, at a constant pressure of 150 mm Hg, with a solution of 10% buffered neutral formalin containing 17.5% barium sulphate, 3.5% sodium citrate and 2% Berlin blue. The proximal tibiotarsi were dissected and fixed in 70% ethanol and infiltrated with Polymaster resin (Mawhinny and Ellis, 1983). The blocks of tissue were cut into 1 mm thick slabs and examined.

**Immunolocalisation of c-myc**

A mouse c-myc monoclonal antibody (Cambridge Research Biochemicals, Cheshire, UK; batch no. 03716 or 90071) raised to a synthetic highly conserved peptide consisting of residues 171 to 188 of the human p62c-myc sequence (Evan et al., 1985) was used to detect c-myc protein within the chick growth plate. Western blotting of extracts of chicken chondrocytes indicated that this antibody bound primarily to a 62/64 kDa peptide with minor staining of 50 and 45 kDa peptides (data not shown). Samples of the proximal bone extremity, including the growth plate, articular cartilage, cartilaginous epiphysis and metaphysis were briefly immersed in 5% polyvinyl alcohol (Sigma, Dorset, UK) and chilled immediately in n-hexane at −70°C (Loveridge et al., 1991). Sections were cut on a cryostat (Bright Instruments, Huntingdon, UK) and c-myc protein was localised using a standard indirect peroxidase procedure (Farquharson et al., 1992a). After fixation in ice-cold acetone the sections (10 μm) were decalcified in 0.25 M EDTA in 50 mM Tris-HCl (pH 7.4). Endogenous peroxidases were blocked by hydrogen peroxide/methanol, which was followed by several rinses in PBS. Sections were blocked with 5% normal goat serum, incubated for 1 hour at room temperature with the c-myc antibody (5 μg/ml), washed extensively and incubated for a further 1 hour at room temperature with peroxidase-labelled goat anti-mouse IgG (Dako, Bucks, UK) diluted 1:50 with PBS. After washing, sections were reacted with 3,3-diaminobenzidine and hydrogen peroxide. As control procedures, an appropriate dilution of normal mouse serum (1:3,000 to give a similar IgG

**Fig. 1.** Histology of the normal and dyschondroplastic growth plate. In the normal proximal tibiotarsus (a), the growth plate (P) is penetrated by vessels from the epiphysis (E) and metaphysis (M). In the normal growth plate the transitional chondrocytes occupy a narrow avascular band between the metaphyseal and epiphyseal vessels. Dyschondroplastic cartilage is an accumulation of the transitional chondrocytes and is avascular (b), the metaphyseal vessels (arrowheads) are blunt ending and appear unable to penetrate the cartilage matrix. There are some areas of revascularisation/repair where the vessels have a more normal morphological appearance. This photomicrograph represents a grade 6 lesion. Bars, 2 mm.
amples of the proximal bone extremity from 4 dyschondroplastic (grade 4) and 4 normal growth plates, including the articular cartilage, the epiphyseal growth plate and the metaphysis, were fixed in neutral-buffered formalin, dehydrated through a graded ethanol series and embedded in paraffin wax. Sections (5 μm) were cut and TGF-β3 was localized (Heine et al., 1987) using avidin-biotin-peroxidase kits (Vector Laboratories, Burlinghame, CA). The sections were dewaxed, treated with hydrogen peroxide/methanol to block endogenous peroxides and then treated with hyaluronidase. Sections were blocked with 1.5% normal goat serum/0.5% BSA, incubated overnight at 40°C with affinity-purified anti-sera at 3-5 μg/ml, washed extensively and incubated with biotinylated goat anti-rabbit IgG and avidin-enzyme complex. Sections were reacted with 3,3-diaminobenzidine and hydrogen peroxide and counterstained with Mayer’s haematoxylin. Primary anti-sera was replaced with normal rabbit IgG as a control in serial sections.

Alkaline phosphatase activity
Unfixed, undecalcified sections (10 μm) were reacted for 1 min
at 37°C in the following medium (Loveridge et al., 1991; Farquharson et al., 1992c): 2 mM α-naphthyl acid phosphate (Sigma), 2 mM magnesium chloride and 1 mg/ml Fast Blue RR (Sigma) in 0.1 mM barbitone buffer, pH 9.4. The sections were then rinsed in 0.1 mM acetic acid, washed in several changes of distilled water and mounted in Aquamount (BDH). Alkaline phosphatase (ALP) activity was measured by microdensitometry using the following machine settings: x40, objective; mask, A2 (10 μm diameter); and wavelength, 585 nm; 7 normal and 7 dyschondroplastic chicks were examined and a minimum of 10 readings were made from 2 serial sections of each growth plate. The results are presented as the mean integrated absorbance × 100 (MIA × 100) ± s.e.m. and statistical analysis was by Student’s t-test.

**Effect of 1,25-dihydroxyvitamin D**

Addition of 1,25-dihydroxyvitamin D (1,25(OH)₂D₃) to the diet from hatching is known to reduce markedly the incidence of ATD (Rennie et al., 1993). In a separate experiment 10 chicks were fed diets containing 5 μg/kg 1,25(OH)₂D₃ for the last 3 days prior to death and the incidence and severity of the lesion and the levels of ALP and c-myc were compared with those of unsupplemented birds.

**RESULTS**

The gross pathology of the ATD lesion (grade 6) compared with a normal avian growth plate is shown in Fig. 1. This clearly shows that the lesion consists of avascular unmineralised cartilage, which forms between the descending epiphysial blood supply and metaphyseal vessels. This area consisted primarily of chondrocytes that were in a transitional state between proliferation and hypertrophy.

Proliferating chondrocytes contained appreciable levels of c-myc protein, which were similar in both normal and dyschondroplastic chicks (Figs 2A, 3B). However, the levels of c-myc protein were much higher in the transitional chondrocytes (Figs 2A, 3B). Both nuclear and cytoplasmic staining were observed. In the dyschondroplastic chicks the c-myc protein content of the transitional chondrocytes was significantly lower (21.5%; P<0.01; range 33.3 to 50.4) compared to that in normal chicks (Figs 2B,C, 3B; range 48.3 to 62.1). Areas where there was some metaphyseal invasion of the growth plate showed increased levels of c-myc protein (Fig. 2D).

Alkaline phosphatase activity in the transitional chondrocytes was only around half that of normal chicks (Fig. 2D).

Immuno-localisation of TGF-β3 showed that this growth factor was either absent or present only at low levels in the transitional chondrocytes of dyschondroplastic chicks compared to those in normal birds (Figs 4A,B). In the normal chick, TGF-β3 was associated with chondroclasts (Fig. 4C) but most of the chondrocytes on the periphery of the dyschondroplastic cartilage were negative for TGF-β3 and this area was not invaded by metaphyseal vessels (Fig. 4D). However, the limited sites of vascular invasion were associated with the presence of TGF-β3 within the chondrocytes (Fig. 4E).

In a further experiment, adding 1,25(OH)₂D₃ to the diet for 3 days prior to death reduced the level of ALP activity and increased c-myc content in normal chicks (Fig. 5). It also decreased the severity of the dyschondroplastic lesion compared with control chicks (unsupplemented: 5 normals, 4 grade 3 and 1 grade 1; 1,25(OH)₂D₃ supplemented: 6 normals, 1 grade 3, 2 grade 2, 1 grade 1). 1,25(OH)₂D₃ also increased the levels of c-myc protein in the chicks with ATD (Fig. 5).

**DISCUSSION**

Chondrocyte differentiation results in hypertrophy and mineralisation and is associated with alterations in extracellular matrix synthesis including production of type X collagen (Kwan et al., 1986), a decrease in the proteoglycan/glycosaminoglycan content (Reddi et al., 1978), and an increase in alkaline phosphatase (ALP) activity (de Bernard et al., 1986). In ATD, chondrocytes do not complete the process of differentiation, resulting in an accumulation of non-mineralised and avascular cartilage. Type X collagen production is reduced (Bashey et al., 1989) and the decrease in the level of glycosaminoglycans, as assessed by Alcian blue staining, between the proliferating and transitional chondrocytes was only around half that...
seen in normal birds (Farquharson et al., 1992b). We have previously reported that ATD is not associated with increases in chondrocyte proliferation or decreases in chondroclastic activity (Farquharson et al., 1992c), so it would seem that factors that regulate chondrocyte differentiation are probably involved in the development of this syndrome. Although vitamin D metabolites are thought to be involved in chondrocyte differentiation (Corvol et al., 1978; Schwartz et al., 1988; Kato et al., 1990) and feeding 1,25(OH)2D3 prevents ATD (Rennie et al., 1993), the mechanisms whereby they regulate this process are not known. Thus the study of ATD provides insight into the mechanisms of normal chondrocyte differentiation and their control by vitamin D metabolites.

Alkaline phosphatase activity is elevated in the transitional chondrocytes of chicks with dyschondroplasia, confirming our earlier report (Farquharson et al., 1992c). TGF-β is known to inhibit alkaline phosphatase activity (Rosen et al., 1988; Loveridge, 1990) as well as regulate proteoglycan production (Skantze et al., 1985), and collagen synthesis (O’Keefe et al., 1988) within chondrocytes. In the normal growth plate the active form of TGF-β is found primarily in differentiating and hypertrophic chondrocytes (Jingushi et al., 1990; Thorp et al., 1992). In ATD, there is a marked decrease in the number of transitional chondrocytes staining positive for TGF-β3 and the intensity of the staining is reduced, indicating either a decrease in production and/or activation or an increased rate of turnover of this growth factor. This suggests that TGF-β3 activity is associated with the processes that result in chondrocyte differentiation. TGF-β3 levels are reduced in the transitional chondrocytes of the dyschondroplastic growth plate, which also show increased ALP activity and an inhibition of the fall in glycosaminoglycan content associated with differentiation (Reddi et al., 1978; Farquharson et al., 1992b).

In the normal avian growth plate most hypertrophic chondrocytes are positively stained for TGF-β3 and invading metaphyseal vessels are rich in resorbing chondroclasts (Thorp et al., 1992). In contrast, dyschondroplastic lesions are avascular (Thorp, 1988a), and few of the chondrocytes contained TGF-β3 and thus the matrix surrounding them is likely to contain only small amounts of this growth factor. Small foci of repair, identified by the presence of metaphyseal vessels, are found at the periphery of the dyschondroplastic lesions. Some of the chondrocytes adjacent to these vessels stained positively for TGF-β3, suggesting that TGF-β3 is being expressed during the vascular invasion of metaphyseal cartilage in accord with the concept that TGF-β3 is important for the induction of angiogenesis (Carrington et al., 1988). Most chondroclasts located on dyschondroplastic matrix showed little or no staining for TGF-β3, which suggests that the TGF-β3 present in chondroclasts may be partly matrix derived, not solely the product of TGF-β3 mRNA within these cells (Sandberg et al., 1988).

Studies with isolated cells have implicated the product of the c-myc proto-oncogene in the competence phase of the cell cycle (Kelly et al., 1983) and suggest that c-myc protein is down-regulated during cell differentiation (Coppola and Cole, 1986); furthermore, enforced overexpression of v-myc inhibits chondrocyte differentiation (Quarto et al., 1992). However, studies on the level of c-myc expression in cells maintained in situ suggest that this may not occur in vivo. In the differentiating crypt cells of the colon (Stewart et al., 1986) and in differentiating lens epithelium (Nath et al., 1987) c-myc protein levels are elevated. Furthermore, studies on cardiac (Izumo et al., 1988) and skeletal muscle (Whitelaw and Hesketh, 1992) imply the c-myc protein in cell hypertrophy, which, in chondrocytes, characterizes differentiation. In the chick growth plate, the hypertrophying (transitional) chondrocytes have levels of c-myc protein that are 2.5 times that of the proliferating cells (Farquharson et al., 1992a). Moreover, Manolagos et al. (1987) have reported that cells with high levels of c-myc protein have a higher expression of the receptor for 1,25(OH)2D3.

In ATD, c-myc protein content is significantly reduced in the transitional chondrocytes compared with normal animals. In contrast, in the proliferative chondrocytes there is no significant difference in the c-myc protein content in normal and ATD chicks. This provides further evidence that chondrocyte differentiation and concomitant hypertrophy is characterised by increases in the cellular level of c-myc protein. Cytoplasmic as well as nuclear staining has been previously observed in a number of immunocytochemical studies (Hendy-Ibbs et al., 1987, Ninomiya et al., 1991) and the first of these suggested that relocation of c-myc protein in the cytoplasm could be involved in cell maturation and differentiation.

Quantification of these changes showed that in moderate cases (grades 3-4) of dyschondroplasia there was a 20% decrease in c-myc protein content. This relatively modest change may at first suggest that c-myc protein is not of great importance in chondrocyte differentiation. However, if c-myc protein is one of the initiating factors even small changes would be amplified during the cascade of events associated with differentiation. It may be that there is a threshold level of c-myc protein below which the expression of the 1,25(OH)2D3 receptor (Manolagos et al., 1987) is not sufficient to allow differentiation to proceed or that changes in the intracellular location may be important (Hendy-Ibbs et al., 1987). The effect of modest changes in c-myc protein might be exacerbated, in the broiler chick, because of the rapid progress of chondrocytes (approx. 21 h; Thorp, 1988b) through the growth plate; the incidence of ATD is negligible in the slower growing laying birds. Thus during rapid growth, only slight changes in factors such as c-myc protein can retard chondrocyte differentiation.

The lack of mineralisation that occurs in ATD (Riddell et al., 1971) may be related to the lower amounts of c-myc protein found in the transitional chondrocytes of the affected chicks. Mineralisation of the growth plate cartilage is generally considered to be initiated by matrix vesicles (Ali et al., 1970; Matsuzawa and Anderson, 1971; Boskey, 1981), which are thought to be formed by a number of processes, including apoptosis (Kardos and Hibbard, 1982; Akisaka and Gay, 1985). Recent evidence has strongly suggested that c-myc protein is a potent inducer of apoptosis (Evan et al., 1992; Shi et al., 1992) and thus a decrease in c-myc protein levels could lead to a lack of matrix vesicles and a failure in mineralisation.
We have previously reported a marked increase in TGF-β3 and the c-myc protein with the onset of chondrocyte differentiation (Thorpe et al., 1992; Farquharson et al., 1992a). As shown by the present study, the failure of chondrocyte hypertrophy and mineralisation in dyschondroplasia is associated with marked reductions in TGF-β3 and c-myc protein. Together, these observations provide strong evidence that TGF-β3 and the product of the c-myc proto-oncogene are involved in chondrocyte differentiation and may be early markers of this process. 1,25(OH)$_2$D$_3$ modu-


