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Transforming Growth Factor-β1, -β2 and -β3 in cartilage and bone cells during endochondral ossification in the chick

BARRY H. THORP[1,*], IRENE ANDERSON[1] and SONIA B. JAKOWLEW[2]

[1]*AFRC Institute of Animal Physiology and Genetics, Edinburgh Research Station, Roslin, Midlothian, EH25 9PS, Scotland*
[2]*Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892, USA*

*To whom correspondence should be addressed

Summary

The localization of TGF-β1, -β2 and -β3 was studied in the growth plate, epiphysis and metaphysis of the tibiotarsus of three-week-old chicks. The different TGF-β isoforms were localized to hypertrophic chondrocytes, chondroclasts, osteoblasts and osteoclasts using immunohistochemical staining analysis with specific TGF-β antibodies. TGF-βs in osteoclasts and chondroclasts were restricted to those cells located on the respective matrices. TGF-β3 localization was mainly cytoplasmic in the transitional (early hypertrophic) chondrocytes, but nuclear staining was also detected in some proliferating chondrocytes. The cell-specific localization of these TGF-β isoforms supports the hypothesis that TGF-β has a role in the coupling of new bone formation to bone and cartilage matrix resorption during osteochondral development and suggests that TGF-β may be a marker of chondrocyte differentiation. TGF-β localization preceded a marked increase in type II collagen mRNA expression in transitional chondrocytes, suggesting a role for TGF-β in the induction of synthesis of extracellular matrix.

Key words: endochondral ossification, cartilage, TGF-β, bone, chicken, type II collagen, chondrocyte, osteoblast, osteoclast.

Introduction

The growth and maintenance of the skeleton is controlled by interactions between the cells that form cartilage and bone (chondroblasts and osteoblasts), those that maintain it (chondrocytes and osteocytes) and those that remove it (chondroclasts and osteoclasts). Long bones grow by endochondral ossification which is a sequence of events that includes chondrocyte proliferation and differentiation, matrix calcification, vascular invasion, removal of cartilage matrix, and bone formation. Bone remodelling follows bone formation and continues throughout life in response to various stimuli. Remodelling consists of bone resorption followed by further bone formation, a linkage referred to as "coupling" (Parfitt, 1982). It is hypothesized that local factors released from the matrix during bone resorption are responsible for the recruitment of osteoblasts to sites of subsequent bone formation. At the cellular level, there is precise temporal and spatial control of proliferation, differentiation, migration and matrix synthesis, but little is known about the control and coordination of these processes at the molecular level.

Bone represents the most abundant source of TGF-β in the body (Seyedin et al., 1985a) and the concentration of TGF-β in bone is second only to that of platelets (Assoian et al., 1983). Recent studies have shown that TGF-β exists in a latent form in bone (Canalis et al., 1988; Orr et al., 1990), which can be activated by acid treatment in vitro (Jennings and Mohan, 1990). Bone formation is stimulated by TGF-β in vivo (Noda and Camilliere, 1989; Joyce et al., 1990). In addition, TGF-β has been shown to regulate chondrocyte and osteoblast proliferation and differentiation in vitro (Centrella et al., 1987; Massague et al., 1986; Seyedin et al., 1985a) and is produced by growth plate chondrocytes (Rosier et al., 1989). Furthermore, TGF-β is a potent regulator of cell functions associated with bone formation in vitro (Ibbotson et al., 1989; ten Dijke et al., 1990) and can also inhibit osteoclast formation and activity (Bonewald and Mundy, 1990). These studies suggest that TGF-β might have a role in the control of bone formation and remodelling (Centrella et al., 1989), and in the modulation of growth plate chondrocytes during endochondral ossification (Gelb et al., 1990; Rosier et al., 1989).

An experimental model of endochondral bone formation in the rat has localized TGF-β1 to chondrocytes in calcifying cartilage and osteoblasts (Carrington et al., 1988). Coordinate expression of TGF-β1, -β2, -β3 and -β4 has been demonstrated in chicken embryo chondro-

cytes both in vitro and in vivo (Jakowlew et al., 1991). The present study shows that, in growing three-week-old chicks, TGF-β1, -β2 and -β3 are localized to transitional and hypertrophic chondrocytes and osteoblasts, cells that are involved in the calcification of extracellular matrix. TGF-β localization in transitional chondrocytes precedes a marked increase in type II collagen mRNA expression, suggesting a role for TGF-β in the induction of synthesis of extracellular matrix. In addition, high levels of expression of the three TGF-β isoforms are found in 'clast' cells which are actively engaged in removing cartilage and bone matrices. This suggests that the TGF-β isoforms might be involved in the coupling of cartilage and bone matrix resorption to new bone formation in vivo in the chicken.

Materials and methods

Preparation of TGF-β-isoform-specific antibodies

Rabbit polyclonal antibodies were generated against synthetic peptides corresponding to unique regions of human TGF-β1 and TGF-β2 and chicken TGF-β3 as previously described (Jakowlew et al., 1991). Anti-P 1-30(1), raised against a peptide corresponding to 30 amino acids at the aminoterminal of mature human TGF-β1 (Flanders et al., 1988) and anti-P 50-75(2), generated to a peptide corresponding to amino acids 50-75 of mature human TGF-β2 (Flanders, 1990), were used to detect chicken TGF-β1 and -β2, respectively, since these isoforms of human and chicken TGF-β show 100% homology at the amino acid level. Anti-P 50-60(3), generated against a peptide corresponding to amino acids 50-60 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 (Flanders et al., 1988, 1990; Flanders, 1990).

Preparation of type II collagen probe

A 1 kb *Bam*HI-*Sma*I restriction fragment of chicken type II collagen cDNA (gift from Linda Sandell) (Sandell et al., 1984), was subcloned into the T7 RNA polymerase vector pGEM 1 (Promega Biotec). Plasmid templates were linearized and antisense and sense RNA transcripts were synthesized using DIG-UTP (Boehringer Mannheim).

Preparation and staining of tissues for TGF-β1, -β2 and -β3

Proximal tibiotarsi were dissected from three-week-old broiler chicks, including the articular cartilage, cartilaginous epiphysis, growth plate and metaphysis. The tissue was fixed in neutral-buffered formalin, dehydrated through graded alcohols and embedded in paraffin wax. 5 μm sections were cut and TGF-β1, -β2 and -β3 were 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 4°C with affinity-purified antisera at 3-5 μg/ml, washed extensively and incubated with biotinylated goat anti-rabbit IgG and avidin-enzyme complex. Sections were stained with 3,3'-diaminobenzidine and hydrogen peroxide and counterstained with Mayer's hematoxylin. Primary antisera were replaced with normal rabbit IgG as a control in duplicate sections.

Preparation and in situ hybridization of tissues

Proximal tibiotarsi were fixed in fresh PBS containing 4% (w/v) paraformaldehyde, dehydrated, embedded in paraffin wax and 5 μm sections were cut. Glass slides were acid washed, coated with a solution of poly-L-lysine (Sigma) and sections mounted. Sections were rehydrated through graded alchols, incubated in prehybridization solution [deionised formamide 5.0 ml, SSC (20×) 2.0 ml, Denhardt's (50×) 0.2 ml, Herring sperm DNA (10 mg/ml heat denatured) 0.5 ml, yeast tRNA (10 mg/ml) and dextran sulphate (50%) 2.0 ml] for 1 hour, washed and incubated overnight in hybridization solution [deionised formamide 5.0 ml, SSC (20×) 2.0 ml, Denhardt's (50×) 0.2 ml, Herring sperm DNA (10 mg/ml heat denatured) 0.5 ml, yeast tRNA (10 mg/ml) and dextran sulphate (50%) 2.0 ml] containing 227 μg/ml of either sense or antisense probe. Following hybridization, slides were washed, incubated in 2% normal sheep serum and probe was detected immunologically using an anti-digoxigenin conjugate to alkaline phosphatase (Boehringer Mannheim). The slides were developed in a solution containing 45 μl nitroblue tetrazolium chloride [75 mg/ml in 70% (v/v) dimethyl formamide], 35 μl x-phosphate (50 mg/ml in dimethyl formamide) and 2.4 mg levamisole in 10 ml 0.1 M Tris buffer pH 8.2.

Results

Growth plate chondrocytes

Using specific TGF-β antibodies, expression of immunoreactive TGF-β1, -β2 and -β3 was detected in most hypertrophic and transitional chondrocytes in the three-week-old chick growth plate (Fig. 1A-C). Little expression of these different TGF-β isoforms was detected in the zones of resting or proliferating chondrocytes. While virtually all growth plate chondrocytes in the transitional zones expressed TGF-β3, only some proliferating chondrocytes expressed this isoform, and this staining was frequently localized to the nucleus. Many of the chondrocytes entering the transitional zone had a flattened appearance and, in most of these cells, expression of TGF-β3 was also localized to the nucleus (Fig. 1B). In contrast, the cytoplasm of the majority of rounded chondrocytes in the transitional zone stained intensely for TGF-β3 (Fig. 1C). No morphological differences were apparent between the majority of the hypertrophic chondrocytes that showed positive staining for TGF-β3 and the few hypertrophic chondrocytes that did not stain (Fig. 1D). TGF-β3 was also highly expressed in hypertrophic chondrocytes found in small cartilage spicules in the metaphysis. The distribution of TGF-β2 staining among the transitional and hypertrophic chondrocytes was similar to that of TGF-β3, except for the complete absence of staining in proliferating chondrocytes (data not shown). Although staining using the TGF-β1 antibody was detected in the cytoplasm of most hypertrophic chondrocytes in the same location as that of TGF-β2 and -β3, staining for TGF-β1 was consistently less intense than for TGF-β2 and -β3 (data not shown).

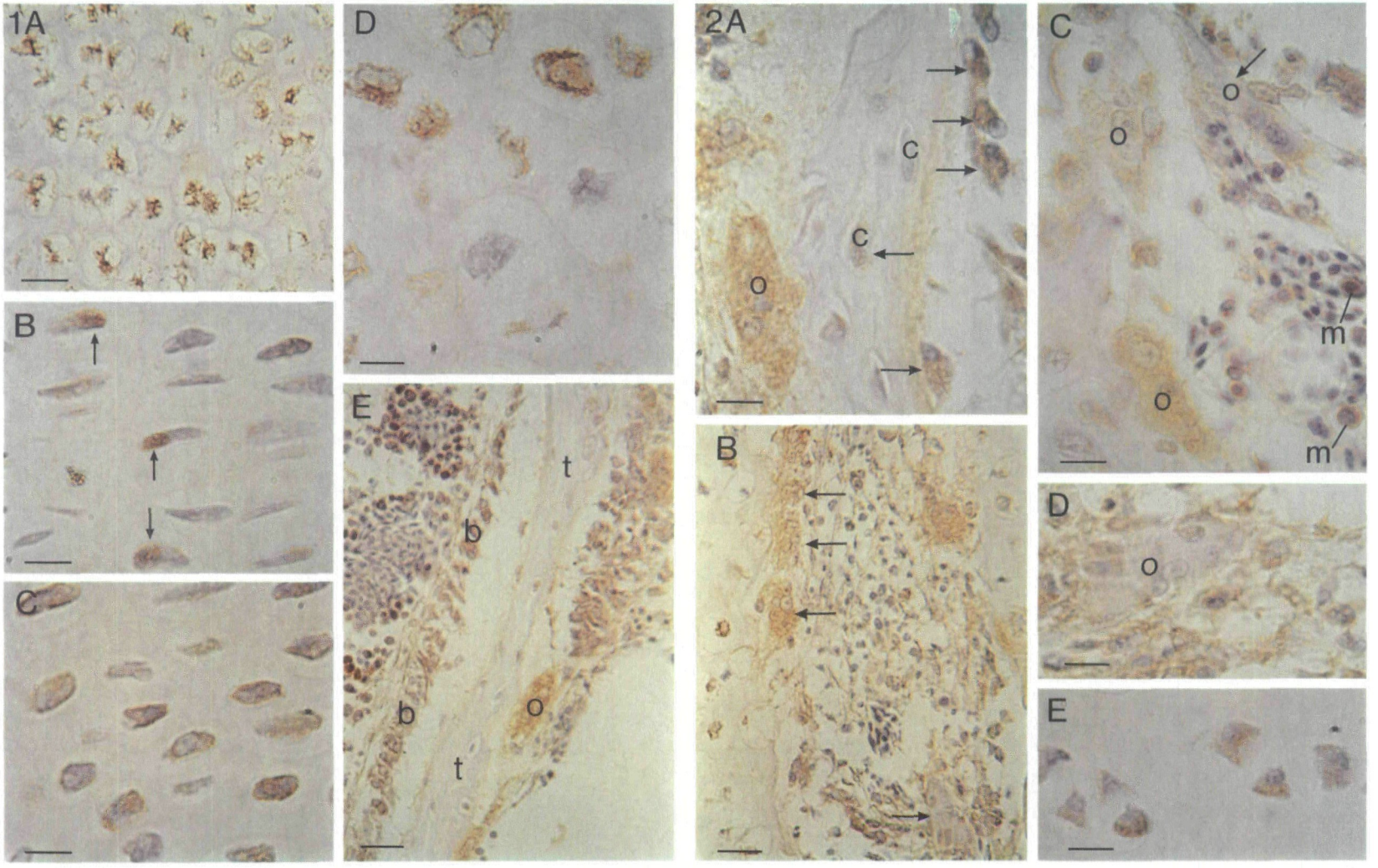

Fig. 1. Localization of TGF-β3 in sections of proximal tibiotarsus. Three-week-old chick bone tissue sections were reacted with anti-P 50-60(3) antibodies and stained as described in Materials and Methods. (A) Hypertrophic chondrocytes of the growth plate show positive staining for TGF-β3 (bar=20 μm). (B) Chondrocytes entering the transitional zone have a flattened appearance and many stain positively for TGF-β3. Arrow denotes localization of TGF-β3 mainly to the nucleus (bar=12 μm). (C) Almost all growth plate chondrocytes in the transitional zones show positive staining for TGF-β3 localized mainly to the cytoplasm (bar=12 μm). (D) Some hypertrophic chondrocytes show little or no staining for TGF-β3. No morphological differences were noted between these cells and those showing positive staining for TGF-β3 (bar=12 μm). (E) The trabeculae (t) of bone in the metaphysis covered by bone lining cells (b) and an osteoclast (o) show positive staining for TGF-β (bar=50 μm).

Fig. 2. Localization of TGF-β3 in proximal tibiotarsus. Three-week-old chick bone tissue sections were reacted with anti-P 50-60(3) antibodies and stained as described in Materials and Methods. (A) Bone lining cells showing positive staining for TGF-β3 are plump, rounded cells (denoted by arrows) located on the osteoid and are designated osteoblasts (bar=12 μm). (B) Chondroclasts in the metaphysis (denoted by arrows) in contact with the matrix or in resorption lacunae stain intensely for TGF-β3, while those not in direct contact with the matrix show little or no staining for TGF-β3 (bar=50 μm). (C) Osteoclasts (o) on the matrix show positive staining for TGF-β3, while osteoclasts located away from the matrix (denoted by arrow) show little or no staining for TGF-β3. Large round mononuclear cells (m) in the marrow show intense staining for TGF-β3 (bar=12 μm). (D) Chondroclast from the metaphyseal region not on the cartilage matrix with little or no staining for TGF-β3 (bar=12 μm). (E) Epiphyseal chondrocytes near the periphery of the epiphysis, adjacent to the perichondrium and growth plate, show positive staining for TGF-β3 localized mainly to the cytoplasm (bar=12 μm).

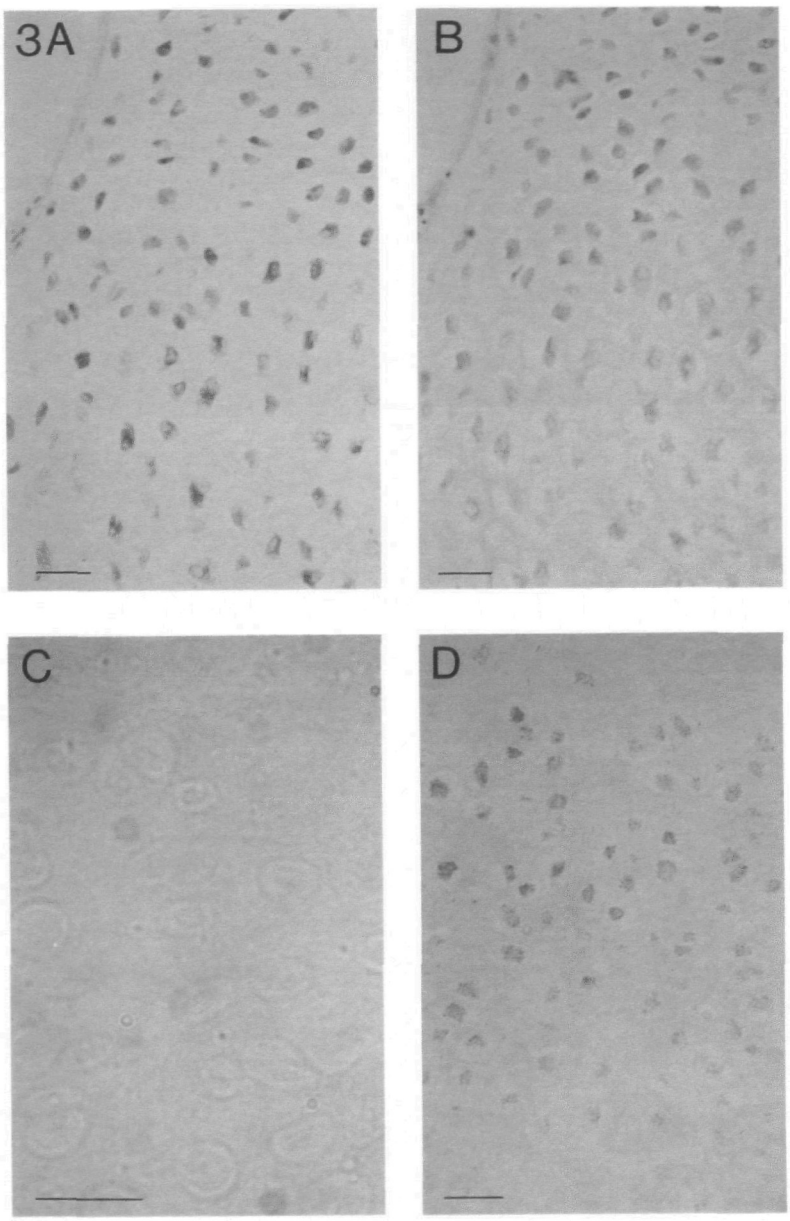

Fig. 3. (A) Localization of TGF-β3 in proximal tibiotarsus. Three-week-old chick bone tissue sections were reacted with anti-P 50-60(3) antibodies and stained as described in Materials and Methods. Primary antisera was replaced with normal rabbit IgG as a control in a duplicate section (B), and none of the chondrocytes are stained (bar=12 μm). Proximal tibiotarsi were reacted with either the sense (C) or antisense (D) probe to type II collagen as described in the Materials and Methods. The rounded transitional chondrocytes reacted with the antisense probe (D) hybridize strongly with type II collagen mRNA, whereas chondrocytes reacted with the sense probe (C) are negative (bar=12 μm).

Osteoblasts and osteocytes

Although most of the cells on the surface of bone showed staining for all three TGF-β isoforms, staining for TGF-β3 was generally more intense than that for TGF-β1 and -β2. Here, as in the growth plate, staining for TGF-β1 was very weak. Many of the cells (Fig. 1E) that expressed TGF-β were plump cuboidal cells located on the osteoid and considered to be osteoblasts (Fig. 2A); TGF-β was localized to the cytoplasm of most of these cells. Although there were a few cells that did not express TGF-β, morphologically these cells could not be distinguished from the osteoblasts. While most of the osteocytes located near the osteoid surface showed staining for TGF-β, osteocytes located deep in the bone matrix were frequently negative for TGF-β staining. In general, those osteocytes that showed staining for TGF-β were usually larger in size than those that did not stain.

Osteoclasts (chondroclasts)

In the metaphysis, strong staining for all three TGF-β isoforms was observed in many osteoclasts (Fig. 1E) and chondroclasts (Fig. 2B) for all three isoforms of TGF-βs. There was little difference in the intensity of staining for the different TGF-β isoforms, but, as before, staining for TGF-β1 was slightly less intense. However, there was marked variation in staining between individual clast cells (Fig. 2C); while most of those osteoclasts and chondroclasts that were not in direct contact with the matrix showed little or no staining for TGF-β1, -β2 and -β3, the majority of clasts in contact with the matrix or in resorption lacunae, stained intensely for all three TGF-βs (Fig. 2B-D). In addition, there were some clasts where staining was localized to small areas in the cytoplasm adjacent to the matrix or to the nucleus.

Epiphyseal chondrocytes and perichondrial cells

Most epiphyseal chondrocytes near the periphery of the epiphysis, adjacent to the perichondrium and growth plate stained positively for TGF-β3 (Fig. 2E). These chondrocytes were considered to be differentiating because there was less cartilage matrix between these cells than between those chondrocytes towards the center of the epiphysis and, as a result, they were likely to have been recently derived from the perichondrium due to appositional growth. Throughout the remainder of the epiphyseal cartilage, staining was detected in the cytoplasm of approximately half of the epiphyseal chondrocytes, but was not as intense as staining near the periphery. In the cartilaginous epiphysis, fewer chondrocytes showed staining for TGF-β1 and -β2 than for TGF-β3 and staining, in general, was less intense. Although the perichondrium and periostium contained many fibroblasts which showed staining for TGF-β3, some cells also showed staining for TGF-β1 and -β2 (data not shown).

Bone marrow

Many of the cells in the haematopoetic marrow stained positively for TGF-β1, -β2 and -β3. The most intense and distinct staining was seen in a few fairly large, round, mononuclear cells which are thought to be blasts (Fig. 2C). Some of these cells may be thromboblasts, which are precursors of thrombocytes, the avian equivalent of platelets. There is no well-established means of easily identifying these cells as yet.

Controls TGF-β

There was no staining of the cartilage cells, bone cells or the bone marrow cells in the tissue sections when normal rabbit serum was used as a control (Fig. 3A).

Type II collagen mRNA

Use of an antisense type II collagen probe indicted abundant expression of Type II collagen mRNA in most of the rounded chondrocytes in the transitional zone of the growth plate (Fig. 3C). In contrast, no expression of Type II collagen mRNA was detected in the proliferating zone. While TGF-β was first detected in transitional chondrocytes that had a partially flattened appearance (Fig. 3B), increased expression of type II collagen mRNA occurred only after the transitional chondrocytes had become rounded. The level of expression of type II collagen mRNA declined distal to the transitional zone and there was little or no expression in the fully hypertrophied cells. No hybridization was detected in any of these chondrocytes when the sense probe was used (Fig. 3C).

Discussion

Our results suggest that there may be several roles for the TGF-β isoforms during endochondral ossification in the chicken. We observe significant differences in the cellular localization of the TGF-β isoforms in differentiating chondrocytes. For example, in those few flattened proliferating chondrocytes that express TGF-β3, expression is usually localized to the nucleus. In contrast, in rounded transitional chondrocytes, localization of TGF-β3 is specific to the cytoplasm. Very little is known about the intracellular compartmentalization of most growth factors. It is known that most growth factors function in endocrine, paracrine or autocrine capacities, but some growth factors, such as fibroblastic growth factor, are thought to regulate the nucleus of the cell of origin and, thus, may have an intracrine role (Logan, 1990). The nuclear localization of TGF-β suggests that it may be able to serve a similar role. However we have no information of whether the identified TGF-β proteins are in a latent or active form.

Our data also show that the appearance of TGF-β in growth plate chondrocytes coincides with changes in the morphology of the cells as they graduate from the proliferating zone to the transitional zone. The transitional chondrocytes of the avian growth plate stain intensely for TGF-β2 and -β3 as well as for type II collagen mRNA. Because expression of TGF-β2 and -β3 in transitional chondrocytes precedes the marked expresson of type II collagen mRNA, TGF-β2 and -β3 may be involved in the induction of Type II collagen

production in the chicken. This expression coincides with the differentiation of chondrocytes from proliferation to hypertrophy. In the rat, TGF-β has been shown to stimulate type II collagen synthesis in chondrocytes in vitro (Seyedin et al., 1985b). In the human foetus, TGF-β2 and -β3 are widely expressed in areas of chondrogenic activity (Gatherer et al., 1990). While only a low level of TGF-β1 mRNA is detected in cartilage where type II collagen mRNA is abundant (Sandberg et al., 1988), our data are consistent with the idea that the TGF-βs may have a role in inducing increased synthesis of type II collagen mRNA in vivo. Recent studies have shown that exogenous addition of TGF-β2 is more effective than TGF-β1 in inducing chondrogenesis and osteogenesis in the rat (Joyce et al., 1990); in addition type II collagen is induced by TGF-β.

Our data show that while there is co-expression of TGF-β1, -β2 and -β3 in most areas of bone and cartilage in chickens, e.g. in fully hypertrophic chondrocytes, only some cells appear to contain just one TGF-β isoform, TGF-β3, e.g. a few of the proliferating chondrocytes. This is in contrast to the mouse embryo where the mRNAs for TGF-β1, -β2 and -β3 are expressed in bone, but show distinct temporal and spatial patterns (Pelton et al., 1990; Schmid et al., 1991). TGF-β1 has been localized to the proliferating and upper hypertrophic zones of the rat growth plate (Jingushi et al., 1990). In the rat, TGF-β2 has been shown to be a more potent stimulator of osteogenesis and chondrogenesis than TGF-β1 (Joyce et al., 1990). In the mouse, it has been suggested that TGF-β3 may be more important in the early differentiation process of endochondral bone formation (Schmid et al., 1991) and the active growth and differentiation of chondroblasts (Millan et al., 1991). The localization of TGF-β3 in the present study suggests that this may also be true in the chicken. The relatively weak staining pattern for TGF-β1 in the chicken suggests this isotype may be of less importance during bone growth in this species.

We have localized TGF-β to hypertrophic chondrocytes and osteoblasts and both cell types show staining for TGF-β. TGF-β produced by these cells is thought to be stored in a latent form in the respective matrices (Carrington et al., 1988). The functional role of TGF-β in these two cell types may be similar as they both produce a matrix that becomes mineralized and is subsequently remodelled by "clast" cells. In addition to expression of TGF-β in osteoblasts, some of the osteocytes that appear to have only recently been encased in bone matrix also express TGF-β. Smaller osteocytes located deeper in the matrix show much weaker staining for TGF-β. This might suggest that only those osteogenic bone cells that are actively secreting bone matrix may be expressing TGF-β.

The mechanism by which differentiating growth plate chondrocytes induce the production of TGF-β remains uncertain. In vitro, fibroblast growth factor (FGF) increases production of TGF-β in chondrocytes (Gelb et al., 1990), and it has been shown that TGF-β can have a potent inhibitory effect on chondrocyte proliferation in culture (Vivien et al., 1990). The TGF-βs are only expressed in growth plate chondrocytes when these cells have ceased to proliferate and begin to hypertrophy. Transitional chondrocytes are a greater distance fom the epiphyseal cartilage, and are less well vascularized than proliferating chondrocytes, and the resulting differences in the microenvironment may contribute to the induction of TGF-β expression.

Our data suggest that the TGF-β isoforms may play a role in coupling new bone formation to bone and cartilage matrix resorption during endochondral ossification and remodelling in the chicken. Furthermore, these isoforms may be markers of chondrocyte differentiation. The localization of TGF-β3 to the nucleus of some proliferating chondrocytes suggests the possibility of intracrine activity. The release of one or more of these TGF-βs by the chondro/osteoclasts during matrix resorption may result in the recruitment and activation of osteoblasts for new bone formation. Furthermore TGF-βs in osteoclast and chondroclasts were restricted to those cells located on the respective matrices and may indicate that they are actively engaged in resorption.

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