REVIEW

Thyroid hormone actions on cartilage and bone: interactions with other hormones at the epiphyseal plate and effects on linear growth

G R Williams, H Robson¹ and S M Shalet²

Molecular Endocrinology Group, Department of Medicine, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN and Departments of ¹Clinical Research and ²Endocrinology, Christie Hospital NHS Trust, Manchester M20 4BX, UK

(Requests for offprints should be addressed to G R Williams, Molecular Endocrinology Group, Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK)

Introduction

Normal linear growth is determined by variable contributions from genetic, nutritional and endocrine factors and continues until bone maturation and epiphyseal closure in early adulthood. The major systemic hormones that influence growth during childhood include thyroid hormone (T_3), growth hormone (GH), insulin-like growth factors (IGF) and glucocorticoids, whereas an increasing contribution from sex steroids develops during adolescence (Underwood & Van Wyk 1992, Weiss & Refetoff 1996). Recently, two signalling proteins, Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP), have also been found to be crucial for the co-ordinated regulation of the pace of growth plate chondrocyte differentiation (Lanske *et al.* 1996, Vortkamp *et al.* 1996, Wallis 1996). These molecules form an integrated negative feedback loop that regulates the rate of endochondral ossification. However, it is not known how systemic hormones and other local factors that act on the growth plate may regulate expression of Ihh and PTHrP or influence activity of the feedback loop to alter the rate of chondrocyte differentiation during growth.

In this review, we focus on the actions of T_3 and its interactions with other hormone signalling pathways in the control of growth. The field highlights a poorly studied area that has important applications to understanding skeletal development and the regulation of linear growth. There are large gaps in our knowledge concerning the actions of T_3 in bone and cartilage, and fundamental questions have yet to be answered. For example, a current point of controversy is whether the major skeletal effects of T_3 result from its direct effects on the skeleton or via interactions with GH and IGF-1. Recent evidence documenting the expression of T_3 receptors in osteoblasts and chondrocytes indicates that direct T_3 effects on the skeleton are likely to be important. Despite this, the target genes that are directly responsive to T_3 in bone cells have not been identified. Furthermore, the precise localisation of T_3 -responsive cells within the growth plate and skeleton

and the ontogeny of T_3 receptors in developing cartilage and bone are unknown. Thus it is not clear whether T_3 acts directly and independently on both osteoblasts and osteoclasts, in addition to chondrocytes, or whether the effects of thyroid hormones on bone turnover and growth are mediated primarily by one cell lineage and subsequently by other secondary events.

The aim of this review is to summarise the effects of T_3 on bone and growth plate cartilage and to discuss the interactions between T_3 and other hormones and their possible relationship to the fundamentally important Ihh/PTHrP feedback loop. The roles of other important growth factors and cytokines are beyond the scope of this review. In a discussion of this type, it is important to be aware that much of our knowledge is derived from studies using a variety of animal species, including rodents, cattle, pigs, rabbits and birds, in addition to humans. Thus data can be conflicting, as there are basic differences between species in the growth plate ossification process and *in vitro* models may not reflect the intact organism. For example, rodent growth plates fuse in late adulthood, whereas this occurs towards the end of adolescence in man. In birds, the system may differ further, as the avian growth plate is more fibrous than its mammalian counterpart. Despite these reservations, there are fundamental similarities in endochondral ossification across species that do allow cautious but valid interpretation of the available data.

Clinical and physiological effects of thyroid hormones on the skeleton

Effects of thyroid hormones on growth

T_3 is a primary determinant of normal postnatal somatic growth and skeletal development, and an important regulator of bone and mineral metabolism in the adult (Allain & McGregor 1993, Compston 1993). Before puberty, thyroid hormone may be the major prerequisite for normal maturation of bone (Underwood & Van Wyk 1992). In contrast, it is not clear if T_3 is important

for intrauterine fetal growth, as infants with congenital thyroid aplasia are of normal size at birth and have normal central nervous system development. This may reflect a compensatory contribution from maternal thyroid hormones that cross the placenta in low concentrations, as continued maturation in the neonatal period can only be maintained by early thyroxine (T_4) replacement.

Untreated childhood hypothyroidism, however, results in profound growth retardation and delayed skeletal maturation. Linear growth is almost completely halted, but can be resumed quickly by replacement of thyroid hormones to produce a rapid catch-up period of growth (Fisher & Plok 1995, Boersma & Wit 1997). In contrast, whereas thyrotoxicosis in children is associated with increased bone turnover and accelerated growth rates, the advanced skeletal maturation that also occurs may lead to short stature (Underwood & Van Wyk 1992, Allain & McGregor 1993, Weiss & Refetoff 1996). In adulthood, thyrotoxicosis increases bone turnover (Franklyn *et al.* 1994) and accelerates bone loss even in the presence of adequate oestrogens (Mosekilde *et al.* 1990) whereas, in hypothyroidism, excessive T_4 replacement has been linked with osteoporosis (Diamond *et al.* 1991, Schneider *et al.* 1994), particularly in previously thyrotoxic patients rendered hypothyroid by iodine-131 treatment (Duncan *et al.* 1994, Franklyn *et al.* 1994).

Resistance to thyroid hormone

The syndrome of resistance to thyroid hormone (RTH) (Refetoff *et al.* 1967) offers further confirmation of the importance of T_3 for growth and skeletal development. RTH is an autosomal dominant condition caused by point mutations of the thyroid hormone receptor (T_3R) β gene (T_3R α is normal in all cases) that generate receptors which display impaired responses to T_3 and antagonise the actions of wild-type T_3R α and β proteins (Refetoff *et al.* 1993). The condition is characterised by a heterogeneous phenotype that is manifest by variable degrees of tissue hypothyroidism in the presence of increased circulating concentrations of free T_4 , free T_3 and thyroid-stimulating hormone. The index family contained patients with stippled epiphyses similar to those documented in profoundly hypothyroid children during the 1930s. A recent analysis of RTH families indicated that short stature occurs in up to 16% of affected children, with delayed bone age in 47% (Weiss & Refetoff 1996). These observations highlight the variable phenotype of RTH as, in 84% and 53% of cases, growth and bone age respectively, were apparently normal.

The reasons for such variability are not completely understood, but recent genetic evidence now indicates fundamental roles for both T_3R α and β during growth and skeletal development. In T_3R α knockout mice, there is growth arrest and delayed bone maturation, with disorganisation of epiphyseal growth plate chondrocytes,

fewer hypertrophic cells and disrupted endochondral bone formation (Fraichard *et al.* 1997). T_3R α null mice have markedly reduced thyroid hormone production rates and severe biochemical hypothyroidism. Remarkably, the growth arrest and delayed bone maturation were rescued after T_3 replacement, 1 $\mu\text{g}/\text{day}$ for 1 week (Fraichard *et al.* 1997). In T_3R β null mice, in contrast, there is disruption of the pituitary–thyroid feedback axis and profound hearing loss, but little effect on skeletal development and growth (Forrest *et al.* 1996a,b). The data from knockout studies suggest that T_3R α has the critical role in thyroid hormone production after weaning, whereas T_3R β exerts a negative regulatory role on hormone production and release (Fraichard *et al.* 1997, Forrest *et al.* 1996a,b). Taken together, these findings make it clear that either T_3R α or T_3R β can mediate the actions of T_3 in the epiphysis and skeleton, but the profound growth disruption seen in T_3R α null mice results from biochemical hypothyroidism. The data emerging from rodents may, in part, explain the phenotypic variation seen in human RTH. Thus the heterogeneity of growth and bone age delay in RTH may reflect the diversity of mutant T_3R β proteins found in this condition and their abilities to cause variable degrees of ‘target tissue hypothyroidism’.

Direct actions of thyroid hormones on the skeleton

Thyroid hormone actions in bone

Only a few studies have investigated T_3 actions in bone. High-affinity T_3 binding sites were identified more than 10 years ago in rat osteoblast lineage cells (Rizzoli *et al.* 1986) and T_3 stimulation of osteoclastic bone resorption was first demonstrated more than 20 years ago in rat cells (Mundy *et al.* 1976). Since then, further studies have suggested that rat, mouse and human osteoblasts are primary T_3 target cells and that osteoclast responses may be secondary, or coupled, to these primary T_3 actions (Rizzoli *et al.* 1986, Sato *et al.* 1987, Kasono *et al.* 1988, Krieger *et al.* 1988, LeBron *et al.* 1989, Egrise *et al.* 1990, Allain *et al.* 1992, Allain & McGregor 1993, Britto *et al.* 1994, Klaushofer *et al.* 1995). Furthermore, expression of T_3Rs in human osteoblasts and osteoclasts at sites of active bone remodelling has been demonstrated recently by immunohistochemistry (Abu *et al.* 1997). Thus both osteoblast and osteoclast activities are affected by abnormalities in thyroid status leading to a net imbalance in bone turnover.

We have shown that rat osteoblastic cells express functional T_3R proteins in patterns that vary in cells that express phenotypes of differing osteoblast maturity (Williams *et al.* 1994, Bland *et al.* 1997). In addition, 9-*cis* retinoic acid receptors (RXR), which bind to target gene promoters as high-affinity heterodimers with T_3R (Glass 1994) and help to mediate the actions of T_3 , are co-expressed in rat osteoblastic cells (Bland *et al.* 1997).

The related receptors for 1,25(OH)₂-vitamin D₃ (VDR) and all-*trans* retinoic acid (RAR) are additional alternative heterodimer partners for RXR, T₃R and each other (Rosen *et al.* 1993, Glass 1994, Schrader *et al.* 1994) and are also co-expressed in rat osteoblastic cells (Williams *et al.* 1995, Bland *et al.* 1997). All these observations support the notion that bone is primarily responsive to T₃, but that responsiveness is complex and may vary with development and vitamin D or retinoid status. In spite of this, no clearly documented transcriptionally activated T₃ target genes have yet been defined in bone cells, although interleukins-6 and -8 (Siddiqi *et al.* 1996) and IGF binding proteins (IGFBPs)-2 (Schmid *et al.* 1992) and -4 (Glantschnig *et al.* 1996) have been implicated in preliminary studies in human, rat and murine species respectively. Findings of a recent study also suggest that IGF-I may be regulated directly by T₃ in rat vertebral, but not femoral, osteoblasts (Van Auker *et al.* 1997).

The epiphyseal growth plate

In the normal epiphyseal plate, chondrocytes are organised into layers (Fig. 1). At the distal epiphyseal end, chondroblast progenitor cells occur singly and in small clusters to form the reserve cell zone. Progressing towards the metaphysis, and adjacent to the reserve zone, small flat proliferating cells form discrete columns and constitute the proliferative zone. The proliferative cells then lose their ability to divide and undergo a process of differentiation to produce enlarging hypertrophic chondrocytes, which are present within the hypertrophic zone. These hypertrophic cells, situated in the lower region of provisional cartilage calcification, secrete matrix that undergoes mineralisation and is then partially resorbed by chondroclasts or osteoclasts. The largest, most distal hypertrophic chondrocytes undergo apoptosis to leave a cartilaginous framework. The remaining mineralised matrix forms a scaffold for apposition of metaphyseal trabecular bone by invading osteoblasts at the resorption front, and constitutes the primary spongiosum. Invading osteoblasts are derived from bone-marrow stromal cells which enter the primary spongiosum via capillaries that advance from the adjacent marrow cavity.

Thyroid hormone effects on growth plate architecture

Lewinson *et al.* (1989) studied the effects of hypothyroidism and interactions between T₃ and GH on rat tibial growth plate morphology. In hypothyroid rats, the width of the epiphyseal plate is reduced as a consequence of the presence of fewer proliferative cells and a reduction in size of hypertrophic cells compared with controls. In addition, mineralisation of the cartilage-marrow interface and absent resorptive activity in this region result in there being no new metaphyseal bone formation and markedly reduced subchondral primary spongiosum metaphyseal

trabecular bone in hypothyroid animals. Few, if any, chondroclasts or bone marrow stromal cells are seen. Treatment of hypothyroid rats with T₄ reverses these changes. In contrast, treatment of hypothyroid rats with GH does not result in recovery of the disrupted growth plate architecture unless co-treatment with T₄ is instigated. In this case, GH augments the positive response to T₄. Similar effects are also seen in articular cartilage, although GH does not enhance the actions of T₄. The important findings from this study (Lewinson *et al.* 1989) confirm the direct actions of thyroid hormone on the growth plate and exclude the possibility that thyroid responses are mediated via increased GH secretion.

In a follow-up study, the responses of rat mandibular condyle cartilage to hypothyroidism, T₄ replacement and GH were documented (Lewinson *et al.* 1994). The condylar bone responds differently to hypothyroidism and undergoes hyperostosis (osteopetrosis) of the subchondral region, suggesting that the endochondral osteogenic response to thyroid hormone varies according to anatomical site and may be dependent on the particular cells that respond to T₃ in each location. In condylar cartilage, unlike in the tibial growth plate, remodelling is not completely blocked and some cartilage scaffold persists, as the cartilage-marrow interface is not mineralised (Lewinson *et al.* 1994). Despite differences in responses of the two sites to hypothyroidism, their response to replacement with T₄ or GH or both hormones remains similar and requires thyroid hormone to be fully realised. At both sites there is diminished cellularity of the zones containing immature cells in hypothyroidism; these cells are chondrocyte progenitor cells and chondroblasts within the condyle, or reserve and proliferative chondrocytes in the growth plate (Lewinson *et al.* 1989, 1994). This key observation is likely to be the basis for the fewer hypertrophic cells and reduced differentiation capacity of proliferative cells that is seen in T₃ deficiency (Carrascosa *et al.* 1992, Ballock & Reddi 1994, Quarto *et al.* 1992). Both processes would result in the reduced linear growth that occurs in juvenile hypothyroidism.

Further considerations suggest that other mechanisms may also contribute to the actions of T₃ at the epiphyseal plate. Terminal differentiation of proliferating chondrocytes into hypertrophic cells is the primary event that characterises endochondral ossification and linear growth. There is a several-fold increase in hypertrophic cell volume that correlates with both the rate of long bone growth and the associated mineralisation of cartilaginous matrix. This mineralised scaffold is the site for new bone formation by osteoblasts, which arrive from the adjacent marrow with invading blood vessels. As the epiphyseal plate is sealed by a mineralised bone marrow-growth plate interface in hypothyroid animals (Lewinson *et al.* 1989), it is possible that an important action of T₃ is to promote or maintain vascular invasion and osteoblast migration to facilitate new bone formation.

Figure 1 Diagrammatic representation of the layered structure of an epiphyseal growth plate. The epiphysis is situated at the top of the figure and the metaphysis, containing the bone marrow cavity, is located below the cartilaginous growth plate. The sites of expression of thyroid hormone receptor (T₃R) isoforms α1 and β1 are shown on the right-hand side of the figure: regions of +++ strong, ++ intermediate, + weak, and – absent immunostaining for both T₃R isoforms are shown. Vertical lines show the limits of staining for T₃R within the reserve and proliferative zones. The location of T₃R expression in this diagram summarises the findings from our own preliminary studies (Robson *et al.* 1997), which are shown in Fig. 2.

Thyroid hormone actions on chondrocytes in vitro

Cultured human fetal epiphyseal chondrocytes possess specific nuclear binding sites for T₃, with appropriate binding affinities in the nanomolar range (Carrascosa *et al.* 1992). These cells respond to T₃ *in vitro*, with increased alkaline phosphatase activity after 9 days of treatment. Similarly, treatment of rat epiphyseal chondrocytes with T₃ for 96 h increased alkaline phosphatase

activity and IGF-I receptor mRNA expression, in addition to decreasing cell proliferation (Ohlsson *et al.* 1992). In that study, IGF-I inhibited the alkaline phosphatase response to T₃. These data suggest the presence of functional nuclear receptors for T₃ in chondrocytes, but it is unclear whether the documented effects of T₃ are primary T₃R-mediated responses, as the times chosen in both studies were beyond those optimally required to demonstrate transcriptional activation of target genes by T₃.

What is clear, however, is that thyroid hormones are absolutely required for normal chondrocyte differentiation. Indeed, rat cartilage responses to GH *in vivo* require thyroid hormones (Lewinson *et al.* 1989, 1994). *In vitro*, in studies of three-dimensional aggregates of rat growth plate resting zone chondrocytes, T_4 stimulated their differentiation and the columnar organisation of hypertrophic chondrocytes (Ballock & Reddi 1994). Furthermore, other studies of primary cultured cartilage cells in rat, chicken and porcine species have shown that thyroid hormones induce expression of a hypertrophic phenotype (Burch & Lebovitz 1982a,b, Burch & McCarty 1984, Burch & Van Wyk 1987, Bohme *et al.* 1992, 1995, Quarto *et al.* 1992, Ballock & Reddi 1994), although the mechanisms by which this is achieved and the particular cells that respond to T_3 have not been characterised. More recently, these findings were extended by studies of T_3 -induced hypertrophy of bovine fetal growth plate chondrocytes in subsets of primary cultured cells derived from maturationally distinct prehypertrophic proliferative cells (Alini *et al.* 1996). These studies revealed that the most immature cells were directly responsive to T_3 and that T_3 could support matrix assembly, hypertrophic differentiation (alkaline phosphatase activity and collagen type X expression) and matrix calcification in serum-free cultures. Again, the molecular mechanisms responsible for these responses were not investigated, as cells were studied after 5–20 days of treatment. Thus it is unknown which genes are direct targets for T_3 during the initiation of hypertrophic chondrocyte differentiation.

Localisation of T_3R in rat tibial growth plate cartilage

In a preliminary study (Robson *et al.* 1997), we determined the expression of the T_3R $\alpha 1$ and $\beta 1$ proteins in the rat growth plate by means of immunohistochemistry (Fig. 2). In the reserve zone, all chondrocyte progenitor cells exhibited specific nuclear staining for T_3R $\alpha 1$ and $\beta 1$, whereas most proliferative zone chondrocytes stained weakly for both T_3R s. In contrast, chondrocytes throughout the hypertrophic zone to the region of provisional cartilage calcification did not express either T_3R isoform. In the metaphyseal primary spongiosum, the nuclei of invading osteoblasts stained strongly positive for both T_3R variants. Similarly, all osteoblasts and osteocytes in adjacent, highly active, metaphyseal and epiphyseal bone also stained strongly for T_3R $\alpha 1$ and $\beta 1$, whereas osteoclasts were negative. These data suggest that the primary target cells for T_3 within the growth plate are progenitor chondroblasts, immature proliferating chondrocytes and primary spongiosum osteoblasts, together with osteoblasts and osteocytes at adjacent sites of active bone turnover. Thus the localisation of T_3R expression in this region correlates well with the known effects of T_3 at the epiphyseal plate and enables us to consider how

thyroid hormone actions may integrate with other signalling pathways that act on the epiphyseal plate to stimulate linear growth.

Interactions between thyroid and other hormones and their effects on the skeleton

Interactions between T_3 and GH

Thyroid hormone is permissive for skeletal growth in that it is required for the expression of many of the skeletal actions of GH (Lewinson *et al.* 1989, Nilsson *et al.* 1994). Thus in hypothyroid children there is blunting of the GH response to provocative stimuli; this probably results from indirect effects of T_3 on pituitary GH secretion, rather than from direct T_3 actions at the hypothalamus (Underwood & Van Wyk 1992, Weiss & Refetoff 1996). The exact contribution of the change in GH status to the growth disorder seen in hypothyroidism is unclear however. GH does exert direct effects on the growth plate, as shown in several species (Loveridge & Farquharson 1993, Ohlsson *et al.* 1993, Nilsson *et al.* 1994, Spagnoli & Rosenfeld 1996), and expression of GH receptors has been clearly documented in human and rat epiphyseal plate chondrocytes (Werther *et al.* 1993). GH increases the width of the growth plate proliferative and hypertrophic zones *per se* in rats, and also stimulates the commitment of resting layer chondrocytes to a proliferative phenotype (Hunziker *et al.* 1994), although this also requires the presence of other pituitary hormones (Gevers *et al.* 1996). Expression of the GH receptor in rat epiphyseal growth plate chondrocytes is likely to be developmentally regulated, with expression in the hypertrophic zone being apparent from birth, and expression in the resting and proliferative zones occurring later when the GH-dependent growth period commences (Ohlsson *et al.* 1993, Werther *et al.* 1993).

Interactions between T_3 and IGF-I

In addition, thyroid hormones influence the IGF-I signalling pathway. In the rat, T_3 increases hepatic expression of IGF-I mRNA (Wolf *et al.* 1989) and enhances the direct effects of IGF-I on cartilage (Ohlsson *et al.* 1992, Lewinson *et al.* 1994). Thus hypothyroid cartilage may be refractory to the induction of IGF-I synthesis by GH; in fact, IGF-I immunostaining within condylar cartilage is reduced in hypothyroid rats and requires thyroid hormone for its restoration (Lewinson *et al.* 1994). In humans with hypothyroidism, there are reductions in GH-binding protein, IGF-I, IGF-II, IGFBP-3 and IGF bioactivity; these changes are reversed after thyroid hormone replacement (Miell *et al.* 1993). Furthermore, in studies of cultured rat and mouse osteoblastic cells, it has been suggested that IGFBPs-2 (Schmid *et al.* 1992) and -4

(Glantschnig *et al.* 1996) may be directly responsive to T_3 , whereas IGF-I receptor mRNA is induced in rat chondrocytes after 96 h of T_3 treatment (Ohlsson *et al.* 1992). Although the actions of GH and IGF-I are closely related, their direct effects on growth plate chondrocytes are likely to differ. Whilst GH receptors are expressed in the resting zone, IGF-I mRNA and protein are not, although IGF-I expression within the remainder of the rat growth plate may be regulated by GH (Ohlsson *et al.* 1993, Nilsson *et al.* 1994). The localisation of IGF-I receptors within discrete layers of the epiphyseal growth plate has not been reported, so IGF-I target cells cannot be identified with certainty, although it is likely that receptors are located predominantly in proliferating cells (Loveridge & Farquharson 1993).

It is apparent that the interplay between GH, IGF-I and T_3 at the growth plate is complex. It has been proposed that GH stimulates differentiation of resting zone chondrocyte precursor cells and that IGF-I stimulates their subsequent clonal expansion. The role of T_3 is postulated to terminate clonal expansion and proliferation, and to induce terminal hypertrophic chondrocyte differentiation (Ohlsson *et al.* 1993).

Oestrogen effects on growth and expression of its receptor in bone

In humans, oestrogens are essential for the growth spurt at puberty and for epiphyseal growth plate fusion. The effects of oestrogen are not well defined, but are likely to be both indirect and direct, as oestrogens influence GH secretion in man (Nilsson *et al.* 1994), and human chondrocytes have been shown to express oestrogen receptors (ER) in intact bone (Braidman *et al.* 1995) and *in vitro* (Rosner *et al.* 1982, Dayani *et al.* 1988). In addition, expression of ER protein has been demonstrated in osteocytes, but not osteoblasts, within intact human, porcine and guinea pig bone slices, using immunohistochemical techniques (Braidman *et al.* 1995), whereas low levels of ER have been documented in primary cultures of human and murine osteoblasts (Pensler *et al.* 1990, Ikegami *et al.* 1993).

Interactions between T_3 and oestrogen

Early studies of cultured rat osteoblastic cells were suggestive of interactions between T_3 and oestrogens in bone

(Egrise *et al.* 1990). Recent studies have investigated interactions between oestrogen and T_3 *in vivo* using the ER agonist, oestradiol, the partial agonist/antagonist, tamoxifen, and the pure antagonist, ICI 182 780, in the ovariectomized-thyroidectomized rat (DiPippo *et al.* 1995, DiPippo & Powers 1997). These studies have suggested that the effects of oestrogen on the skeleton may result from a direct antagonism of the normal actions of T_3 . In particular, tamoxifen appeared to act as a T_3 antagonist in terms of its ability to increase tibial length. These interactions have been proposed to be mediated by cross-talk or competition between T_3R and ER at the level of DNA binding in target gene promoters, as both receptors recognise identical consensus hormone response-element binding-site sequences. Some support for this at the molecular level has been obtained *in vitro* (Shao *et al.* 1995, Zhu *et al.* 1996, Klinge *et al.* 1997).

Oestrogen resistance

The effects of oestrogen on the skeleton are likely to be important in both sexes in humans, given the skeletal abnormalities (delayed epiphyseal closure, eunuchoid proportions, osteoporosis) seen both in a male patient with oestrogen resistance caused by a homozygous mutation of the ER (Smith *et al.* 1994) and in a second male with aromatase deficiency (Morishima *et al.* 1995). The clear-cut epiphyseal plate abnormality and bone loss seen in oestrogen resistance contrast with the complexities of the skeletal phenotype seen in RTH syndromes, as described earlier. In the single case of an ER mutation causing recessive hormone resistance (Smith *et al.* 1994), the homozygous defect resulted in complete absence of a functional ER protein, thereby contrasting with the heterozygous dominant-negative nature of RTH. For this reason, it is difficult to compare the skeletal phenotypes between the two conditions; however, it is intriguing to speculate that variability in the RTH phenotype may in part result from abnormal interactions between mutant T_3R β and normal ER proteins.

Androgen effects on growth and expression of its receptor in cartilage

Androgens are also important for male linear growth and epiphyseal maturation and closure. Testosterone binding

Figure 2 Longitudinal sections (original magnification $\times 400$) of decalcified rat proximal tibiae, showing immunolocalisation of T_3R $\alpha 1$ (left panels) and T_3R $\beta 1$ (right panels) within distinct zones of the growth plate, using isoform-specific rabbit polyclonal antibodies against human T_3R $\alpha 1$ and T_3R $\beta 1$ proteins (Macchia *et al.* 1990, Falcone *et al.* 1992), which we have demonstrated to recognise rat T_3R isoforms specifically in bone cells, using western blotting (Williams *et al.* 1994, Bland *et al.* 1997). The upper panels show reserve (RZ), proliferative (PZ) and proximal hypertrophic (HZ) zones; the lower panels show the distal hypertrophic zone (HZ) and metaphyseal primary spongiosum (MPS). Nuclei staining positive for T_3R $\alpha 1$ or T_3R $\beta 1$ proteins were visualised using diaminobenzidine in sections counterstained with haematoxylin. Marked specific nuclear staining was seen in all cells of the RZ, whereas most PZ chondrocytes also stained positive for both T_3R s. In contrast, chondrocytes throughout the HZ did not stain for either T_3R isoform. In the MPS, nuclei of invading osteoblasts stained strongly positive for both T_3R $\alpha 1$ and $\beta 1$ proteins. Arrows indicate examples of positive-staining nuclei in PZ chondrocytes and MPS invading osteoblasts.

sites have been described in epiphyseal chondrocytes from many species, including humans (Carrascosa *et al.* 1990), and differing responses to both oestrogen and testosterone between the sexes have been reported *in vitro* in humans, rabbits and rats (Corvol *et al.* 1987, Carrascosa *et al.* 1990, Somjen *et al.* 1991, Schwartz *et al.* 1994). 5 α -Reductase activity has also been detected in rat and human epiphyseal chondrocytes (Ackerman & Hamilton 1976, Blanchard *et al.* 1991), and aromatase activity is expressed in human cartilage (Blanchard *et al.* 1991). Androgens stimulate mineralisation of rat bone matrix implants *in vitro* (Kapur & Reddi 1989), increase rat tibial growth plate width when injected directly (Ren *et al.* 1989), and may promote rat chondrocyte differentiation (Schwartz *et al.* 1994), although others have documented increased proliferative responses of rat chondrocytes to both testosterone and oestrogen *in vivo* and *in vitro* (Somjen *et al.* 1991).

Interactions between sex steroids and GH/IGF-I signalling

Both androgens and oestrogens have also been reported to interact with the IGF-I pathway in rabbit chondrocytes (Itagane *et al.* 1991), although others have shown that testosterone does not affect IGF-I expression in the liver or alter IGF and IGF-I receptor expression in the growth plate of rats (Phillip *et al.* 1992). Androgens and oestrogens differentially regulate GH receptor mRNA expression (induced by testosterone, inhibited by oestrogen) in rabbit liver and growth plates, and both hormones stimulate GH secretion (Yu *et al.* 1996). There are no reports, however, of interactions between androgens and T₃ in skeletal cells or on the control of linear growth in any species.

1,25(OH)₂-vitamin D₃ effects on growth and expression of its receptor in cartilage

Vitamin D deficiency in man produces abnormal expansion of the growth plate as a result of widening of the hypertrophic zone; these features are also seen in the epiphyses of VDR knockout mice (Yoshizawa *et al.* 1997). This could be due either to a delay in hypertrophic chondrocyte apoptosis or to acceleration of proliferating chondrocyte differentiation to hypertrophic cells; evidence from *in vitro* studies implicates the latter mechanism. Treatment of rabbit growth plate chondrocytes with 1,25(OH)₂-vitamin D₃ results in reduced alkaline phosphatase activity and a delay in differentiation to the hypertrophic phenotype (Kato *et al.* 1990). These effects are opposite to those induced by T₃. Thus 1,25(OH)₂-vitamin D₃ inhibits precocious chondrocyte hypertrophy and its deficiency destroys the balance of inhibition and stimulation of chondrocyte maturation, leading to widening of the hypertrophic zone in rachitic growth plates. Nuclear receptors for 1,25(OH)₂-vitamin D₃ have been demonstrated in murine, rat and rabbit chondrocytes *in vitro* (Jikko *et al.* 1993, Klaus *et al.* 1994, Balmain *et al.*

1995, Akiyama *et al.* 1996) and in sections of chicken and rat growth plates (Klaus *et al.* 1991, Berry *et al.* 1996). They are expressed throughout the growth plate region, but are most concentrated in proliferative and hypertrophic cells (Klaus *et al.* 1991, Berry *et al.* 1996). Studies on murine cells *in vitro* suggest that the recruitment of chondrocytes from progenitor stem cells and the progression of chondrocyte differentiation are both inhibited by 1,25(OH)₂-vitamin D₃ (Akiyama *et al.* 1996).

Retinoid effects on growth and expression of their receptors in cartilage

Retinoids exert profound effects on the developing skeleton and have important roles in limb bud development, anterior/posterior axis orientation, chondrogenesis, growth plate maturation and chondrocyte apoptosis. Retinoids are potent teratogens that cause intrauterine abnormalities of limb formation and promote epiphyseal plate closure in animal studies (Standeven *et al.* 1996). Expression of retinoid receptors has been widely documented in developing cartilage and their ontogeny has been implicated in control of endochondral bone formation during mouse embryogenesis (Noji *et al.* 1989, Dolle *et al.* 1990, Ruberte *et al.* 1990, Mendelsohn *et al.* 1991, Lohnes *et al.* 1993, Luo *et al.* 1995, Sucov *et al.* 1995). The major effects of retinoids on skeletal formation occur during chondrogenesis, and RAR α is required for initiation of chondrocyte differentiation (Cash *et al.* 1997). Northern blotting analysis of retinoid receptor expression in cultured rat chondrocytes has revealed expression of RAR α and γ and RXR α and β mRNAs, whereas RAR β and RXR γ transcripts are not expressed (Hagiwara *et al.* 1996). Retinoids regulate collagen gene transcription and suppress expression of the chondrocyte phenotype, in cultured rat and rabbit cells, that is characterised by collagen type II expression (Ballock *et al.* 1994, Benya & Padilla 1986). All-*trans* retinoic acid inhibits cartilage matrix synthesis (Horton *et al.* 1987), and both all-*trans* retinoic acid and 9-*cis* retinoic acid decrease rat chondrocyte proliferation in culture (Hagiwara *et al.* 1996). Thus retinoids promote the differentiation of cultured chondrocytes to cells with a more osteoblast-like phenotype (Gentili *et al.* 1993).

Interactions between T₃ and 1,25(OH)₂-vitamin D₃ and retinoids

Whereas T₃R, VDR, RAR and RXR proteins are each expressed in cartilage, there is no formal documentation to show which of the various receptor isoforms are co-expressed in individual chondrocytes, as has been shown in rat osteoblastic cells (Williams *et al.* 1994, Bland *et al.* 1997). Despite this, antagonistic interactions between 9-*cis* retinoic acid and 1,25(OH)₂-vitamin D₃ during chick limb bud chondrogenesis, which implicate RXR and VDR heterodimer formation, have been documented

Figure 3 In the upper part of the figure, some of the signals that contribute to progression of a chondrocyte from the reserve zone through proliferation and on to hypertrophy are shown; a representation of the growth plate regions in which various hormone receptors and the Ihh/PTHrP feedback loop components are expressed is shown in the lower part. The known and proposed actions of T₃ are shown at the top of the figure. Reserve zone chondrocytes differentiate to proliferating cells by a process that is stimulated by GH and may be affected by T₃, as reserve cells express T₃Rs. Proliferating cells undergo clonal expansion, a process that is stimulated by IGF-1 and probably inhibited by T₃, and begin to express the PTH/PTHrP receptor before commitment to hypertrophy. Terminal differentiation towards the hypertrophic phenotype is induced by T₃ and committed cells synthesise and secrete Ihh. Ihh acts on perichondrial cells to stimulate synthesis and release of PTHrP. PTHrP acts on uncommitted proliferating cells that express the PTH/PTHrP receptor to inhibit cell differentiation, thereby delaying cell maturation and reducing Ihh synthesis to complete a feedback control loop (Lanske *et al.* 1996, Vortkamp *et al.* 1996). In addition, T₃ probably acts directly on primary spongiosum osteoblasts to facilitate their invasion from the bone marrow vasculature. The known extent of expression of growth hormone receptor (GHR) (Ohlsson *et al.* 1993, Werther *et al.* 1993), T₃ receptor α 1 and β 1 (T₃R) (Robson *et al.* 1997), vitamin D receptor (VDR) (Klaus *et al.* 1991, Berry *et al.* 1996), oestrogen receptor (ER) (Braidman *et al.* 1995), and IGF-1 (Loveridge & Farquharson 1993, Ohlsson *et al.* 1993) proteins within the growth plate are shown for comparison with the region in which components of the feedback loop are expressed. The IGF-1 receptor (IGF-R) has been suggested to be expressed within the proliferative zone (Loveridge & Farquharson 1993). A detailed analysis of the growth plate regions and individual chondrocytes that co-express various combinations of these molecules will facilitate development of a molecular understanding of the regulation of endochondral bone formation.

recently (Tsonis *et al.* 1996). Furthermore, a recent report suggested that T₃-induced terminal differentiation of rat growth plate chondrocytes is inhibited by 1,25(OH)₂-vitamin D₃ and retinoids (Zhou *et al.* 1997), indicating that interactions between these hormones are likely to play a part in the mediation of T₃ action in cartilage, as we have shown in osteoblastic cells (Williams *et al.* 1994, Williams *et al.* 1995, Bland *et al.* 1997).

Ihh/PTHrP feedback loop

Ihh is a member of a conserved family of secreted proteins that have key roles in embryonic patterning in many

organisms. In *Drosophila*, the Hedgehog protein regulates embryonic segmentation and anterior/posterior polarity during development. In vertebrates, three Hedgehog family members have been identified and these include the Ihh protein, which is expressed at high levels in prehypertrophic chondrocytes. PTHrP is structurally homologous to PTH, binds to a common PTH/PTHrP surface receptor and is produced in numerous tissues, including the growth plate perichondrium, from where it is secreted to act on receptors expressed in growth plate chondrocytes. Recent studies using chicken and mouse tissues have convincingly demonstrated that Ihh and

PTHrP target PTH/PTHrP receptor expressing growth plate chondrocytes in a negative feedback loop to regulate the co-ordinated progression of chondrocyte maturation and hypertrophy (Lanske *et al.* 1996, Vortkamp *et al.* 1996). The feedback loop is shown schematically in Fig. 3, where its relationship to other hormone signalling pathways that act on the growth plate is also shown.

Thus prehypertrophic chondrocytes that are committed to the hypertrophic phenotype express and secrete Ihh, which regulates the rate of hypertrophic differentiation. Ihh acts directly on cells within the adjacent perichondrium to stimulate production and release of PTHrP. PTHrP is secreted, and acts, via its receptor on prehypertrophic uncommitted proliferative cells, to maintain cells in a proliferative state and delay or inhibit the onset of hypertrophy (Lee *et al.* 1996). The level of Ihh expression by committed cells has been proposed to regulate the number of cells leaving the proliferative stage to enter hypertrophy. As cells progress to full hypertrophy, the level of expression of Ihh diminishes and the negative feedback loop is attenuated, thereby allowing terminal differentiation to be completed; the more Ihh is secreted, the fewer cells are committed to the hypertrophic state, and *vice versa*. Thus both PTHrP and Ihh signalling pathways converge on PTH/PTHrP receptor expressing cells, and both signalling molecules act to maintain chondrocyte proliferation and delay hypertrophic differentiation (Lanske *et al.* 1996, Vortkamp *et al.* 1996, Wallis 1996).

Possible relationship between T_3 and Ihh/PTHrP signalling

These actions are opposite to those of T_3 , which facilitates the differentiation of growth plate chondrocytes and the recruitment of cells to the proliferating zone (Lewinson *et al.* 1989, Carrascosa *et al.* 1992, Quarto *et al.* 1992, Ballock & Reddi 1994, Lewinson *et al.* 1994). At present, the relationship between T_3 signalling and the activity of the Ihh/PTHrP feedback loop is not known, although T_3 R proteins appear to be expressed in regions of the growth plate that may overlap with areas in which Ihh and PTH/PTHrP are expressed. It is possible, therefore, that T_3 may induce terminal hypertrophic chondrocyte differentiation by inhibiting the expression or actions of Ihh or the PTH/PTHrP receptor, both of which must be considered as potential T_3 target genes in the epiphyseal growth plate.

Conclusions

The physiological and clinical effects of thyroid hormones on linear growth are well established. Growth plate responses to thyroid hormones have been detailed in histological studies and the effects of T_3 on chondrocyte differentiation have been documented by cell biological

methods. Surprisingly, however, specific populations of T_3 target cells in growth plates have not yet been definitively identified and the molecular mechanisms of T_3 action in cartilage have not been studied. These issues present a major challenge, as the actions of T_3 at the growth plate involve highly complex interactions with other, incompletely defined, hormone signalling pathways.

It will be important, therefore, to localise precisely the expression of T_3 R in epiphyseal chondrocytes in relation to target cells for other interacting hormones (Fig. 3) and to determine how T_3 R expression is regulated during growth plate development and by other hormones. Such studies will facilitate the development of strategies for identification of T_3 target genes associated with chondrocyte differentiation, and should help to define the molecular basis for interactions between T_3 and other endocrine pathways in cartilage. Ultimately, a detailed analysis of the mechanism of T_3 action in growth plate chondrocytes will contribute significantly to our understanding of the factors that regulate endochondral ossification during long bone growth.

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