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**Micronutrients and longitudinal growth**

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Although the supply of macronutrients such as calcium and phosphorus are known to be important in skeletal growth, the role of micronutrients is, as yet, poorly defined. Bone is a complex heterogeneous tissue which supports the musculature and, thus, its growth and development are intimately connected with overall body growth. It also serves as a Ca and P reservoir which can be accessed during disturbances in mineral homeostasis. For instance, acidic diets lead to an increase in urinary Ca with a consequent increase in parathyroid hormone secretion leading to a loss of Ca from the skeleton (Abu-Damir *et al.* 1991).

Cellular activity is, in part, dependent on the surrounding matrix (Nathan & Sporn, 1991) but current methods for investigating the biochemistry of bone cells rely on the use of isolated cells (see Zanelli & Loveridge, 1990) and these cannot account for the interactions between cell types or between the cells and their extracellular matrix. Over the last few years we have developed approaches allowing the investigation of bone cells maintained *in situ*. This has led to sensitive methods for investigating the effect of hormones and growth factors (see Zanelli & Loveridge, 1990; Loveridge *et al.* 1992) on the biochemistry of bone cells which have been applied to particular skeletal disorders (Farquharson *et al.* 1992*b,c*). The present review will focus on the cell biology, biochemistry and endocrinology of longitudinal bone growth and the possible roles of various micronutrients.

THE GROWTH PLATE

During longitudinal growth, chondrocytes undergo a series of well-defined stages which are characterized by changes in proliferation, shape and size, and synthesis and deposition of extracellular matrix components (Brighton, 1978; Hunziker *et al.* 1987; Loveridge *et al.* 1992). Because of the spatial relationships within the growth plate it is possible to easily identify cells at these stages. The rate of longitudinal growth is dependent on a combination of the proliferative rate, which adds new cells to the growth plate, and the rate of maturation or hypertrophy which results in increased matrix synthesis and, thus, the expansion of the growth plate. For example, the proliferation rate in the chick growth plate is only twice that of the rat even though the rate of

longitudinal growth of the broiler chick is approximately five times higher. The regulation of bone growth through chondrocyte proliferation and maturation is not fully understood despite many studies with cell and explant culture and whole-body experiments (see Centrella & Canalis, 1985; Isaksson *et al.* 1987; Loveridge *et al.* 1990, 1992).

#### *Resting and proliferative zones*

Resting chondrocytes (the stem, germinal or reserve layer) have a fibroblastic phenotype and a low mitotic rate. During growth some cells are committed to differentiate and enter the proliferating zone. Growth hormone (GH) is considered to be the main regulator of postnatal bone growth but there is controversy regarding its action. Traditionally it has been assumed that GH acts by increasing hepatic IGF-I production (Salmon & Daughaday, 1957), but direct effects of GH on chondrocytes *in vivo* and *in vitro* have been reported (Isaksson *et al.* 1987). Resting chondrocytes respond to GH, whereas IGF-I acts on the more committed progenitor cell suggesting that locally produced IGF-I is a second effector in GH action. Receptors to IGF-I are found predominantly on proliferating chondrocytes, whereas the stem cells have the highest concentration of GH receptors. However, some workers have failed to find stimulation and growth of chondrocytes by GH (Makower *et al.* 1989). Thus, the relative contributions of the direct or indirect effects of GH have still to be elucidated. Our preliminary studies (Loveridge *et al.* 1993) show that short-term administration of a GH antiserum did not alter IGF-I levels or the rate of proliferation. However, it reduced the size of the proliferative zone, adding strong support to the hypothesis that GH acts to 'commit' chondrocytes to proliferate.

In addition to regulating chondrocyte proliferation (Nilsson *et al.* 1986), possibly through increased glucose 6-phosphate dehydrogenase (*EC* 1.1.1.49) activity (Farquharson & Loveridge, 1990), IGF-I also stimulates matrix synthesis of collagen and proteoglycans (Guenther *et al.* 1982). The covalently-linked collagen types II and IX are the predominant collagen types in this zone with less produced by hypertrophic chondrocytes (Leboy *et al.* 1988).

#### *Transitional or prehypertrophic zone*

Maturation to the hypertrophic state results in the chondrocytes becoming more voluminous and spherical with increases in rough endoplasmic reticulum and Golgi apparatus. Maturing chondrocytes initiate changes in the distribution and concentration of major extracellular matrix components such as collagen types II, IX, X and XI, fibronectin and proteoglycans (see Boskey, 1989).

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is found in the transitional chondrocytes (Thorpe *et al.* 1992). Its major role is the inducement of a differentiated phenotype (Seyedin *et al.* 1987) as evidenced by the increased amounts of TGF- $\beta$  mRNA, protein and receptor in the hypertrophic chondrocytes. In cell culture TGF- $\beta$  increases DNA synthesis and decreases type II and X collagen production, decreases alkaline phosphatase (*EC* 3.1.3.1; ALP) activity, and increases or decreases proteoglycan synthesis (see Loveridge *et al.* 1992).

Fibroblast growth factor (FGF) has a similar location in the growth plate to that of TGF- $\beta$  and these growth factors synergistically stimulate chondrocyte differentiation.

Crabb *et al.* (1990) suggested that the c-myc oncogene may underlie the synergy observed between TGF- $\beta$  and FGF which is consistent with the high levels of c-myc-proto-oncogene found in proliferating and differentiating growth plate chondrocytes (Farquharson *et al.* 1992a).

### *Hypertrophic zone*

Ultrastructural examination of hypertrophic chondrocytes maintained *in situ* (Hunziker *et al.* 1984) has indicated that terminal hypertrophic chondrocytes play a major role in regulating ossification. Little is known, however, about chondrocyte death. Hunziker *et al.* (1984) proposed that capillary invasion of the most distal hypertrophic chondrocytes leads to monocyte invasion and active destruction of the cell (Farnum & Wilsman, 1987). This is contrary to the hypotheses that hypertrophic chondrocytes die due to low oxygen tension and inadequate diffusion of metabolites or that they persist after capillary penetration.

Mineralization is restricted to the extracellular matrix surrounding the hypertrophic chondrocytes. ALP has long been associated with mineralization and inhibition of ALP impairs calcification (Register & Wuthier, 1984), although its exact role is unclear. Active ALP is found only in the mineralizing and pre-mineralizing chondrocytes (de Bernard *et al.* 1986; Farquharson *et al.* 1992c) suggesting that activation is a key event during calcification.

Collagen type X is unique to the matrix surrounding hypertrophic chondrocytes where it constitutes 20% of the total collagen production (Bashey *et al.* 1991) and has been associated with mineralization. Other proposed roles for type X include inhibition of calcification, stimulation of angiogenesis and targeting cells for osteoclastic removal.

Many other matrix proteins are thought to play a role in mineralization (Boskey, 1989). These include chondrocalcin, proteoglycans, osteonectin, osteocalcin and phosphoproteins. Their role may be to act either as nucleators (Neuman & Neuman, 1953) or inhibitors of crystal growth, possibly depending on whether they are attached to collagen or are in solution. Matrix vesicles are considered to be the initial centres of nucleation in the calcification process (Bonucci, 1990). They are enriched in Ca, Ca-binding phospholipids and ALP which could trigger the precipitation of the initial hydroxyapatite crystals via the formation of Ca-phospholipid-phosphate complexes.

## MICRONUTRIENTS AND THE GROWTH PLATE

The term micronutrient includes both vitamins and trace elements and the role of these will be discussed further.

### *Vitamins*

*Vitamin D.* The effect of sunlight on maintaining skeletal integrity has been recognized since ancient times and vitamin D is one of the most important factors in bone metabolism. Part of this effect is related to the regulation of Ca homeostasis, but 1,25-dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D<sub>3</sub>) also plays a role in chondrocyte differentiation. Growth plate chondrocytes bind both 1,25(OH)<sub>2</sub>D<sub>3</sub> (Suda *et al.* 1985) and 24,25-dihydroxycholecalciferol (Corvol *et al.* 1980) the two most potent vitamin D metabolites. 1,25(OH)<sub>2</sub>D<sub>3</sub> is known to regulate a number of factors important in growth

plate metabolism. Collagen is the primary organic component and its synthesis is regulated by  $1,25(\text{OH})_2\text{D}_3$  (Minghetti & Norman, 1988), while the fibrillar matrix is stabilized by covalent crosslinks formed by lysyl oxidase which is also regulated by this hormone.

Vitamin D metabolites also regulate TGF- $\beta$  (Pfeilschifter & Mundy, 1987) and proto-oncogene expression (Minghetti & Norman, 1988), both of which are important in the transition from proliferation to differentiation (Farquharson *et al.* 1992b). They modify responses to parathyroid hormone (Bradbeer *et al.* 1988) and regulate other matrix proteins (Hinek & Poole, 1988) and alkaline phosphatase activity (Schwartz *et al.* 1988). In general it causes an increase in avian and mammalian cartilage growth (Burch *et al.* 1988).

*Vitamins A, C and K.* The effects of vitamin A on bone metabolism were first reported by Mellanby (1947). It is now considered to be essential during limb bud development in the fetus (Brickell & Tickle, 1989) and it may have a role in the remodelling of the skeleton. Vitamin C is important in the activity of lysyl oxidase which is involved in the cross-linking of the collagen molecules. However, because of the relatively rapid turnover of collagen within the growth plate, the importance of this vitamin in growth plate function is unclear. Vitamin K is important in the  $\gamma$ -carboxylation of osteocalcin, a protein relatively unique to bone which is capable of binding Ca.

#### *Trace elements*

The term 'trace element' was used to describe any mineral which occurred in living tissues in concentrations too small to be accurately measured by early analytical techniques and, although an increasing number of trace elements are being designated as essential to animal health and development, there is constant debate over what criteria should be used to assess essentiality (Underwood & Mertz, 1987). A large number of trace elements affect longitudinal growth and bone metabolism (Beattie & Avenell, 1992), but for the purposes of the present review we will confine attention to the major influences on the growth plate.

*Copper.* The essentiality of Cu in bone metabolism and in maintenance of skeletal integrity has been demonstrated in many species. Skeletal abnormalities can result from a primary Cu deficiency or by secondary Cu deficiency when induced by ammonium tetrathiomolybdate in rats or molybdenum in cattle (Davis & Mertz, 1987).

Histological findings in both primary and secondary hypocuprosis include thickening of the epiphyseal growth plate cartilage, reduced desposition of the bone matrix, thinning of the cortical bone and osteoporosis with impaired osteoblastic activity. Biochemically, Cu is an essential cofactor of the enzyme lysyl oxidase which is involved in the mechanism of crosslinking of collagen fibres, necessary to provide the bone structure with its strength and rigidity.

*Molybdenum.* The actual, minimum requirement of Mo remains unknown, but a level of  $<0.02$  mg/kg in the diet has been estimated for chicks and rats (Mills & Davis, 1987), providing that dietary factors which interfere with Mo utilization, such as tungsten, are absent. More widely recognized is the toxic effect of the element, although this is dependent on species and age as well as the chemical form. In addition, Mo can interact with other nutrients, and the interaction which is most significant is that with Cu and sulphur which markedly reduces bioavailability of Cu (Mills & Davis, 1987).

At high concentrations, Mo induces changes in the epiphyseal growth plate through its effects on Cu metabolism, but it is unclear whether or not Mo can induce changes independent of its effects on Cu status. Our recent studies indicate that Mo causes a significant reduction in longitudinal bone growth both in the presence and absence of induced Cu deficiency. Growth plate width was increased only in Cu-deficient animals and was primarily related to a failure in chondrocyte maturation. Mo also inhibited both glucose-6-phosphate dehydrogenase activity and cell proliferation. Thus, Mo can induce changes in longitudinal bone growth which are distinct from those resulting from Cu deficiency.

*Manganese.* Among the most marked effects of Mn deficiency are the skeletal abnormalities, including retarded bone growth, defective skull development and enlargement of the joints (Hurley & Keen, 1987). Retarded longitudinal growth appears to be related to an inhibition of endochondral ossification primarily resulting from defects in the proteoglycan content of the growth plate, particularly in sulphation of chondroitin sulphate. This in turn is related to the activation of the glycosyl transferase enzymes which are activated by Mn and are necessary for the formation of the polysaccharide and the linkage between the sugar and the protein.

#### SUMMARY

The present review has concentrated on the control of longitudinal growth and the relative importance of certain micronutrients. By far the most significant of these is 1,25(OH)<sub>2</sub>D<sub>3</sub> which is now being recognized, not only for its role in maintaining Ca homeostasis, but also its major role in chondrocyte differentiation within the growth plate.

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