RECENT RESEARCH ON THE GROWTH PLATE

Impact of inflammatory cytokines on longitudinal bone growth

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Abstract

Children with inflammatory diseases usually display abnormal growth patterns as well as delayed puberty. This is a result of several factors related to the disease itself, such as malnutrition, hypercortisolism, and elevated levels of pro-inflammatory cytokines. These factors in combination with glucocorticoid treatment contribute to growth retardation during chronic inflammation by systemically affecting the major regulator of growth, the GH/IGF1 axis. However, recent studies have also shown evidence of a direct effect of these factors at the growth plate level. In conditions of chronic inflammation, pro-inflammatory cytokines are upregulated and released into the circulation. The most abundant of these, tumor necrosis factor α, interleukin 1β (IL1β), and IL6, are all known to directly act on growth plate cartilage to induce apoptosis and thereby suppress bone growth. Both clinical and experimental studies have shown that growth retardation can partly be rescued when these cytokines are blocked. Therefore, therapy modulating the local actions of these cytokines may be effective for preventing growth failure in patients with chronic inflammatory disorders. In this review, we report the current knowledge of inflammatory cytokines and their role in regulating bone growth.

Key Words
- cytokines
- inflammation
- growth plate
- chondrocyte
- growth retardation

Normal regulation of bone growth

The process of longitudinal bone growth is complex and tightly regulated by several factors. Hormones play a major role in the regulation of longitudinal bone growth and most central are the growth hormone/insulin-like growth factor 1 (GH/IGF1) axis, thyroid hormones, and sex steroids (Wit & Camacho-Hubner 2011). Nutrition is also an important regulator of growth, where poor nutrition leads to stunted growth (Gat-Yablonski et al. 2011, Marcovecchio & Chiarelli 2013).

Longitudinal bone growth is the result of a process called endochondral ossification, by which the embryonic cartilaginous model of most bones is gradually replaced by calcified bone (Mackie et al. 2008). Growth occurs at the growth plate, a thin layer situated at both ends between the diaphysis and the epiphysis of all long bones (Kronenberg 2003). The growth plate contains only one cell type, the chondrocyte, distributed at different levels of differentiation within the resting, proliferative, and hypertrophic zones (Fig. 1). Chondrocytes are recruited...
from the resting layer to the proliferative layer, where they actively proliferate and thereafter undergo hypertrophy. The newly formed cartilage is invaded by blood vessels and bone cell precursors, which remodel the hypertrophic zone cartilage into bone (Mackie et al. 2008).

The GH/IGF1 axis is the major regulator of longitudinal bone growth (Fig. 1). GH is secreted from the pituitary gland under the control of GH-releasing hormone (GHRH) and plays an important role only in postnatal bone growth. GH deficiency leads to growth retardation, which has been seen in GH receptor-knockout mice, while high levels of GH have the opposite effect, by causing gigantism (Pass et al. 2009). The systemic actions of GH are thought to be mediated by IGF1 (Le Roith et al. 2001). IGF1 is produced systemically by the liver and plays an important role during both embryonic and postnatal growth in rodents and humans (Le Roith et al. 2001, Martensson et al. 2004). Igf1-knockout mice show growth retardation and low levels of IGF1 in serum; this is also one of the most frequently observed abnormalities in patients with chronic inflammatory diseases, and perhaps a reason for the often observed growth retardation in these patients (Lupu et al. 2001, Simón 2010).

The original somatomedin hypothesis states that GH has only an indirect effect on the growth plate by stimulating the production of IGF1 from the liver, which in turn exerts its effects on the growth plate (Salmon & Daughaday 1957). Those experiments were carried out both in vivo in rats as well as in vitro in costal cartilage (Le Roith et al. 2001). However, several studies have also reported a direct effect of GH on the growth plate (Isaksson et al. 1982, Isgaard et al. 1988). In a study by Wang et al. (2004), GH was demonstrated to act both directly on resting/stem-like chondrocytes to stimulate proliferation, as well as indirectly, through IGF1, to promote chondrocyte hypertrophy. This observation does not exclude the possibility that the direct effect might be mediated by an increase in the local production of IGF1, which in turn stimulates growth plate chondrocytes. The GH receptor can also be detected in the chondrocytes of all zones of the growth plate and, therefore, a direct effect of GH has been suggested (Parker et al. 2007). Such a direct effect of GH is supported by a study where local injection of GH into the growth plate accelerated bone growth compared with the contralateral bone (Isaksson et al. 1982).

IGF1 has also been found to be produced by chondrocytes in the proliferative zone and its expression is increased upon stimulation with GH (Nilsson et al. 1986). This finding indicates that IGF1 has a specific role in the differentiation of chondrocytes through autocrine/paracrine mechanisms. Mice with a targeted deletion of Igf1 in chondrocytes have reduced body length, while serum IGF1 levels are normal, which highlights the importance of locally produced IGF1 in growth plate chondrocytes for the normal regulation of longitudinal bone growth (Govoni et al. 2007).

**Impact of inflammation on bone growth**

Growth is often impaired in children with chronic inflammatory diseases, such as inflammatory bowel disease (IBD), Crohn’s disease (CD), ulcerative colitis (UC), and juvenile idiopathic arthritis (JIA). Growth retardation occurs in up to 56% of children with CD and up to 10% of children with UC (Abraham et al. 2012), and several publications have reported short stature in JIA patients (Simón 2010). The degree of growth impairment is variable and can range from a mild decrease in growth velocity to severe forms of short stature, which can be defined as a body height more than two S.D. below the mean of the population. Children suffering from chronic inflammation are exposed to several factors contributing to growth failure such as malnutrition, glucocorticoid (GC) therapy, and pro-inflammatory cytokines (Ahmed & Savendahl 2009, Ahmed et al. 2013). Altogether, these factors act both systemically and locally at the growth plate level to suppress bone growth (Fig. 2).

It is well known that nutritional problems contribute to growth retardation in children with inflammatory diseases and this is mainly a result of an imbalance between consumed calories and energy requirements. The degree of malnutrition seems to be correlated with disease
severity (Simon 2010). However, based on the results of experiments carried out with pair-fed controls in a rat model of colitis, malnutrition should only account for about 60% of the final growth impairment, whereas the remaining reduction in growth would result from the inflammatory process itself (Ballinger 2000). Elevated levels of circulating pro-inflammatory cytokines can reduce energy uptake and metabolism as well as induce protein catabolism. Studies in mice have shown a significant decrease in IGF1 levels as well as a reduction in GH receptor expression in the growth plate during food restriction (Gat-Yablonski et al. 2008, Pando et al. 2012). Furthermore, malnutrition often leads to delayed sexual development that indirectly results in growth retardation linked with negative effects on the GH/IGF1 axis (Ezri et al. 2012).

During early onset of inflammation, the levels of circulating endogenous GCs are increased via the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Hardy et al. 2012). GCs are steroid hormones that bind to and activate the glucocorticoid receptor (GR), playing an important role during early development. The growth-suppressing effects of GCs can be mediated through both systemic and local mechanisms. GCs suppress the GH/IGF1 axis through inhibiting GH secretion (Mushtaq 2002) and downregulating GH receptors in the liver, thereby inhibiting IGF1 production and activity (Kritsch et al. 2002). The effect of GCs on pubertal development has also been studied but the results vary and are still debated (Kinouchi et al. 2012). Direct effects of GCs on growth plate cartilage include inhibition of chondrocyte proliferation and mineralization, as well as increased apoptosis (Chrysis et al. 2002).

Cytokines, normally upregulated under conditions of chronic inflammation, can act individually or in combination to suppress longitudinal bone growth (MacRae et al. 2006a). They not only have direct local effects at the growth plate level, but can also act systemically by suppressing IGF1 (Martensson et al. 2004, MacRae et al. 2006b). Cytokines are signaling molecules that mediate intercellular and intracellular communication and the most abundant cytokines that are upregulated in inflammatory diseases such as IBD and JIA are tumor necrosis factor α (TNFα), interleukin 1β (IL1β), and IL6 (MacRae et al. 2006a). Cytokines, such as IL1 or TNFα, have also been shown to inhibit the production of sex steroids by acting directly on the gonads or through suppression of gonadotropin-releasing hormone (GnRH) secretion (Hong et al. 2004).

Systemic effects of cytokines have been reported in transgenic mice overexpressing TNFα (TNF) and IL6, both models show growth retardation (De Benedetti et al. 1997, Li & Schwarz 2003). The IL6-overexpressing mice exhibit a defective growth phenotype with a size reduction of 50-70% compared with normal non-transgenic littermates. The growth retardation in these mice was associated with decreased levels of IGF1 and IGF1-binding protein 3 (IGFBP3), yet with normal distribution of GH pituitary cells and GH production (De Benedetti et al. 1997, De Benedetti et al. 2001). This finding is consistent with the situation in patients with JIA where high circulating levels of IL6 are negatively correlated with IGF1 and IGFBP3 levels (Gaspari et al. 2011). On the other hand, IL1β and IL6 have been shown to have the ability to stimulate GH secretion while TNFα does not seem to have this effect (Mainardi et al. 2002). TNFα and IL1β also have the potential to act directly at the growth plate level by decreasing chondrocyte proliferation and hypertrophy as well as by increasing apoptosis (Martensson et al. 2004). TNFα and IL1β have been shown to inhibit IRS1, Akt, and MAPK phosphorylation and to induce a state of IGF1 resistance in several different cell types. However, little is known about the mechanism involving growth plate chondrocytes (Pass et al. 2009, Farquharson & Ahmed 2013). Generally, IL6 has been considered to act systemically suppressing growth by altering the GH/IGF1 axis, although more recent studies have shown that IL6 also has direct effects on the growth plate chondrocytes (Nakajima et al. 2009, Fernandez-Vojvodich et al. 2013).

It is still unclear by which cellular mechanisms these pro-inflammatory cytokines act on the GH/IGF1 axis and the growth plate chondrocytes. Both IL1α and IL1β bind to...
the plasma membrane receptor IL1R1 that is expressed in all three zones of the growth plate in fetal rat metatarsal bones as well as in mice (Martensson et al. 2004, Simsma-Maziel et al. 2013). IL1R1 has recently been reported by Simsma-Maziel et al. (2013) to be expressed in ATDC5 chondrocytes in all stages of differentiation. They further showed that IL1β activates the p38 MAPK and NFκB pathways in ATDC5 cells throughout the stages of proliferation and differentiation. In the ATDC5 chondrocyte cell line, IL1β also reduces proteoglycan synthesis, and cell numbers, as well as mRNA expression of aggrecan, type II collagen, and type X collagen.

It has been suggested that members of the suppressor of cytokine signaling family (SOCS), especially SOCS2, may play a central role in cytokine-induced growth retardation (Ahmed & Farquharson 2010). SOCS proteins are signaling proteins that have the ability to downregulate cytokine and growth factor signaling by inhibiting the JAK/STAT signaling pathway. SOCS2 is a unique GH receptor inhibitor, as shown by the phenotype of the knockout mice, which grow 30–40% bigger than their normal littermates. Socs2-knockout mice have increased longitudinal bone growth and body length as well as wider proliferative and hypertrophic growth plate zones (Pass et al. 2009, 2012).

Pro-inflammatory cytokines and their action on the growth plate

There are only a few studies that have reported local effects of pro-inflammatory cytokines at the growth plate level. These studies have mostly focused on TNFα, IL1β, and IL6 and have reported evidence that individual cytokines have varying local effects on the growth plate chondrocytes in both organ and cell culture experiments. We first reported that TNFα can suppress longitudinal bone growth through direct actions on the growth plate cartilage in a model of cultured fetal rat metatarsal bones (Martensson et al. 2004). TNFα-treated bones grew slower in comparison with control bones, but only when exposed to a high concentration (100 ng/ml) of TNFα (Fig. 3a). The mechanisms behind this growth suppression included decreased chondrocyte proliferation and hypertrophy as well as increased apoptosis (Martensson et al. 2004). These findings were later supported by MacRae et al. (2006b), who also reported growth suppression when metatarsal bones were exposed to TNFα. TNFα was also found to reduce proteoglycan synthesis, and cell number, as well as mRNA expression of aggrecan, type II collagen, and type X collagen, when studied in the ATDC5 chondrocyte cell line (MacRae et al. 2006b). We have recently reported that
TNFα is produced endogenously throughout the growth plate and that treatment with etanercept, a soluble TNF receptor, leads to improved longitudinal bone growth of cultured fetal rat metatarsal bones (Fernandez-Vojvodich et al. 2012). Altogether, locally produced TNFα seems to play a role in the normal regulation of bone growth, while the negative effects of TNFα on chondrocytes have only been seen at very high concentrations.

Supraphysiological concentrations of IL1β have been reported to suppress longitudinal bone growth in cultured fetal rat metatarsal bones in the same way as TNFα does, through direct actions on the growth plate cartilage (Fig. 3b; Martensson et al. 2004). The mechanisms behind this effect include decreased chondrocyte proliferation, hypertrophy, and increased apoptosis. Treatment with anakinra, an IL1 antagonist that suppresses the actions of IL1β, improves bone growth in this experimental model (Fernandez-Vojvodich et al. 2012). IL1β was reported to be produced endogenously in growth plate chondrocytes, indicating that this cytokine also has a role in the normal regulation of bone growth (Fernandez-Vojvodich et al. 2012). Indeed, suppression of locally produced IL1β with anakinra was found to improve bone growth. IL1 receptor type 1 (IL1R1)-knockout mice have recently been reported to have normal growth, although their growth plates were narrower and had higher proteoglycan content when compared with those of WT animals (Simsa-Maziel et al. 2013). IL1β is produced by growth plate chondrocytes, and both this cytokine and its receptor seem to play a role in the normal regulation of bone growth. Furthermore, at high concentrations, IL1β impairs bone growth by directly targeting the growth plate, an effect that can be prevented with specific anti-cytokine treatment.

In the studies with metatarsal bones mentioned previously, IL6 did not have any effects on growth plate chondrocytes or growth (Martensson et al. 2004, MacRae et al. 2006b). However, contrary to the general consideration that IL6 only affects growth systemically, Nakajima et al. (2009) showed in a later study that IL6 inhibits the early differentiation of ATDC5 chondrocytes by inhibiting cartilaginous nodule formation and decreasing expression of type II collagen, aggrecan, and type X collagen. Interestingly, when fetal metatarsal bones were cultured with IL6 in combination with its soluble receptor IL6 Rα, bone growth was clearly decreased (Fernandez-Vojvodich et al. 2013; Fig. 3c). This growth retardation was associated with a decrease in the cell density of the proliferative zone as well as decreases in the length and area of the hypertrophic zone, thus providing further support for a local effect of IL6 on the growth plate.

Interactions between pro-inflammatory cytokines

Usually more than one cytokine is upregulated in inflammatory diseases, and therefore growth retardation might be the result of a synergistic effect of two or more cytokines. Interestingly, IL1β and TNFα have been shown to act in synergy to decrease longitudinal growth (Martensson et al. 2004). Growth inhibition was observed at far lower concentrations when these cytokines were combined rather than added separately. This synergistic effect could partly be prevented by treatment with anti-IL1β, anti-TNFα, or IGF1 (Martensson et al. 2004; Fig. 4). Similarly MacRae et al. (2006b) reported a 59% growth reduction in metatarsal bones treated with a combination of TNFα and IL1β. Catch-up growth has not been observed after combined treatment with TNFα and IL1β, indicating that even a short period of exposure to these cytokines may have an irreversible negative effect on longitudinal bone growth. It has also been shown that both anakinra and etanercept have the capacity to increase growth in a dose-dependent manner in rat metatarsal bones exposed to cytokines (Fernandez-Vojvodich et al. 2011). Indeed, a combination of these biologics together with IGF1 also induced improved longitudinal bone growth in cultured fetal metatarsal bones (Fernandez-Vojvodich et al. 2011). IL6 when given in combination with IL1β and TNFα was found to reduce bone growth; however, this effect was similar to the combination without IL6, which indicates that IL6 does not add to the synergism.
Impact of GC treatment on bone growth

Besides the endogenous GCs that increase under conditions of chronic inflammation, exogenous GCs such as dexamethasone or prednisolone are commonly used clinically because of their high therapeutic efficacy as anti-inflammatory or immunosuppressant agents. It has been estimated that about 5–10% of children require some form of GC treatment during their childhood (Warner 1995).

GCs may have beneficial effects on bone growth in patients with chronic inflammation thanks to their effective suppression of cytokine levels and inflammation (Marcovecchio et al. 2012). However, long-term GC therapy is associated with severe negative side effects including osteoporosis and growth impairment (Deshmukh 2007).

Interestingly, growth retardation had already been reported in patients with JIA before corticosteroids became commonly used as anti-inflammatory drugs (Czernichow 2009). Furthermore, growth impairment has been reported in systemic and polyarticular JIA patients never treated with corticosteroids (Polito et al. 1997). This indicates that other factors, not only GCs, contribute to the deleterious effects of chronic inflammation on bone growth.

The systemic effects of GCs are well known, and even topically administered dexamethasone eye drops have been demonstrated to dose-dependently inhibit bone growth and negatively influence several other bone parameters in young rabbits (Kugelberg et al. 2005). Evidence for local effects of GCs on the growth plate had emerged from both in vitro and in vivo studies. Results from in vitro experiments have shown that the GC dexamethasone directs the differentiation of mesoblastic precursor cells into mature chondrocytes by upregulating SOX9, the transcription factor that determines chondrogenesis (Locke et al. 2004). SOX9 activates SOX5 and SOX6 to trigger early stages of differentiation when chondrocytes proliferate to form columns, and additionally suppresses their terminal differentiation (Ikeda et al. 2004). In contrast, high-dose GC treatment has also been reported to induce undesired cell death in growth plate chondrocytes causing growth retardation in young rats (Chrysis et al. 2003). The first evidence of apoptosis in growth plate chondrocytes after GC treatment was reported by the detection of TUNEL-positive cells, increased BAX, as well as decreased BCL2 expression, and parathyroid hormone-related hormone (Sanchez & He 2002, Chrysis et al. 2003).

GC-induced apoptosis in chondrocytes is mainly regulated through activation of the caspase cascade, which includes caspase 8 and 9, and suppression of the Akt–phosphatidylinositol 3-kinase signaling pathway (Chrysis et al. 2005, MacRae et al. 2007, Zaman et al. 2012, 2014). In addition, it has also been reported that GC treatment differentially regulates BCL2 family proteins such as BAX, BID, and BAK (BAK1) to trigger undesired apoptosis in proliferative chondrocytes (Zaman et al. 2014). Interestingly, young mice lacking Bax are protected from GC-induced bone growth retardation (Zaman et al. 2012), which is indicative of a physiological role of the apoptotic machinery in the regulation of bone growth.

In children treated with GCs, permanent growth retardation as well as partial catch-up growth has been reported after cessation of treatment (Simon et al. 2002, Bechtold & Roth 2009). Patients with cystic fibrosis show prolonged and permanent growth retardation after GC treatment (Lai et al. 2000). The potential for catch-up growth seems to be highly dependent on the dose as well as the duration of the GC treatment (Bechtold & Roth 2009). In a model of ex vivo-cultured postnatal rat metatarsal bones, partial catch-up growth was also demonstrated after cessation of GC exposure (Chagin et al. 2010).
Prevention of growth retardation caused by chronic inflammation

Immunomodulatory biological drugs offer a relatively new type of anti-inflammatory treatment consisting of proteins that selectively inhibit the effects of cytokines. These drugs, targeting TNFα, IL1β, or IL6, have shown promising results and are potential therapies that can restore longitudinal bone growth in children suffering from chronic inflammatory diseases.

Several studies have reported a significant decrease in disease activity and improved bone growth in patients treated with different anti-TNF agents (Schmeling et al. 2003, Tynjala et al. 2006, Fernandez-Vojvodic et al. 2007, Malik et al. 2012). Etanercept, a recombinant fusion protein based on the p75 receptor for TNF (TNFR2) and the Fc region of human IgG1, acts as a soluble receptor through TNF-driven inflammation, which plays a key role in the arthritic process (Lovell et al. 2008). Etanercept treatment was reported to improve both disease activity and growth in a small mixed population of seven prepubertal and pubertal girls with refractory JIA and growth retardation (Schmeling et al. 2003). Those effects were accompanied by a discontinuation of oral GCs, a reduction in circulating IL6, and increased levels of IGFI and IGFBP3. In another study, etanercept improved linear bone growth in the majority of members of a group of prepubertal and pubertal patients with JIA who were not responding to conventional therapy (Fernandez-Vojvodic et al. 2007). Furthermore, etanercept has been reported to improve bone mineral density status in those JIA children in whom disease activity was suppressed by the treatment (Simionini et al. 2005). Other TNFα antagonists such as infliximab, a chimeric anti-TNF antibody, and adalimumab, a fully humanized anti-TNF antibody, have also been shown to clinically improve growth in children with CD (Malik et al. 2012, Altowati et al. 2013).

Anti-IL1 agents have also shown promising effects in treating inflammatory conditions. Anakinra is a recombinant form of the human IL1 receptor antagonist that competes with IL1 when binding to the IL1R1. Anakinra is frequently used for the treatment of rheumatoid arthritis in adults, whereas in children, it has been used less often and preferably for the treatment of systemic onset JIA (Pascual et al. 2005).

Even though the biological drugs have revolutionized the treatment of several chronic inflammatory conditions in both adults and children, other drugs such as methotrexate are still being used as a first choice or in combination with anti-cytokine treatments.

GH treatment has been shown to improve height velocity and lean body mass in children with severe JIA and could, therefore, potentially be used to improve growth in these patients (Simon et al. 2007). In another study, GH treatment was reported to be most effective in rescuing bone growth in those childhood JIA patients with moderate disease activity (Bechtold et al. 2007).

There has been much interest in developing selective GR modulators, a novel class of drugs maintaining the anti-inflammatory properties of GCs while limiting their negative side effects (Owen et al. 2007). AL-438 is a specific non-steroidal ligand for GR, which has full anti-inflammatory effect, but reduced negative side effects on osteoblasts and metatarsal bone growth when studied in vivo (Owen et al. 2007). Whether selective GR modulators can be used in vivo to effectively suppress inflammation without compromising longitudinal bone growth has not yet been documented.

Conclusion and future perspectives

Conditions of chronic inflammation result in several events that have a negative effect on longitudinal bone growth. Malnutrition is commonly seen in patients with inflammatory diseases and is known to impair bone growth. Growth is further suppressed by the use of GCs as anti-inflammatory drugs. Elevated levels of pro-inflammatory cytokines, such as TNFα, IL1β, and IL6, also contribute to growth retardation through both systemic actions on the GH/IGF1 axis and local actions on the growth plate. High concentrations of these cytokines suppress growth in a dose-dependent manner by decreasing chondrocyte proliferation and hypertrophy while increasing apoptosis. On the other hand, the inhibition of these cytokines with biological drugs has been shown to increase growth in both experimental and clinical studies. Cytokines are also produced locally by growth plate chondrocytes, indicating a function in the normal regulation of bone growth. Although cytokines are important, other yet unidentified factors may contribute to the pathogenesis of long-bone growth retardation in patients with chronic inflammatory disorders.

Even though the use of biologics has decreased the number of patients with severe growth retardation, growth impairment is still an issue in patients with more severe forms of chronic inflammatory diseases where malnutrition and high-dose GC treatment may be contributing factors. The development of new specific cytokine inhibitors or antagonists is therefore important to enable more effective prevention of cytokine-induced
growth retardation. Another future perspective is the possibility of identifying new targets to prevent GC-induced growth retardation.

Today, the most effective way to reduce growth retardation in children with chronic inflammation is to control the inflammation with the current drugs available, while at the same time reducing the duration of treatment as well as the dosage. It is also important to have an early diagnosis, as attempts to rescue bone growth in children have to start before epiphyseal closure.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Received in final form 28 March 2014
Accepted 3 March 2014
Accepted Preprint published online 7 April 2014