NEW TREATMENT STRATEGIES FOR GROWTH FAILURE CAUSED BY CHRONIC INFLAMMATION IN CHILDREN

Paola Fernández Vojvodich

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Front cover:
Collagen type X immunostaining in a foetal rat metatarsal bone.

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To my beloved daughters,

♥ Isabel and Laura ♥
ABSTRACT

Chronic inflammation during childhood often leads to impaired bone growth and reduced height in adulthood. Interleukin-1β (IL-1β), interleukin-6 (IL-6) and the tumour necrosis factor-α (TNF-α) are pro-inflammatory cytokines up-regulated during chronic inflammation. At the same time, experimental studies indicate that those cytokines affect growth by interfering with the Growth Hormone (GH)/Insulin-like Growth Factor I (IGF-I) axis. Moreover, IL-1β and TNF-α decrease murine bone growth in vitro by acting at the growth plate level.

To counteract growth retardation in children with severe forms of JIA, GH therapy has been used, with beneficial effects on growth and final height in some patients. On the other hand, the introduction of biologic agents has revolutionized the treatment of rheumatoid arthritis in children and adults. Anti-TNF therapy not only decreases disease activity but may also improve growth in some juvenile idiopathic arthritis (JIA) patients. However, there is still a group of children in which the GH or anti-TNF therapy does not affect growth positively. For this reason, it is necessary to investigate new treatment strategies to prevent and/or treat growth failure in those patients.

**First**, the effects of TNF-α antagonism on longitudinal growth in paediatric JIA patients were studied, differentiating any response from the normal pubertal growth spurt. From this study it can be concluded that TNF inhibition with etanercept (TNF soluble receptor) improves growth in a majority of JIA patients independent of puberty. Nevertheless, there are still patients who do not respond to TNF-inhibition and therefore are in need of alternative treatment modalities to optimize their growth.

**In the second study**, it was hypothesized that biologic agents may rescue foetal rat metatarsal bones from cytokine-induced growth impairment and that IGF-I may potentiate such an effect. Indeed, both anakinra (IL-1 receptor antagonist) and etanercept efficiently and dose-dependently prevent cytokine-induced bone growth impairment in cultured bones. Combinations of anakinra or etanercept with IGF-I further improved bone growth.

**In the third study**, it was found that IL-1β and TNF-α are produced by growth plate chondrocytes and that both cytokine antagonists improve growth of cultured foetal rat metatarsal bones suggesting that these cytokines play a physiological role in the normal regulation of longitudinal bone growth.

**In the fourth study**, the local effects of IL-6 on growth plate chondrocytes of foetal rat metatarsal bones were studied. It was found that in the presence of its receptor, IL-6 decreases in vitro bone growth and it further suppresses growth when combined with IL-1β or TNF-α. Furthermore, IL-6 is produced by growth plate chondrocytes in vitro after stimulation with IL-1β+TNF-α, which may partially explain the synergistic inhibitory effect of those cytokines on murine bone growth.

**In conclusion**, pro-inflammatory cytokines, normally up-regulated in children suffering from chronic inflammatory diseases, act in a synergistic way targeting growth plate chondrocytes and thereby decrease longitudinal bone growth. Biological agents blocking the actions of pro-inflammatory cytokines may improve bone growth.
LIST OF PUBLICATIONS


IV. Fernández-Vojvodich P, Zaman F, Sävendahl L. 2012: Interleukin-6 act locally at the growth plate to decrease longitudinal growth in fetal rat metatarsal bones. *Manuscript*
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<td>ACR</td>
<td>American College of Rheumatology</td>
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<tr>
<td>ALS</td>
<td>Acid labile subunit</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CRF</td>
<td>Chronic renal failure</td>
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<td>ECM</td>
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<td>GP</td>
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<td>IBD</td>
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<td>Janus kinase</td>
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<td>JCA</td>
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<td>Juvenile idiopathic arthritis</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<tr>
<td>M6P/IGF-IIR</td>
<td>Mannose 6-phosphate/IGF type II receptor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MMTTV</td>
<td>Mouse mammary tumour virus</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>OB-R</td>
<td>Leptin receptor</td>
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<td>PPR</td>
<td>PTH/PTHrP receptor</td>
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<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
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<td>PZ</td>
<td>Proliferative zone</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
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<td>Reverse transcriptase polymerase chain reaction</td>
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<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
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<td>RZ</td>
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<td>SDS</td>
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<td>SoJIA</td>
<td>Systemic onset juvenile idiopathic arthritis</td>
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<td>T3</td>
<td>Triiodothyronine</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>TIMPs</td>
<td>Tissue inhibitor of metalloproteinases</td>
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<tr>
<td>TNF-R1</td>
<td>Tumour necrosis factor - receptor type 1</td>
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<td>Tumour necrosis factor alpha</td>
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<td>Thyroid hormone receptor</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<tr>
<td>TUNEL</td>
<td>TdT-mediated deoxy-UTP nick end labelling</td>
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<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WNT</td>
<td>Wingless-type MMTV integration site family</td>
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<tr>
<td>yr</td>
<td>year</td>
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<tr>
<td>ΔhSDS</td>
<td>Change in height SDS</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
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1 INTRODUCTION

1.1 ENDOCHONDRAL OSSIFICATION

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. During endochondral ossification, chondrocytes proliferate, undergo hypertrophy and die; the cartilage extracellular matrix they construct is then invaded by blood vessels, bone marrow cells, osteoclasts and osteoblasts, the last of which deposit bone matrix on remnants of cartilage matrix (Mackie, Ahmed et al. 2008).

Bone growth is influenced by several autocrine/paracrine and endocrine factors, which will be briefly presented and illustrated in Figure 1.

1.1.1 Autocrine/paracrine regulators of growth

1.1.1.1 Wingless-type MMTV integration site family (WNTs)

WNTs are expressed in the surrounding tissue of the early mesenchymal condensations that will become the cartilage template of the new bone (Guo, Day et al. 2004). High WNT signalling enhances direct ossification of mesenchymal cells whereas low WNT signalling allows chondrogenesis and subsequent endochondral bone formation (Day, Guo et al. 2005).

1.1.1.2 Bone Morphogenetic Proteins (BMPs)

BMP signalling is crucial for endochondral ossification because it promotes commitment of mesenchymal cells into the chondrogenic lineage, as well as proliferation and survival of chondrocytes in the cartilage anlagen (Yoon, Ovchinnikov et al. 2005).

1.1.1.3 Fibroblast Growth Factors (FGFs)

FGF signalling decreases the rate of chondrocyte proliferation whereas it stimulates hypertrophic differentiation. FGF antagonizes BMP signalling in the control of chondrocyte proliferation. FGFs are produced by cells in the perichondrium while FGFRs are expressed in proliferative and hypertrophic chondrocytes (Minina, Kreschel et al. 2002).

1.1.1.4 Indian Hedgehog (IHH)

IHH is considered the master regulator of both chondrocyte and osteoblast differentiation during endochondral bone formation (Kronenberg 2003). IHH is expressed in cells undergoing the transition from proliferating to hypertrophic chondrocytes. The receptors for IHH are located in the perichondral region flanking the IHH expression domain, extending slightly further toward the end of the cartilage elements; however, periartricular epiphyseal chondrocytes respond to IHH stimulation by synthesizing PTHrP. PTHrP then acts on proliferative chondrocytes to inhibit their differentiation into hypertrophic chondrocytes, thus forming a feedback loop that
stabilizes the rate of chondrocyte differentiation, and consequently, the height of the proliferative columns (Vortkamp, Lee et al. 1996).

In addition, IHH signalling on the perichondral cells adjacent to the IHH-producing prehypertrophic cells of the growth plate induce them to convert to osteoblasts, which form part of the primary ossification centre (St-Jacques, Hammerschmidt et al. 1999).

1.1.1.5 Parathyroid hormone related protein (PTHrP)

PTHrP is expressed by periarticular epiphysial chondrocytes of the growth plate and plays a fundamental role in keeping proliferating chondrocytes in the proliferative stage. The PTH/PTHrP receptors (PPRs) are expressed at low levels by proliferating chondrocytes and at high levels by prehypertrophic/early hypertrophic chondrocytes. Under PTHrP signalling, chondrocytes keep proliferating in the proliferative pool. When chondrocytes are no longer sufficiently stimulated by PTHrP, they stop proliferating and synthesize IHH (Vortkamp, Lee et al. 1996; Kronenberg 2003).

1.1.1.6 Components of the extracellular matrix

The ECM of the growth plate cartilage is composed mainly of collagens and proteoglycans, but it also contains other non-collagenous proteins. Collagen type II is the major collagen in cartilage, and it is present throughout the growth plate, whereas collagen type X is expressed only in the hypertrophic zone (Hausler, Helmreich et al. 2002).

Collagen type II is of foremost importance, because without it, endochondral ossification would not occur (Talts, Pfeifer et al. 1998). In addition, proteolytic degradation of collagen type II induces chondrocyte hypertrophy as well as expression of MMP-13 (collagenase 3) (Tchetina, Kobayashi et al. 2007), which is crucial for remodelling the ECM in the transition zone of the growth plate. MMP-13 induces collagen type II degradation, chondrocyte differentiation, collagen type X synthesis and calcium incorporation (Wu, Tchetina et al. 2002).

Another metalloproteinase, MMP-9 (gelatinase B) is highly expressed in osteoclasts and it promotes apoptosis, vascularization and ossification of the hypertrophic cartilage (Vu, Shipley et al. 1998). VEGF is an angiogenic factor expressed in hypertrophic chondrocytes which works on specific receptors located in the perichondrium promoting migration of endothelial cells towards the GP cartilage (Zelzer, Glotzer et al. 2001).

In contrast, tissue inhibitor of metalloproteinases (TIMPs) are expressed throughout the growth plate to modulate the effect of MMPs (Haeusler, Walter et al. 2005).

The components of cartilage matrix are not only important for their structural roles, but some also exert specific receptor mediated effects on cell behaviour. Integrins are receptors that mediate chondrocyte-matrix interactions; without them, chondrocytes are smaller, unable to differentiate, undergo apoptosis and in general, bone growth is stunted (Hirsch, Lunsford et al. 1997).
Figure 1: Endocrine and paracrine/autocrine factors that regulate endochondral ossification.

Longitudinal bone growth occurs at the growth plate (GP) by endochondral ossification. The GP contains only one cell type, the chondrocyte, at different levels of differentiation, distributed within the resting (RZ), proliferative (PZ) and hypertrophic (HZ) zones. Numerous endocrine and paracrine/autocrine factors regulate the different processes that take place during endochondral ossification; i.e. chondrogenesis; proliferation; hypertrophy; extracellular matrix (ECM) synthesis/mineralization; apoptosis and ossification.

GCs: Glucocorticoids; GH: Growth Hormone; IGFs: Insulin-like Growth Factors; WNTs: Wingless-type mouse mammary tumour virus integration site family; BMPs: Bone Morphogenetic Proteins; PTHrP: Parathyroid hormone related protein; FGFs: Fibroblast Growth Factors; IHH: Indian Hedgehog; MMP: matrix metalloproteinase.

1.1.2 Endocrine regulators of growth

1.1.2.1 Glucocorticoids (GCs)

GCs play an important role in early development, but the constant exposure to GCs in chronic inflammatory diseases is detrimental to longitudinal bone growth (Simon, Fernando et al. 2002). In vitro experiments have shown that the GC dexamethasone directs the differentiation of stem cells into mature chondrocytes by upregulation of Sox9, the transcription factor that determines chondrogenesis (Locker, Kellermann et al. 2004). On the other hand, it inhibits GP’s chondrocyte proliferation by impairing the GH-induced stimulation of local secretion and paracrine action of
IGF-I (Jux, Leiber et al. 1998) and it stimulates chondrocyte apoptosis mainly in terminal hypertrophic chondrocytes concomitantly with a reduction in PTHrP expression (Chrysis, Ritzen et al. 2003).

1.1.2.2 **Growth Hormone (GH)**

GH has been known for many years to be an important endocrine regulator of longitudinal growth. The indirect actions of GH on longitudinal bone growth are mediated by IGF-I, which was formerly known as sulphation factor or somatomedin (Salmon and Daughaday 1957; Daughaday, Hall et al. 1972; Klapper, Svoboda et al. 1983), which may be produced systemically (mainly in the liver) or locally by GP proliferative chondrocytes (Nilsson, Isgaard et al. 1986).

However, direct actions of GH at the GP level have been suggested, as the growth hormone receptor (GHR) is expressed by GP chondrocytes (Cruickshank, Grossman et al. 2005). GH may act directly on the growth plate resting zone to promote generation of chondrocyte precursors, as suggested by experiments in which ghr gene deletion decreased both number and size of growth plate chondrocytes, in contrast to Igf1 null mice which exhibited only reduced chondrocyte size. This observation does not discard the possibility, however, that GH actions are mediated by local production of IGF-II, which is elevated in igf1 null mice (Wang, Zhou et al. 2004).

1.1.2.3 **Insulin-like Growth Factors (IGFs)**

The insulin-like growth factor (IGF) system plays an essential role in the regulation of cell growth, proliferation and survival, and affects nearly every organ system in the body. This family includes three ligands (IGF-I, IGF-II and insulin), their cell surface receptors (IGF-IR, M6P/IGF-IIIR, IR and the hybrid IR/IGF-IR), six high affinity binding proteins (IGFBP-1 to 6) which prolong the half-life of the IGFs and modulate their bioavailability and activity, and the IGFBP proteases (Annunziata, Granata et al. 2011).

The two major pathways that are activated by the IGF-1R are the MAPK (specifically ERK-1/2) and the PI3-K pathways. The ERK-1/2 pathway is associated with cell proliferation, because of its ability to drive the cyclin loop; whereas the PI3-K pathway is associated with enhancing specific cellular events, including hypertrophy and differentiation (O'Connor, McCusker et al. 2008).

The metabolic actions of IGF-I on growth plate ECM synthesis and chondrocyte proliferation were long time ago recognized (Salmon and DuVall 1970). IGF-I is produced by GP proliferative chondrocytes even in the absence of systemic GH stimulus (Nilsson, Isgaard et al. 1986), highlighting the autocrine/paracrine role of this growth factor.

1.1.2.4 **Leptin**

Leptin is a hormone secreted mainly by white adipose tissue that regulates the appetite and therefore body weight (Cinti, Frederich et al. 1997). The leptin receptor, OB-R, shows many features of the class I cytokine receptor, and it is expressed in several tissues such as the lung and kidney but also the choroid plexus and hypothalamus (Tartaglia, Dembski et al. 1995). Moreover, OB-R is expressed in cartilaginous skeletal growth centres in which leptin enhances chondrocyte
proliferation and ECM synthesis in parallel with increased IGF-1 receptor levels (Maor, Rochwerger et al. 2002). In addition, leptin acts systemically by stimulating GH secretion in pituitary cells (Accorsi, Munno et al. 2007).

1.1.2.5 Estrogens

Increased levels of estrogen during puberty results in an initial growth spurt followed by growth plate fusion in both young men and women. Experimental studies in rabbits have shown that estrogen accelerates the process of chondrocyte proliferation, advancing GP senescence (exhaustion of the proliferative capacity of GP chondrocytes, accompanied by a reduction in the size of chondrocytes) followed by GP fusion. The increased proliferation upon estrogen treatment is accompanied by increased levels of IGF-I, suggesting the involvement of the GH/IGF-I axis (Weise, De-Levi et al. 2001).

1.1.2.6 Androgens

Most of the effects of androgens on bone growth are mediated through estrogen, as hypertrophic growth plate chondrocytes express aromatase P450 (CYP19), which converts testosterone into estrogen (Oz, Millsaps et al. 2001).

Androgens may stimulate longitudinal growth through direct effects on GP, as dihydrotestosterone, a non-aromatizable androgen, can stimulate proliferation and differentiation in GP chondrocytes. This effect is linked to IGF-I upregulation (Krohn, Haffner et al. 2003). However, androgens do not affect growth in foetal rat metatarsal bones, despite the expression of androgen receptors in this model (Chagin, Vannesjo et al. 2009).

1.1.2.7 Thyroid hormone

Thyroid hormone inhibits proliferation, whereas it stimulates hypertrophic differentiation of GP chondrocytes by interacting with thyroid hormone receptors (TRs) expressed mainly in resting and proliferative chondrocytes, but which can also be found in prehypertrophic GP chondrocytes (Robson, Siebler et al. 2000). Both triiodothyronine (T3, the active form of thyroid hormone) and thyroxine (T4, the pro-hormone) are capable of inducing chondrocyte differentiation, as the deiodinases that catalyze the conversion of T4 into T3 are expressed in the GP (Miura, Tanaka et al. 2002). Furthermore, T3 inhibits PTH/PTHrP receptor mRNA expression in the ATDC5 chondrogenic cell line, suggesting indirect actions as well.

1.1.2.8 Retinoids

The effects of retinoic acid (vitamin A) deficiency (Wolbach and Hegsted 1952) and excess (Wolbach and Hegsted 1953) on GP chondrocyte maturation, mineralization, vascularization and bone growth have long since been observed. Different retinoid receptors (RARs) are expressed throughout the GP but mainly in HZ and perichondrium. On the other hand, endogenous retinoids are expressed in perichondrium (Koyama, Golden et al. 1999). Retinoic acid activates the promoter of collagen type X, demonstrating the direct effect of retinoids on chondrocyte differentiation (Cohen, Lassova et al. 2006).
1.1.2.9 Vitamin D

Vitamin D is the major regulator of mineral homeostasis. Vitamin D deficiency in children (rickets) leads to impaired longitudinal bone growth associated with increased GP HZ and defective GP mineralization. The HZ widening is the result of defective apoptosis of hypertrophic chondrocytes in parallel to normal proliferation (Donohue and Demay 2002). Moreover, in vitro studies have revealed that calcitriol (vitamin D3, the active metabolite) induces proliferation of cultured GP chondrocytes via stimulation of local IGF-I synthesis (Krohn, Haffner et al. 2003). The direct effects of vitamin D at the growth plate level are mediated by vitamin D receptors (VDR) expressed in GP chondrocytes, to promote vascularization and osteoclastogenesis (Masuyama, Stockmans et al. 2006).

1.2 CHRONIC INFLAMMATION AND GROWTH

It has long been observed that children who suffer from chronic inflammatory diseases such as JIA or IBD, CRF, cystic fibrosis, or diabetes often experience growth retardation and decreased adult height (Czernichow 2009). In JIA, the prevalence of short stature ranges from 10.4% in children with the polyarticular form to 41.0% in patients with the systemic form (Gaspari, Marcovechio et al. 2011). In the same way, growth failure is temporary in 40-50% of IBD cases, but can be prolonged in 10-20% in sufferers of CD (Cezard, Touati et al. 2002).

Figure 2: The pathophysiology of growth in chronic inflammatory diseases.

Malnutrition, GC therapy and elevated circulating levels of pro-inflammatory cytokines are factors that contribute to the growth retardation observed in children suffering chronic inflammatory diseases. They may affect bone growth both systemically and locally at the growth plate level.

IL: Interleukin; GC: Glucocorticoid; GH: Growth Hormone; IGF-I: Insulin-like Growth Factor.
Possible pathways leading to growth retardation in chronic diseases include caloric deprivation, due to too little substrate available to the child or an excessive need for and an overconsumption of substrate, as well as the inefficient management of body components needed for growth (Underwood 1999). In addition, long-term GC therapy contributes to the pathogenesis of growth retardation (Simon, Fernando et al. 2002). Nevertheless, growth impairment is also observed in systemic and polyarticular JIA patients who have never received corticosteroid treatment (Polito, Strano et al. 1997) and malnutrition accounts for 60% of the final growth impairment in a rat model of colitis, leaving the remaining loss in growth to the inflammatory process itself (Ballinger, Azooz et al. 2000).

The most common up-regulated pro-inflammatory cytokines found in tissues or biological fluids from IBD (Funakoshi, Sugimura et al. 1998) or JIA (Mangge, Kenzian et al. 1995; Kutukculer, Caglayan et al. 1998; Yilmaz, Kendirli et al. 2001; Saxena, Aggarwal et al. 2005; Macrae, Wong et al. 2007) patients are IL-1β, TNF-α and IL-6, and they can affect growth both systemically, by disrupting the GH/IGF-I axis, or locally, at the growth plate level.

Figure 2 illustrates the pathophysiology of growth during chronic inflammatory diseases.

1.2.1 Systemic effects of IL-1β, TNF-α and IL-6 on the GH/IGF-I axis

It has been shown that the pro-inflammatory cytokine IL-6 is not only produced by immune cells but also by the folliculostellate cells in the normal anterior pituitary (Spangelo, MacLeod et al. 1990), and it stimulates GH secretion in a paracrine manner (Mainardi, Saleri et al. 2002). However, transgenic mice over-expressing IL-6 exhibit a defective growth phenotype associated with decreased IGF-I levels, yet normal GH production (De Benedetti, Alonzi et al. 1997). The decreased levels of IGF-I appear to be secondary to decreased IGFBP-3 levels and thereby increased IGF-I clearance (De Benedetti, Meazza et al. 2001). Moreover, these mice exhibit reduced trabecular and cortical bones and smaller GPs, with reduced HZs and impaired development of ossification centres as well as decreased osteoblast but increased osteoclast activity (De Benedetti, Rucci et al. 2006). On the other hand, in a rat model of colitis, growth impairment is related to increased levels of IL-6 and GH but decreased levels of IGF-I and IGFBP-3 as compared to controls (Ballinger, Azooz et al. 2000) indicating a state of GH resistance.

Regarding TNF-α, this cytokine up-regulates both IL-6 and GH mRNA expression in ovine pituitary cell cultures (Nash, Brandon et al. 1992); however, GH production is decreased when pituitary cells are co-cultured with TNF-α and galanin, a peptide synthesized in GHRH neurons involved in the physiological regulation of GH secretion. Transgenic mice over-expressing the human TNF-α develop an erosive polyarthritis and exhibit reduced growth (Li and Schwarz 2003).

Regarding IL-1β, it stimulates GH release from pig pituitary cells in vitro (Mainardi, Saleri et al. 2002) probably by stimulation of interleukin-6 release (Spangelo, Jarvis et al. 1991; Spangelo, Judd et al. 1991); but in combination with galanin, GH is decreased. Interestingly, reduction in IL-1 signalling by IL-1R1 suppression or central IL-1Ra over-expression in mice result in a low bone mass phenotype, including bone growth impairment associated with increased osteoclastogenesis (Bajayo, Goshen et al. 2005).
Finally, both IL-1β and TNF-α have been shown to induce a state of GH resistance as both cytokines synergize in an additive way to decrease GHR expression and IGF-I production in primary cultures of rat hepatocytes (Wolf, Bohm et al. 1996).

1.2.2 Local effects of IL-1β, TNF-α and IL-6 at the GP level

Our group demonstrated the first evidence that IL-1β and TNF-α act locally, at the growth plate level, to inhibit longitudinal growth in cultured foetal rat metatarsal bones (Martensson, Chrysis et al. 2004). This observation was corroborated later when IL-1β and TNF-α again inhibited longitudinal growth in postnatal mouse metatarsals cultured in vitro (MacRae, Farquharson et al. 2006). The mechanisms behind growth retardation in those experiments included decreased chondrocyte proliferation and hypertrophy and increased apoptosis (Martensson, Chrysis et al. 2004; MacRae, Farquharson et al. 2006).

In vitro studies have shown that IL-1β decreased proliferation and sulphate incorporation but increased collagen production and alkaline phosphatase activity in cells derived from the RZ of rat costochondral cartilage (Horan, Dean et al. 1996). Additional studies in the murine chondrogenic ATDC5 cell line demonstrated that both IL-1β and TNF-α decrease the synthesis of ECM components such as Collagen II, X and aggrecan (MacRae, Farquharson et al. 2006).

In the previously mentioned studies, IL-6 had no effect on chondrocytes or growth; however, in a posterior study in cultured ATDC5 cells, IL-6 inhibited early differentiation and reduced the expression of type II collagen, aggrecan and type X collagen, when it was added earlier in culture (Nakajima, Naruto et al. 2009).

The receptors for IL-1β and TNF-α: IL-1R1 and TNF-R1, respectively, are expressed by GP chondrocytes in foetal rat metatarsal bones at all levels of differentiation (Martensson, Chrysis et al. 2004). Moreover, TRAF, an adaptor protein for both IL-1R1 and TNF-R1, regulates cell survival, differentiation and proliferation by the activation of downstream effectors including NF-kB and MAPKs. Among the MAPKs, pro-inflammatory cytokines induce mainly p38 MAPK and JNK; however, ERK1/2 may also be induced by TNF-R1 and IL-1R1 activation (O'Connor, McCusker et al. 2008).

On the other hand, IGF-IR is also expressed by GP chondrocytes in growing rats at all levels of differentiation (Cruickshank, Grossman et al. 2005). It is possible that receptors for both cytokines and growth factors are co-expressed in GP chondrocytes, which would result in intracellular crosstalk, as ERK1/2 is a common intracellular signalling component among the distinct ligand-activated pathways; thereby inducing a state of IGF-I or cytokine resistance (O'Connor, McCusker et al. 2008).

1.3 GROWTH PROMOTING THERAPIES TARGETING THE GH/IGF-I AXIS

Children with JCA suffering growth impairment exhibited low IGF-I levels but low to normal GH secretion which was not correlated to the IGF-I levels, irrespectively of GC treatment or disease’s subtype (Allen, Jimenez et al. 1991). In that study, the authors suggested that those children may present a state of IGF-I resistance and that reconstitution of IGF-I levels by means of improved nutrition and a better control of the disease activity should be prioritized before any GH intervention. Nevertheless, in a placebo-controlled cross-over study of children with JCA and severe growth retardation, GH treatment increased growth velocity in the majority of patients
independently of the initial GH status (Saha, Haapasaari et al. 2004). Furthermore, GH treatment was effective as well in children with severe JIA and under GC therapy, with improved height velocity and lean mass, although no benefit on fat and bone mass was observed in this study (Simon, Prieur et al. 2007).

Even though GH treatment is beneficial in most growth-retarded JIA patients, the extent of disease activity have a significant influence on height outcome, since those patients with a moderate disease activity are the most profited from GH treatment (Bechtold, Ripperger et al. 2007). For that reason, new treatment strategies are needed to promote growth in those patients with severe inflammation.

1.4 NEW IMMUNO-MODULATORY BIOLOGICAL DRUGS

Immunomodulatory biological drugs targeting IL-1β, TNF-α or IL-6 are potential therapies that could restore longitudinal bone growth in children suffering chronic inflammatory diseases.

1.4.1 Anti-TNF agents

Etanercept is a recombinant fusion protein based on the p75-receptor for TNF (TNF-R2) and the Fc-part of human IgG1. It acts as a soluble receptor through competitive inhibition of the TNF-receptor on the cell surface, thereby diminishing TNF-driven inflammation which plays a key role in the arthritic process. Etanercept is approved for the use in paediatric patients suffering from JIA (Enbrel® 2008). In patients with polyarticular JIA, it leads to a significant decrease in disease activity with sustained improvement and few side-effects as verified in an eight-year follow-up study (Lovell, Reiff et al. 2008).

Other TNF antagonists are: Infliximab (Remicade®, a chimeric anti-TNF antibody) and adalimumab (Humira®, a fully human anti-TNF antibody). To detect the potential off-target activities of the three TNF inhibitors clinically used, protein biochips containing a printed serial dilution of TNF-α and about 384 different human proteins were used. Infliximab showed the highest degree of specificity, whereas adalimumab and etanercept showed more potential off-target binding events (Feyen, Lueking et al. 2008).

Even though anti-TNF agents have revolutionized RA treatment in adults and children, other DMARDs, such as methotrexate, are still used as a first choice or in concomitance to anti-TNF drugs in JIA children. However, this anti-metabolite that inhibits the purine synthesis enzyme, dihydrofolate reductase, has proven to decrease bone growth in young rats through its direct effects on the GP-- including decreased chondrocyte and osteoblast proliferation and collagen type II expression, as well as increased chondrocyte and osteocyte apoptosis (Xian, Cool et al. 2007). On the contrary, improved bone growth has been reported in JIA patients treated with either etanercept or other TNF-antagonists. In a small mixed population (n=7) of prepubertal and pubertal girls with refractory JIA and growth retardation, etanercept treatment improved both disease activity and growth. Those effects were accompanied by a discontinuation of oral GCs, a reduction of circulating IL-6 and increased levels of IGF-1 and IGFBP-3 (Schmeling, Seliger et al. 2003). Furthermore, etanercept improved bone mineral status in 15 out of 20 JIA children who responded to etanercept after one year of treatment regarding the control of disease activity, in contrast to the ones who did not respond (Simonini, Giani et al. 2005).
1.4.2 Anti-IL-1 agents

**Anakinra** (Kineret®) is a recombinant form of the human IL-1 receptor antagonist which competes with IL-1 when binding to the IL-1RI. It is approved for the treatment of rheumatoid arthritis in adult patients failing to respond to one or more disease modifying anti-rheumatic drugs (Amgen 2006).

Anakinra is preferably used in the treatment of systemic onset JIA (SoJIA), a subtype of JIA characterized by initial systemic symptoms, including fever and/or rash, which precede the development of arthritis by months or even years. Indeed, IL-1β expression and production is enhanced in SoJIA patients, especially during the febrile phase of the disease. Anakinra treatment led to complete remission in 7 out of 9 SoJIA patients who were refractory to other therapies (Pascual, Allantaz et al. 2005). In a larger study, 15 out of 20 SoJIA patients showed at least 30% improvement, according to the ACR pediatric core set criteria, during the first three months of anakinra treatment. Clinical systemic features, including fever and rash, were resolved in 14 cases within the first three months (Lequerre, Quartier et al. 2008).

Two newer IL-1 antagonists have recently been approved for the treatment of CAPS (cryopyrin-associated periodic syndromes): **Canakinumab** (Ilaris®), a fully human IL-1β antibody, and **rilonacept** (Arcalyst®), a fusion protein consisting of the ligand-binding domain of the IL-1 receptor and the IL-1-receptor accessory protein bound to human IgG1.

In a phase II, multicenter, open-label study evaluating dosing and preliminary safety and efficacy of canakinumab in 25 SoJIA patients with active systemic features, this drug showed a promising preliminary safety and efficacy profile in this limited cohort. Indeed, 15 of 25 patients (60%) achieved an ACR 50% improvement, whereas 4 achieved the inactive disease status (Ruperto, Quartier et al. 2012). Thanks to favourable pharmacokinetics and better acceptance from children, it can be speculated that canakinumab will replace anakinra in the future.

1.4.3 Anti-IL-6 agents

**Tocilizumab** (Actemra®) is a humanized anti-IL-6R monoclonal antibody that binds both membrane and soluble IL-6R thereby preventing IL-6 actions during chronic inflammation (Mihara, Kasutani et al. 2005).

Tocilizumab has shown excellent and rapid effectiveness against SoJIA in a randomized, double-blind, placebo-controlled, withdrawal phase III trial involving 56 children (aged 2–19 years) with disease refractory to conventional treatment (Yokota, Imagawa et al. 2008). In addition, it has shown to be safe and effective as monotherapy in children with polyarticular JIA intractable to conventional methotrexate therapy. Interestingly, the effectiveness observed in this study was independent of the serum levels of IL-6 before treatment (Imagawa, Yokota et al. 2012). In both studies, no effect on growth has been reported, even though anecdotic catch-up growth has been observed in 4 children with SoJIA previously (Yokota, Miyamae et al. 2005).
2 AIMS

The general aim of this thesis has been to improve understanding of the local effects of pro-inflammatory cytokines at the growth plate level in order to develop more specific strategies for treating growth disruption secondary to chronic inflammatory diseases.

More specifically, the aims have been:

1. To study the potential of a TNF-α antagonist to rescue growth independently of the pubertal growth spurt, in children with JIA (Paper I).

2. To optimize the use of cytokine antagonists to prevent cytokine-induced growth retardation in a model of cultured foetal rat bones (Paper II).

3. To investigate the local production of IL-1β and TNF-α by growth plate chondrocytes and their effects on growth in cultured foetal rat bones (Paper III).

4. To investigate the local effects of IL-6 at the growth plate level, in the presence of its soluble receptor in a model of cultured foetal rat bones (Paper IV).
3 METHODOLOGICAL CONSIDERATIONS

3.1 RETROSPECTIVE CLINICAL STUDY (PAPER I)

In the first article, I analyzed in a retrospective way the records of JIA patients who had received etanercept treatment in our rheumatologic clinic (Astrid Lindgren Children’s Hospital, Solna, Sweden). From 52 records in total, 21 were excluded mainly because of interrupted treatment, advanced puberty, or concomitant GH therapy.

As the aim of the study was to examine the contribution of puberty to the effect of one year etanercept treatment on longitudinal growth, we collected historical information about pubertal signs (breast development in girls and penile/scrotal enlargement and/or presence of pubic hair in boys) and obtained the parental self-reported heights in order to calculate the mid-parental target height for all the patients.

Once the pubertal status was established, the records were divided into 2 groups, the prepubertal patients (n=20), who started etanercept treatment at least 1 year before onset of puberty, and the pubertal patients (n=11) which included adolescents in early/midpuberty (girls and boys younger than 13.3 and 13.6 years, respectively and with pubertal signs and growth velocities ≥ 5 cm/year at start of etanercept treatment).

To establish the level of growth retardation in the included 31 subjects, the child’s height SDS was calculated the year before and the year after the start of etanercept treatment according to height SDS standards (Karlberg and Taranger 1976) and by use of piecewise linear regression. Furthermore, each child was compared to his/her own target height SDS [the child’s height SDS subtracted by the mid-parental height SDS (the sum of the father’s and mother’s height plus 13 cm if it is a boy or minus 13 cm if it is a girl, all divided by 2)].

To evaluate how growth was affected by etanercept, the growth patterns before and after treatment were compared in relation to the standard values for age and sex for each patient. More precisely, I calculated the change in height SDS (ΔhSDS) the year before (from time point -1 yr to 0) and the first year after (time point 0 to +1 yr) the treatment was initiated. “Responders” were those patients who improved their ΔhSDS after etanercept treatment was initiated (i.e. positive difference) and “Non-responders” were those patients who did not improve (i.e. negative or no difference). In this way, even the patient who lost height SDS, but to a lesser extent than before etanercept, would be considered a “Responder”. On the other hand, a patient who continued to gain a height SDS, but to a lesser extent than before the etanercept treatment, was considered a “Non-responder”. Catch-up growth is defined as an acceleration of the growth rate following a period of growth retardation caused by a secondary deficiency, such as acute malnutrition or severe illness (Mosby's Medical Dictionary, 8th edition. © 2009, Elsevier). In that sense, response in Paper I is related to catch-up growth induced by etanercept treatment.

3.2 METATARSAL ORGAN CULTURE (PAPERS II, III AND IV)

For my experimental studies, the foetal rat metatarsal organ culture model was used since, unlike isolated cells in culture, this model preserves the histological architecture of the growth plate, and thus, the intercellular interactions and local
microenvironments found in vivo. At the same time, such organ cultures facilitate the study of direct effects of various compounds in the absence of systemic factors.

Briefly, the three middle metatarsal bone rudiments were dissected from Sprague Dawley rat foetuses on embryonic day 20 (e20) and cultured separately in 24-well culture dishes at 37°C in a humidified atmosphere containing 5% CO₂. Each well contained 0.5 ml MEM supplemented with 0.3 mg/ml L-glutamine, 0.2% BSA, 0.05 mg/ml ascorbic acid, 1 mM sodium glycerophosphate, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was changed every 2-3 days.

The e20 foetal rat metatarsal bones are cartilage anlagen surrounded by a narrow bone collar in the centre. They are around 2.3 mm long at the moment of dissection and they increase their length by approximately 50% after 7 days in culture.

Foetal rat metatarsal bones are ideal to study the actions of cytokines versus postnatal bones since they do not present a bone marrow containing cytokine-producing cells from the immune system that could interfere with my studies.

This model was employed in Papers II, III and IV (included in this thesis) to study longitudinal bone growth, cytokine production (RT-PCR), expression (IHC) and secretion (ELISA), chondrocyte proliferation (BrdU incorporation), apoptosis (TUNEL), hypertrophy (Collagen type X IHC) and indirectly, extracellular matrix degradation (quantification of GAG released to the medium).

All studies were approved by the local ethics committee in Stockholm.

### 3.3 MEASUREMENT OF LONGITUDINAL GROWTH (PAPERS II, III AND IV)

Digital pictures of each metatarsal bone were taken on the first day (designated as day 0) and subsequently 2, 5, 7, 9 and 12 days later using a Hamamatsu C4742–95 digital camera attached to a Nikon SMZ-U microscope. The longitudinal growth of the metatarsals was measured by using the Image-Pro® Plus software (Media Cybernetics Inc., Berkshire, UK). Growth at the different time-points was calculated as the percent increase in bone length from the day of dissection (day 0=baseline) (Papers II and III) or growth velocity [bone length increase/time (µm)/day] (Paper IV).

### 3.4 CELL PROLIFERATION ASSAY (BrdU) (PAPERS II, III AND IV)

For this assay, 5-bromo-2'-deoxyuridine (BrdU) was added into the culture medium 2.5 hours prior to fixation; in order to let the thymidine analogue, BrdU, be incorporated into the newly synthesized DNA by cells entering and progressing through the DNA synthesis (S) phase of the cell cycle. Afterwards, bones were formaldehyde-fixed, decalcified and paraffin-embedded to prepare serial sections of the tissue. Later, the slides were preheated, deparaffinized and rehydrated. Next, antigen retrieval was performed to increase the sensitivity of the immunostaining by incubating the slides in hot citrate buffer.

Detection of BrdU-positive cells was performed according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK) as this kit contained a specific monoclonal antibody that identified those cells that were proliferating during the period of BrdU incubation. As the secondary antibody, the fluorescent conjugated antibody FITC was used to detect the bound mouse anti-BrdU antibody, and the fluorescent stain DAPI was used to identify the nucleus of all chondrocytes since it
binds to the A-T rich regions in DNA in all cells. By use of a fluorescence microscope, proliferative cells were identified (FITC positive cells) and the percent of the total cell number (DAPI positive cells) was calculated. Eventual positive cells within the perichondrium and primary ossification centre were excluded from analysis.

### 3.5 APOPTOSIS ASSAY (TUNEL) (PAPERS II, III AND IV)

It has been demonstrated that terminal hypertrophic chondrocytes in the GP die by apoptosis, characterized by rapid cellular condensation, followed by endothelial cell penetration into the vacated lacuna (Farnum and Wilsman 1989). The TUNEL assay allows for detection of apoptosis at the individual cell level as the terminal transferase TdT catalizes the addition of nucleotides to the 3' terminus of DNA fragments generated in response to apoptotic signals. These nucleotides are biotin-labelled and can be detected by streptavidin conjugated to Alexa Fluor® 546 (Invitrogen Inc.). By use of a fluorescence microscope, apoptotic cells (Alexa positive cells) are identified and the percent of the total cell number (DAPI positive cells) is calculated. In my studies, positive cells within the perichondrium and primary ossification centre were excluded from analysis.

### 3.6 ALCIAN BLUE BINDING ASSAY (GAG QUANTIFICATION) (PAPERS II, III AND IV)

The ECM of the growth plate cartilage is composed mainly of collagens and proteoglycans. Aggrecan is a large aggregating proteoglycan specific for cartilage and it consists of a core protein covalently bound to side chains of GAGs, mainly chondroitin sulphate, which is characterized by its negatively charged carboxyl and sulphate groups (Heinegard and Oldberg 1989). On the other hand, alcian blue is a tetravalent cation (basic) dye used to stain acidic polysaccharides. The four charges allow the dye to bind to negatively charged polymers such as GAGs at high ionic strength.

In my experiments, the GAGs released into the medium by foetal rat metatarsal bones were quantified, as pro-inflammatory cytokines are known to induce ECM degradation in articular chondrocytes. I performed the Alcian blue-binding assay (Wieslab™ sGAG quantitative kit, Euro-Diagnostica, Sweden) according to the manufacturer’s instructions and expressed GAG release in relation to metatarsal bone wet weight.

### 3.7 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) (PAPER III)

This method was used to identify endogenous production of IL-1β and TNF-α by growth plate chondrocytes (Paper III). The first step was to extract total RNA from snap-frozen cartilaginous parts and whole foetal rat metatarsal bones by use of the RNA mini kit (BioRad). This method relies on the property of silica-covered mini columns to adsorb nucleic acids depending on the pH and the salt content of the buffer used. Briefly, the tissue was homogenized and cells were lysated. After addition of alcohol, the samples were applied to the mini columns. Three quick washes, which include a DNase I digest, followed. Samples were ready to use after
they were eluted with a warmed elution solution into RNase free micro tests tubes included in the kit.

Afterwards, I applied the iScript™ cDNA Synthesis Kit (BioRad, Cat. # 170-8891) to obtain DNA since it contained Reverse Transcriptase, which is a DNA polymerase enzyme that transcribes single stranded RNA into single stranded DNA and then synthesizes a second strand of DNA complementary to the reverse-transcribed single-stranded cDNA after degrading the original mRNA due to its RNaseH activity.

The end-products thus obtained were subjected directly to PCR in a Perkin-Elmer GeneAmp PCR System 2400. PCR is used to amplify a specific region of a DNA strand (the DNA target). The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

### 3.8 Immunohistochemistry (IHC) (PAPERS III AND IV)

Immunohistochemical stainings were performed to detect endogenous cytokine production (Paper III) and collagen type X expression (Papers III and IV) in foetal rat metatarsal bones.

To perform IHC, bones were formaldehyde-fixed, decalcified and paraffin-embedded to prepare serial sections of the tissue. Later, the slides were preheated, deparaffinized and rehydrated. Afterwards, antigen retrieval was performed to increase the sensitivity of the immunostaining by incubating the slides in hot citrate buffer. Next, inactivation of endogenous peroxidase activity was performed by incubating the slides with hydrogen peroxide, as the method for the development of colour uses the peroxidase enzymatic system (DAB). In the case of collagen type X IHC, additional incubation with hyaluronidase was performed in order to digest the GAG hyaluronan, and thereby exposed the antigenic site in the collagen molecule.

The next step in IHC was the incubation of samples with serum from a species unrelated to the primary or secondary antibodies or the tissue in study in order to block non-specific hydrophobic interactions. In the case of IL-1β and TNF-α IHC (Paper III), an additional blocking step of the endogenous biotin was performed.

The primary antibodies to detect TNF-α and IL-1β used in Paper III were the rabbit anti-rat TNF antibody (U-CyTech BV, Utrecht University, The Netherlands) and goat anti-rat IL-1β antibody (AF-501-NA, R&D Systems, Minneapolis, MN) followed by appropriate secondary biotin-labeled antibodies: donkey anti-rabbit antibody (705-066-147) and donkey anti-goat (705-066-147) from Jackson ImmunoResearch Lab, West Grove, PA, respectively. In each assay, controls for specificity of the cytokine stainings were included, based on parallel staining studies, omitting the primary antibodies and using a primary isotype-matched immunoglobulin of irrelevant antigenspecificity (negative rabbit IgG control, XO936, DAKO, Glostrup, Denmark, DAKO Cytomatation, Glostrup, Denmark or goat anti human IL-4, AF-504, R&D Systems).
For collagen type X detection in Papers III and IV, the primary antibody used was the monoclonal antibody X53 against human recombinant type X (Quartett GmbH, Berlin, Germany) as the primary antibody. The polyclonal rabbit anti-mouse Immunoglobulins biotiynilated (DAKO) was used as the secondary antibody.

The complex formed by biotin in the secondary antibodies with avidin and horseradish peroxidase in the ABC solution (Vectastain Elite kit, Vector) was later identified by the reaction with DAB and hydrogen peroxide, which resulted in a permanent dark-brown colour in which the protein of interest was found. The slides were counterstained with Mayer’s haematoxylin (Paper III) or Alcian blue (Papers III and IV).

3.9 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (PAPER IV)

IL-6 production was investigated in the conditioned media of foetal rat metatarsal bones treated with IL-1β and/or TNF-α by use of the Quantikine ELISA rat immunoassay (R&D systems, Minneapolis, MN) which employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat IL-6 had been pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells and any rat IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the Stop Solution was added. The intensity of the colour measured was in proportion to the amount of rat IL-6 bound in the initial step. The sample values were then read from the standard curve.
4 RESULTS AND DISCUSSION

4.1 GROWTH IMPROVEMENT WITH ETANERCEPT IS INDEPENDENT OF PUBERTY (PAPER I)

The aim of my first study was to identify the contribution of anti-TNF therapy on longitudinal growth of JIA patients independently of puberty. A previous study in a small mixed population (n=7) of prepubertal and pubertal girls with refractory JIA and growth retardation demonstrated for the first time that etanercept treatment improved both disease activity and growth velocity, highlighting the importance of disease control on growth restoration (Schmeling, Seliger et al. 2003). However, it was not clear if the catch-up growth observed in those patients was due to the use of etanercept, the TNF antagonist, which may have prevented those children from the negative effects of this cytokine on growth, or, the fact that most of those children had undergone puberty, which is usually accompanied by growth acceleration. It has been shown that girls with JIA have a later menarche than both their mothers and the normal female population, particularly if they have received GC therapy (Rusconi, Corona et al. 2003). I therefore analyzed separately the prepubertal from the pubertal children in my study.

During the year before instauration of etanercept treatment, height SDS decreased by 0.3±0.1 SDS in prepubertal patients (n=20) and by 0.2±0.2 SDS in pubertal patients (n=11). In contrast, during the first year of etanercept treatment, height SDS increased by 0.2±0.1 SDS (p=0.001 vs. the year before etanercept) in prepubertal patients and by 0.2±0.1 SDS (p=0.071 vs. the year before etanercept) in pubertal patients. After analyzing each individual patient, it was found that 17 of 20 prepubertal and 8 of 11 pubertal patients were “responders” to etanercept treatment with regards to growth. Moreover, the growth response was inversely correlated to the need of intraarticular GC injections (p=0.001).

Taken as a group, the prepubertal patients exhibited catch-up growth; in that sense, our results indicate that growth improvement with etanercept is independent of puberty. Although the growth improvement in the pubertal group did not reach statistical significance in our study, the majority of those patients showed catch-up growth after etanercept treatment too, albeit these children were less growth retarded at start of etanercept treatment (-1.3±0.3 vs. -2.1±0.3 height SDS in the prepubertal group).

In parallel to the development of the present study, another group in Finland confirmed that refractory JIA pre/midpubertal patients treated with etanercept or infliximab improved their growth and reduced disease activity. This effect was predominant in the most growth-retarded children, who decreased their need of GCs after initiation of anti-TNF treatment (Tynjala, Lahdenne et al. 2006). Another study has shown that etanercept treatment, with or without methotrexate, contributes to the restoration of normal growth in children with JIA (Giannini, Ilowite et al. 2010), however, in this study, no consistent effect of disease control on height was observed for any of the treatment groups over the course of the study.

Furthermore, a subsequent study in polyarticular-course JIA patients demonstrated that etanercept + methotrexate treatment resulted in a significant increase of bone mineral content and lean to fat mass ratio, in contrast to methotrexate treatment
only. Improvement of disease activity and a clear tapering of corticosteroid dose were observed in both groups. Moreover, decreased IL-6 and increased osteoprotegerin plasma levels were observed in the etanercept + methotrexate group (Billiau, Loop et al. 2010).

A common pattern in the previously mentioned studies (Tynjala, Lahdenne et al. 2006; Billiau, Loop et al. 2010), including this one (Vojvodich, Hansen et al. 2007) was the reduction of GC administration as a consequence of the improvement of disease activity following etanercept treatment. This observation may suggest that growth improvement after etanercept treatment is due to the reduced exposure to GCs, as these agents are well known to inhibit longitudinal growth by direct actions at the GP level (Chrysis, Ritzen et al. 2003). Additional in vitro experiments testing etanercept at the GP level were needed to understand the cause and effect relationship between etanercept and bone growth, and this is what was covered in the next study.

4.2 BIOLOGICAL AGENTS EFFICIENTLY PREVENT CYTOKINE-INDUCED BONE GROWTH IMPAIRMENT (PAPER II)

In vitro experiments have demonstrated that IL-1β and TNF-α, two of the main cytokines up-regulated during chronic inflammation, exert direct effects on the GP, impairing longitudinal bone growth by decreasing chondrocytes’ proliferation and hypertrophy as well as increasing apoptosis (Martensson, Chrysis et al. 2004; MacRae, Farquharson et al. 2006). Indeed, both IL-1RI and TNF-RI receptors, which mediate the actions of IL-1β and TNF-α, respectively, are expressed by GP chondrocytes at all levels of differentiation in the foetal rat metatarsal bone (Martensson, Chrysis et al. 2004). The aim of my second study was to investigate the potential of clinically-used biological agents against IL-1β and TNF-α to counteract the growth retardation induced by the combination of these two cytokines, and if IGF-I may potentiate such an effect.

In the present study, I found that the biologic agents, anakinra and etanercept, each have the capacity to dose-dependently rescue the growth of cytokine-exposed foetal rat metatarsal bones. The biologics also restored chondrocyte proliferation, prevented ECM degradation, as well as rescued growth plate chondrocytes from undergoing apoptosis. Furthermore, when IGF-I was used in combination with etanercept or anakinra, it rescued metatarsal bones from cytokine-induced growth retardation in an additive way.

In the previous study conducted by our group, cytokine antibodies added in 10-fold excess in relation to the cytokine concentration or IGF-I, could partially reverse the deleterious effect of IL-1β plus TNF-α on growth (Martensson, Chrysis et al. 2004). However, it has now become evident that the antibodies used had a relatively low affinity and that IL-1β or TNF-α signalling was not entirely blocked (R&D Systems 2009; R&D Systems 2010). Paper II demonstrated that effective blockage of those pro-inflammatory cytokines with anakinra or etanercept rescues longitudinal bone growth and protects growth plate chondrocytes from the deleterious effects of the combination of IL-1β plus TNF-α. Nonetheless, the second study also showed that an early treatment start with etanercept leads to a more efficient growth rescuing effect than if introduced after the initiation of cytokine exposure.

Another study in postnatal mouse metatarsal bones, where growth retardation was induced by culturing them with synovial fluid rich in cytokines (including IL-1β and TNF-α) from a JIA patient, failed to restore growth despite co-incubation with
IGF-I or specific antibodies against IL-1β and TNF-α in adequate concentration. This suggested that other factors in the biological fluid played a predominant role in the growth inhibition observed (Macrae, Wong et al. 2007). The use of synovial fluid instead of individual cytokines in those experiments brought one closer to the in vivo situation, but the simplified experimental model employed in Paper II let us observe the direct harmful effects of IL-1β and TNF-α at the GP level as well as the benefits of inhibiting them by use of biological drugs.

Growth improvement after anti-TNF therapy is associated to a reduction of GC administration in JIA patients (Tynjala, Lahdenne et al. 2006; Vojvodich, Hansen et al. 2007; Billiau, Loop et al. 2010) and it was hypothesized that anti-TNF treatment may act indirectly to restore growth in those subjects by reducing exposure to GCs or by systemic effects at the GH/IGF-I axis (Macrae, Wong et al. 2007). However, Paper II demonstrates that etanercept prevents the direct actions of TNF-α at the GP level.

The dose- and time-dependent effect of etanercept in cytokine-exposed metatarsal bones suggests that a higher dose and/or an earlier introduction of etanercept treatment might more effectively prevent growth failure in children with chronic inflammatory diseases. In addition, the dose-dependent growth rescuing effect of anakinra in cytokine-exposed metatarsal bones suggests that therapies targeting IL-1-actions have the potential to rescue bone growth in paediatric patients suffering from chronic inflammatory disorders. However, the results gathered are only experimental and thereby need to be validated in controlled clinical studies.

In Paper II, the strategy of combining the growth promoting agent IGF-I with etanercept or anakinra was also explored; this strategy improved the growth of cytokine-treated foetal rat metatarsal bones in an additive way. IGF-I is today clinically available as mecasemin (recombinant human IGF-1), but it is only approved for the long term treatment of growth failure in children with severe primary IGF-deficiency (Fintini, Brufani et al. 2009). Based on the present data, we speculate that combination therapy with etanercept and IGF-I might effectively rescue bone growth in paediatric patients with chronic inflammatory diseases. However, such a treatment must be regarded experimental and should only be initiated within clinical trials carefully monitoring any adverse reactions including immunologic interactions.

4.3 IL-1β AND TNF-α ARE PRODUCED BY GROWTH PLATE CHONDROCYTES (PAPER III)

In Paper II, it was demonstrated that anakinra and etanercept have the capacity to rescue cultured foetal rat metatarsal bones from cytokine-induced growth impairment. Interestingly, when a high concentration of anakinra or etanercept was added in combination with IL-1β and TNF-α, bone growth even slightly exceeded that in control bones. Based on those findings, it was then hypothesized that pro-inflammatory cytokines might be endogenously produced by growth plate chondrocytes acting locally to modulate normal bone growth. To test this hypothesis, the local cytokine expression in cultured foetal rat metatarsal bones was investigated and then the bones were challenged to anakinra and/or etanercept while the longitudinal bone growth was monitored.

In this study, it was found that IL-1β and TNF-α were indeed produced by growth plate chondrocytes, and that suppression of locally produced TNF-α and/or IL-1β led to improved bone growth. This is the first demonstration of a functional role of locally
expressed IL-1β and TNF-α in the normal growth plate. Other studies have found that IL-1β and TNF-α are locally expressed by articular chondrocytes in healthy young human cartilage (Middleton, Manthey et al. 1996; Moos, Fickert et al. 1999) which supports these findings.

Mechanistic studies identified different roles for the endogenously produced IL-1β and TNF-α in the normal regulation of longitudinal bone growth. For instance, etanercept increased proliferation within the PZ, and cell number within the RZ, although proliferation was decreased in this zone. These results suggest that endogenous TNF-α might decrease chondrocyte proliferation within the PZ, but increase proliferation within the RZ. In line with these findings, TNF-α reduced chondrocyte proliferation in the differentiated phenotype of the murine ATDC5 chondrogenic cell line (MacRae, Farquharson et al. 2006) whereas it stimulated [3H] thymidine incorporation in the undifferentiated phenotype of the same cell line (Horiguchi, Akiyama et al. 2000). Furthermore, anakinra treatment tended to increase cell density within the HZ while etanercept had an opposite effect. Altogether, I believe that endogenously produced TNF-α and IL-1β may act through different mechanisms to suppress longitudinal bone growth by selectively targeting different zones of the growth plate.

Morphometric analysis demonstrated that anakinra and etanercept induced non-significant increments of HZ area and height as well as a slight reduction of GAG released into the medium. In combination, those effects were synergistic in an additive way. These results suggest that endogenously produced IL-1β and TNF-α play a normal role to control the height of the HZ and to induce ECM degradation. These findings are supported by previous observations in postnatal mouse metatarsal bones, in which IL-1β and/or TNF-α inhibited bone growth, an effect which was accompanied by a reduction of the size of the hypertrophic growth plate zone (MacRae, Farquharson et al. 2006). On the other hand, cytokines, such as IL-1α and TNF-α, have been shown to stimulate chondrocyte production of MMP-13 (Shi, Schmitt-Talbot et al. 2004), known to be important for the ossification of the hypertrophic zone during endochondral bone formation (Inada, Wang et al. 2004).

Although our findings are significant and attempts have been made to include appropriate controls, the present study has some limitations. First of all, we have applied an ex vivo model. In an in vivo model, there may be counter-regulatory feedback mechanisms, which could reduce the impact of local blockage of cytokine actions in the growth plate. Secondly, our studies have been performed in foetal bones. It is possible that endogenously produced pro-inflammatory cytokines only play a regulatory role during bone development, and therefore are of minor importance for postnatal growth. Lastly, we have studied rat bones and differences between the species may exist.

4.4 IL-6 ACTS DIRECTLY AT THE GROWTH PLATE LEVEL TO DECREASE LONGITUDINAL BONE GROWTH (PAPER IV)

IL-6 can be found in high concentrations in serum and synovial fluid of JIA patients (Kutukculer, Caglayan et al. 1998; Yılmaz, Kendirli et al. 2001; Saxena, Aggarwal et al. 2005; Macrae, Wong et al. 2007), and over-expression of this cytokine in transgenic mice results in impaired bone growth that involves alterations in the GH/IGF-I axis (De Benedetti, Alonzi et al. 1997). In contrast to IL-1β and TNF-α,
direct effects of IL-6 on GP chondrocytes were not proven (Martensson, Chrysis et al. 2004; MacRae, Farquharson et al. 2006). The present study aimed to investigate the direct effects of IL-6 in foetal rat metatarsal bones, in the presence of the soluble IL-6 receptor, IL-6Rα.

In Paper IV, it was found that IL-6 decreased longitudinal bone growth when IL-6Rα was present in the culture medium. Mechanistic studies revealed a significant reduction in the PZ’s cell density associated with non-significant reduction of chondrocyte proliferation and increased apoptosis in this zone. In addition, IL-6 decreased HZ height and area together with a non-significant increase in GAG release. Supporting these findings, previous studies have shown that mice that over-express IL-6 are smaller than their wild-type littermates, and they also exhibit reduced HZs (De Benedetti, Rucci et al. 2006). Moreover, the decreased chondrocyte differentiation observed in my studies is supported by previous findings in the ATDC5 chondrogenic cell line, in which IL-6 inhibited cartilaginous nodule formation and markedly reduced the expression of type II collagen, aggrecan and type X collagen when introduced early during culture (Nakajima, Naruto et al. 2009).

In Paper IV, the interactions between IL-6 and the already-studied IL-1β and TNF-α were investigated at the GP level. I found that IL-6+IL-6Rα synergized with either IL-1β or TNF-α to further decrease HZ height and area, and ultimately, bone growth. Moreover, IL-6 and its soluble receptor, together with IL-1β, reduced PZ cell density and in combination with TNF-α, it reduced RZ area. However, when IL-6 was added to the already detrimental IL-1β+TNF-α combination, bone growth was not further decreased.

To further explore the suppression of IL-6+IL-6Rα activity in the presence of IL-1β+TNF-α, I investigated IL-6 production by foetal rat metatarsal bones treated with IL-1β and/or TNF-α and found that even though production of IL-6 mediated by IL-1β or TNF-α alone did not differ from the control, in combination, both cytokines synergistically increased the levels of IL-6 in the conditioned media. In an attempt to block the actions of endogenously-produced IL-6, I treated foetal rat metatarsal bones with the IL-6 antibody added to the detrimental IL-1β+TNF-α combination for 12 days, but growth could not be re-constituted (data not shown).

It has been shown before that both IL-1β and TNF-α stimulates IL-6 mRNA expression in gingival fibroblasts by a mechanism involving the MAPK, yet not the NF-kB signalling pathway, as only pharmacological inhibitors against the p38 and the JNK MAPKs significantly inhibited IL-1β and TNF-α-induced mRNA expression of IL-6 (Palmqvist, Lundberg et al. 2008). Moreover, experiments in the SW982 human synovial sarcoma cell line have demonstrated that IL-1β stimulates the production of IL-6, whereas IL-6 plus its soluble receptor stimulate production of IL-1RI in those cells. Interestingly, SW982 cells treated with IL-1β plus the soluble IL-6R only, enhances IL-1β-mediated MMP production, as IL-6 is already present in the medium, which explains the synergism observed between IL-1β and IL-6 (Suzuki, Hashizume et al. 2010). Based on this knowledge, the synergism between IL-1β plus IL-6+IL-6Rα observed in Paper IV can be explained by an increased sensitivity to IL-1β in the foetal rat metatarsal bones mediated by IL-6+IL-6Rα. In the same way, the increased production of endogenous IL-6 mediated by IL-1β+TNF-α treatment in metatarsal bones may enhance the sensitivity to IL-1β, thereby exhibiting synergistic effects. However, addition of IL-6+IL-6Rα to the already nocive IL-1β+TNF-α treatment might
not contribute anymore to the enhancement of IL-1RI expression, or it may encounter a limitation in the IL-1β substrate.

Several clinical studies in which anti-TNF treatment restored growth in children with JIA found that growth improvement was associated with a reduction of circulating IL-6 (Schmeling, Seliger et al. 2003; Billiau, Loop et al. 2010). Tocilizumab, a humanized anti-IL-6R monoclonal antibody that prevents IL-6 from binding to IL-6R (Mihara, Kasutani et al. 2005), has been shown to be safe and effective when used in combination with methotrexate to treat patients with moderate to severe active RA who are responding inadequately or who are intolerant to TNF antagonists (Emery, Keystone et al. 2008). The use of tocilizumab in children with SoJIA has shown a sustained clinical improvement and a favourable risk-benefit profile. The findings of this study might represent a step forward in the control of a disease that has been previously proven as difficult to manage (Yokota, Imagawa et al. 2008). Nevertheless, more experimental and clinical studies are needed in order to determine the contribution of this or future anti-IL-6 drugs, to counteract growth retardation in JIA patients.
5 CONCLUSIONS

From the individual studies, the following conclusions have been reached:

Etanercept is capable to improve linear bone growth and reduce the need for intraarticular glucocorticoid injections in a majority of prepubertal and pubertal JIA patients who are non-responsive to conventional therapy. The minority of JIA patients who do not respond to anti-TNF treatment may be candidates for therapeutic agents that target other pro-inflammatory cytokines or therapies targeting the GH/IGF-I axis. (Paper I)

Anakinra and etanercept dose-dependently improve in vitro growth of foetal rat metatarsal bones exposed to pro-inflammatory cytokines. From this finding, it can be speculated that increased dosing of biological agents might be clinically effective to improve bone growth in children with chronic inflammatory diseases. The experimental data also suggest that an alternative approach combining treatment with biologics and IGF-I may be effective to further improve growth in those patients. However, such treatment strategies need to be evaluated within controlled clinical trials. (Paper II)

Pro-inflammatory cytokines IL-1β and TNF-α are produced within the growth plate of foetal rat metatarsal bones and suppression of their activities leads to improved longitudinal bone growth. Further studies may clarify if anti-cytokine therapy has the capacity to increase postnatal bone growth in vivo. (Paper III)

IL-6 is endogenously produced by cultured foetal rat metatarsal bones upon stimulation with IL-1β and TNF-α. Added exogenously, IL-6, in the presence of its soluble receptor, decreases in vitro bone growth demonstrating direct actions of IL-6 at the growth plate level. Moreover, it synergizes with IL-1β and TNF-α to further decrease bone growth. (Paper IV)

In conclusion, the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, up-regulated during chronic inflammatory diseases in children, exert direct effects on growth plate chondrocytes and interact in a synergistic way to decrease longitudinal bone growth. Biological agents targeting any of these cytokines may prevent damage to the growth plate in those patients while at the same time the inflammatory condition itself ameliorates. In addition, IGF-I treatment may be effective to further improve growth in those patients; however, such treatment strategies need to be evaluated within controlled clinical trials.
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