Glucocorticoid-induced effects on the growth plate and the IGF system

Glucocorticoïd-geïnduceerde effecten op de groeischijf en het IGF systeem

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 15 april 2003 des middags te 14.30 uur

door

Jeske Johanna Smink

Geboren op 22 februari 1975, te Amersfoort

Promotor : Prof. Dr. R. Berger

Co-promotoren : Dr. S.C. van Buul-Offers
Dr. J.A. Koedam

Paranimfen : Drs. H. Vermeer
Dr. F.M. Verheijen

Cover: Section of the proximal tibial growth plate of a 4-week-old normal mouse
Printed by: Optima Grafische Communicatie, Rotterdam
ISBN 90-393-3302-5

The studies presented in thesis were carried out at the Department of Pediatric Endocrinology, Wilhelmina Children's Hospital of the University Medical Center Utrecht (Utrecht, The Netherlands), within the Graduate School for Developmental Biology.

The printing of this thesis was financially supported by: Fleur BV, EBCC BV, OctoPlus, J.E. Jurriaanse foundation, Dr. Ir. van de Laar foundation and Novo Nordisk Farma BV.

"Discovery consists of seeing what everybody has seen
and thinking what nobody has thought"
(A. von Szent-Gyorgyi)

voor mijn ouders

Contents

List of abbreviations

ALS	acid-labile subunit
BMP	bone morphogenetic protein
Cbfa1	core binding factor α-1
CD-RAP	cartilage-derived retinoic acid-sensitive protein
DXM	dexamethasone
ECM	extracellular matrix
ER	estrogen receptor
FGF	fibroblast growth factor
FGF-R	fibroblast growth factor receptor
GC	glucocorticoid
GH	growth hormone
GHR	growth hormone receptor
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HC	hydrocortisone
HIF-1	hypoxia-inducible factor 1
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGF-IR	type I IGF receptor
IGF-IIR	type II IGF receptor
IGFBP	IGF-binding protein
Ihh	Indian hedgehog
MMP	matrix metalloproteinase
PCNA	proliferating cell nuclear antigen
PRDL	prednisolone
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
T	thyroid hormone
TR	thyroid hormone receptor
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

Chapter 1

General introduction

LONGITUDINAL GROWTH

Longitudinal growth results in an increase in body stature and depends on many factors, including environmental influences, nutrition, genetic factors, systemic hormones and growth factors. Longitudinal growth in man consists of different phases. The highest overall growth rate occurs during fetal development. Postnatally, the highest longitudinal growth rate is achieved just after birth, followed by a slower growth rate during mid-childhood. A growth spurt follows during puberty, until the age of peak height velocity has been reached and longitudinal growth ceases [1]. Responsible for longitudinal bone growth are the growth plates located at the proximal and distal ends of the long bones, by a process called endochondral ossification.

ENDOCHONDRAL OSSIFICATION

During endochondral ossification in the growth plates, the chondrocytes (cartilage cells in the growth plate), follow a highly coordinated program of proliferation, differentiation, maturation and eventually apoptosis, leaving the cartilaginous scaffold on which new bone is formed. These processes result in longitudinal bone growth [2-4].

Development of the growth plate

The process of endochondral ossification begins during the formation of the long bones in the embryo. In the embryo, mesenchymal cells condense, differentiate into chondrocytes and form the cartilaginous model of the bone. The chondrocytes start to hypertrophy in the diaphysis of the bone, osteoclasts (bone resorbing cells) and blood capillaries start to invade the diaphysis and the initial bone is formed in the primary ossification centre. This primary ossification centres spreads outwards to the ends of the bones. A secondary ossification centre develops in the epiphyseal cartilage at the ends of the bones. The primary and secondary ossification centre remain separated by a layer of cartilage, the growth plate, which is located between the epiphyseal and methaphyseal bone (Fig. 1). During postnatal growth, endochondral ossification continues in these growth plates, resulting in longitudinal bone growth [3,4]. After puberty, growth velocity rapidly decreases and the growth plates fuse (not in rodents), resulting in cessation of longitudinal bone growth.

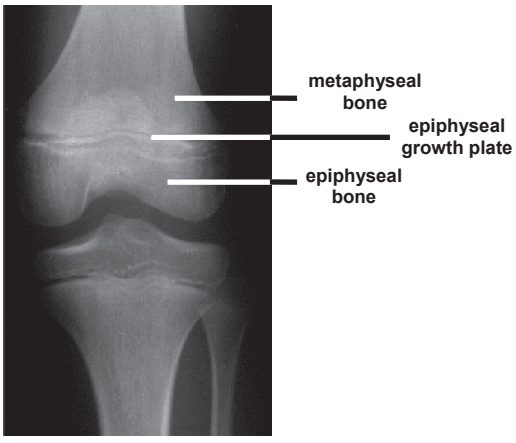

Figure 1 The growth plate as visible on a X-ray. X-ray of a child's long bone, showing the upper leg (femur) and the lower leg (tibia). The epiphyseal growth plate is visible as a thin line, located between the metaphyseal and epiphyseal bone.

Structure of the epiphyseal growth plate

The growth plate is an organized structure, consisting of three functionally and structurally distinct layers, the resting, proliferative and hypertrophic zone (Fig. 2). The chondrocytes in these different zones are at different stages of differentiation. In all three zones, the growth plate chondrocytes are embedded in a cartilaginous extracellular matrix (ECM) [4,5].

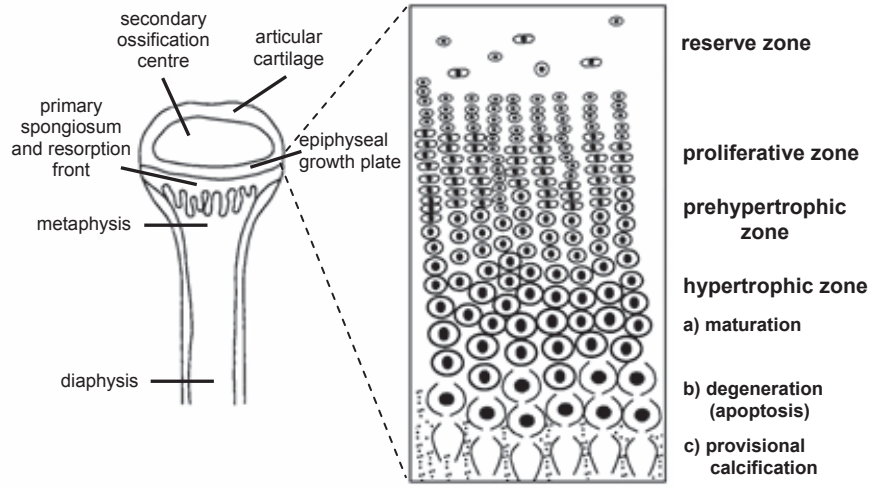

Figure 2 Structure of the epiphyseal growth plate. Schematic representation of a growing long bone, showing the major structures of the bone. The right hand panel shows a detailed view of the growth plate and the organization in the different zones, as indicated (derived from 6).

The resting zone

The most immature chondrocytes are located at the distal epiphyseal end of the growth plate, in the resting zone. Resting chondrocytes are small, spherical progenitor cells that divide rarely and lie as single cells or in small clusters in a dense cartilaginous ECM consisting largely of type II collagen and proteoglycans [5,6]. The resting chondrocytes act as stem-like cells, as removal of the growth plate *in vivo* in rabbits, leaving only the resting zone, resulted in a complete regeneration of the growth plate [7]. In the same study, resting zone cartilage placed ectopically alongside the proliferative zone, induced a 90-degree shift in the orientation of nearby proliferative chondrocytes. This indicates that resting chondrocytes also direct the alignment of chondrocyte columns parallel to the long axis of the bone [7].

The proliferative zone

In the proliferative zone, chondrocytes display a flattened shape and they divide rapidly, followed by the two daughter cells lining up along the long axis of the bone. This clonal expansion results in organized longitudinal chondrocyte columns, a characteristic feature of the growth plate. The increase in the number of proliferative chondrocytes contributes to longitudinal bone growth [5]. After a finite number of cell divisions, the proliferating chondrocytes loose their ability to divide and start to enlarge as they mature to the hypertrophic phenotype [5]. The ECM between the proliferative chondrocyte columns consists mainly of longitudinally oriented collagen II fibrils.

The hypertrophic zone

The transition zone between the proliferative and hypertrophic zones, where proliferative chondrocytes start to round up and begin to enlarge, is often referred to as the prehypertrophic zone [4,5]. The hypertrophic chondrocytes continue to enlarge up to five- to tenfold, which substantially contributes to the increase in longitudinal bone growth, and eventually they die by apoptosis (programmed cell death). In the hypertrophic zone, the secreted ECM differs

from the other zones and is rich in type X collagen. In the lower part of the hypertrophic zone, the ECM becomes mineralized in the zone of provisional calcification [5].

The terminal hypertrophic chondrocytes (most distal hypertrophic chondrocytes) undergo apoptosis [8] and leave empty lacunae which are separated by the ECM (Fig. 3). This cartilaginous scaffold is the template on which new bone will be formed. The formation of new bone requires angiogenesis, the invasion of blood vessels from the metaphyseal bone at the base of the hypertrophic zone [9]. Angiogenesis and apoptosis are associated processes in the growth plate [9].

Chondroclasts or osteoclasts (bone resorbing cells) partially resorb the calcified ECM and blood vessels enter this resorption front. The invading blood vessels allow the osteoblasts to invade and to lay down new metaphyseal trabecular bone, completing the process of endochondral ossification [4,6,9,10].

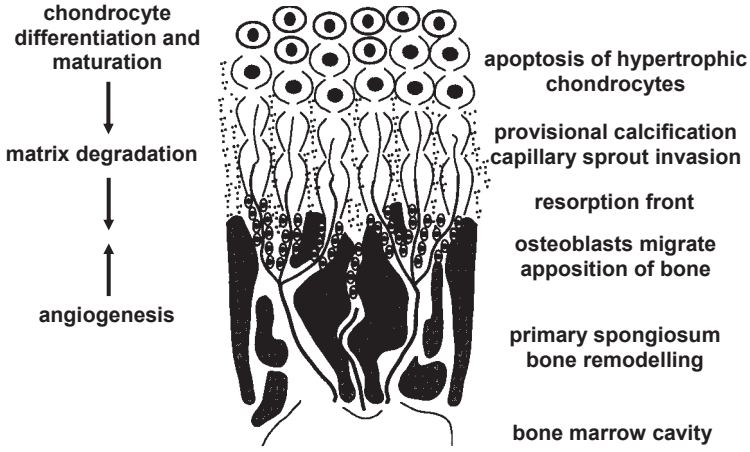

Figure 3 Processes in the hypertrophic zone of the growth plate. Schematic representation of the lower part of the growth plate and the metaphyseal bone. Chondrocytes mature in the hypertrophic zone and die by apoptosis, followed by matrix degradation. This facilitates the invasion of blood vessels and osteoblasts into the lower part of the growth plate, to lay down new bone (derived from 6).

The Extracellular Matrix (ECM)

The chondrocytes of the different zones are embedded in an extracellular matrix (ECM), which also exhibits a structural organization. The ECM is produced by the chondrocytes and mainly consists of collagens and proteoglycans. The collagens type II, IX and XI are expressed in all three zones of the growth plate, whereas collagen type X is only present in the hypertrophic zone [5,11]. The proteoglycans, which require sulphation for their activation and cross-linking to the ECM, are also present in all three zones. The ECM interacts with the chondrocytes through cell surface adhesion receptors, the integrins [12], which is important for chondrocyte differentiation and survival [13].

ECM remodelling enzymes, as matrix metalloproteinases (MMPs), are important players in endochondral ossification. They are crucial in the modelling and degradation of the ECM, and in the process of angiogenesis [14]. Furthermore, degradation of the ECM also releases growth factors which may be present in the ECM, which in turn may have their effect on growth plate chondrocytes.

GROWTH FACTORS

Different growth factors play an important role in endochondral ossification. Besides their local effects on the growth plate, they also exert systemic effects, which will not be discussed. Among these growth factors are vitamin D_3, parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), sex steroids, thyroid hormones, vascular endothelial growth factor (VEGF) and

components of the growth hormone (GH) - insulin-like growth factor (IGF) axis. These factors also interact with each other and are responsible for the correct regulation of longitudinal bone growth.

Vitamin D_3

Vitamin D_3 plays an important role in bone formation, as vitamin D_3 receptor inactivation in mice resulted in severe impairment of bone formation and growth retardation. In these mice the growth plate was enlarged, due to widening of the hypertrophic zone, suggesting a role for vitamin D_3 in the differentiation of chondrocytes [15]. Vitamin D_3 is thought to stimulate the differentiation of proliferating chondrocytes into hypertrophic chondrocytes and to inhibit the terminal differentiation of hypertrophic chondrocytes. Chondrocytes thus respond differently to vitamin D_3, depending on their differentiation stage in the growth plate [16,17].

Indian hedgehog - Parathyroid hormone-related peptide negative feedback-loop

Parathyroid hormone-related peptide (PTHrP) is also important in the regulation of differentiation of chondrocytes. In PTHrP and in PTHrP/PTH-receptor knock-out mice, the growth plate displayed reduced chondrocyte proliferation, premature hypertrophic differentiation and advanced endochondral ossification, resulting in dwarfism [18-20]. In contrast, overexpression of PTHrP in mice resulted in delayed endochondral ossification, with persistent chondrocyte proliferation, also resulting in dwarfism [21]. These data strongly suggest a role for PTHrP in the transition zone between the proliferative and hypertrophic zone. In this transition zone, the PTHrP/PTH-receptor is indeed expressed [22,23]. PTHrP expression is stimulated by Indian hedgehog (Ihh), a morphogen that is also a regulator of chondrocyte differentiation [24]. Overexpression of Ihh suggested that Ihh blocks hypertrophic differentiation and delays or prevents endochondral ossification, mediating its effects via PTHrP, as reviewed by Vortkamp [25]. Ihh stimulates PTHrP expression, which acts on the PTHrP/PTH receptor, which in turn maintains chondrocyte proliferation, inhibits hypertrophic differentiation and inhibits Ihh expression. In this negative feedback-loop, PTHrP and Ihh regulate the rate of chondrocyte proliferation and hypertrophic differentiation [23,25].

Bone Morphogenetic Proteins

The bone morphogenetic proteins (BMPs) are part of the TGF-β superfamily and play a major role in the control of chondrogenesis. They were originally identified as bone-derived factors able to induce formation of ectopic cartilage and bone [4]. In the embryonic chick limb, BMPs were shown to regulate the progression of chondrocyte differentiation into the prehypertrophic phenotype and to be able to increase apoptosis [26]. Several of the BMPs, BMP-2, -4, -6 and -7, and the BMP receptors are also expressed in the proliferative and prehypertrophic zones of the postnatal growth plate [27-30]. The expression in these zones, suggests that the BMPs might play a similar role in the postnatal growth plate as shown in the embryonic growth plate.

Fibroblast Growth Factors

The fibroblast growth factors (FGFs) consist of at least 21 members, which interact with at least 4 receptors (FGF-R) and play a role in endochondral ossification [31,32]. Several *in vivo* data suggest that the FGF system inhibits longitudinal bone growth. In FGF-2 (bFGF) transgenic mice, longitudinal bone growth was decreased [33], whereas FGF-R3 knock-out mice displayed increased longitudinal bone growth [34,35]. Activating mutations in FGF-R3 in humans causes the most common form of dwarfism, achondroplasia [56]. The FGFs are suggested to act at the local level of the growth plate by decreasing chondrocyte proliferation, chondrocyte hypertrophy and cartilage matrix production [36]. FGF-2 and FGF-R3 are also suggested to stimulate angiogenesis of the growth plate [9].

Thyroid hormones

Thyroid hormone (T3), and its precursor (T4), are essential regulators of longitudinal bone growth [37]. In the growth plate, resting and proliferative chondrocytes express both thyroid hormone receptors (TR) α and β, suggesting that the resting and proliferative chondrocytes are the primary targets of thyroid hormone [38]. Thyroid hormones are suggested to stimulate hypertrophic differentiation of chondrocytes. Treatment of mice with T4 indeed stimulated hypertrophic differentiation [39]. This is confirmed in the TRα knock-out mice, which showed growth arrest, delayed bone formation and failure of hypertrophic chondrocyte differentiation[40,41]. The TRβ isoform can compensate for TRα in the growth plate, but is itself dispensable in the growth plate [37]. Besides the actions of T3 on the chondrocytes itself, it is also required for angiogenesis [42].

Sex steroids

Sex steroids are crucial for the pubertal growth spurt and cessation of growth in adulthood [6]. In a human male patient with a mutation in the estrogen receptor α (ERα), growth continued into adulthood, as the growth plates did not fuse and there was no evidence of a pubertal growth spurt [43]. This discovery led to the suggestion that estrogen, and not androgen, is the main regulator of pubertal growth in both sexes [44]. ERα knock-out mice displayed a similar phenotype as the human patient, whereas ERβ knock-out mice did not have this phenotype. This indicated that mainly ERα mediates the effects of estrogens on the growth plate [45]. However, besides ERα, ERβ [46,47] and the androgen receptor [48,49] are also expressed in the growth plate. The actions of androgens in chondrocytes is still not clear, however, they may be involved in establishing gender differences in the skeleton [48]. Besides the presence of sex steroid receptors in the growth plate, key enzymes involved in sex steroid synthesis are also expressed in the growth plate, indicating that intracrinology might be a major source of local steroid delivery [50].

Vascular Endothelial Growth Factor

One of the last stages in endochondral ossification is the invasion of the growth plate by bone-forming osteoblasts, which requires vascular invasion or angiogenesis. As the growth plate itself is avascular and resistant to angiogenesis, it has been suggested that hypertrophic chondrocytes express activators of angiogenesis, whereas resting and proliferative

chondrocytes express inhibitors of angiogenesis [6,9,51]. One of the suggested angiogenic activators is vascular endothelial growth factor (VEGF) [6,9], which is a chemo-attractant for endothelial cells and one of the important growth factors for endothelial cells [52]. VEGF is expressed by hypertrophic chondrocytes [9,53-55]. Inactivation of VEGF by administration of a soluble receptor in mice resulted in suppression of vascular invasion, trabecular bone formation and an increase of the width of the hypertrophic zone, resulting in decreased longitudinal bone growth [53]. This established a key role for VEGF in angiogenesis during endochondral ossification [9,53].

Growth hormone - insulin-like growth factor axis

Of all the growth factors, the components of the growth hormone (GH) - insulin-like growth factor (IGF) axis are suggested to be main regulators of postnatal longitudinal bone growth [57,58], which will be discussed in detail below.

THE INSULIN-LIKE GROWTH FACTOR SYSTEM

The insulin-like growth factors (IGFs) were originally identified as factors stimulating sulphation of the proteoglycan chondroitin, mediating the "sulphation factor activity" of GH. The IGFs are structurally homologous to proinsulin, resulting in their names [59,60]. The IGFs are part of the IGF system, which comprises of two ligands, IGF-I and IGF-II, two IGF receptors, and six IGF-binding proteins, IGFBP-1 to -6.

The IGFs are potent mitogenic, anti-apoptotic and differentiating promoting growth factors and in high doses also have insulin-like metabolic actions [61]. They are produced by multiple tissues, and can act both in an endocrine and autocrine/paracrine fashion. Their gene expression is tissue specific and developmentally regulated [61].

GH

Growth hormone (GH) has no apparent embryonic role, but is a major growth regulator of postnatal development [62,63]. This is illustrated in GH knock-out mice, which displayed severe postnatal growth retardation and delayed bone development, which became apparent 2 weeks postnatally [63]. In humans, GH deficiency and insensitivity, including GH receptor defects, also result in dwarfism [64-66]. GH has IGF-I independent, stimulating effects on longitudinal bone growth [58] and *in vitro* on the proliferation of chondrocytes [67]. Besides its IGF-I independent effects, many of the actions of GH are mediated by IGF-I. GH is also suggested to stimulate the proliferation and differentiation of chondrocytes by local IGF-I production [68].

IGF-I

IGF-I is expressed at low levels embryonically and is induced postnatally [69-71]. Exogenously added IGF-I has been shown to have a stimulatory effect on longitudinal bone growth [72-74] and to stimulate chondrocytes at all stages of differentiation [68]. IGF-I is suggested to play a role both in embryonic and postnatal longitudinal growth, as shown in IGF-I knock-out mice. These mice, some of which survive into adulthood, displayed severe growth retardation and delayed bone development, resulting in dwarfism [75,76]. The growth retardation already occurred during embryonic development and continued during postnatal development, as was

also evident in the human counterpart of the IGF-I knock-out mouse [77]. In IGF-I knock-out mice, the decreased longitudinal bone growth was due to a reduced chondrocyte proliferation [58] and a decrease in linear dimension of hypertrophic chondrocytes [58,78].

IGF-II

In contrast to IGF-I, IGF-II is expressed at high levels embryonically, beginning at postimplantation stages [79,80]. Its serum levels decrease postnatally in rodents, whereas levels remain high in humans [81]. IGF-II is essential for normal embryonic development and is suggested not to be involved in postnatal development, as IGF-II knock-out mice were growth retarded at birth, but displayed a normal postnatal growth rate [82,83]. However, exogenously added IGF-II has been shown to have a stimulatory effect on longitudinal bone growth postnatally [72,74]. IGF-I and IGF-II both have independent and essential activities on longitudinal growth, as the double knock-out mice had a growth deficiency which was approximately the sum of the single knock-outs growth deficiency [76].

The IGF receptors

The actions of both IGFs are mediated by the type I IGF receptor (IGF-IR) [84]. The IGF-IR is a tyrosine kinase receptor with high homology to the insulin receptor [85]. It is a transmembrane glycoprotein, consisting of 2 extracellular, ligand-binding α subunits and 2 intracellular β subunits, containing tyrosine-kinase domains [85]. The IGF-IR has a high affinity for IGF-I, a slightly lower affinity for IGF-II and a weak affinity for insulin, which is tissue-dependent [86]. IGF-IR is, as IGF-I, also suggested to play a role in both embryonic and postnatal longitudinal growth, as shown in IGF-IR knock-out mice [75,76]

The type II IGF receptor (IGF-IIR) is a single-chain transmembrane glycoprotein with no tyrosine kinase domains, also known as the cation-independent mannose-6-phosphate receptor [87]. It binds IGF-I with a much lower affinity than IGF-II and does not bind insulin [88]. The IGF-IIR is suggested to be important for clearance of IGF-II from the extracellular environment, although it may also mediate some of the actions of IGF-II [89]. IGF-IIR knock-out mice, which generally die at birth, are larger and showed that IGF-IIR is essential for embryonic development and growth regulation [90].

IGFBPs

The IGFBPs (IGFBP-1 to -6) are crucial modulators of both IGFs and they can act as endocrine as well as autocrine/paracrine factors [61,91]. Each of the IGFBPs is expressed in a tightly regulated developmental- and tissue-specific manner. The IGFBPs modulate IGF activity by i) acting as transport proteins of the IGFs in the circulation, ii) prolonging the half-lives of the IGFs, iii) providing tissue-and cell-type specific localization of the IGFs and iv) competing for the interaction of the IGFs with their receptors [61]. The IGFBPs have different affinities for the IGFs and can either enhance or inhibit IGF activity. Their biological activities depend on possible cell surface or ECM association and on posttranslational modifications as phosphorylation, glycosylation and proteolysis by specific IGFBP proteases, which alter their affinity for the IGFs [61].

The major pool of IGFs in the circulation is bound to the IGFBPs. IGFBP-3 is responsible for the majority of the IGF-binding capacity in serum. IGFBP-3 forms a 150 kDa complex

together with IGF and a third component, the acid-labile subunit (ALS). The role of ALS appears to increase the molar mass of the IGF/IGFBP-3 complex, in order to limit the access of circulating IGF to various tissues [91]. The other IGFBPs circulate as binary complexes with the IGFs in serum [91].

Knock-out models of the different IGFBPs resulted only in small morphological alterations, which were suggested to be caused by functional compensation by the other IGFBPs [92]. Therefore, to study the functions of the IGFBPs, one should use transgenic animals and *in vitro* and *in vivo* techniques.

IGFBP-1 mainly inhibits, but can also enhance, IGF actions [61], and exerts its major role in the female reproductive system, as shown both *in vitro* and *in vivo* and in transgenic animals [93]. Both *in vitro* and *in vivo* studies showed that IGFBP-2 has mainly inhibitory effects on IGF actions [94], *e.g.* IGFBP-2 was shown to inhibit GH-stimulated growth *in vivo* [95]. IGFBP-2 transgenic mice showed that the main target of IGFBP-2 is bone, as it is a potent negative regulator of bone growth and remodeling [96,97]. IGFBP-3 has been shown to both enhance [98,99] and inhibit [100,101] IGF-dependent actions *in vitro* [61]. IGFBP-3 administration *in vivo* showed that IGFBP-3 is a negative regulator of growth [102]. In contrast, IGFBP-3 transgenic mice showed selective organomegaly of the spleen, liver and heart [103]. IGFBP-4, in contrast to IGFBP-1, -2, -3, and -5, only functions as an inhibitor of IGF actions, as shown by several *in vitro* studies [104-106]. IGFBP-4 plays an important role in bone formation and resorption, as shown *in vivo* [107,108]. As IGFBP-4, IGFBP-5 also plays an important role in bone [107-109]. Recently, IGFBP-5 has also been identified to form a ternary complex with IGF and ALS in the circulation, as IGFBP-3 [110]. IGFBP-6 functions as an inhibitor of IGF action, both *in vitro* and *in vivo* [61].

Besides the IGF-dependent functions, more evidence is emerging that the IGFBPs also have direct, IGF-independent, effects on cellular functions [111]. These IGF-independent functions involve specific receptors or binding to integrins. IGFBP-1 was shown to bind with its RGD sequence to $\alpha_5\beta_1$ integrins (fibronectin receptor), which mediated IGFBP-1 stimulated cell migration [112] and it was also involved in cell detachment and subsequent apoptosis [113]. The role of the RGD sequence in IGFBP-2 has not been elucidated yet, although it is proposed to bind also to $\alpha_5\beta_1$ integrins [114]. Some of the IGF-independent actions of IGFBP-3 and -5 are thought to be mediated through specific signaling receptors [112]. IGFBP-3 can inhibit cell growth and it induces apoptosis, by its own presumed receptor [115]. IGFBP-5 functions as a growth factor in bone, as shown both *in vitro* and *in vivo* [116]. For IGFBP-4 and -6 no clear IGF-independent functions have yet been described.

The IGFBPs thus have overlapping and unique functions, which involve cell proliferation and differentiation in both an IGF-dependent and -independent fashion.

The somatomedin hypothesis

In 1957, the "somatomedin hypothesis" was proposed to understand the regulation of somatic growth. It stated that pituitary-derived GH stimulated the liver production of a growth factor named sulphation factor, renamed as somatomedin (IGF-I), which in turn regulated longitudinal growth in an endocrine fashion [59] (Fig. 4A). In the mid-eighties, the original somatomedin hypothesis was extended with an additional direct effect of GH and IGF-I on the growth plate [117], resulting in "the dual effector theory" [62] (Fig. 4B). Local GH injections in the tibia of rats had shown that GH could also stimulate longitudinal bone growth directly [118]. GH would stimulate resting chondrocytes and IGF-I production in the growth plate, which in

turn (together with the endocrine IGF-I) would stimulate the clonal expansion of proliferative chondrocytes. The GH receptor (GHR) is indeed expressed by chondrocytes. In neonatal rabbits and humans the GHR is detected in the proliferative and hypertrophic zone [119,120] and its expression is extended to the resting zone during postnatal development [121,122]. In IGF-I knock-out mice, the width of the resting zone was increased, which was suggested to be caused by increased GH levels [78].

The somatomedin hypothesis has recently been adapted again, due to new evidence obtained from liver-specific IGF-I knock-out mice. These mice had reduced IGF-I serum levels (25% of normal), but showed normal growth rates [123]. However, double knock-out mice of the liver-specific IGF-I knock-out mouse and the ALS knock-out mouse, had further reduced levels of circulating IGF-I and did show a decrease in longitudinal bone growth. This suggested that a threshold of circulating IGF-I is necessary for normal longitudinal bone growth [124], although autocrine/paracrine IGF-I levels are suggested to be still the major player in the growth plate (Fig. 4C). These data show that both endocrine as well as autocrine/paracrine IGF-I are important in the regulation of longitudinal bone growth.

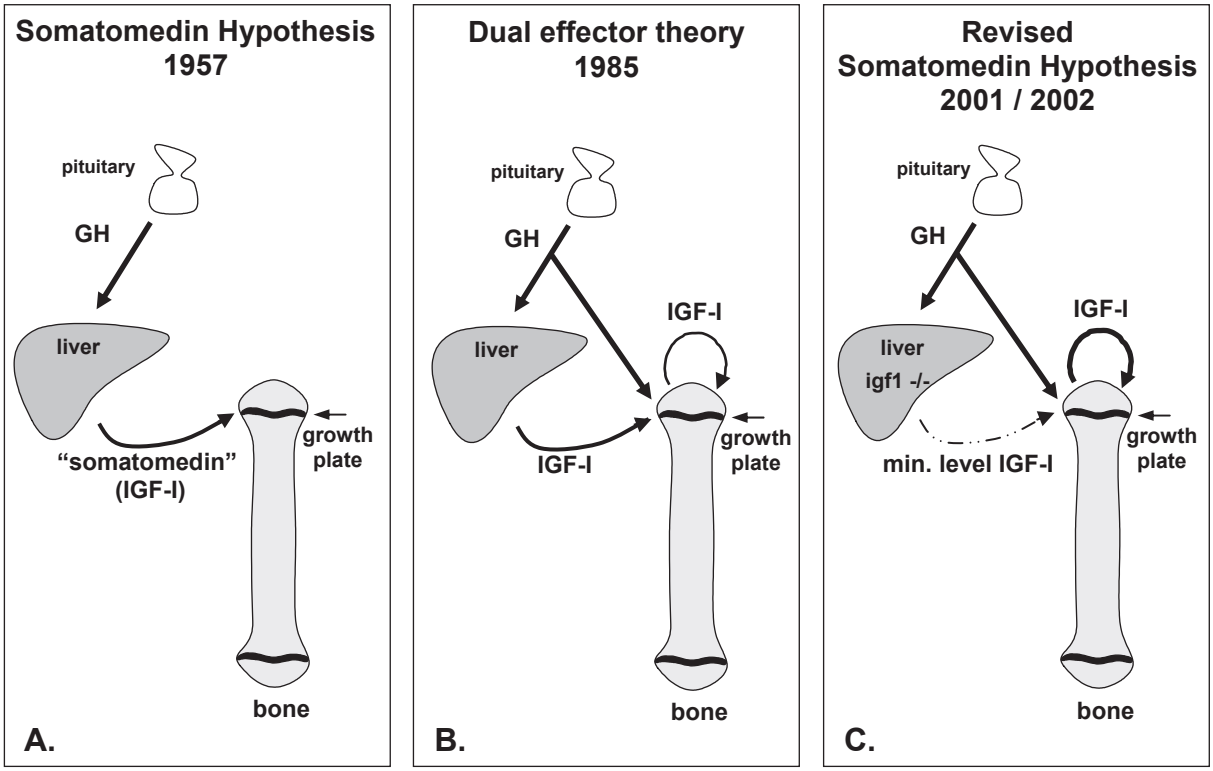

Figure 4 Evolving concepts in the somatomedin hypothesis. (**A**) The original somatomedin hypothesis proposed that GH controlled longitudinal growth in an endocrine fashion, by stimulating the liver-production of somatomedin (IGF-I), which in turn mediated longitudinal bone growth. (**B**) In the eighties, GH and IGF-I were also shown to have a direct effect on longitudinal bone growth, (with IGF-I also being produced locally) besides their endocrine effect, resulting in the dual effector theory. (**C**) Recently it was shown that minimal (min.) endocrine levels of IGF-I are required for normal longitudinal growth, although autocrine/paracrine actions of IGF-I still remain important (adapted from 125).

The IGF system in the growth plate

It has been suggested that IGF-I and IGF-II both have a unique and complementary role in stimulating longitudinal bone growth at the local level [125]. They are indeed both expressed in the growth plate. IGF-I was detected in the proliferative zone in the growth plates of postnatal mice [58], whereas in rats, IGF-I was detected in proliferative and hypertrophic

chondrocytes [126,127]. In fetal cows [128] and sheep [129], IGF-I was also detected in proliferative and hypertrophic chondrocytes. Together with the decreased proliferation and hypertrophic differentiation in IGF-I knock-out mice [58,78], this might indicate that IGF-I both stimulates proliferation and differentiation of chondrocytes. The IGF-IR is expressed in all zones of the growth plate [68,128]. Expression of IGF-II was primarily shown in the proliferative zone in both fetal and postnatal animals [128-131]. Although postnatally, endocrine IGF-II levels are low in rodents, IGF-II is expressed in growth plate chondrocytes [130,131], indicating a possible role for IGF-II in postnatal growth.

The IGFBPs are also present in growth plate chondrocytes. In fetal cows, IGFBP-3, -4 and -5 were predominantly expressed by proliferative chondrocytes, whereas IGFBP-2 was equally expressed in all zones [128]. In fetal sheep, IGFBP-2 to -5 were detected in the reserve and proliferative zone and little in the hypertrophic zone [129]. IGFBP-1 was not detected in the growth plate and only low levels of IGFBP-6 [129]. *In vivo* studies on murine growth plates only showed expression of IGFBP-5 and -6, which rapidly declined with age. Only low levels of IGFBP-6 were still detectable postnatally [130]. Several factors, including IGF-I and IGF-II, modulate the expression of IGFBPs by chondrocytes *in vitro* [132,133]. This suggests that the IGFBPs are also involved in the local regulation of longitudinal bone growth.

GLUCOCORTICOIDS

Glucocorticoids (GCs) are steroid hormones secreted from the adrenal glands in response to various stimuli and they are implicated in carbohydrate and protein metabolism. GCs diffuse across cell membranes and bind to the intracellular GC receptor (GR), which mediates their actions [1].

GC action

In the cytoplasm, the GR forms a heterocomplex with several chaperone proteins as hsp90 and immunophilins [134]. Upon binding with GCs, the chaperone proteins release from the GR and a nuclear localization signal and a DNA-binding domain are exposed. The GR-GC complex translocates to the nucleus [135], where it forms homodimers. Subsequently, the homodimers bind to specific consensus DNA sequences, the Glucocorticoid Response Elements (GREs), and activate gene expression [135].

Besides the activation of genes, GCs are also known to be involved in the transcriptional repression of genes. GC-induced repression of genes is not mediated via GREs, but is suggested to involve several other mechanisms, as transcriptional interference via interactions with other transcription factors as AP-1 and NF-κB or involvement of other DNA binding sites [136]. Inhibition of gene transcription by GCs does not require homodimerization of the GR [136]. Knock-out mice in which the GR cannot form homodimers and thus cannot activate but only repress genes, survive [137], whereas GR knock-out mice, in which both repression and activation is abolished, are not viable [138]. This indicates the importance of negative gene regulation by GCs [137,139]. Besides these genomic actions of GCs, they are also known to display rapid non-genomic actions, which are suggested to be mediated via GC-membrane associated receptors [140].

Use of GCs

Due to the potent and multiple inhibitory effects of GCs on the immune system, including lymphoid tissues as thymus and spleen, they are widely used as anti-inflammatory and immunosuppressive drugs in all kinds of diseases [1]. They are very effective to treat diseases like asthma, rheumatoid arthritis, chronic inflammatory or autoimmune diseases, as well as after organ transplantations.

For these applications in the clinic, synthetic GCs as prednisolone, dexamethasone and others are commonly used. These synthetic GCs have a greater GC activity than the naturally occurring cortisol, making them more suitable for clinical applications [1]. However, the use of GCs may result in several severe side-effects. Among these side-effects are suppression of the Hypothalamus-Pituitary-Adrenal (HPA) axis, skin thinning, metabolic changes, osteoporosis and growth retardation [141]. Growth retardation caused by GC treatment in children is becoming a greater problem, as the use of GCs in the treatment of mild-to-moderate asthma expands [142].

GC-induced growth retardation

GCs have been shown to induce not only growth retardation in man, but also in several experimental animal models, such as mice [143,144], rats [145], chickens [146], rabbits [147] and piglets [148,149], creating the possibility to study the effects of GCs in detail.

GCs are shown to act locally to inhibit growth, suggesting a mechanism intrinsic to the growth plate [150,151]. Local GC infusion in rabbits only decreased tibial length of the treated tibia, whereas the contralateral vehicle-treated tibia was not affected [150]. The GR has indeed been shown to be present in the growth plate, in the proliferative and hypertrophic zones [152], as well as in the resting zone [153,154], indicating that the chondrocytes of the different zones are GC target cells.

Several *in vitro* studies on growth plate chondrocytes have shown the susceptibility of proliferative chondrocytes to GCs [155,156]. GC treatment decreased chondrocyte proliferation, due to an increase in cell doubling times with a reduced number of S phase cells [157]. *In vivo* studies also showed effects of GCs on total growth plate width [144,151,158] and on the different zones of the growth plate. GCs are suggested to inhibit the growth of resting chondrocytes, which would result in fewer proliferative chondrocytes [159]. They also decrease the proliferation of chondrocytes in the proliferative zone [158,160] and increase the number of apoptotic chondrocytes in the hypertrophic zone [151,160]. In addition, angiogenesis activity, which is closely linked to apoptosis, is also suggested to be diminished by GCs [160]. Furthermore, they also affect the cartilaginous ECM, by inhibiting ECM production [158] and mineralization [161], which is confirmed in a recent *in vitro* study [153]. GCs thus disturb endochondral ossification at several stages, such as proliferation, apoptosis and angiogenesis, resulting in inhibition of longitudinal bone growth.

Catch-up growth

It is well established that growth retardation, caused by different diseases, malnutrition or GC treatment, could be of a temporary state. When the cause of the growth retardation is restored or removed, catch-up growth can occur; a phase of accelerated linear growth toward the "original" growth curve [162].

Catch-up growth after GC treatment is suggested to be a mechanism intrinsic to the growth plate [163]. Recent experiments showed that GCs inhibit proliferation of chondrocytes, but conserved their proliferative potential. The normal senescence of the growth plate is suggested to depend on the cumulative number of divisions chondrocytes have undergone [164]. Inhibition of proliferation by GCs would slow senescence and after GC treatment is terminated, the affected growth plate would be less senescent, resulting in catch-up growth. In recent experiments, accelerated senescence, caused by estrogen treatment, indeed showed exhaustion of proliferative potential of chondrocytes and consequently earlier fusion of the growth plates in rabbits [165].

Nevertheless, it should be borne in mind that catch-up growth is often not complete, depending on severity and duration of the GC therapy, but also on the age at onset of the treatment and at the termination of the treatment [142,159]. If children are treated for prolonged periods with GCs, permanent growth retardation is inevitable [142].

Glucocorticoids and the IGF system

For several years already, the adverse effects of GCs on growth have been suggested to involve impaired action of the GH-IGF system [155,166,167]. The IGFs have also been suggested to be able to counteract the adverse effects of GCs. This was shown *in vivo* for GH [168], *in vitro* for IGF-I [155] and for IGF-II in human IGF-II transgenic mice, which showed partial protection of the skeleton against the adverse effects of GCs [143].

Several studies, however, showed conflicting data concerning effects of GCs on serum levels of the IGFs and IGFBPs. GCs have been reported to alter the pulsatile secretion of GH [142,169], although no effects on GH have also been reported [155,170]. IGF-I plasma levels can be normal, decreased or increased during GC treatment [142], in both humans [169-171], as well as in experimental animal models [143,146,172]. However, serum IGF bioactivity has been shown to be decreased by GCs [167,173], this could be due to changes in levels of inhibitors of IGF action, the IGFBPs [174,175]. Analyses of GC effects on IGFBP serum levels, however, also showed conflicting data [169,171,175-177]. Serum levels of the IGF system therefore provide limited insight into the mechanism of GC-induced growth retardation and the possible involvement of the IGF system [177].

Because GC-induced growth retardation has been shown to occur at the local level of the growth plate [150,151], the effects of GCs on the IGF system in the growth plate appear to be much more important than the effects on serum levels [155,167], although no conclusive studies are yet performed. Several *in vitro* studies have indicated that GCs indeed regulate components of the IGF system in chondrocytes. In cultured rat growth plate chondrocytes, GCs suppressed GH-induced IGF-I and IGF-IR expression, although basal IGF-IR expression was not affected [156]. In rabbit costal chondrocytes, IGFBP-5 levels were inhibited and IGFBP-3 levels were increased upon GC treatment [133], whereas in fetal ovine growth plate chondrocytes, IGFBP-2 and -5 levels were inhibited by GCs [132].

No *in vivo* data, however, are available concerning the possible involvement of the local IGF system in the growth plate in GC-induced growth retardation. Besides this deficit in studies on GC-induced growth retardation and the local IGF system, the data available of *in vitro* studies on epiphyseal chondrocytes are scattered. Some have studied the IGFBPs, others only IGF-I. Also, IGF-II has been left out as a possible player in GC-induced growth retardation. Therefore, as a first step to unravel the involvement of the IGF system in

GC-induced growth retardation, one should study the effects of GCs on the IGF system at the local level of the growth plate, both *in vitro* and *in vivo*.

OUTLINE OF THIS THESIS

Several studies indicate a possible involvement of the components of the IGF axis in GC-induced growth retardation. However, effects of GCs on the endocrine IGF system have been shown to provide little information on the mechanism of GC-induced growth retardation. Furthermore, GCs are known to act locally at the level of the growth plate. Although a few *in vitro* studies in chondrocytes on the effects of GCs on the IGF system exist, these are incomplete and the involvement of the IGF system in GC-induced growth retardation still remains elusive. We aimed to elucidate the role of the IGF system in GC-induced growth retardation at the local level of the growth plate, using different experimental animal models.

We first studied in **chapter 2** the role of IGFBPs in the regulation of growth, in lymphoid tissues of hIGF-II transgenic mice, using non-radioactive *in situ* hybridization. In **chapter 3**, we investigated the *in vivo* effects of a long-term GC treatment of 4 weeks in 3-week-old mice, on the expression of the IGF system in the growth plates. To further elucidate a possible protective role of IGF-II against GC-induced growth retardation, we also performed in parallel a study using human IGF-II transgenic mice. We extended this study in **chapter 4**, where we performed a short-term GC treatment of 1 week in 3-week-old mice and analyzed the effects on general growth as well as on the growth plate and the effects on expression of the IGF system. In **chapter 5**, we used prepubertal (6-week-old) piglets to study the short-term effects of GC treatment on the growth plate, apoptosis and angiogenesis. To study the effects of GCs on angiogenesis in more detail, we studied in **chapter 6** the effects of GCs on VEGF, an important regulator of angiogenesis, in growth plate chondrocytes *in vitro*, derived from neonatal piglets. In **chapter 7**, we returned to the IGF system and studied the effects of GCs on proliferation and the IGF system in the same chondrocytes as used in chapter 6. In an addendum of chapter 7, we performed a preliminary study on the regulation of IGFBP-2 by GCs at the transcriptional level. In **chapter 8**, the possible involvement of the IGF system in GC-induced growth retardation at the local level of the growth plate is discussed, using the results described in **chapters 2** to **7**.

REFERENCES

1. Greenspan FS, Strewler G. Basic and clinical endocrinology. 5th ed. New Jersey: Prentice-Hall International Inc., 1997.
2. de Crombrugghe B, Lefebvre V, Nakashima K 2001 Regulatory mechanisms in the pathways of cartilage and bone formation. *Curr Opp Cell Biol,* **13:** 721-727.
3. Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R 1995 Toward a molecular understanding of skeletal development. *Cell,* **80:** 371-378.
4. Cancedda R, Descalzi CF, Castagnola P 1995 Chondrocyte differentiation. *Int Rev Cytol,* **159:** 265-358.
5. Hunziker EB 1994 Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Micr Res Techn,* **28:** 505-519.
6. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
7. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD, Baron J 2002 The role of the resting zone in growth plate chondrogenesis. *Endocrinology,* **143:** 1851-1857.
8. Horton WE, Jr, Feng L, Adams C 1998 Chondrocyte apoptosis in development, aging and disease. *Matrix Biol,* **17:** 107-115.

9. Gerber H-P, Ferrara N 2000 Angiogenesis and bone growth. *Trends Cardiovasc Med,* **10:** 223-228.
10. Yin M, Gentili C, Koyama E, Zasloff M, Pacifici M 2002 Antiangiogenic treatment delays chondrocyte maturation and bone formation during limb skeletogenesis. *J Bone Miner Res,* **17:** 56-65.
11. Horton WA. Morphology of connective tissue: cartilage. In: McCusick V, editor. Connective tissue and its heritable disorders. New York: Wiley-Liss Inc., 1993: 641-675.
12. Ruoslahti E 1991 Integrins. *J Clin Invest,* **87:** 1-5.
13. Svoboda KK 1998 Chondrocyte-matrix attachment complexes mediate survival and differentiation. *Micr Res Techn,* **43:** 111-122.
14. Werb Z 1997 ECM and cell surface proteolysis: regulating cellular ecology. *Cell*, **91:** 439-442.
15. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizau A, Matsumoto T, Kato S 1997 Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet,* **16:** 391-396.
16. Boyan BD, Sylvia VL, Dean DD, Schwartz Z 2001 24,25-(OH)(2)D(3) regulates cartilage and bone via autocrine and endocrine mechanisms. *Steroids,* **66:** 363-374.
17. Boyan BD, Sylvia VL, Dean DD, Del Toro F, Schwartz Z 2002 Differential regulation of growth plate chondrocytes by 1alpha,25-(OH)(2)D(3) and 24R,25-(OH)(2)D(3) involves cell-maturation-specific membrane-receptor-activated phospholipid metabolism. *Crit Rev Oral Biol Med,* **13:** 143-154.
18. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC 1994 Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J Cell Biol,* **126:** 1611-1623.
19. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VLJ, Kronenberg HM, Mulligan RC 1994 Lethal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev,* **8:** 277-289.
20. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra AB, Jüppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrP receptor in early development and indian-hedgehog-regulated bone growth. *Science,* **273:** 663-666.
21. Weir EC, Philbrick WM, Amling M, Neff LA, Baron R, Broadus AE 1996 Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc Natl Acad Sci U S A,* **93:** 10240-10245.
22. Lee K, Lanske B, Karaplis AC, Deeds JD, Kohno H, Nissenson RA, Kronenberg HM, Segre GV 1996 Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology,* **137:** 5109-5118.
23. Van der Eerden BCJ, Karperien M, Gevers EF, Löwik CWGM, Wit JM 1999 Expression of indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. *J Bone Min Res,* **15:** 1045-1055.
24. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ 1996 Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science,* **273:** 613-622.
25. Vortkamp A 2001 Interaction of growth factors regulating chondrocyte differentiation in the developing embryo. *Osteoarthritis Cartilage,* **9 (suppl A):** S109-S117.
26. Zou H, Wieser R, Massague J, Niswander L 1997 Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev,* **11:** 2191-2203.
27. Iwasaki M, Le AX, Helms JA 1997 Expression of indian hedgehog, bone morphogenetic protein 6 and gli during skeletal morphogenesis. *Mech Dev,* **69:** 197 -202.
28. Bostrom MP, Lane JM, Berberian WS, Missri AA, Tomin E, Weiland A, Doty SB, Blaser D, Rosen VM 1995 Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. *J Orthop Res,* **13:** 357-367.
29. Houston B, Thorp BH, Burt DW 1994 Molecular cloning and exprssion of bone morphogenetic protein-7 in the chick epiphyseal growth plate. *J Mol Endocrinol,* **13:** 289-301.
30. Yazaki Y, Matsunaga S, Onishi T, Nagamine T, Origuchi N, Yamamoto T, Ishidou Y, Imamura T, Sakou T 1998 Immunohistochemical localization of bone morphogenetic proteins and the receptors in epiphyseal growth plate. *Anticancer Res,* **18:** 2339-2344.
31. Jingushi S, Scully SP, Joyce ME, Sugioka Y, Bolander ME 1995 Transforming growth factor β and fibroblast growth factors in rat growth plate. *J Orthop Res,* **13:** 761-768.
32. Petersen KG, Werner S, Chen G, Williams LT 1992 Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development,* **114:** 233-243.

33. Coffin JD, Florkiewicz RZ, Neumann J, Mort-Hopkins T, Dorn GW, Lightfoot P, German R, Howles PN, Kier A, O'Toole BA, Sase J, Gonzalez AM, Baird A, Doetschman T 1995 Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol Biol Cell,* **6:** 1861-1873.
34. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P 1996 Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell,* **84:** 911-921.
35. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM 1996 Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat Genet,* **12:** 390-397.
36. Mancilla EE, De Luca F, Uyeda JA, Czerwiec FS, Baron J 1998 Effects of fibroblast growth factor-2 on longitudinal bone growth. *Endocrinology,* **139:** 2900-2904.
37. Harvey CB, O'Shea PJ, Scott AJ, Robson H, Siebler T, Shalet S, Samarut J, Chassande O, Williams GR 2002 Molecular mechanisms of thyroid hormone effects on bone growth and function. *Mol Genet Metab,* **75:** 17-30.
38. Robson H, Siebler T, Stevens DA, Shalet S, Williams GR 2000 Thyroid hormone acts directly on growth plate chondrocytes to promote hypertrophic differentiation and inhibit clonal expansion and cell proliferation. *Endocrinology,* **141:** 3887-3897.
39. Smeets T and Van Buul-Offers SC 1986 Influence of growth hormone and thyroxine on cell kinetics in the proximal tibial growth plate of Snell dwarf mice. *Cell Tissue Kinet,* **19:** 161-170.
40. Fraichard A, Chassande O, Plateroti M, Roux JP, Trouillas J, Dehay C, Legrand C, Gauthier K, Kedinger M, Malaval L, Rousset B, Samarut J 1997 The T3R alpha gene encoding a thyroid hormone receptor is essential for postnatal development and thyroid hormone production. *EMBO J,* **16:** 4412-4420.
41. Gauthier K, Plateroti M, Harvey CB, Williams GR, Weiss RE, Refetoff S, Willott JF, Sundin V, Roux JP, Malaval L, Hara M, Samarut J, Chassande O 2001 Genetic analysis reveals different functions for the products of the thyroid hormone receptor alpha locus. *Mol Cell Biol,* **21:** 4748-4760.
42. Lewinson D, Harel Z, Shenzer P, Silbermann M, Hochberg Z 1989 Effect of thyroid hormone and growth hormone on recovery from hypothyroidism of epiphyseal growth plate cartilage and its adjacent bone. *Endocrinology,* **124:** 937-945.
43. Smith EP, Boyd J, Frank GR, Takahashi M, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in man. *N Engl J Med,* **331:** 1056-1060.
44. Grumbach MM 2000 Estrogen, bone, growth and sex: a sea change in conventional wisdom. *J Pediatr Endocrinol Met*, **13:** 1439-1455.
45. Vidal O, Lindberg MK, Hollberg K, Baylink DJ, Andersson G, Lubahn DB, Mohan S, Gustafsson JA, Ohlsson C 2000 Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. *Proc Natl Acad Sci U S A,* **97:** 5474-5479.
46. Van der Eerden BCJ, Gevers EF, Löwik CWGM, Karperien M, Wit JM 2002 Expression of estrogen receptor α and β in the epiphyseal plate of the rat. *Bone,* **30:** 478-485.
47. Van der Eerden BCJ, Karperien M, Wit JM 2001 The estrogen receptor in the growth plate: implications for pubertal growth. *J Pediatr Endocrinol Met,* **14:** 1527-1533.
48. Van der Eerden BCJ, van Til NP, Brinkmann AO, Löwik CWGM, Wit JM, Karperien M 2002 Gender differences in expression of androgen receptor in tibial growth plate and metaphyseal bone of the rat. *Bone,* **30:** 891-896.
49. Noble B, Routledge J, Stevens H, Hughes I, Jacobson W 1999 Androgen receptors in bone-forming tissue. *Horm Res*, **51:** 31-36.
50. Van der Eerden BCJ, van de Ven J, Löwik CWGM, Wit JM, Karperien M 2002 Sex steroid formation in the tibial growth plate of the rat. *Endocrinology,* **143:** 4048-4055.
51. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, and Werb Z 1998 MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell,* **93:** 411-422.
52. Ferrara N 1999 Molecular and biological properties of vascular endothelial growth factor. *J Mol Med,* **77:** 527-543.
53. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N 1999 VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med,* **5:** 623-628.
54. Garcia-Ramirez M, Toran N, Andaluz P, Carrascosa A, Audi L 2000 Vascular endothelial growth factor is expressed in human fetal growth cartilage. *J Bone Miner Res,* **15**: 534-540.
55. Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE 1999 Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. *J Anat,* **194:** 519-524.

56. Vajo Z, Francomano CA, Wilkin DJ 2000 The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: the achondroplasia family of skeletal dysplasias, Muenke craniosynostosis, and Crouzon syndrome with acanthosis nigricans. *Endocr Rev,* **21:** 23-39.
57. Butler AA, LeRoith D 2001 Control of growth by the somatropic axis: growth hormone and the insulin-like growth factors have related and independent roles. *Annu Rev Physiol,* **63:** 141-164.
58. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A 2001 Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol,* **229:** 141-162.
59. Salmon WD, Daughaday WH 1957 A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage *in vitro*. *J Lab Clin Med,* **49:** 825-826.
60. Van den Brande JL 1999 A personal view on the early history of the insulin-like growth factors. *Horm Res,* **51 (suppl 3):** 149-175.
61. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev,* **16:** 3-34.
62. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC 1998 Growth hormone and bone. *Endocr Rev,* **19:** 55-79.
63. Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE, Baumann G, Kopchick JJ 1997 A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci U S A,* **94:** 13215-13220.
64. Savage MO, Burren CP, Blair JC, Woods KA, Metherell L, Clark AJ, Camacho-Hubner C 2001 Growth hormone insensitivity: pathophysiology, diagnosis, clincial variation and future perspectives. *Horm Res,* **55 (suppl 2):** 32-35.
65. Wit JM, Drayer NM, Jansen M, Walenkamp MJ, Hackeng WH, Thijssen JH, Van den Brande JL 1989 Total deficiency of growth hormone and prolacting, and partial deficiency of thyroid stiumulating hormone in two Dutch families: a new variant of hereditary pituitary deficiency. *Horm Res,* **32:** 170-177.
66. Woods KA, Savage MO 1996 Laron syndrome: typical and atypical forms. *Baillieres Clin Endocrinol Metab,* **10:** 371-387.
67. Lindahl A, Isgaard J, Nilsson A, Isaksson OGP 1986 Growth hormone potentiates colony formation of epiphyseal chondrocytes in suspension culture. *Endocrinology,* **118:** 1843-1848.
68. Hunziker EB, Wagner J, Zapf J 1994 Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes in vivo. *J Clin Invest,* **93:** 1078-1086.
69. Bondy CA, Werner H, Roberts CT, Jr, LeRoith D 1990 Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. *Mol Endocrinol,* **4:** 1386-1398.
70. Streck RD, Pintar JE 1992 The embryonic pattern of rat insulin-like growth factor-I gene expression suggests a role in induction and early growth of the liver. *Endocrinology,* **131:** 2030-2032.
71. Streck RD, Wood TL, Hsu MS, Pintar JE 1992 Insulin-like growth factor I and II and insulin-like growth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic and fetal limbs. *Dev Biol,* **151:** 586-596.
72. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Bloemen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
73. Smeets T, Van Buul-Offers SC 1983 The influence of growth hormone, somatomedins, prolactin and thyroxine on the morphology of the proximal epiphysis and growth plate of Snell dwarf mice. *Growth,* **47:** 160-173.
74. Van Buul-Offers SC, Hoogerbrugge CM, Branger J, Feijlbrief M, Van den Brande JL 1988 Growth-stimulating effects of somatomedin-/insulin-like peptides in Snell dwarf mice. *Horm Res,* **29:** 229-236.
75. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (*Igf-1*) and type 1 IGF receptor (*Igf1r*). *Cell,* **75:** 59-72.
76. Baker J, Liu JP, Robertson EJ, Efstratiadis A 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* **75:** 73-82.
77. Woods KA, Camacho-Hubner C, Savage MO, Clark AJ 1996 Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *N Engl J Med,* **335:** 1363-1367.
78. Wang J, Zhou J, Bondy CA 1999 IGF1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J,* **13:** 1985-1990.
79. Lee JE, Pintar JE, Efstratiadis A 1990 Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development,* **110:** 151-159.

80. Stylianopoulou F, Efstratiadis A, Herbert J, Pintar JE 1988 Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. *Development,* **103:** 497-506.
81. D'Ercole AJ 1996 Insulin-like growth factors and their receptors in growth. *Endocrinol Metab Clin North Am,* **25:** 573-590.
82. DeChiara TM, Efstratiadis A, Robertson EJ 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature,* **345:** 78-80.
83. DeChiara TM, Robertson EJ, Efstratiadis A 1991 Parental imprinting of the mouse insulin-like growth factor II gene. *Cell,* **64:** 849-859.
84. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B 1997 The IGF-I receptor in cell growth, transformation and apoptosis. *Biochim Biophys Acta,* **1332:** F105-F126.
85. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, Jacobs S, Francke U, Ramachandran J, Fujita-Yamaguchi Y 1986 Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J,* **5:** 2503-2512.
86. Rechler MM, Nissley SP 1985 The nature and regulation of the receptors for insulin-like growth factors. *Annu Rev Physiol,* **47:** 425-442.
87. Nissley SP, Kiess W, Sklar MM. The insulin-like growth factor-II/mannose-6-phosphate receptor. In: LeRoith D, editor. Insulin-like growth factors: cellular and molecular aspects. Boca Raton: CRC Press, 1991: 111-150.
88. Nissley P, Kiess W 1991 Reciprocal modulation of binding of lysosomal enzymes and insulin-like growth factor-II (IGF-II) to the mannose 6-phosphate/IGF-II receptor. *Adv Exp Med Biol,* **293:** 311-324.
89. Kornfeld S 1992 Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. *Annu Rev Biochem,* **61:** 307-330.
90. Wang ZQ, Fung MR, Barlow DP, Wagner EF 1994 Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature,* **372:** 464-467.
91. Rajaram S, Baylink DJ, Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev,* **18:** 801-831.
92. Pintar J, Schuller A, Bradshaw S, Cerro J, Grewal A. Genetic disruption of IGF binding proteins. In: Takano K, Hizuka N, Takahashi S-I, editors. Molecular mechanisms to regulate the activities of Insulin-like Growth Factors. Amsterdam: Elsevier Science, 1998: 65-70.
93. Lee PD, Giudice LC, Conover CA, Powell DR 1997 Insulin-like growth factor binding protein-1: recent findings and new directions. *Proc Soc Exp Biol Med,* **216:** 319-357.
94. Wolf E, Lahm H, Wu M, Wanke R, Hoeflich A 2000 Effects of IGFBP-2 overexpression *in vitro* and *in vivo*. *Pediatr Nephrol,* **14:** 572-578.
95. Hoeflich A, Nedbal S, Blum WF, Erhard M, Lahm H, Brem G, Kolb HJ, Wanke R, Wolf E 2001 Growth inhibition in giant growth hormone transgenic mice by overexpression of insulin-like growth factor-binding protein-2. *Endocrinology,* **142:** 1889-1898.
96. Hoeflich A, Wu M, Mohan S, Foll JL, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb H, Wolf E 1999 Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. *Endocrinology,* **140:** 5488-5496.
97. Hoeflich A, Pavicic T, Nedbal S, Schmidt C, Weher U, Rambeck W, Wolf E, Eckstein F 2002 Effects of IGFBP-2 and GH overexpression on bone growth in transgenic mice (abstract). *GH and IGF Res,* **12:** 224-225.
98. Angelloz-Nicoud P, Binoux M 1995 Autocrine regulation of cell proliferation by the insulin like growth factor (IGF) and IGF binding protein-3 protease system in a human prostat carcinoma cell line (PC-3). *Endocrinology,* **136:** 5485-5492.
99. Cohen P, Lamson G, Okajima T, Rosenfeld RG 1993 Transfection of the human insulin-like growth factor binding protein-3 gene into Balb/c fibroblasts inhibits cellular growth. *Mol Endocrinol,* **7:** 380-386.
100. Cornell HJ, Embery G, Herington AC 1986 Association of insulin like growth factors with metabolically inactive and active carrier bound complexes in sera. *J Endocrinol,* **241:** 744-749.
101. Conover CA, Ronk M, Lombana F, Powell DR 1990 Structural and biological characterization of bovine insulin like growth factor binding protein-3. *Endocrinology,* **127:** 2795-2803.
102. Van Buul-Offers SC, Reijnen-Gresnigt MG, Bloemen R, Hoogerbrugge C, Van den Brande JL 1995 Co-administration of IGF-binding protein-3 differentially inhibits the IGF-I-induced total body and organ growth of Snell dwarf mice. *Prog Growth Factor Res,* **6:** 377-383.
103. Murphy LJ, Molnar P, Lu X, Huang H 1995 Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. *J Mol Endocrinol,* **15:** 293-303.

104. Mohan S, Bautista CM, Wergedal J, Baylink DJ 1989 Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: a potential local regulator of IGF action. *Proc Natl Acad Sci U S A,* **86:** 8338-8342.
105. Culouscou JM, Shoyab M 1991 Purification of a colon cancer cell growth inhibitor and its identification as insulin-like growth factor binding protein-4. *Cancer Res,* **51:** 2813-2819.
106. LaTour D, Mohan S, Linkhart TA, Baylink DJ, Strong DD 1990 Inhibitory insulin-like growth factor-binding protein: cloning, complete sequence, and physiological regulation. *Mol Endocrinol,* **4:** 1806-1814.
107. Mohan S, Baylink DJ 1996 Insulin-like growth factor system components and the coupling of bone formation to resorption. *Horm Res,* **45 (suppl 1):** 59-62.
108. Mohan S, Farley JR, Baylink DJ 1995 Age-related changes in IGFBP-4 and IGFBP-5 levels in human serum and bone: implications for bone loss with aging. *Prog Growth Factor Res,* **6:** 465-473.
109. Jones JI, Gockerman A, Busby WH Jr, Camacho-Hubner C, Clemmons DR 1993 Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. *J Cell Biol,* **121:** 679-687.
110. Twigg SM, Baxter RC 1998 Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *J Biol Chem,* **273:** 6074-6079.
111. Hwa V, Oh Y, Rosenfeld RG 1999 The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev,* **20:** 761-787.
112. Baxter RC 2000 Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *Am J Physiol Endocrinol Metab,* **278:** E967-E976.
113. Perks CM, Newcomb PV, Norman MR, Holly JM 1999 Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells. *J Mol Endocrinol,* **22:** 141-150.
114. Schuett BS, Langkamp M, Elmlinger MW, Ranke MB 2002 Integrin mediated IGF-independent action of IGFBP-2 and IGFBP-2 fragments (abstract). *GH and IGF Res,* **12:** 275.
115. Lee K-W, Cohen P 2002 Nuclear effects: unexpected intracellular actions of insulin-like growth factor binding protein-3. *J Endocrinol,* **175:** 33-40.
116. Miyakoshi N, Richman C, Kasukawa Y, Linkhart TA, Baylink DJ, Mohan S 2001 Evidence that IGF-binding protein-5 functions as a growth factor. *J Clin Invest,* **107:** 73-81.
117. Green H, Morikawa M, Nixon T 1985 A dual effector theory of growth-hormone action. *Differentiation,* **29:** 195-198.
118. Isaksson OGP, Jansson J-O, Gause IAM 1982 Growth hormone stimulates longitudinal bone growth directly. *Science,* **216:** 1237-1239.
119. Barnard R, Haynes KM, Werther GA, Waters MJ 1988 The ontogeny of growth hormone receptors in the rabbit tibia. *Endocrinology,* **122:** 2562-2569.
120. Werther GA, Haynes KM, Barnard R, Waters MJ 1990 Visual demonstration of growth hormone receptors on human growth plate chondrocytes. *J Clin Endocrinol Metab,* **70:** 1725-1731.
121. Edmondson SR, Baker NL, Oh J, Kovacs G, Werther GA, Mehls O 2000 Growth hormone receptor abundance in tibial growth plates of uremic rats: GH/IGF-I treatment. *Kidney Int,* **58:** 62-70.
122. Gevers EF, Van der Eerden BCJ, Karperien M, Raap AK, Robinson ICAF, Wit JM 2002 Localization and regulation of the growth hormone receptor and growth hormone-binding protein in the rat growth plate. *J Bone Miner Res,* **17:** 1408-1419.
123. Sjögren K, Liu J-L, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Isaksson OG, Jansson J-O, Ohlsson C 1999 Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proc Natl Acad Sci U S A,* **96:** 7088-7092.
124. Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu J-L, Ooi GT, Setser J, Frystyk J, Boisclair YR, LeRoith D 2002 Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest,* **110:** 771-784.
125. LeRoith D, Bondy CA, Yakar S, Liu J-N, Butler A 2001 The somatomedin hypothesis: 2001. *Endocr Rev,* **22:** 53-74.
126. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology,* **141:** 2847-2853.
127. Nilsson A, Carlsson B, Isgaard J, Isaksson OGP, Rymo L 1990 Regulation by GH of insulin-like growth factor-I mRNA expression in rat epiphyseal growth plate as studied with in-situ hybridization. *Endocrinology,* **125:** 67-74.
128. Olney RC, Mougey EB 1999 Expression of the components of the insulin-like growth factor axis across the growth plate. *Mol Cell Endocrinol,* **156:** 67-71.

129. de los Rios P, Hill DJ 1999 Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of the ovine fetus: possible functional implications. *Can J Physiol Pharmacol,* **77:** 235-249.
130. Wang E, Wang J, Chin E, Zhou J, Bondy CA 1995 Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology,* **136:** 2741-2751.
131. Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M 1993 Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology,* **132:** 1158-1167.
132. de los Rios P, Hill DJ 2000 Expression and release of insulin-like growth factor binding proteins in isolated epiphyseal growth plate chondrocytes from the ovine fetus. *J Cell Physiol,* **183:** 172-181.
133. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
134. Pratt WB, Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev,* **18:** 306-360.
135. Beato M, Herrlich P, Schütz G 1995 Steroid hormone receptors: many actors in search of a plot. *Cell,* **83:** 851-857.
136. Webster JC, Cidlowski JA 1999 Mechanisms of glucocorticoid-receptor-mediated repression of gene expression. *Trends Endocrinol Met,* **10:** 396-402.
137. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schütz G 1998 DNA binding of the glucocorticoid receptor is not essential for survival. *Cell,* **93:** 531-541.
138. Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schimid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schütz G 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev,* **9:** 1608-1621.
139. Karin M 1998 New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell,* **93:** 487-490.
140. Borski RJ 2000 Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends Endocrinol Met,* **11:** 427-436.
141. Hanania NA, Chapman KR, Kesten S 1995 Adverse effects of inhaled corticosteroids. *Am J Med,* **98:** 196-208.
142. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
143. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
144. Altman A, Hochberg Z, Silbermann M 1992 Interactions between growth hormone and dexamethasone in skeletal growth and bone structure of young mice. *Calcif Tissue Int,* **51:** 298-304.
145. Ortoft G, Gronbaek H, Oxlund H 1998 Growth hormone administration can improve growth in glucocorticoid-injected rats without affecting the lymphocytopenic effect of the glucocorticoid. *Growth Horm IGF Res,* **8:** 251-264.
146. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
147. Sheagren JN, Jowsey J, Bird DC, Gurton ME, Jacobs JB 1977 Effect on bone growth of daily versus alternate-day corticosteroid administration: an experimental study. *J Lab Clin Med,* **89:** 120-130.
148. Fritz PC, Ward WE, Atkinson SA, Tenenbaum HC 1998 Tamoxifen attenuates the effects of exogenous glucocorticoid on bone formation and growth in piglets. *Endocrinology,* **139:** 3399-3403.
149. Guo C-Y, Ward W, Cairns P, Atkinson S 2000 Comparative response in growth and bone status to three dexamethasone treatment regimens in infant piglets. *Pediatr Res,* **48:** 238-243.
150. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
151. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
152. Silvestrini G, Mocetti P, Ballanti P, Di Grezia R, Bonucci E 1999 Cytochemical demonstration of the glucocorticoid receptor in skeletal cells of the rat. *Endocrine Res,* **25:** 117-128.
153. Siebler T, Robson H, Shalet SM, Williams GR 2002 Dexamethasone inhibits and thyroid hormone promotes differentiation of mouse chondrogenic ATDC5 cells. *Bone,* **31:** 457-464.
154. Abu EO, Horner A, Kusec V, Triffitt JT, Compston JE 2000 The localization of the functional glucocorticoid receptor α in human bone. *J Clin Endocrinol Metab,* **88:** 883-889.

155. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
156. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
157. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.
158. Annefeld M 1992 Changes in rat epiphyseal cartilage after treatment with dexamethasone and glycosaminoglycan-peptide complex. *Pathol Res Pract,* **188:** 649-652.
159. Gafni RI, Baron J 2000 Catch-up growth: possible mechanisms. *Pediatr Nephrol,* **14:** 616-619.
160. Sanchez CP, He Y-Z 2002 Alterations in the growth plate cartilage of rats with renal failure receiving corticosteroid therapy. *Bone,* **30:** 692-698.
161. Silbermann M, Maor G 1978 Mechanisms of glucocorticoid-induced growth retardation: impairment of cartilage mineralization. *Acta Anat,* **101:** 140-149.
162. Boersma B, Wit JM 1997 Catch-up growth. *Endocr Rev,* **18:** 646-661.
163. Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD, Cutler GBJ 1994 Catch-up growth after glucocorticoid excess: a mechanism intrinsic to the growth plate. *Endocrinology,* **135:** 1367-1371.
164. Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S, Baron J 2001 Catch-up growth is associated with delayed senescence of the growth plate in rabbits. *Pediatr Res,* **50:** 618-623.
165. Weise M, De-Levi S, Barnes KM, Gafni RI, Abad V, Baron J 2001 Effects of estrogen on growth plate senescence and epiphyseal fusion. *Proc Natl Acad Sci U S A,* **98:** 6871-6876.
166. Robson H, Siebler T, Shalet S, Williams GR 2002 Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. *Pediatr Res,* **52:** 137-147.
167. Mehls O, Himmele R, Hömme M, Kiepe D, Klaus G 2001 The interaction of glucocorticoids with the growth hormone-insulin-like growth factor axis and its effects on growth plate chondrocytes and bone cells. *J Pediatr Endocrinol Met,* **14:** 1475-1482.
168. Mauras N 2001 Growth hormone therapy in the glucocorticoidsteroid-dependent child: metabolic and linear defects. *Horm Res,* **56 (suppl 1):** 13-18.
169. Miell JP, Taylor AM, Jones J, Holly JMP, Gaillard RC, Pralong FP, Ross RJM, Blum WF 1993 The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF-binding proteins in normal male volunteers. *J Endocrinol,* **136:** 525-533.
170. Ogueh O, Miell JP, Jones JC, Jones JS, Alaghband-Zadeh J, Johnson MR 2000 Antenatal dexamethasone and the growth hormone - insulin-like growth factor axis. *Hum Reprod,* **15:** 1403-1406.
171. Kapila P, Jones J, Rees L 2001 Effect of chronic renal failure and prednisolone on the growth hormone-insulin-like growth factor axis. *Pediatr Nephrol,* **16:** 1099-1104.
172. Luo J, Murphy LJ 1989 Dexamethasone inhibits growth hormone induction of insulin-like growth factor-I (IGF-I) messenger ribonucleic acid (mRNA) in hypophysectomized rats and reduces IGF-I mRNA abundance in the intact rat. *Endocrinology,* **125:** 165-171.
173. Caufriez A, Copinschi G 1986 Somatomedins and steroids. *Horm Res,* **24:** 185-188.
174. Unterman TG, Phillips LS 1985 Glucocorticoid effect on somatomedins and somatomedin inhibitors. *J Clin Endocrinol Metab,* **61:** 618-626.
175. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
176. Bang P, Degerblad M, Thoren M, Schwander J, Blum W, Hall K 1993 Insulin-like growth factor (IGF) I and II and IGF binding protein (IGFBP) 1, 2 and 3 in serum from patients with Cushing's syndrome. *Acta Endocrinol,* **128:** 397-404.
177. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.

Chapter 2

Insulin-like growth factor (IGF) II induced changes in expression of IGF binding proteins in lymphoid tissues of hIGF-II transgenic mice

J.J. Smink, J.G. Koster, B.I. Hendriks-Stegeman and S.C. van Buul-Offers.

Department of Pediatric Endocrinology, University Medical Center Utrecht, Utrecht, The Netherlands.

Endocrinology 1999, **140:** 5876-5882

ABSTRACT

Overexpression of human insulin-like growth factor II (IGF-II) in transgenic mice does not result in increased overall body growth. The IGF-II overexpression, however, specifically causes growth of the thymus and not of the spleen. We address the question whether the observed differences in growth induction in lymphoid tissues by IGF-II can be related to differences in local IGF-binding protein (IGFBP) production, using non-radioactive *in situ* hybridization and Northern blot analysis.

IGFBP-2, -4 and -5 are expressed in both lymphoid tissues of normal mice. The spleen additionally expressed IGFBP-3 and IGFBP-6. IGFBP-1 expression was not detected. Although the expression *pattern* of the IGFBPs did not change upon IGF-II overexpression, the level of expression changed in a specific manner for each IGFBP. In both the thymus and the spleen of transgenic mice, IGFBP-2 and -5 gene expression was slightly increased, whereas the level of IGFBP-4 expression was not altered. In the spleen, IGFBP-6 expression was not altered by IGF-II overexpression, whereas IGFBP-3 expression was strongly increased.

The differences in IGFBP expression, and the difference in response of these IGFBPs to IGF-II overexpression in thymus and spleen suggests an important role for these proteins in growth regulation of both lymphoid tissues. We speculate that an increase of IGFBP-3 expression together with changes in expression of other IGFBPs, inhibits IGF-II stimulated growth in the spleen by an autocrine/paracrine pathway.

INTRODUCTION

The insulin-like growth factors (IGF-I and -II) are potent mitogenic and differentiation promoting growth factors [1]. Furthermore, the IGFs can inhibit cell death, induce differentiation and stimulate differentiated functions in several cell types [2]. The IGFs play an important role in pre- and postnatal growth. In rodents, IGF-I is presumed to be important in pre- and postnatal growth, whereas IGF-II only seems to be important in prenatal growth [3,4].

IGFs are produced in multiple tissues and can act both in an endocrine and autocrine/paracrine fashion. The activity of IGFs is regulated at various levels, resulting in a complex regulation of IGF bioactivity [2,5]. Their intracellular effects are mediated predominantly via the type I IGF receptor [6].

IGFs are present in the circulation and throughout the extracellular space bound to members of high affinity IGF binding proteins (IGFBPs). The availability of the IGFs for their receptor is modulated by these IGFBPs, of which six are cloned and characterized [7,8]. IGFBP genes are widely expressed in the developing tissues of rodents [9], sheep [10] and humans [11]. The IGFBPs act mainly as autocrine and/or paracrine factors at or close to their sites of synthesis [2]. The individual IGFBPs differ in their tissue distribution and may either inhibit or potentiate IGF activity. Furthermore, they differ in their IGF-binding capacity [2,12]. IGFBP-3 is the major serum carrier of IGFs, in a complex with an acid-labile subunit (ALS) [13].

Transgenic mice with recombinant human IGF-II under the control of the H-2K^b promoter show increased IGF-II serum levels, whereas overall body growth is not affected [14]. The hIGF-II transgene is highly expressed in the two lymphoid tissues, the thymus and the spleen, causing thymic but no splenic growth [14-16].

Because there are indications that the IGFBPs regulate actions of locally produced IGFs in lymphoid tissues [5,17], we studied the IGFBP expression in the spleen and the thymus of normal and the hIGF-II transgenic mice *in situ*. The observed differences in IGFBP expression and the difference in response to IGF-II overexpression suggest an important role for the IGFBPs in growth regulation. The IGFBP-3 gene, expressed in the spleen only, is most susceptible to IGF-II overexpression. We speculate that IGFBP-3 plays a prominent paracrine role with respect to the inhibition of the hIGF-II transgene bioactivity in the spleen, by either an IGF-dependent or IGF-independent mechanism.

MATERIALS AND METHODS

Materials

All restriction enzymes and modifying enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany), as well as the digoxigenin-UTP, anti-digoxigenin Fab-fragments, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphatase, blocking reagent, the Tripure isolation reagent and the Agarose Gel DNA extraction kit. Nylon membranes were purchased from QIAGEN (Westburg, The Netherlands). [α^{32}P]dCTP (10 mCi/ml) and the RediPrime Random Primer labeling mixture were obtained from Amersham (Buckinghamshire, UK). Polyvinyl alcohol was obtained from Aldrich (Milwaukee, MI). Euparal mounting medium was purchased from Klinipath (Duiven, The Netherlands).

Mouse IGFBP-1 to -6 complementary DNAs (cDNAs) were kindly provided by Prof. Dr. S. L. S. Drop and Dr. J.W. van Neck (Department of Pediatrics, subdivision of Pediatric Endocrinology, Rotterdam, The Netherlands). GAPDH cDNA was kindly provided by Dr. H. van Teeffelen (Department of Physiological Chemistry, Utrecht, The Netherlands).

Mice and tissue preparation

hIGF-II transgenic mice were generated by introduction of a human IGF-II gene into FVB/N control mice as described in detail by Van Buul-Offers and colleagues [14]. Throughout the study, the line designated 5'-74 (TgII), was used for our experiments. As controls, normal FVB/N mice were used. The animals were kept under standardized laboratory conditions. The mice were killed by decapitation after ether anaesthesia at the age of 4 weeks. Thymus and spleen were dissected, frozen in liquid nitrogen and stored at -80°C. The protocol received approval of the committee for Animal Experiments of the Medical Faculty, University of Utrecht.

Probes

Digoxigenin-labeled complementary RNA (cRNA) probes

Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP as substrate [18]. cDNAs encoding human IGF-II [19] and mouse IGFBP-1, -2, -3, -4, -5 and -6 cDNAs, corresponding to amino acid position 100-133, 98-258, 137-204, 131-205, 88-182, 83-140 respectively [9], were used as templates for the synthesis of antisense and/or sense digoxigenin-labeled RNA probes.

[α^{32}P]dCTP-labeled cDNA probes

Twenty nanograms of gel-purified inserts of plasmids containing mouse IGFBP-2, -3 and -5 cDNA, were radiolabeled with 50 µCi [α^{32}P]dCTP, using random primed DNA labeling as described by the manufacturer.

In situ hybridization

Tissues used for *in situ* hybridization were fixed for 18 hours in 4% (wt/vol) paraformaldehyde at 4°C, washed in PBS, dehydrated through a series of ethanol and embedded in paraffin.

Paraffin tissue sections (10 µm) were dewaxed, hydrated, rinsed in PBS and treated with proteinase K (0.07 units/ml) for 30 minutes at 37°C and subjected to an acetylation treatment [20]. Sections were rinsed in 2xSSC and kept in this solution until the start of the hybridization. Hybridization was performed in a solution containing 50% formamide, 2xSSC, 1x Denhardt's solution, 1 µg/µl yeast RNA and 10% dextran-sulphate and the digoxigenin labeled cRNA probe at a concentration of 500-1500 pg/µl. Sections were hybridized overnight at 53°C. After hybridization, sections were washed with 50% formamide in 2xSSC at 53°C for 30 minutes and treated with RNase A (1 unit/ml) for 30 minutes at 37°C. Subsequently, sections were rinsed in 2xSSC, treated with 10% lamb serum for 30 minutes and incubated with sheep antidigoxigenin Fab-fragments coupled to alkaline phosphatase (1:500) for 2 h at room temperature.

Chromogenesis was performed with 0.38 mg/ml nitroblue tetrazolium and 0.19 mg/ml 5-bromo-4-chloro-3-indolyl-phosphatase in the presence of 6% (wt/vol) polyvinylalcohol [21], resulting in a blue precipitate. Sections were counterstained with nuclear fast red, dehydrated through a series of ethanol and mounted with Euparal.

Three to four different animals of each strain were used per analyzed IGFBP messenger RNA (mRNA) and for each probe, the *in situ* hybridization was repeated 5 to 6 times.

Northern blot analysis

RNA extraction

Total RNA was extracted from frozen spleen and thymus from 4-week-old normal (FVB/N) and hIGF-II transgenic mice, using Tripure solution reagent according to the procedures of the manufacturer, based on the single step acid guanidinium thiocyanate method [22] .

Northern blot analysis

Twenty micrograms of total RNA was separated by electrophoresis in a 1% (wt/vol) agarose/2.2M formaldehyde gel in 1x 3-(morpholino) propanesulphonic acid (MOPS) buffer and transferred to a 0.2 µm nylon membrane and crosslinked by UV radiation (1200 µJ).

The membranes were prehybridized for 2 hours at 60°C in a solution containing 0.1% SDS, 3xSSC, 5xDenhardt's solution, 10% dextran-sulphate and 50 µg/ml denaturated salmon sperm DNA. Hybridization was performed at 60°C overnight in the same solution, containing the [α^{32}P]dCTP-labeled probe.

Following hybridization, the membranes were washed to a stringency of 0.2xSSC, 0.1% (wt/vol) SDS. Membranes were analyzed using the GS-363 Molecular Imager (Biorad, Hercules, CA) and subsequently quantified using the Molecular Analyst software program, version 1.4 (Biorad). The signals were also visualized by autoradiography on Fuji RX X-ray film.

Tissues of at least three different animals of each strain were analyzed.

Statistical analysis

Data of the Northern blot analyses were analyzed with Student's two-tailed test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

IGFBP expression patterns and changes therein upon IGF-II overexpression

Thymus

Representative thymus sections of 4-week-old normal and hIGF-II transgenic mice, analyzed with *in situ* hybridization using specific IGFBP probes, are shown in Fig. 1.

Control *in situ* hybridizations of thymus sections of transgenic mice with non-radioactive human IGF-II RNA probe showed very strong staining in the medulla and in a small number of cells in the cortex (Fig. 1B), whereas hardly any IGF-II transcripts could be detected in the thymus of FVB/N non-transgenic mice (Fig. 1A). As a control for specific hybridization, sections were hybridized with the corresponding sense RNA probes, which showed no signals (see *e.g.*, Fig. 1C). In the thymus of normal mice no expression of IGFBP-1, -3, and -6 could be detected (Fig. 1, D-F). IGFBP-2, -4 and -5 transcripts were present, each showing a unique expression pattern (Fig. 1, G-J). Transcripts of IGFBP-2 were present in groups of cells, mainly at the boundary of the medulla and the cortex and, at a low level, in the medulla (Fig. 1G). IGFBP-4 was exclusively detected in the medulla (Fig. 1H). A similar expression pattern was seen for IGFBP-5 (Fig. 1J); however the number of cells expressing IGFBP-5 was lower than for IGFBP-4. IGFBP-4 and -5 transcripts were detected in single positive cells and not in groups of positive cells as seen for IGFBP-2.

To study the influence of IGF-II overexpression on IGFBP expression, thymus sections of 4-week-old hIGF-II transgenic mice were analyzed with *in situ* hybridization (Fig. 1, K-M). No expression of IGFBP-1, -3, or -6 could be detected (data not shown). The expression patterns of IGFBP-2, -4 and -5 in the transgenic thymus was similar to that found in the thymus of normal mice (Fig. 1, K-M). However, the level of expression was slightly increased

for IGFBP-2, (Fig. 1, G and K), and to a higher extent for IGFBP-5 (Fig 1, J and M). IGFBP-4 transcripts were present at the same level as in normal mice. Although the shown data suggests a lower level of IGFBP-4 mRNA in the transgenic thymus (Fig. 1L), most of the performed experiments showed no difference in levels of expression. However, the picture shown was chosen to illustrate the expression pattern.

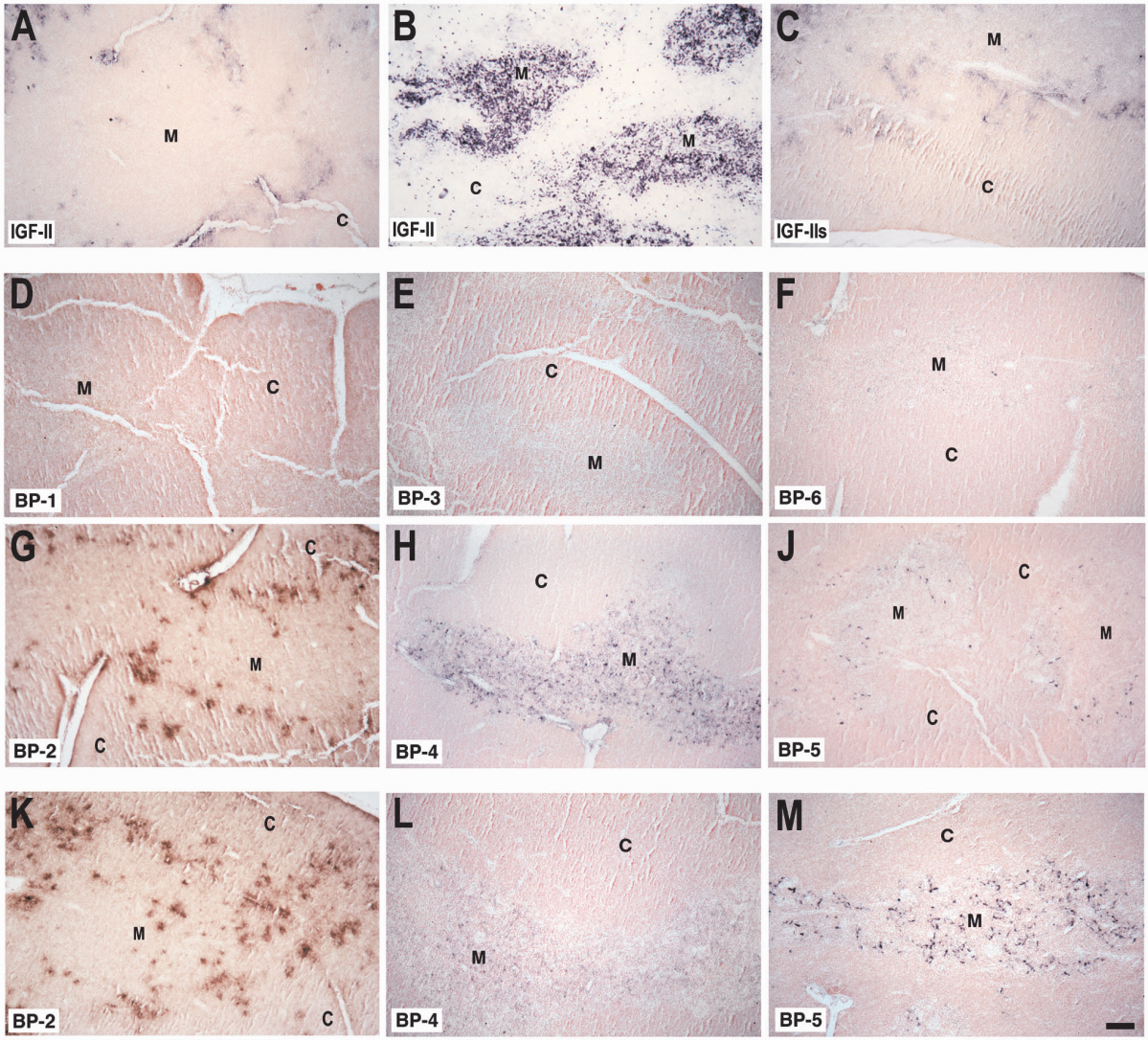

Figure 1 IGFBP expression in the thymus. Expression patterns of IGF-II and IGFBP-1 to -6 mRNA in representative sections of the thymus of 4-week-old normal (FVB/N) (**A**; **D-J**) and hIGF-II transgenic (**B**; **K-M**) mice, as analyzed by *in situ* hybridization, under brightfield illumination. (**C**) A representative section of the transgenic thymus hybridized with an IGF-II sense RNA probe (IGF-IIs). Sections were hybridized with antisense digoxigenin-labeled cRNA probes, specific for human IGF-II and mouse IGFBP-1 to -6 as indicated. All sections were counterstained with nuclear fast red. The mRNA signal is shown as a blue precipitate under brightfield illumination. The signal of IGFBP-2 mRNA (**G** and **K**) is shown as a dark brownish precipitate, due to the used mounting medium. **M**, medulla; **C**, cortex. Magnification, 100 times. The black bar in the right conner refers to the actual size of 100 μm.

Spleen

Representative spleen sections of 4-week-old normal and hIGF-II transgenic mice, analyzed using *in situ* hybridization with specific mouse IGFBP probes are shown in Fig. 2.

Control *in situ* hybridizations of spleen sections of the transgenic mice with non-radioactive hIGF-II RNA probe showed high IGF-II transgene expression in the white pulp and a few positively stained cells in the red pulp and/or marginal zone (Fig. 2B). Spleen sections of the non-transgenic FVB/N control mice showed a very weak IGF-II expression in

the red pulp (Fig. 2A). In the spleen of normal mice, no IGFBP-1 gene expression could be detected (data not shown). IGFBP-2, -3, -4, -5 and -6 mRNAs were present, each showing a unique expression pattern (Fig. 2, C, D, G-J). Control sections hybridized with the appropriate sense probes exhibited no signal (data not shown).

IGFBP-2 is expressed at a high level in groups of cells in the red pulp (RP) and at a very low level in the white pulp (WP) (Fig. 2C). IGFBP-3 transcripts were detected, at a low level, in the inner lining of the marginal zone (MZ) and in some cells in the white pulp, whereas no IGFBP-3 transcripts could be detected in the red pulp (Fig. 2D). IGFBP-4 transcripts were detected all through in the white pulp only (Fig. 2G).

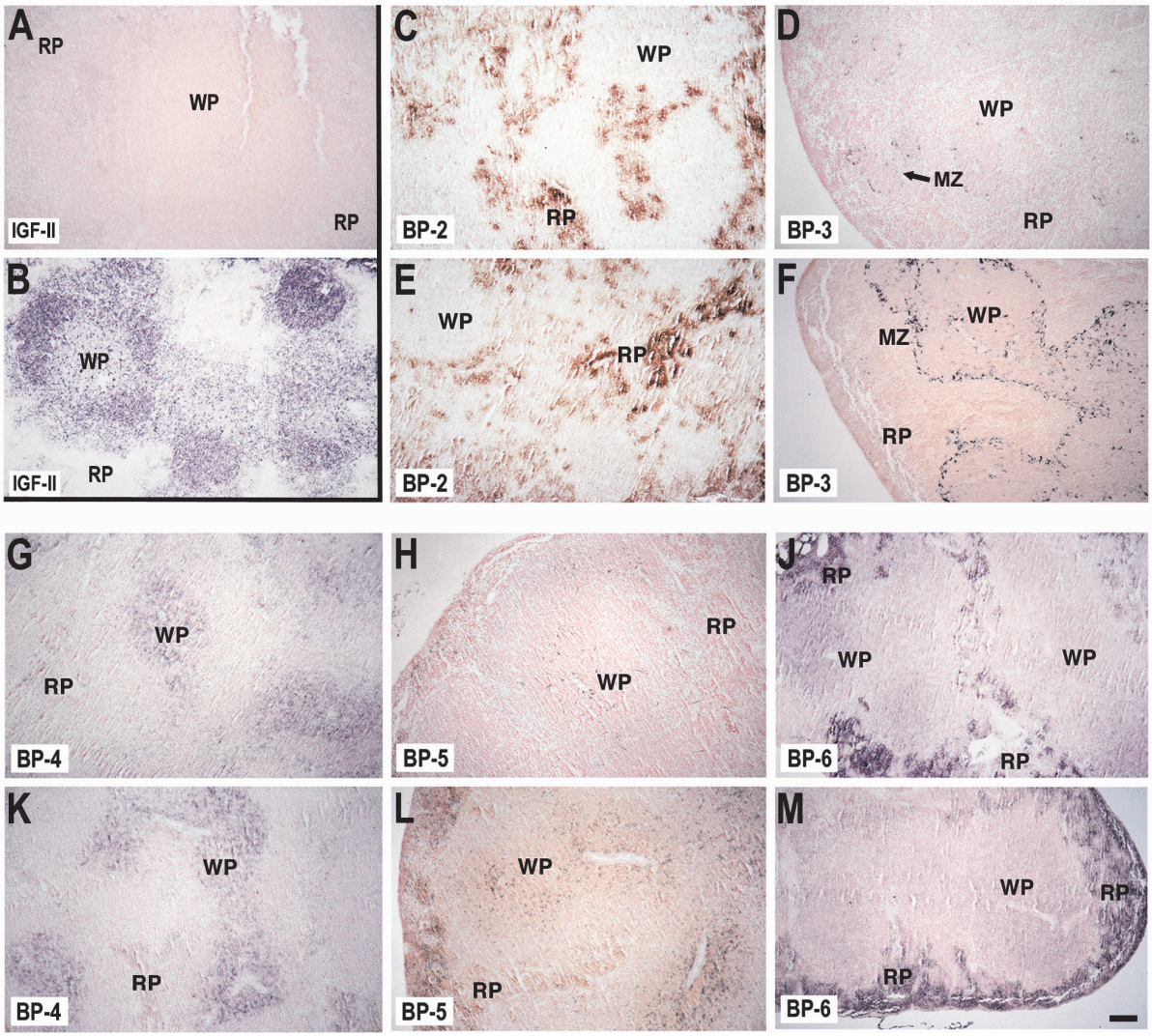

Figure 2 IGFBP expression in the spleen. Expression and localization of IGF-II and IGFBP-2 to -6 mRNA in representative sections of the spleen of 4-week-old normal mice (**A**; **C-D**; **G-J**) and hIGF-II transgenic mice (**B**; **E-F**; **K-M**) by *in situ* hybridization, under brightfield illumination. Sections were hybridized with antisense digoxigenin-labeled cRNA probes, specific for human IGF-II and mouse IGFBP-1 to -6 as indicated. Sections were further treated as described in Fig. 1. **WP**, white pulp; **RP**, red pulp; **MZ**, marginal zone. Magnification: 100 times. The black bar in the right conner refers to the actual size of 100 μm.

IGFBP-5 transcripts were detected in the white pulp in sparsely scattered cells and in cells concentrated around the veins. IGFBP-5 expression was also detected at a low level at the exterior part of the red pulp (Fig. 2H). At the exterior part of the red pulp, also a considerable amount of IGFBP-6 transcripts were detected, whereas lower amounts were detected in the other parts of the red pulp and in the white pulp (Fig. 2J).

To study the influence of IGF-II overexpression on IGFBP expression, spleen sections of 4-week-old hIGF-II transgenic mice were analyzed using *in situ* hybridization (Fig. 2, E, F, K-M). No expression of IGFBP-1 could be detected in the transgenic spleen (data not shown). Although the level of expression of some IGFBPs changed upon IGF-II overexpression, the pattern of expression remained the same, as was observed in the thymus. IGFBP-4 and -6 transcripts were detected at the same level as in normal mice (*cf.* Fig. 2, G and K and *cf.* Fig. 2, J and M, respectively). On the contrary, the level of expression of IGFBP-2, -3 and -5 was increased as compared to the expression level in the normal spleen.The expression of IGFBP-2 was slightly increased in the white pulp, whereas the level of expression in the red pulp was not altered (*cf.* Fig. 2, C and E). The expression of IGFBP-5, detected in the red pulp at the edge of the section and in the white pulp, showed an increase in level of expression (Fig. 2, H and L). IGFBP-3 expression in the marginal zone and the white pulp was strongly increased (Fig 2, D and F).

Quantification of changes in IGFBP-3 expression

To quantify the changes in IGFBP-3 expression upon IGF-II overexpression, Northern blot analysis was performed. Total RNA was extracted from the spleen and the thymus of 4-week-old normal and hIGF-II transgenic mice and subsequently hybridized with a mouse IGFBP-3 [α^{32}P]dCTP-labeled cDNA probe, a representative experiment is shown in Fig. 3A. This revealed a transcript of 2.6 kb (Fig. 3A), corresponding to an IGFBP-3 transcript length as described previously [23]. This transcript was only present in the spleen of normal and hIGF-II transgenic mice (Fig. 3A, lanes 4 and 1, respectively) and not in the thymus of either mice strain (Fig. 3A, lanes 2 and 3, respectively). Quantification of the detected IGFBP-3 transcripts in the normal spleen (n=3) and hIGF-II transgenic spleen (n=5), after normalization with the GAPDH mRNA signal, showed a statistically significant increase in level of expression in the transgenic spleen of 1.75 ± 0.11-fold (P<0.01) (Fig. 3B).

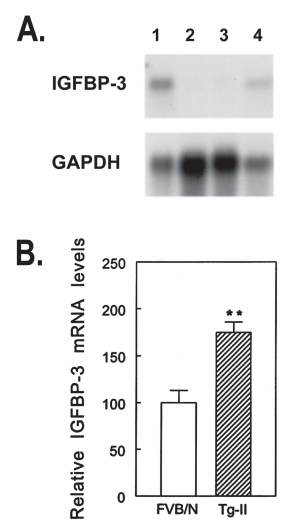

Figure 3 Quantitative analysis of changes in IGFBP-3 expression. Total RNA extracted from the spleen and the thymus of 4-week-old normal and hIGF-II transgenic mice was analyzed using Northern blot analysis. The membrane was hybridized with a cDNA probe specific for mouse IGFBP-3. The filter was subsequently hybridized with a GAPDH probe as a control for the amount of transferred RNA in each sample. Representative Northern blot data are shown in panel A. (**A**) The RNA in lanes 1 and 4 were derived from the spleen; in lanes 2 and 3 from the thymus. The RNAs in the lanes 2 and 4 were derived from normal mice; in lanes 1 and 3 from hIGF-II transgenic mice. (**B**) Data obtained from Northern blots of the spleen, which have been quantified by densitometry. The open bar represents IGFBP-3 mRNA levels of the spleen of normal mice (FVB/N) and the cross-hatched bar of hIGF-II transgenic mice (Tg-II). The data are expressed as a percentage of the IGFBP-3 mRNA level detected in the spleen of normal mice, which has been attributed a value of 100%. Means ± S.E.M. are given. The numbers of animals is FVB/N: 3; Tg-II: 5. **P<0.01 compared with the mean normal spleen value.

An increase in expression of IGFBP-2 and -5 in the thymus and spleen of hIGF-II transgenic mice is shown using *in situ* hybridization, although not as strong as seen for IGFBP-3 in the spleen. Quantification of IGFBP-2 and -5 expression using Northern blot analysis showed the presence of IGFBP-2 and -5 mRNA in the spleen and the thymus of normal and hIGF-II transgenic mice, however, no statistically significant differences were detected (data not shown).

DISCUSSION

Besides the endocrine actions of IGFs, these hormone peptides can exert actions locally in a variety of tissues and cell types. Tissue-specific regulation of the IGF-bioactivity may be accomplished by the presence of IGFBPs, which are differentially expressed in various tissues [2]. The relative concentration of each IGFBP changes, depending on the physiological and pathological environment [12,24]. In hIGF-II transgenic mice, both the spleen and the thymus express the hIGF-II gene at a high level [14,16], which might therefore induce a change in local IGFBP expression. Furthermore, it was shown that both IGF receptors are present in both organs, enabling IGF-II bioactivity [16]. However, the thymus displayed growth in response to this IGF-II overexpression, whereas the size of the spleen was unaffected, although both organs displayed an increase in $CD4^+$ T cells [15].

To answer the question whether the difference in response to IGF-II overexpression was related to differences in IGFBP expression, we studied the expression of the six IGFBP genes in both lymphoid tissues of normal and hIGF-II transgenic mice .

Normal IGFBP expression patterns in lymphoid organs

Each IGFBP gene has a specific, unique expression pattern in both lymphoid tissues, as shown in this study using non-radioactive *in situ* hybridization analysis. In both lymphoid tissues, IGFBP-2, -4 and -5 transcripts were detected. In addition to these genes, the spleen expressed the IGFBP-3 and -6 gene. IGFBP-4 and -5 displayed a similar expression pattern, in both tissues. On the contrary, IGFBP-2 transcripts showed a different localization, and were mainly present in the red pulp of the spleen and at the boundary of the medulla/cortex in the thymus. IGFBP-6 transcripts were mainly detected in the red pulp of the spleen and at a low concentration in the white pulp, colocalizing with IGFBP-2, -4 and -5 transcripts. IGFBP-3 transcripts showed a completely different localization, and were present mainly in the marginal zone. Colocalization and differences in localization of certain IGFBPs was previously shown at the tissue level in human fetus, mouse kidney and mouse skeleton [11,25,26]. In both the spleen and the thymus, the IGFBP transcripts are detected in mature lymphocytes, based on the localization and morphology of the positively stained cells. IGFBP-3 transcripts, however, are predominantly present in the macrophages of the marginal zone, based on staining results with an antibody specific for the detection of macrophages (MOMA) present in the marginal zone (Koster, personal communication), although a low expression in lymphocytes in the white pulp is also shown.

The observed differences in IGFBP expression in the spleen and the thymus of normal mice suggest a putative difference in mediating local IGF activity in these tissues.

IGF-II induced changes in IGFBP expression

Previous findings in the literature already suggested a direct relationship between IGFs and IGFBPs at the serum level [27-30], especially of IGF-II on IGFBP-3 in Pit-1 deficient Snell dwarf mice [30]. In this study a direct relationship between IGF-II and a number of IGFBPs is shown at tissue level, varying in different tissues. In spleen and thymus, IGFBP-2 and -5 mRNA are positively correlated with IGF-II mRNA, as previously shown also at the protein level for IGFBP-2 in serum of PEPCK-IGF-II transgenic mice [27] and for IGFBP-5 in human serum [28]. IGFBP-4 mRNA does not seem to be correlated with IGF-II mRNA levels, in accordance with data concerning the protein in human serum [29]. IGFBP-6 expression, only present in the spleen, seems also not to be correlated with levels of IGF-II mRNA, as shown

here in this study. The IGF-II-induced changes in IGFBP expression are summarized in Table 1.

Table 1 IGF-II-induced changes

Tissue	Growth	mRNA			
		IGF-II	IGFBP-2	IGFBP-3	IGFBP-5
Thymus	+	⇑⇑⇑⇑	↑	-	⇑
Spleen	-	⇑⇑⇑⇑	↑	⇑⇑	⇑

Changes of growth and IGF-II and IGFBP expression in the thymus and the spleen of 4-week-old hIGF-II transgenic mice, as compared to normal mice, are indicated. The size of the arrow(s) is representative for the observed increase in level of expression. The increase in IGFBP-2 and -5 is based on the *in situ* results, while the increase of IGFBP-3 is based on the *in situ* and the Northern blot analysis.

Previous studies in IGF-I transgenic mice have shown an *in vivo* temporally and spatially relationship between IGF-I mRNA and IGFBP-5 mRNA, in the brain [31]. This previous study with IGF-I transgenic mice and our study with IGF-II transgenic mice, indicate the possibility of tissue-specific direct regulation of the expression of IGFBPs by their ligands, the IGFs.

In the spleen, the IGFBP-3 gene seems to be most susceptible to IGF-II overexpression, which is confirmed by Northern blot analysis. hIGF-II transgenic Snell dwarf mice also show an increase in IGFBP-3 mRNA in the spleen and an increase in IGFBP-3 serum levels, as compared to normal Snell dwarf mice [14] (unpublished results). In these hIGF-II transgenic Snell dwarf mice no IGF-I is present [14,24]. Furthermore, IGF-I serum levels are not elevated in normal hIGF-II transgenic mice [14], indicating that the increase of IGFBP-3 in hIGF-II transgenic normal and Snell dwarf mice cannot be caused by IGF-I. In addition, IGF-II treatment of dwarf mice results in a pronounced increase of IGFBP-3 in serum [30].

Role of IGFBP-3 in growth regulation

As discussed above, the main difference between the spleen and the thymus in terms of regulation of IGFBP expression by IGF-II is the considerable increase in IGFBP-3 expression in the macrophages of the marginal zone of the spleen. The number of these macrophages in the marginal zone is not increased in the spleen of hIGF-II transgenic mice, indicating that the increase in IGFBP-3 expression is due to constitutive IGF-II expression and not due to changes in morphology of the marginal zone. The presence and upregulation of IGFBP-3 is independent of GH and/or IGF-I, since similar results were obtained in hIGF-II transgenic Snell dwarf mice (unpublished results). The IGF-II-induced increase in IGFBP-3 expression can have effects on several mechanisms involving growth regulation. First, it has been demonstrated *in vitro* [32-35] and *in vivo* [36], that IGFBP-3 can act as a negative growth regulator of cell proliferation by sequestering the IGF-II. Thus, the spleen-specific enhanced IGFBP-3 expression might inhibit the growth-inducing effect of the IGF-II transgene in the spleen by sequestering IGF-II and thereby diminishing the IGF-II bioactivity. Observations in IGFBP-3 transgenic mice [37] seem to contradict this speculation, as in these mice an increase of IGFBP-3 serum levels results in growth of the spleen. However, no IGFBP-3 mRNA could be detected in the spleen of these mice, excluding high levels of IGFBP-3 in the spleen. Furthermore, the enhanced IGFBP-3 serum levels were of the binary complex. As this IGF-IGFBP-3 complex can cross the capillary endothelium, enhanced serum levels can transport more IGF-I or -II into the spleen causing enhanced IGF-bioactivity. Thus, in these animals, IGFBP-3 probably acts mainly by an endocrine pathway, instead of the proposed autocrine/paracrine way of action in our transgenics.

Besides the growth-inhibitory effect of IGFBP-3 caused by regulating the availability of free IGFs, IGFBP-3 can also induce growth arrest [38-40] and apoptosis, mediated through a pathway independent of the IGF-IGF-receptor interaction [41]. The relatively high concentrations of IGFBP-3 in the transgenic spleen could have such an IGF-II independent activity.

Therefore, although the influence of other IGFBPs on the IGF-II bioactivity should not be neglected, the considerable increase in IGFBP-3 mRNA and selective expression in the spleen, suggest that this IGFBP plays a prominent role in the reduction of the growth promoting effect of IGF-II in the spleen.

In conclusion, in this study it has been shown that the thymus and the spleen display a tissue-specific IGFBP expression. In all likelihood, it is the difference in IGFBP expression that causes a difference in local IGF-II bioactivity, resulting in a strong growth promoting effect of IGF-II in the thymus and hardly any in the spleen. This suggests that the growth-inducing effect of IGF-II depends on its local environment.

ACKNOWLEDGEMENTS

Thanks are due to Mrs. M.G. Gresnigt and Mr. R.J. Bloemen for technical assistance, to Mrs. I. van de Brink for taking care of the animals, to Dr. J.A. Koedam for critical reading the manuscript and to Mr. R. Hövig in help with photographic illustrations.

REFERENCES

1. Cohick WS, Clemmons DR 1993 The insulin-like growth factors. *Annu Rev Physiol,* **55:** 131-153.
2. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev,* **16:** 3-34.
3. Baker J, Liu JP, Robertson EJ, Efstratiadis A 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* **75:** 73-82.
4. Van Buul-Offers SC 1996 Insulin-like growth factor-II in the cycle of life. *Biomedical Reviews,* **5:** 65-71.
5. Clark R 1997 The somatogenic hormones and insulin-like growth factor-I: stimulators of lymphopoieses and immune function. *Endocr Rev,* **18:** 157-179.
6. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B 1997 The IGF-I receptor in cell growth, transformation and apoptosis. *Biochim Biophys Acta,* **1332:** F105-F126.
7. Rajaram S, Baylink DJ, Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev,* **18:** 801-831.
8. Clemmons DR 1997 Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev,* **8:** 45-62.
9. Schuller AGP, Zwarthoff EC, Drop SLS 1993 Gene expression of the six insulin-like growth factor binding proteins in the mouse conceptus during mid- and late gestation. *Endocrinology,* **132:** 2544-2550.
10. Delhanty PJD, Han VKM 1993 The expression of insulin-like growth factor (IGF) II and IGF binding protein-2 genes during ovine development. *Endocrinology,* **132:** 41-52.
11. Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K 1996 IGF-binding protein mRNAs in the human fetus: tissue and cellular distribution of developmental expression. *Horm Res,* **45:** 160-166.
12. Mohan S, Baylink DJ 1996 Editorial: insulin-like growth factor (IGF)-binding proteins in serum. Do they have additional roles besides modulating the endocrine IGF actions? *J Clin Endocrinol Metab,* **81:** 3817-3820.
13. Baxter RC 1995 Insulin-like growth factor binding proteins as glucoregulators. *Metabolism,* **44:** 127.
14. Van Buul-Offers SC, de Haan K, Reijnen-Gresnigt MG, Meinsma D, Jansen M, Oei SL, Bonte EJ, Sussenbach JS, Van den Brande JL 1995 Overexpression of human insulin-like growth factor-II in transgenic mice causes growth of the thymus. *J Endocrinol,* **144:** 491-502.
15. Kooijman R, Van Buul-Offers SC, Scholtens LE, Schuurman H-J, Van den Brande JL, Zegers BJM 1995 T cell development in insulin-like growth factor-II transgenic mice. *J Immunol,* **15:** 5736-5745.
16. Van der Ven LTM, Roholl PJM, Reijnen-Gresnigt MG, Bloemen RJ, Van Buul-Offers SC 1997 Expression of insulin-like growth factor II (IGF-II) and histological changes in the thymus and spleen of transgenic mice overexpressing IGF-II. *Histochem Cell Biol,* **107:** 193-203.

17. Van Buul-Offers SC, Kooijman R 1998 The role of growth hormone and insulin-like growth factors in the immune system. *Cell Mol Life Sci,* **54:** 1083-1094.
18. Melton D, Krieg P, Rebaglati M, Maniatis T, Zinn K, Green MR 1984 Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res,* **12:** 7035-7056.
19. Jansen M, Van Schaik FMA, Van Tol H, Van den Brande JL, Sussenbach JS 1985 Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. *FEBS Lett,* **179:** 243-246.
20. Wilkinson DG. *In situ* hybridization: a practical approach. Oxford: Oxford University Press, 1992.
21. De Block M, Debrouwer D 1993 RNA-RNA *in situ* hybridization using digoxigenin-labeled probes: the use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. *Anal Biochem,* **215:** 86-89.
22. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* **162:** 156-159.
23. Molnar P, Murphy LJ 1994 Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. *J Mol Endocrinol,* **13:** 59-67.
24. Van Buul-Offers SC, Bloemen RJ, Reijnen-Gresnigt MG, Van Leiden HA, Hoogerbrugge CM, Van den Brande JL 1994 Insulin-like growth factor I and II and their binding proteins during postnatal development of dwarfed Snell mice before and during growth hormone and thyroxine therapy. *J Endocrinol,* **143:** 191-198.
25. Lindenbergh KD, Rosato RR, van NJ, Nauta J, van KM, Groffen C, Zwarthoff EC, Drop SL 1997 Gene expression of the insulin-like growth factor system during mouse kidney development. *Mol Cell Endocrinol,* **132:** 81-91.
26. Wang E, Wang J, Chin E, Zhou J, Bondy CA 1995 Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology,* **136:** 2741-2751.
27. Wolf E, Kramer R, Blum WF, Föll JL, Brem G 1994 Consequences of postnatally elevated insulin-like growth factor-II in transgenic mice: endocrine changes and effects on body and organ growth. *Endocrinology,* **135:** 1877-1886.
28. Mohan S, Libanati C, Dony C, Lang K, Srinivasan N, Baylink DJ 1995 Development, validation, and application of a radioimmunoassay for insulin-like growth factor binding protein-5 in human serum and other biological fluids. *J Clin Endocrinol Metab,* **80:** 2638-2645.
29. Honda Y, Landale EC, Strong DD, Baylink DJ, Mohan S 1996 Recombinant synthesis of insulin-like growth factor binding protein-4 (IGFBP-4): development, validation, and application of a radioimmunoassay for IGFBP-4 in human serum and other biological fluids. *J Clin Endocrinol Metab,* **81:** 1389-1396.
30. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Blomen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
31. Ye P, D'Ercole J 1998 Insulin-like growth factor I (IGF-I) regulates IGF binding protein-5 gene expression in the brain. *Endocrinology,* **139:** 65-71.
32. Anelloz-Nicoud P, Binoux M 1995 Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostat carcinoma cell line (PC-3). *Endocrinology,* **136:** 5485-5492.
33. Cohen P, Lamson G, Okajima T, Rosenfeld RG 1993 Transfection of the human insulin-like growth factor binding protein-3 gene into Balb/c fibroblasts inhibits cellular growth. *Mol Endocrinol,* **7:** 380-386.
34. Kaicer E, Blat C, Imbenotte J, Troalen F, Cussenot OI, Calvo F, Harel L 1993 IGF binding protein-3 secreted by the prostate adenocarcinoma cells (PC-3) differential effect on PC-3 and normal prostate cell growth. *Growth Regul,* **3:** 180-189.
35. Perkel VS, Mohan S, Baylink DJ, Linkhart TA 1990 An inhibitory insulin-like growth factor binding protein (In-IGFBP) from human prostatic cell conditioned medium reveals N-terminal sequence identity with bone derived In-IGFBP. *J Clin Endocrinol Metab,* **71:** 533-535.
36. Van Buul-Offers SC, Reijnen-Gresnigt MG, Bloemen R, Hoogerbrugge C, Van den Brande JL 1995 Co-administration of IGF-binding protein-3 differentially inhibits the IGF-I-induced total body and organ growth of Snell dwarf mice. *Prog Growth Factor Res,* **6:** 377-383.
37. Murphy LJ, Molnar P, Lu X, Huang H 1995 Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. *J Mol Endocrinol,* **15:** 293-303.
38. Mohseni-Zadeh S, Binoux M 1997 Insulin-like growth factor (IGF) binding protein-3 interacts with the type 1 IGF receptor, reducing the affinity of the receptor for its ligand: an alternative mechanism in the regulation of IGF action. *Endocrinology* **138** 5645-5648.

39. Velez-Yanguas MC, Kalebic T, Maggi M, Kappel CC, Letterio J, Uskokovic M, Helman LJ 1996 1alpha, 25-dihydroxy-16-ene-23-yne-26, 27-hexafluorocholecalciferol (Ro24-5531) modulation of insulin-like growth factor-binding-3 and induction of differentiation and growth arrest in a human osteosarcoma cell line. *J Clin Endocrinol Metab,* **81:** 93-99.
40. Moerman EJ, Thweatt R, Moerman AM, Jones RA, Goldstein S 1993 Insulin-like growth factor binding protein-3 is overexpressed in senescent and quiescent human fibroblasts. *Exp Gerontol,* **28:** 361-370.
41. Rajah R, Valentinis B, Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-b1 on programmed cell death through a p53- and IGF-independent mechanism. *J Biol Chem,* **272:** 128181-12188.

Chapter 3

IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice

J.J. Smink, J.G. Koster, M.G. Gresnigt, R. Rooman*, J.A. Koedam and S.C. van Buul-Offers

*Department of Pediatric Endocrinology, University Medical Center Utrecht, Utrecht, The Netherlands, *Department of Pediatrics, Antwerp University Hospital, Belgium*

Journal of Endocrinology 2002, **175**: 143-153

ABSTRACT

Glucocorticoid (GC) treatment in childhood can lead to suppression of longitudinal growth as a side-effect. The actions of GCs are thought to be mediated in part by impaired action of the insulin-like growth factors (IGF-I and IGF-II) and their binding proteins (IGFBP-1 to -6). We have studied the effects of GCs on IGF and IGFBP expression at the local level of the growth plate, using non-radioactive *in situ* hybridization.

We treated 3-week-old normal mice for 4 weeks with dexamethasone (DXM). We also treated human IGF-II (hIGF-II) transgenic mice in order to investigate whether IGF-II could protect against the growth retarding effect of this GC. DXM treatment resulted in general growth retardation in both mice strains, however, only in normal mice was tibial length decreased. In both normal and hIGF-II trangenic mice, the total width of the growth plate was not affected, whereas the width of the proliferative zone decreased as a result of the DXM treatment. Additionally, only in normal mice, the width of the hypertrophic zone thickened.

Only expression of IGF-I, IGF-II and IGFBP-2 could be detected in the growth plates of 7-week-old normal mice. IGFBP-1, -3, -4, -5 and -6 mRNAs were not detected. DXM treatment of normal mice induced a significant 2.4-fold increase in the number of cells expressing IGF-I mRNA, whereas IGF-II and IGFBP-2 mRNA levels were not affected.

In hIGF-II transgenic mice, IGF-I mRNA levels were significantly increased, while endogenous IGF-II and IGFBP-2 mRNAs were unaffected, compared to normal animals. DXM treatment of the hIGF-II transgenic mice induced a further increase of IGF-I mRNA expression, to a similar extent as in DXM-treated normal mice.

The increase of IGF-I due to DXM treatment in normal mice might be a reaction in order to minimize the GC-induced growth retardation. Another possibility could be that the increase of IGF-I would contribute to the GC-induced growth retardation by accelerating the differentiation of chondrocytes, resulting in accelerated ossification. In the growth plates of hIGF-II transgenic mice, the higher basal level of IGF-I, might be responsible for the observed partial protection against the adverse effects of GCs on bone.

INTRODUCTION

Long-term high-dose glucocorticoid (GC) treatment as anti-inflammatory and immunosuppressive therapy is associated with marked skeletal growth retardation in children [1,2]. In experimental animal models, high doses of GCs also have a growth-suppressive effect on longitudinal bone growth [3-7]. GCs have been shown to act locally to inhibit longitudinal growth, suggesting a local mechanism within the growth plate [8].

Locally produced insulin-like growth factor (IGF) axis components, including both IGF-I and -II, their binding proteins (IGFBP)-1 to -6 and their receptors, play a key role in longitudinal bone growth [9,10]. Disruption of the IGF signaling results in growth retardation [11]. Besides being produced by growth plate chondrocytes, exogenously added IGFs have a stimulatory effect on longitudinal bone growth [12,13]. IGF-I and IGF-II both have a unique and complementary role in augmenting longitudinal bone growth [14]. However, there still exists some doubt whether both IGF-I and IGF-II are produced in the growth plate. Several *in vitro*

data have shown expression of both IGFs [10,15], while several *in vivo* studies have shown conflicting results [16-20].

It has been suggested that the growth-inhibiting side-effect of GCs is partially mediated by impaired action of the IGF axis components in *in vitro* studies [21-24]. GCs and IGF axis components have opposite effects on growth, as described above. In addition, GCs regulate the expression of IGFs [21] and IGFBPs [23] in chondrocytes *in vitro*. Few data are available, however, concerning the regulation by GCs *in vivo* [5] and it has been reported that serum levels of IGF axis components provide little insight into the mechanisms of GC-induced growth retardation and the possible involvement of the IGF axis components therein [2]. Therefore, in order to elucidate the possible involvement of IGF axis components in GC-induced growth retardation, it is important to study locally produced IGFs and IGFBPs and their regulation by GCs in the growth plate.

We have previously treated 3-week-old female mice for 4 weeks with 20 µg dexamethasone (DXM)/day or saline (phosphate-buffered saline; PBS) [3]. These mice showed general growth retardation and a significant reduction in tibial length and weight. We have now studied the effects of this GC treatment on growth plate morphology. We have also studied the possible involvement of locally expressed IGFs and IGFBPs in this GC-induced growth retardation, by analyzing their expression in the growth plates of these mice using non-radioactive *in situ* hybridization. This study also served to analyze whether only IGF-II or both IGF-I and IGF-II are expressed by growth plate chondrocytes.

We have previously suggested a possible protective effect of IGF-II against GC-induced growth retardation [3]. It was shown that DXM did not affect tibial length and weight in the same degree in human (h) IGF-II transgenic mice as in normal mice [3]. This suggested that hIGF-II overexpression might be able to partially counteract the growth inhibitory effect of DXM at the level of the growth plate. In order to study the possible role of the hIGF-II transgene in GC-induced growth retardation, we also analyzed the morphology of the growth plates of these mice and the expression patterns of the IGFs and IGFBPs, which were compared with normal PBS- and DXM-treated animals.

MATERIALS AND METHODS

Materials

Restriction enzymes and modifying enzymes, digoxigenin-UTP, anti-digoxigenin Fab-fragments, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl-phosphatase (BCIP) and blocking reagent were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Polyvinyl alcohol was obtained from Aldrich (Milwaukee, WI, USA). Euparal mounting medium was purchased from Klinipath (Duiven, The Netherlands).

Mice and tissue preparation

hIGF-II transgenic mice were generated by introduction of a human IGF-II gene, under the control of the H-2K^b promoter and containing the SV40 small t intron and early polyadenylation signal, into FVB mice as described in detail previously [25]. Throughout the study, the transgenic line designated 5'-74 (TgII) and normal FVB/N mice were used for our experiments. The animals were kept under standardized laboratory conditions.

Three-week-old female FVB and TgII mice were divided into two groups of five mice each. The animals were injected subcutaneously with 0.1 ml vehicle (PBS; pH 7.4) or 20 µg DXM once a day, 5 days a week for 4 weeks (Experiment 2, [3]). The mice were killed by decapitation after ether anesthesia 2 h after the last injection, at the age of 7 weeks. At this age the animals are maturing sexually and are still growing [26]. The protocol received the approval of the committee for Animal Experiments of the University Medical Center Utrecht, the Netherlands.

The tibiae were dissected and fixed in buffered 3.8% formalin for 24 h. Tibiae were subsequently decalcified for 24 h in 0.45 M phosphate-buffered EDTA, pH 8.0, washed in PBS, dehydrated through a series of ethanol and embedded in paraffin in a standardized way to ensure proper orientation.

Morphometry of the growth plate
Growth plate sections were stained with hematoxylin and eosin and photographs of the sections were taken with a Zeiss Axiomat HRC camera equipped with the AxioVision software version 3.0 (Zeiss, München-Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width. Corresponding sections of the tibia of the different groups were used to ensure correct comparison between the different groups. Total width of the growth plate (distance between the epiphysis and the chondro-osseous junction) was determined from one image (magnification X50) per growth plate section, covering the entire transverse area excluding the periphery of the growth plate. Five animals per treatment group were analyzed (two sections per animal). From the images, measurements at 100 µm intervals were performed (about 18 measurements per growth plate) and averaged. The measuring lines were subsequently shortened to the first appearance of regular chondrocyte columns (the boundary between the resting and the proliferative zone) and to the first enlargement of the flattened cells (the boundary between the proliferative and the hypertrophic zones). From the lengths of these lines the widths of the three different zones of the growth plate were calculated.

Probes
Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP as substrate [27]. cDNAs encoding human IGF-I and IGF-II [28,29], cDNA encompassing the 1 kb BamHI – BamHI SV40 fragment [25] and mouse IGFBP-1, -2, -3, -4, -5 and -6 cDNAs (kindly provided by S.L.S. Drop and J.W. van Neck, Department of Pediatrics, Rotterdam, The Netherlands) [30] were used as templates for the synthesis of antisense and sense digoxigenin-labeled cRNA probes. All probes used were specific for the mRNAs analyzed. The human IGF-II probe detects both endogenous mouse IGF-II as well as the hIGF-II transgene mRNA [31]. The SV40 fragment detects specifically the hIGF-II transgene, making it possible to discriminate between the endogenous and transgene IGF-II mRNAs. Probes were checked for possible cross-hybridization using *in situ* hybridization on different types of mouse tissues (spleen, thymus and complete mice embryos) [30,31] for the IGFBP probes, and brain for the IGF probes (C. Reijnders, personal communication). The various probes displayed distinct expression patterns in these tissues. Northern blot analysis of different tissues using the same probes, yielded bands of the expected sizes, verifying the correct identity of the probes.

In situ hybridization
Paraffin tissue sections (10 µm) were cut in a standardized way and mounted on 2% amino-propyl-triethoxy silane/3% glutaraldehyde-coated glass slides. Corresponding sections of the tibia of the different groups were used for the same probes to ensure reliable comparison between the groups. Sections were dewaxed, hydrated, rinsed in PBS and treated with proteinase K (0.21 units/ml) for 30 minutes at 37ºC, treated with 0.2 M HCl for 10 min at room temperature and subjected to an acetylation treatment [32]. Sections were rinsed in 2xSSC and kept in this solution until the start of the hybridization. Hybridization was performed in a solution containing 50% formamide, 2xSSC, 1x Denhardt's solution, 1 µg/µl yeast RNA and 10% dextran sulphate and the digoxigenin-labeled cRNA probe at a concentration of 1000-1500 pg/µl. Sections were hybridized overnight at 51ºC, except for IGF-II where 58ºC was used. After hybridization, sections were washed with 50% formamide in 2xSSC at the hybridization temperature for 30 min and treated with RNase A (1 unit/ml) for 30 min at 37ºC. Subsequently, sections were rinsed in 2xSSC, treated with 10% lamb serum for 30 min and incubated with sheep anti-digoxigenin Fab-fragments coupled to alkaline phosphatase (1:1250) overnight at 4ºC. Chromogenesis was performed with 0.38 mg/ml NBT and 0.19 mg/ml BCIP in the presence of 6% (w/v) polyvinylalcohol [33] at room temperature in the dark, resulting in a blue precipitate. Sections were counterstained with nuclear fast red, dehydrated through a series of ethanol and mounted with Euparal.

Five different animals per treatment group were used per analyzed mRNA. Each analyzed glass slide contained six sections, three of a PBS- and three of a DXM-treated mouse (either a normal or a hIGF-II transgenic mouse). Both strains were analyzed in the same *in situ* hybridization experiment. For quantitative evaluation of the number of chondrocytes expressing mRNA, sections were coded and analyzed using the Image-Pro Plus software program from Media Cybernetics (Silver Springs, MD, USA). The number of positive cells in the growth plates (excluding the periphery of the growth plates) were counted and expressed relative to the number of positive cells in the FVB PBS-treated group (control group).

Statistical analysis
Data are expressed as means ± SEM. The effects of the DXM treatment and the hIGF-II transgene were statistically tested by one-way ANOVA with Bonferroni-Dunn post hoc tests using InStat version 3.00 (GraphPad Software, Inc., San Diego, CA, USA). The effects of the DXM treatment on body and tibial length of both mice strains (FVB and hIGF-II transgenic) were calculated using Student's t-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Growth plate morphology

Normal FVB mice were treated for 4 weeks with 20 µg/day DXM, which resulted in a significant inhibition of body length and weight [3]. Tibial length was also significantly inhibited, to a similar extent as total body length, as summarized in Table 1. DXM treatment of hIGF-II transgenic mice resulted in a similar inhibition of body length as in FVB mice. However, tibial length was not significantly affected in the hIGF-II transgenic mice (Table 1).

DXM treatment of FVB mice had no effect on the total width of the proximal tibial growth plate. However, the width of the proliferative zone was significantly decreased (by 11%), whereas the width of the hypertrophic zone was significantly increased (by 17%). The resting zone was not affected by the DXM treatment (Table 2). hIGF-II transgenic mice showed a significantly larger width of the growth plate compared with untreated FVB mice (12% increase). This increase was caused by a non-significant increase in the width of all three zones of the growth plate. Treatment with DXM caused a significant decrease in the width of the proliferative zone (14%), a similar decrease as that seen in FVB mice. In contrast, in hIGF-II transgenic mice, DXM did not affect the width of the hypertrophic zone (Table 2). As in FVB mice, the resting zone was not affected by the DXM treatment.

Table 1 Effect of dexamethasone (DXM) treatment on body length and tibial length of normal (FVB) and hIGF-II transgenic (TgII) mice. n = 5 in all cases

	Body			**Tibia**		
Group	length(cm)	*P* value[1]	inhibition by DXM (%)	length (mm)	*P* value[1]	inhibition by DXM (%)
FVB PBS	18.06 ± 0.26			17.11 ± 0.23		
FVB DXM	17.24 ± 0.06	<0.02	4.5	16.49 ± 0.10	<0.05	3.6
TgII PBS	17.86 ± 0.07			16.58 ± 0.13		
TgII DXM	17.00 ±0.09	<0.01	4.8	16.27 ± 0.18	NS	1.9

[1]compared with PBS control within the same strain; NS, not significant. Means ± SEM are given.

Table 2 Effect of DXM treatment on total growth plate width and width of the three zones (µm) of the growth plate in normal (FVB) and hIGF-II transgenic (TgII) mice. n = 5 in all cases

Group	**total growth plate**	**resting zone**	**proliferative zone**	**hypertrophic zone**
FVB PBS	168.2 ± 4.9	21.9 ± 1.1	85.6 ± 3.7	59.7 ± 1.8
FVB DXM	170.9 ±1.8	24.4 ± 0.8	76.6 [1] ± 1.1	69.9 [1] ± 1.5
TgII PBS	188.0 [1] ± 2.6	25.6 ± 1.8	93.2 ± 1.5	68.9 ± 1.8
TgII DXM	177.0± 4.6	26.4± 1.1	80.2 [2] ± 1.6	70.3 ± 3.9

[1] *P*<0.05 compared to the normal, PBS group; [2] *P*<0.05 compared to the TgII, PBS group. Means ± SEM are given.

Expression of IGF axis components in the postnatal growth plate

To analyze the endogenous expression of the IGFs and their binding proteins, IGFBP-1 to -6, in the postnatal epiphyseal growth plate of mice, non-radioactive *in situ* hybridization was performed on tibial epiphyseal growth plates of 7-week-old PBS-treated normal mice. At the age of 7 weeks, the mice are maturing sexually and are still growing [26]. As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals in any of the treatment groups (Figs 1E, 2E and 3E).

In the growth plates of these mice, IGF-I, IGF-II and IGFBP-2 transcripts were present, each showing a similar expression pattern. However, no expression of IGFBP-1, -3, -4, -5 and -6 could be detected (data not shown). Using the same probes, we have previously demonstrated the expression of IGFBP-2, -3, -4, -5 and -6 in lymphoid tissues [31].

IGF-I mRNA was predominantly present in hypertrophic chondrocytes and less in late hypertrophic chondrocytes (Fig. 1A). IGF-I transcripts were also detected in proliferative chondrocytes located at the periphery of the growth plate (not shown), but not in proliferative chondrocytes in the central area. Weak expression of IGF-I was present in some of the resting chondrocytes. In addition, IGF-I transcripts were also detected in osteoblasts aligning the trabeculae.

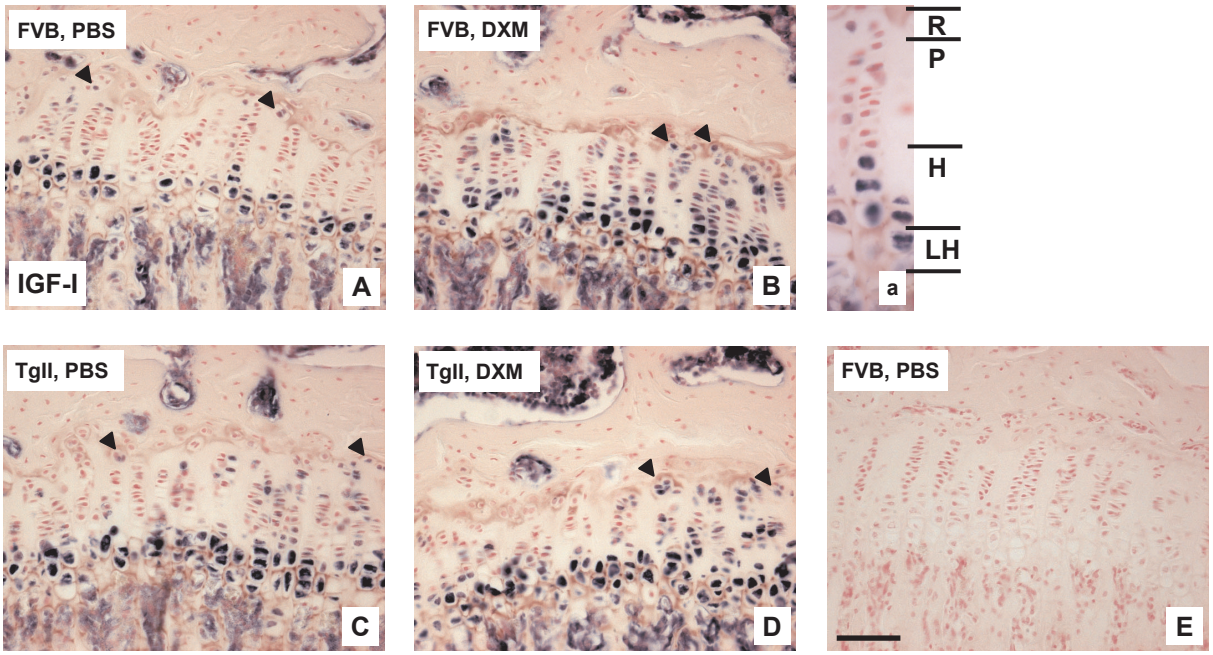

Figure 1 IGF-I expression in the postnatal growth plate. Expression patterns of IGF-I mRNA in representative sections of the growth plates of 7-week-old mice. (**A**) PBS-treated FVB mice, (**B**) DXM-treated FVB mice, (**C**) PBS-treated hIGF-II transgenic mice, (**D**) DXM-treated hIGF-II transgenic mice and (**E**) section of the PBS-treated FVB mice hybridized with an IGF-I sense probe, as analyzed by non-radioactive *in situ* hybridization, under brightfield illumination. Magnification X200. Sections were counterstained with nuclear fast red. The mRNA signal is shown as a blue precipitate under brightfield illumination. (**a**) Detail of signal in PBS-treated FVB mice, magnification X400. **R**, resting zone; **P**, proliferative zone; **H**, hypertrophic zone; **LH**, late hypertrophic zone. Arrowheads indicate IGF-I mRNA in the resting zone. Scale bar, 100 μm.

Like IGF-I, IGF-II was predominantly expressed in the hypertrophic zone. However, in the late hypertrophic zone fewer cells expressed IGF-II than was observed for IGF-I (Fig. 2A). IGF-II mRNA was also present in proliferative chondrocytes located at the periphery of the growth plate (not shown), but not in proliferative chondrocytes in the central area. In contrast to IGF-I, IGF-II mRNA was not detected in the resting zone. In the peripheral sections of the growth plate, more cells expressed IGF-I and IGF-II mRNA (not shown) than in sections of the center of the tibia. In these peripheral sections also, more proliferative chondrocytes showed expression of both IGFs. As IGF-I, IGF-II was also expressed in osteoblasts aligning the trabeculae.

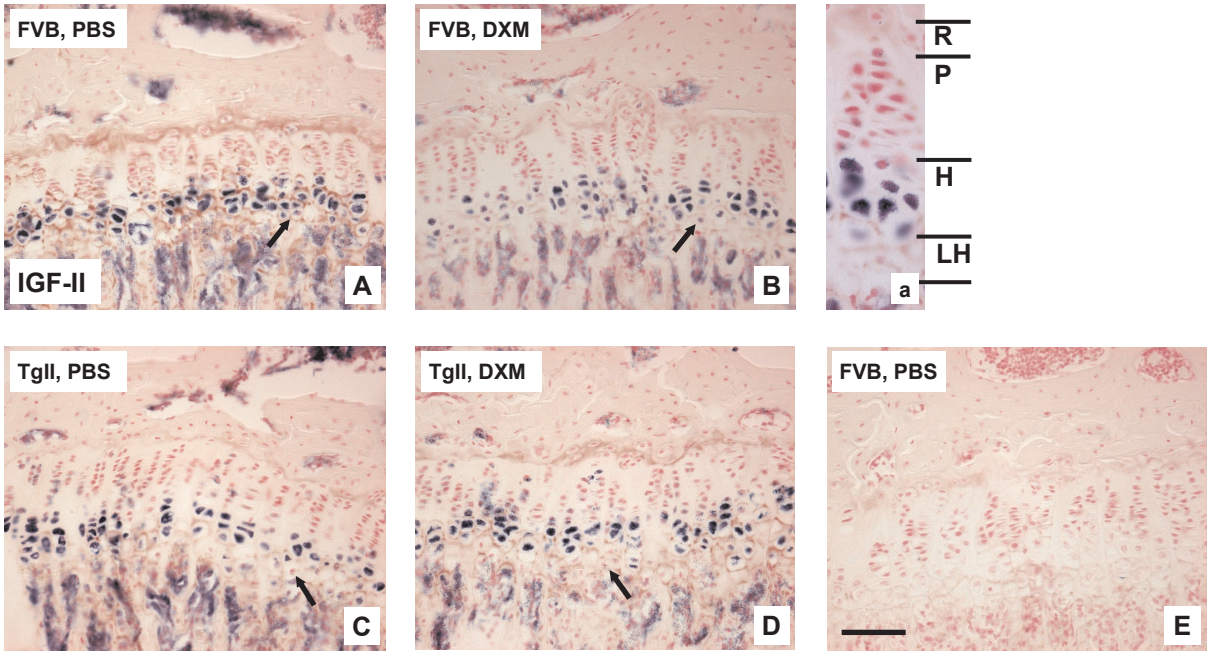

Figure 2 IGF-II expression in the postnatal growth plate. Expression patterns of IGF-II mRNA in representative sections of the growth plates of 7-week-old mice. (**A**) PBS-treated FVB mice, (**B**) DXM-treated FVB mice, (**C**) PBS-treated hIGF-II transgenic mice, (**D**) DXM-treated hIGF-II transgenic mice and (**E**) section of the PBS-treated FVB mice hybridized with an IGF-II sense probe. Sections were treated as described in Fig. 1. Magnification X200. (**a**) Detail of signal in PBS-treated FVB mice, magnification X400. Arrows indicate the lower level of expression in the late hypertrophic (LH) zone, compared to the hypertrophic (H) zone. Scale bar, 100 µm.

Of the IGFBPs, only IGFBP-2 could be detected in the postnatal growth plate (Fig. 3A). IGFBP-2 transcripts were mainly detected in the hypertrophic zone and fewer cells in the late hypertrophic zone expressed IGFBP-2 mRNA. As for IGF-I and IGF-II, IGFBP-2 mRNA was also detected in the proliferative cells located at the periphery of the growth plate, but not in the proliferative cells in the center of the tibia. No IGFBP-2 mRNA was detected in the resting zone. IGFBP-2 was also expressed in osteoblasts aligning the trabeculae.

Effects of DXM treatment on IGF axis components in the growth plate

DXM treatment resulted in a significant 2.4-fold (± 0.3, $P<0.001$) increase in the number of IGF-I-expressing chondrocytes in the epiphysial tibial growth plate (Fig. 1B). In the center of the growth plate, more rows of cells were positive for IGF-I, including proliferative chondrocytes. In contrast, in PBS-treated FVB mice, no proliferative chondrocytes were positive for IGF-I. IGF-I expression in the resting zone was not altered because of the DXM treatment. Although the width of the hypertrophic zone increased because of the DXM treatment, this could only partially account for the increase in IGF-I-expressing cells as, besides hypertrophic chondrocytes, proliferative chondrocytes also expressed IGF-I. The expression patterns and levels of IGF-II (Fig. 2B) and IGFBP-2 (Fig. 3B) were similar to those found in the growth plates of control mice.

As in the control animals, no expression of IGFBP-1, -3, -4, -5, and -6 was detected (data not shown).

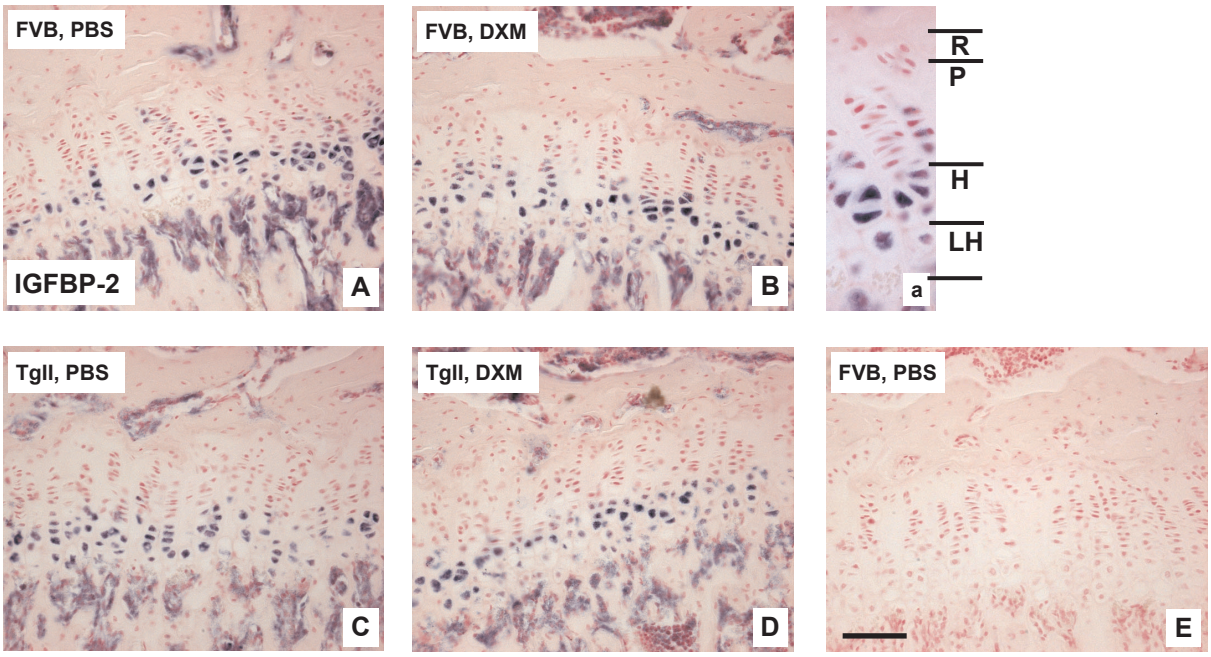

Figure 3 IGFBP-2 expression in the postnatal growth plate. Expression patterns of IGFBP-2 mRNA in representative sections of the growth plates of 7-week-old mice. (**A**) PBS-treated FVB mice, (**B**) DXM-treated FVB mice, (**C**) PBS-treated hIGF-II transgenic mice, (**D**) DXM-treated hIGF-II transgenic mice and (**E**) section of the PBS-treated FVB mice hybridized with an IGFBP-2 sense probe. Sections were treated as described in Fig. 1. Magnification X200. (**a**) Detail of signal in PBS-treated FVB mice, magnification X400. Scale bar, 100 µm.

Expression of IGF axis components in the postnatal growth plate of hIGF-II transgenic mice

To analyze the influence of hIGF-II overexpression on the expression of IGF axis components in the growth plate, sections of the growth plates of PBS-treated hIGF-II transgenic mice were also analyzed. The hIGF-II transgene itself was detected using a transgene specific probe (SV40 probe). The transgene was not detected in the epiphyseal growth plate, although transgene mRNA was detected in osteoblasts aligning the trabeculae (Fig. 4C). The number of IGF-I-expressing cells was significantly increased 1.9-fold (±0.3, $P<0.05$) in hIGF-II transgenic mice (Fig. 1C). This included positive cells in the proliferative zone. In contrast, the number of cells expressing endogenous IGF-II (Fig. 2C) and IGFBP-2 (Fig. 3C) in the growth plate were not changed in hIGF-II transgenic mice as compared to normal mice. No expression of IGFBP-1, -3, -4, -5 and -6 was detected in the postnatal growth plates of hIGF-II transgenic mice (data not shown).

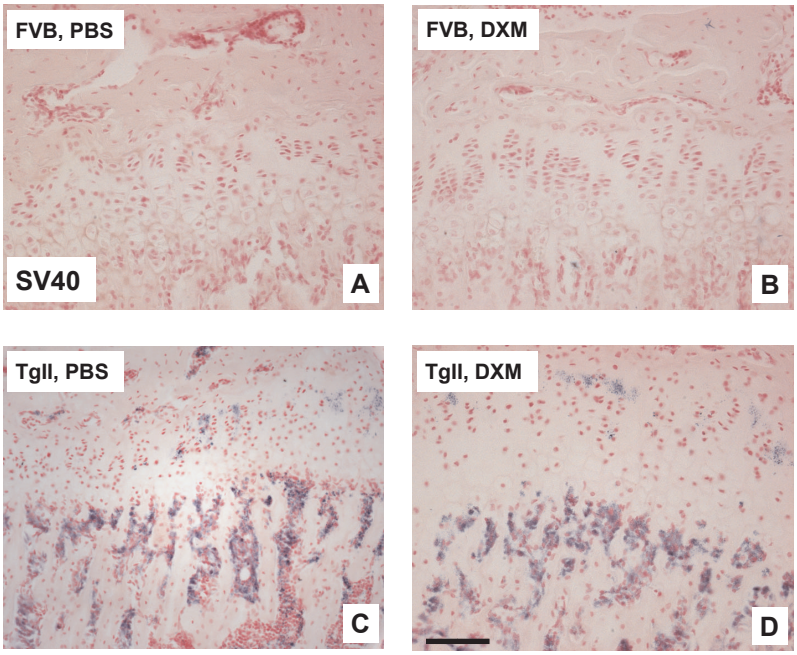

Figure 4 Transgene expression in the postnatal growth plate. Expression patterns of transgene mRNA in representative sections of the growth plates of 7-week-old mice. SV40 is a transgene sprecific probe. (**A**) PBS-treated FVB mice, (**B**) DXM-treated normal mice, (**C**) PBS-treated hIGF-II transgenic mice and (**D**) DXM-treated hIGF-II transgenic mice. Sections were treated as described in Fig. 1. Magnification, X200. Blue staining observed in the extracellular matrix of the growth plate is non-specific. Scale bar, 100 µm.

Effect of DXM treatment on IGF axis components in the growth plates of hIGF-II transgenic mice

When DXM-treated hIGF-II transgenic mice were compared with PBS-treated normal mice, the number of IGF-I-expressing chondrocytes in the epiphyseal tibial growth plate, increased 2.6-fold (±0.3), to a similar level to that observed in DXM-treated normal mice, including proliferative chondrocytes (Fig. 1D). This increase was significant when compared with control normal mice ($P<0.001$), but not significant when compared with PBS-treated hIGF-II transgenic mice. Endogenous IGF-II and IGFBP-2 gene expression were not affected by the DXM treatment (Fig. 2D and 3D respectively). IGFBP-1, -3, -4, -5 and -6 transcripts were not detected in hIGF-II transgenic mice treated with DXM (data not shown). We observed no change in transgene expression levels caused by the DXM treatment (Fig. 4D).

DISCUSSION

There are strong indications that GC-induced growth retardation involves impaired action of the IGF axis components [5,21-24]. In the present study, we analyzed the *in vivo* effects of GCs on the expression of the IGF axis components at the local level of the epiphyseal growth plate, where longitudinal growth is regulated and adverse effects of GCs on growth are expected to be targeted [34]. Besides studying the effects of GCs on the expression of the IGF axis components, the possible protective effect of IGFs against GCs was also studied using hIGF-II transgenic mice.

The analyses performed in this study on the endogenous expression of the IGF axis components in the mouse postnatal growth plate (postnatal day (P) 49), showed expression of IGF-I, IGF-II and IGFBP-2. Until now, there have been conflicting reports whether *both* the IGFs are expressed in the postnatal epiphyseal growth plate. Expression of IGF-I was shown in the rat epiphyseal growth plate (P10, P28, P35) [17,20] and costochondral growth plate [35]. In contrast, Wang *et al*. [16] detected only IGF-II in murine growth plates (P25) and Shinar *et al*. [19] reported similar results in rat growth plates (P10 - P35). Although species differences and age variables will influence the observed expression signals, the conflicting data are more likely

caused by the detection method used. In previous studies [16,19,20], in which radioactive *in situ* hybridization was used, less clear expression of IGF-I and IGF-II was found. This was confirmed by Reinecke *et al*. [17], who also showed a better visible signal of IGF-I expression using non-radioactive *in situ* hybridization than that shown previously by the radioactive method [20]. It is unlikely that the detection of both IGF-I and IGF-II is due to cross-hybridization between the probes used (described in *Materials and Methods*). In addition, there are differences in the expression patterns and responses to DXM treatment and the presence of the hIGF-II transgene, as discussed below.

IGF-I was mainly detected in cells of the hypertrophic zone and fewer positive cells were detected in the late hypertrophic zone. Only a few cells expressing IGF-I were detected in the resting and proliferative zones. This distribution confirms previous data [17]. Local IGF-I synthesis of proliferative chondrocytes was previously thought to stimulate the clonal expansion of chondrocyte columns in the proliferative zone in an autocrine/paracrine manner, as described by the "dual effector theory" [9]. It should be borne in mind, however, that the mitogenic properties of the IGFs have only been demonstrated in cultured cells, and not in *in vivo* models. In our study, only a small number of cells in the proliferative zone expressed IGF-I, whereas IGF-I expression was mainly detected in the hypertrophic zone, suggesting that IGF-I could be involved in the differentiation of proliferative to hypertrophic chondrocytes. In IGF-I knock-out mice, hypertrophic chondrocytes are reduced by 30% in linear dimension, accounting for most of the observed decrease in longitudinal growth [36]. This further strengthens the notion that IGF-I plays an important role in augmenting chondrocyte hypertrophy [14,34,37]. Furthermore, infusion of IGF-I in hypophysectomized rats showed that IGF-I stimulates growth plate chondrocytes at all stages of differentiation, including hypertrophic chondrocytes [12], rather than acting specifically upon proliferative chondrocytes as postulated in the "dual effector theory". Previously, there was uncertainty as to the source of the IGF-I that would promote the chondrocyte hypertrophy [14]. Our study provides strong evidence that the source of the IGF-I is the growth plate itself. However, besides having a role in differentiation of chondrocytes, IGF-I may still play a role in the clonal expansion of proliferative chondrocytes, in a paracrine manner. Our data, however, suggest that the "dual effector theory" should be extended with a role for IGF-I in chondrocyte differentiation. The type I IGF receptor, which mediates the intracellular effects of both IGF-I and IGF-II [38], is mainly expressed in the proliferative and hypertrophic zones of the growth plate [16,34]. However, in the hypertrophic zone, distinctly higher levels of type I IGF receptor mRNA levels are present compared with the proliferative zone [16], supporting the suggestion that in addition to stimulating proliferation, IGFs play a role in differentiation of chondrocytes.

The distribution of IGF-II transcripts confirms previous data, which showed predominant expression in the hypertrophic zone and no (in our study, very low) expression in the terminally differentiated chondrocytes at the chondro-osseous junction [16]. IGF-II has been suggested to play a role in proliferation of chondrocytes, and also in their differentiation [39]. The presence of both IGFs, their distinct expression patterns and the difference in response to GC treatment (see below), strengthens the suggestion that they have a unique and complementary role in regulating bone growth in a paracrine manner [14].

Concerning the IGFBPs, we are the first to detect expression of IGFBP-2 in the postnatal growth plates of sexually maturing mice (P49). The detection of IGFBP-2 is specific and is not due to cross-hybridization with other IGFBPs (described in *Materials and Methods*). This contrasts with the fetal situation where expression of IGFBP-2 to -6 in the growth plate *in vivo* has been reported previously in sheep [18] and cows [10]. However, expression was at such

a low level that detection was only possible using RT-PCR [10,18]. In fetal murine growth plates [16], radioactive *in situ* hybridization only showed expression of IGFBP-5 and -6 in the growth plate, which rapidly declined with age. IGFBP-5 was no longer detectable after embryonic day 18 and only low levels of IGFBP-6 were detectable up to at least P25 [16]. IGFBP-1 has never been detected in the growth plate, neither pre- nor postnatally [10,16,40]. In our study, IGFBP-2 is mainly expressed in the hypertrophic zone in co-localization with IGF-I and IGF-II. Various studies have suggested that IGFBP-2 plays a role in growth and development [41,42]. In addition, a role of IGFBP-2 in the growth plate is suggested by its presence in cultured chondrocytes [24,43]. The specific localization of IGFBP-2 in the pre- and hypertrophic zone of the growth plate and the fact that IGFBP-2 is the only member of the IGFBPs present at this stage of development suggests a modulating role for this IGFBP in chondrocyte differentiation and maturation, in collaboration with the IGFs.

Treatment of normal mice with DXM induced general growth retardation and a decrease in tibial length [3]. We now report that total growth plate width was not affected, while the proliferative zone decreased and the hypertrophic zone increased. In contrast, in hIGF-II transgenic mice, DXM decreased only total body length, whereas tibial length was not affected. The DXM-induced reduction in the proliferative zone of the growth plate remained, whereas the hypertrophic zone was no longer affected in the hIGF-II transgenic mice. Thus, there appears to be a correlation between tibial growth retardation and an increase in the hypertrophic zone of the growth plate. This increase of the hypertrophic zone could be caused by an accelerated differentiation of proliferative chondrocytes. Premature maturation of chondrocytes has previously been shown to lead to growth retardation. In parathyroid hormone-related peptide (PTHrP) receptor knockouts for example, an accelerated chondrocyte differentiation led to premature ossification [44,45]. However, the effect of GCs on the proliferative zone could also be important in GC-induced growth retardation, as shown in *in vitro* studies [21,46].

Treatment of normal mice with DXM also increased the number of chondrocytes expressing IGF-I. The increase of IGF-I-expressing cells can only partially be explained by the increase of the hypertrophic zone as, besides hypertrophic chondrocytes, proliferative chondrocytes also express IGF-I after DXM treatment. In addition, we also reported elevated serum levels of IGF-I [3]. We have shown no effects on IGF-II and IGFBP-2 mRNA levels in the growth plate, and no effects on serum IGF-II and IGFBP-2 levels have been detected [3]. The increase in IGF-I due to GC treatment is surprising, since IGFs are thought to promote growth. It could be that the increase of IGF-I in the growth plate might counteract the adverse effects of GCs on the growth plate in an attempt to diminish growth retardation. The observed increase, however, is not sufficient to completely abolish the observed growth deficit. IGF-I has indeed been shown previously to be able to counteract the anti-proliferative effects of GCs, both *in vitro* as well as *in vivo* [22]. However, this is the first study to describe an upregulation of IGF-I in response to GC treatment, possibly as a compensatory mechanism. Another possibility could be that the increase of IGF-I in the growth plate might result in an increased differentiation of chondrocytes, resulting in thickening of the hypertrophic zone, accelerated ossification and growth retardation. Probably other factors are involved as well, such as, for example the Indian hedgehog-PTHrP negative feedbackloop [47], core binding factor α-1 (Cbfa1), transforming growth factor-β, fibroblast growth factors and others [7,48]. The effects of GC treatment on the type I IGF receptor levels in the growth plate *in vivo* are not known. However, *in vitro* data of chondrocytes showed that DXM had no effect on type I

IGF receptor levels [21]. However, a possible involvement of the type I IGF receptor in GC-induced growth retardation cannot be excluded.

It is noteworthy that the hIGF-II transgenic mice also have increased IGF-I mRNA levels in the growth plate as compared to normal mice, whereas IGF-II and IGFBP-2 levels are not affected. However, this increase in IGF-I has no effect on tibial growth of the hIGF-II transgenic mice [3]. Transgene-specific hybridization showed that the hIGF-II transgene is not expressed in the growth plate, but only in osteoblasts aligning the trabeculae. The transgene in osteoblasts could be responsible for the increase in IGF-I levels in the growth plate in a paracrine manner as has been shown by us for IGFBP expression in lymphoid tissues [31]. In addition, the transgene is also thought to be able to regulate circulating IGF-I as in the hIGF-II transgenic mice serum IGF-I levels are elevated [3]. The hIGF-II transgene in the osteoblasts does not result in an increase of IGFBP-2 in the growth plate, and no effect on IGFBP-2 serum levels has been shown [3]. In contrast, in the lymphoid tissues, the presence of the hIGF-II transgene results in an increase of IGFBP-2 [31]. This difference might be due to the localization of the hIGF-II transgene and IGFBP-2, which are more co-localized in the lymphoid tissues than in the growth plate. This difference in localization apparently does not hamper the effect of the transgene on IGF-I expression. These hIGF-II transgenic mice do show a similar growth retardation to that of the normal mice when treated with DXM. However, while this treatment resulted in a significant reduction in tibial length and weight of normal mice, no significant effects on the tibia and the hypertrophic zone of the growth plate of hIGF-II transgenic mice were observed. The high levels of IGF-I already present in the growth plate of the untreated hIGF-II transgenic mice might be responsible for the observed partial protection against the GCs with regards to growth retardation of the tibia. This is supported by data that showed that increased IGF-I levels, in the presence of GCs, could counterbalance the growth inhibiting effect of the GCs [22].

ACKNOWLEDGEMENTS

Thanks are due to Mrs. I. van de Brink for taking care of the animals.

REFERENCES

1. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
2. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.
3. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
4. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
5. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
6. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
7. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
8. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
9. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC 1998 Growth hormone and bone. *Endocr Rev,* **19:** 55-79.

10. Olney RC, Mougey EB 1999 Expression of the components of the insulin-like growth factor axis across the growth plate. *Mol Cell Endocrinol,* **156:** 67-71.
11. Baker J, Liu JP, Robertson EJ, Efstratiadis A 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* **75:** 73-82.
12. Hunziker EB, Wagner J, Zapf J 1994 Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes in vivo. *J Clin Invest,* **93:** 1078-1086.
13. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Bloemen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
14. LeRoith D, Bondy CA, Yakar S, Liu J-N, Butler A 2001 The somatomedin hypothesis: 2001. *Endocr Rev,* **22:** 53-74.
15. Bhaumick B 1993 Insulin-like growth factor (IGF) binding proteins and insulin-like growth factor secretion by cultured chondrocyte cells: identification, characterization and ontogeny during cell differentiation. *Regul Pept,* **48:** 113-122.
16. Wang E, Wang J, Chin E, Zhou J, Bondy CA 1995 Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology,* **136:** 2741-2751.
17. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology,* **141:** 2847-2853.
18. de los Rios P, Hill DJ 1999 Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of the ovine fetus: possible functional implications. *Can J Physiol Pharmacol,* **77:** 235-249.
19. Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M 1993 Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology,* **132:** 1158-1167.
20. Nilsson A, Carlsson B, Isgaard J, Isaksson OGP, Rymo L 1990 Regulation by GH of insulin-like growth factor-I mRNA expression in rat epiphyseal growth plate as studied with *in situ* hybridization. *Endocrinology,* **125:** 67-74.
21. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
22. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
23. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
24. Smink JJ, Koedam JA, Koster JG, Van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *J Endocrinol,* **174:** 343-352.
25. Van Buul-Offers SC, de Haan K, Reijnen-Gresnigt MG, Meinsma D, Jansen M, Oei SL, Bonte EJ, Sussenbach JS, Van den Brande JL 1995 Overexpression of human insulin-like growth factor-II in transgenic mice causes growth of the thymus. *J Endocrinol,* **144:** 491-502.
26. van Buul SC, Van den Brande JL 1978 The Snell-dwarfmouse I. General growth pattern, before and during growth hormone and thyroxine therapy. *Acta Endocrinol,* **89:** 632-645.
27. Melton D, Krieg P, Rebaglati M, Maniatis T, Zinn K, Green MR 1984 Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res,* **12:** 7035-7056.
28. Jansen M, Van Schaik FMA, Van Tol H, Van den Brande JL, Sussenbach JS 1985 Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. *FEBS Lett,* **179:** 243-246.
29. Jansen M, Van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabbay KH, Nussbaum AL, Sussenbach JS, Van den Brande JL 1983 Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature,* **306:** 609-611.
30. Schuller AGP, Zwarthoff EC, Drop SLS 1993 Gene expression of the six insulin-like growth factor binding proteins in the mouse conceptus during mid- and late gestation. *Endocrinology,* **132:** 2544-2550.
31. Smink JJ, Koster JG, Hendriks-Stegeman BI, Van Buul-Offers SC 1999 Insulin-like growth factor (IGF) II induced changes in expression of IGF binding proteins in lymphoid tissues of hIGF-II transgenic mice. *Endocrinology,* **140:** 5876-5882.
32. Wilkinson DG. *In situ* hybridization: a practical approach. Oxford: Oxford University Press, 1992.

33. De Block M, Debrouwer D 1993 RNA-RNA *in situ* hybridization using digoxigenin-labeled probes: the use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. *Anal Biochem,* **215:** 86-89.
34. Siebler T, Robson H, Shalet S, Williams GR 2001 Glucocorticoids, thyroid hormone and growth hormone interactions: implications for the growth plate. *Horm Res,* **56 (suppl 1):** 7-12.
35. Lin W-W, Oberbauer AM 1999 Spatiotemporal expression of alternatively spliced IGF-I mRNA in the rat costochondral growth plate. *J Endocrinol,* **160:** 461-467.
36. Wang J, Zhou J, Bondy CA 1999 IGF1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J,* **13:** 1985-1990.
37. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A 2001 Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol,* **229:** 141-162.
38. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B 1997 The IGF-I receptor in cell growth, transformation and apoptosis. *Biochim Biophys Acta,* **1332:** F105-F126.
39. Hill DJ 1992 Peptide growth factor interactions in embryonic and fetal growth. *Horm Res,* **38:** 197-202.
40. Luo J, Murphy LJ 1989 Dexamethasone inhibits growth hormone induction of insulin-like growth factor-I (IGF-I) messenger ribonucleic acid (mRNA) in hypophysectomized rats and reduces IGF-I mRNA abundance in the intact rat. *Endocrinology,* **125:** 165-171.
41. Hoeflich A, Wu M, Mohan S, Foll JL, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb H, Wolf E 1999 Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. *Endocrinology,* **140:** 5488-5496.
42. Streck RD, Wood TL, Hsu MS, Pintar JE 1992 Insulin-like growth factor I and II and insulin-like growth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic and fetal limbs. *Dev Biol,* **151:** 586-596.
43. Borromeo V, Bramani S, Holder AT, Carter C, Secchi C, Beattie J 1996 Growth hormone stimulates the secretion of insulin-like growth factor binding protein-2 (IGFBP-2) by monolayer cultures of sheep costal growth plate chondrocytes. *Mol Cell Biochem,* **162:** 145-151.
44. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science,* **273:** 663-666.
45. Jobert AS, Zhang P, Couvineau A, Bonaventure J, Roume J, Le Merrer M, Silve C 1998 Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia. *J Clin Invest,* **102:** 34-40.
46. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.
47. Van der Eerden BCJ, Karperien M, Gevers EF, Lowik CWGM, Wit JM 1999 Expression of indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. *J Bone Min Res,* **15:** 1045-1055.
48. Hering TM 1999 Regulation of chondrocyte gene expression. *Front Biosc,* **4:** d743-d761.

Chapter 4

Short-term glucocorticoid treatment of prepubertal mice decreases growth and IGF-I expression in the growth plate

J.J. Smink, M.G. Gresnigt, N. Hamers, J.A. Koedam, R. Berger* and S.C. van Buul-Offers

*Department of Pediatric Endocrinology and *Metabolic Diseases, University Medical Center Utrecht, Utrecht, The Netherlands*

Conditionally accepted
Journal of Endocrinology

ABSTRACT

The Insulin-like Growth Factor (IGF) system is an important mediator of postnatal longitudinal growth, and the growth inhibiting effects of glucocorticoid (GC) treatment are suggested to be due to impaired action of the IGF system. However, the precise changes of the IGFs and IGF-binding proteins (IGFBPs) in the growth plate, occurring upon short-term GC treatment have not been characterized.

Prepubertal mice treated daily with dexamethasone (DXM) for 7 days, showed significant growth inhibition of total body length and weight and weight of the liver, thymus and spleen, whereas the weight of the kidneys was not affected.

Analysis of the tibial growth plate showed that the total growth plate width significantly decreased to 84.5% of control values, caused by a significant decrease in the proliferative zone. The number of PCNA-positive chondrocytes in the proliferative zone decreased significantly (to 40%) and TUNEL-staining showed a significant 1.6-fold increase in apoptotic hypertrophic chondrocytes.

In the growth plates, both IGF-I and IGF-II, and IGFBP-2 mRNAs were detected, mainly in the proliferative and prehypertrophic zones. DXM treatment significantly decreased the number of chondrocytes expressing IGF-I, whereas the number of chondrocytes expressing IGF-II and IGFBP-2 were not affected. The decrease in IGF-I expression in the growth plate indicates that GC treatment affects IGF-I at the local level of the growth plate, which could contribute to the GC-induced growth retardation.

INTRODUCTION

The process of endochondral ossification, which occurs at the growth plates of the long bones, results in longitudinal growth. During endochondral ossification, chondrocytes within the growth plate proliferate, differentiate, mature and eventually die by apoptosis and are replaced by bone [1]. This process has to be tightly controlled in order to maintain normal growth.

The Insulin-like Growth Factor (IGF) system has been proposed to be the major determinant of postnatal longitudinal growth [2-5]. The IGF system consists of IGF-I, IGF-II, six IGF-binding proteins (IGFBP-1 to -6), which modulate the IGF bioavailability, and the IGF receptors [6]. The IGFs are produced by multiple tissues and can act both in an endocrine and autocrine/paracrine fashion [6]. The IGFBPs act mainly as autocrine/paracrine factors at or close to their sites of synthesis [6].

Whether IGF-I and IGF-II are both expressed by growth plate chondrocytes, was an issue of debate for several years [7-10]. Recently, we and others showed expression of both IGF-I and IGF-II in the growth plate [11-13]. However, due to differences in species and ages of the models used, discrepant results for localization of IGF-I [3,10,13] and IGF-II [8,9,13] were reported. Nevertheless, IGF-I and IGF-II are suggested to each have a unique and complementary role in augmenting longitudinal bone growth [4,13].

Glucocorticoids (GCs), which are widely used as anti-inflammatory and immuno-suppressive drugs, result in growth inhibition as a side-effect in both children [14] and experimental animal models [1,15-17]. GCs act locally to inhibit growth, suggesting a mechanism which is intrinsic to the growth plate [18,19]. GC-induced growth retardation is suggested to be due to impaired action of the components of the IGF system [13,15,20,21]. GCs are known to regulate the expression of the components of the IGF system in the circulation *in vivo* [15,17,22,23]

and in chondrocytes *in vitro* [20,24,25]. While the known *in vivo* data often describe other effects than the effects in the growth plate, *in vitro* data obtained from chondrocytes usually show very short-term effects of GCs for several hours and these results do not reflect growth retardation *in vivo*. In view of the local action of GCs in causing growth retardation, it is important to study the regulation of the IGF system by GCs in the growth plate itself.

At first, to resolve the discrepancy in IGF localization in the growth plate, we investigated the distribution of the IGFs in growth plates of 4-week-old (prepubertal) mice, and compared them with data obtained in our previous study on 7-week-old (postpubertal) mice [13]. To study the possible involvement of the IGF system in GC-induced growth retardation, we have previously studied the effects of dexamethasone (DXM) treatment for 4 weeks on the expression of the IGFs and IGFBPs in the growth plates of postpubertal mice and showed an increased expression of IGF-I in the growth plate, whereas IGF-II and IGFBP-2 mRNA were not affected [13]. During the first week of this treatment, however, growth velocity decreased markedly, while during the further duration of treatment, DXM had little effect on growth [17]. Therefore, to study the effects of GCs on general growth and the IGF system in the growth plate, when the most marked effect on growth is observed, we have treated 3-week-old mice for 7 days with 20 μg/day DXM and analyzed the short-term *in vivo* effects on the growth plate.

MATERIALS AND METHODS

Materials

Restriction enzymes and modifying enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany). DeadEnd™ colorimetric Apoptosis Detection System (TUNEL assay) was obtained from Promega (Leiden, The Netherlands). Monoclonal antibody specific for PCNA (PC-10) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The biotinylated secondary antibody and the Vectastain ABC kit were purchased from Vector Laboratories (Burlingame, CA, USA) and the 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) from Sigma Chemical Co. (St. Louis, MO, USA). PVDF membranes (Immobilon-P) were from Millipore Corp. (Bedford, MA, USA). Polyvinyl alcohol was obtained from Aldrich (Milwaukee, WI, USA). Euparal and DPX mounting medium were purchased from Klinipath (Duiven, The Netherlands).

Mice and tissue preparation

Three-week-old female FVB mice were divided into two groups of five mice each. The mice were selected to ensure equal means and standard deviations for total body length and weight in each group at the start of the experiment. The animals were injected subcutaneously with 0.1 ml vehicle (phosphate buffered saline (PBS), pH 7.4), or with 20 μg dexamethasone (DXM), once a day for 7 days. The mice were killed by decapitation after ether anaesthesia, 2 h after the last injection. The protocol received the approval of the committee for Animal Experiments of the University Medical Center Utrecht, the Netherlands.

The animals were weighed and measured (nose-tail length) at the beginning and the end of the experiment. Organs were immediately removed, frozen in liquid nitrogen and weighed. The tibiae were carefully dissected and cleared from adjacent muscle and immediately fixed in buffered 3.8% formalin for 24 h. Tibiae were subsequently decalcified for 24 h in 0.45 M phosphate-buffered EDTA, pH 8.0, washed in PBS, dehydrated through a series of ethanol and embedded in paraffin in a standardized way to ensure proper orientation.

Paraffin tissue sections (10 μm) were cut in a standardized way and mounted on 2% amino-propyl-triethoxy silane/3% glutaraldehyde coated glass slides. Sections were deparaffinized and hydrated prior to histochemical analyses.

Western Ligand Blot Analysis

Sera of the five mice of each group were pooled and loaded on a 12% gel for non-reducing SDS-PAGE. IGFBPs were visualized by electrotransfer to PVDF membranes followed by incubation with ^{125}I-IGF-II [26]. Molecular weights were calculated using BioRad (Hercules, USA) broad range markers as standard. Bands were quantified by densitometry using the GS-363 Molecular Imager and the Molecular Analyst software program, version 1.5 (Biorad, Hercules, USA).

Determination of serum IGF-I
Sera of the five mice of each group were pooled and serum IGF-I was measured by heterologous radioimmunoassay after Sep-Pak C18 chromatography (Waters Corp., Milford, MA), as described previously [26].

Morphometry of the growth plate
Growth plate sections were stained with hematoxylin and eosin and pictures of the sections were taken with a Zeiss Axiomat HRC camera equipped with the AxioVision software version 3.0 (Zeiss, München-Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width. Corresponding sections of the tibia were used to ensure correct comparison between the different groups. Total width of the growth plate (distance between the epiphysis and the chondro-osseous junction) was determined from the entire transverse area excluding the periphery of the growth plate. Of the images, measurements at 100 µm intervals were performed (about 20 measurements per growth plate) and averaged. The measuring lines were subsequently shortened to the first appearance of regular chondrocyte columns (the boundary between the resting and the proliferative zone) and to the first enlargement of the flattened cells (the boundary between the proliferative and the hypertrophic zones). From the lengths of these lines the widths of the three different zones of the growth plate were calculated. Five animals per treatment group were analyzed (two sections per animal).

Probes
Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP as substrate [27]. cDNAs encoding human IGF-I and IGF-II [28,29] and cDNAs encompassing mouse IGFBP-1, -2, -3, -4, -5 and -6 cDNAs (kindly provided by S.L.S. Drop and J.W. van Neck, Dept. of Pediatrics, Erasmus University Rotterdam, The Netherlands) [30], were used as templates for the synthesis of antisense and sense digoxigenin-labeled cRNA probes. All probes used were specific for the mRNAs analyzed. Probes were checked for possible cross-hybridization using *in situ* hybridization on different types of mouse tissues (spleen, thymus and complete mice embryos) [30,31] for the IGFBP probes, and brain for the IGF probes (C. Reijnders, personal communication). The various probes displayed distinct expression patterns in these tissues. Northern blot analysis of different tissues using the same probes, yielded bands of the expected sizes verifying the correct identity of the probes.

In situ hybridization
Corresponding sections of the tibia of the different groups were used for the same probes to ensure reliable comparison between the groups. The *in situ* hybridization, using digoxigenin-labeled cRNA probes, was performed as described previously [13]. As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals.

Five different animals per treatment group were used per analyzed mRNA. Analysis of the five different animals of both groups was performed in the same *in situ* hybridization, for each analyzed mRNA. Each analyzed glass slide contained six sections, three of a PBS- and three of a DXM-treated mouse. For quantitative evaluation of the number of chondrocytes expressing mRNA, sections were coded and the number of positive cells in a fixed window was determined using the Image-Pro Plus software program (version 4.5) from Media Cybernetics, L.P. (Silver Spring, MD, USA). The total number of all positive cells (including weakly stained cells) in all zones of the growth plate (excluding the periphery of the growth plates) was counted and expressed relative to the total number of cells in the area that was counted.

Immunohistochemistry (PCNA)
Deparaffinized sections were treated with 0.1% Triton X-100 in PBS for 2 min at room temperature. After blocking with 10% normal horse serum, the sections were incubated with primary antibody to Proliferating Cell Nuclear Antigen (PCNA) at a 1:200 dilution for 30 min at 37°C, followed by incubation overnight at 4°C. For negative controls, the first antibody was omitted from this diluent, which showed no signal. Biotinylated secondary antibodies were used at 1:100 dilution and incubated for 30 min. For detection, the avidin-biotin peroxidase complex method in combination with nickel-enhanced DAB as substrate was used. Sections were counterstained with nuclear fast red, dehydrated and mounted with DPX. Counting of the number of positive cells was performed as described for the *in situ* hybridization.

Determination of apoptosis
Apoptotic cell death was determined by the TUNEL reaction, using the DeadEnd colorimetric Apoptosis Detection system, which was performed according to the procedures of the manufacturer with some minor modifications. Sections were fixed with 4% (w/v) paraformaldehyde/PBS for 15 min and treated with 20 µg/ml proteinase K for 10 min at room temperature. Subsequently, sections were postfixed with 4% (w/v) paraformaldehyde/PBS for 5 min. The TdT reaction was performed at 37°C for 60 min. For negative controls,

the TdT enzyme was replaced by water, which did not show any signal. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide/PBS for 10 min at room temperature. Biotinylated nucleotides were detected by Streptavidin-horseradish peroxidase conjugate. DAB staining was performed for 30 min at room temperature in the dark, resulting in an insoluble colored substrate at the site of DNA fragmentation. Subsequently, sections were counterstained with 0.1% light green, dehydrated and mounted with DPX.

For quantitative evaluation of the number of TUNEL-positive chondrocytes in the hypertrophic zone, sections were coded and the number of positive cells in the hypertrophic zone were counted and expressed relative to the total number of cells in the hypertrophic zone, as described for the *in situ* hybridization.

Statistical analysis

Data are expressed as the means ± SEM. The effects of the DXM treatment were statistically tested using Student's t-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

DXM-induced growth retardation

Treatment of 3-week-old prepubertal FVB mice for 7 days with 20 µg/day DXM caused a severe decrease in total body weight and length gain, resulting in a significant reduction in body weight and length. Tibial length and weight were not significantly reduced. The weights of the liver, thymus and spleen were significantly decreased by the DXM treatment, whereas the decrease in kidney weight was not significant (Table 1).

Table 1 Effect of dexamethasone (DXM) treatment on body and organ weight and body length. n = 5 in both groups

		PBS	DXM	*P*-value
Total	body weight (g)	16.7 ± 0.3	13.6 ± 0.6	<0.01
	body length (cm)	15.9 ± 0.1	15.3 ± 0.2	<0.05
	weight gain (g/wk)	6.1 ± 0.1	3.0 ± 0.3	<0.001
	length gain (cm/wk)	1.9 ± 0.1	1.3 ± 0.1	<0.001
Tibia	weight (mg)	70.3 ± 1.0	65.8 ± 2.5	NS
	length (mm)	14.8 ± 0.2	14.4 ± 0.2	NS
Organ	liver (mg)	852.3 ± 19.0	678.9 ± 49.7	<0.05
	kidney (mg)	197.5 ± 6.1	187.3 ± 4.6	NS
	thymus (mg)	100.2 ± 5.9	21.5 ± 1.9	<0.001
	spleen (mg)	82.0 ± 3.0	35.8 ± 2.7	<0.001

P-values are as indicated; NS, not significant, compared to PBS-treated control animals. Means ± SEM are given.

The IGF-I serum levels decreased from 511.8 ng/ml in PBS-treated control animals to 390.4 ng/ml in DXM-treated animals. Western Ligand Blot analysis of normal mice sera showed the expected presence of 4 IGFBPs [17], a doublet of 41.5/38.5 kDa (most probably IGFBP-3), a 30 kDa band (probably IGFBP-2), a 26.5 kDa (probably IGFBP-5) band and a 24 kDa band (probably IGFBP-4). DXM treatment did not affect the IGFBP protein levels (data not shown).

Growth plate morphology

The DXM treatment significantly decreased the total width of the proximal tibial growth plate to 84.5% ± 4.0% of control values ($P<0.05$), caused by a significant decrease in the width of the proliferative zone to 73.8% ± 6.9% of control values ($P<0.01$). The width of the hypertrophic zone showed a small, non-significant, decrease, whereas the resting zone was not affected by the DXM treatment (Table 2 and Fig. 1).

Table 2 Effect of dexamethasone (DXM) treatment on total growth plate width and width of the 3 zones (μm). n = 5 in both groups.

Treatment	total growth plate	resting zone	proliferative zone	hypertrophic zone
PBS	322.6 ± 15.5	32.4 ± 0.4	133.4 ± 5.6	152.3 ± 9.3
DXM	272.6 ± 10.9*	32.5 ± 1.2	98.4 ± 6.8*	140.5 ± 5.4

* $P<0.05$, compared to PBS-treated control animals. Means ± SEM are given.

Immunostaining for Proliferating Cell Nuclear Antigen (PCNA), a marker for proliferating cells, showed a significant decrease in the number of proliferating chondrocytes from 24.8% ± 2.2% in control animals (Fig. 1A) to 9.8% ± 1.5% in DXM-treated animals (Fig. 1B) ($P<0.001$) (expressed as a percentage of the total number of cells in the growth plate). Staining for PCNA was predominant in the proliferative zone, however, some staining in the prehypertrophic and hypertrophic zone was also shown.

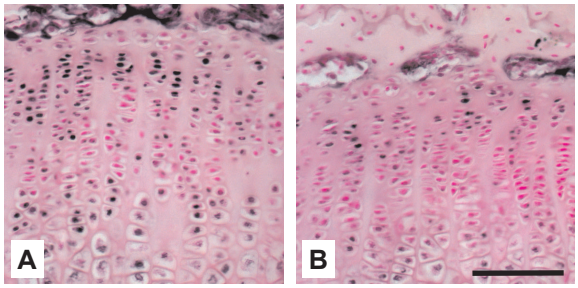

Figure 1 Immunohistochemical analysis of Proliferating Cell Nuclear Antigen (PCNA) in representative sections of tibial growth plates of control (**A**) and DXM-treated mice (**B**). The protein signal is shown as a dark precipitate. Scale bar represents 100 μm.

In the growth plates of the control animals, only the terminal row of hypertrophic chondrocytes contained a low percentage (1.0% ± 0.2%) of apoptotic chondrocytes (expressed as a percentage of the number of cells in the hypertrophic zone). DXM treatment induced a significant 1.6-fold increase to 1.6% ± 0.1% ($P<0.01$) (data not shown).

Effects of DXM treatment on IGF axis components in the growth plate

IGF-I mRNA was predominantly expressed in the proliferative zone and in the early hypertrophic zone (or prehypertrophic zone) of the growth plate, in 4-week-old normal control mice. Weak staining was observed in some of the resting and hypertrophic chondrocytes. In control animals, 56.5% ± 4.7% of the chondrocytes in the growth plate expressed IGF-I (Fig. 2A), which was significantly decreased in all zones of the growth plate, to 35.4% ± 4.0% in the DXM-treated animals (Fig. 2B) ($P<0.01$). The expression pattern (presence in the various zones) of IGF-I was not affected by the DXM treatment.

IGF-II mRNA showed a similar expression pattern as IGF-I and was also predominantly expressed in the proliferative and prehypertrophic zones. Some staining was observed in the resting and hypertrophic zones. IGF-II mRNA was detected in 53.0% ± 2.6% of the

chondrocytes in control animals (Fig. 2C). The number of IGF-II expressing chondrocytes was not affected by the DXM treatment (49.0% ± 7.6%) (Fig. 2D).

IGFBP-2 was the only IGFBP which could be detected in the growth plate; IGFBP-1, -3, -4, -5 or -6 were not detectable. The expression pattern of IGFBP-2 was comparable with that of IGF-I and IGF-II, *i.e.* mainly detected in the proliferative and prehypertrophic zones and a weak expression was detected in the resting zone. No IGFBP-2 mRNA was detected in the hypertrophic zone. In 41.2% ± 7.2% of the chondrocytes, IGFBP-2 mRNA was detected in control animals (Fig. 2E). DXM treatment did not affect the number of IGFBP-2 expressing chondrocytes (44.8% ± 6.3%) (Fig. 2F).

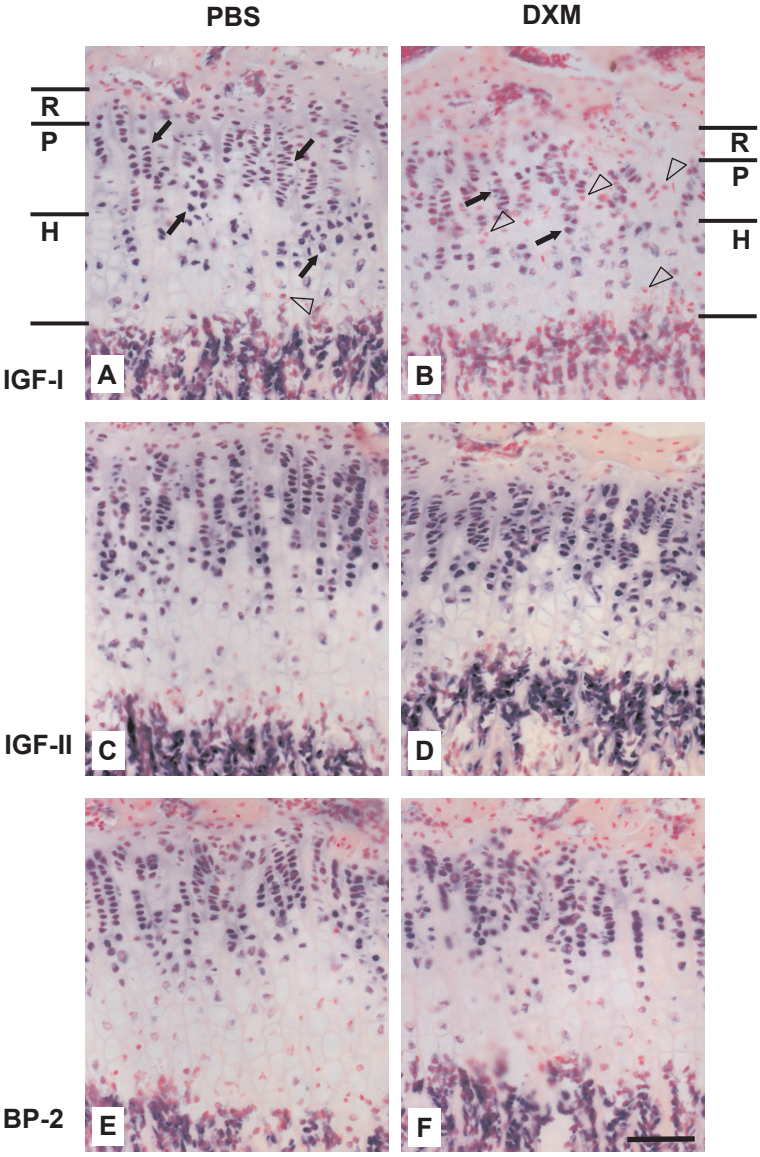

Figure 2 Expression of the IGF system in the postnatal growth plate. Expression patterns of IGF-I mRNA (**A**, **B**), IGF-II mRNA (**C**, **D**) and IGFBP-2 mRNA (**E**, **F**) in representative sections of the growth plates of 4-week-old mice, **A**, **C**, **E**, PBS-treated mice (PBS), **B**, **D**, **F**, DXM-treated mice (DXM), as analyzed by non-radioactive *in situ* hybridization. Sections were counterstained with nuclear fast red. The mRNA signal is shown as a blue precipitate.
R, resting zone; **P**, proliferative zone; **H**, hypertrophic zone. Scale bar represents 100 µm. Arrows indicate examples of chondrocytes expressing IGF-I mRNA, arrowheads indicate examples of chondrocytes expressing no IGF-I mRNA.

DISCUSSION

In our study, we showed that short-term GC treatment of prepubertal mice i) induced growth retardation at body and organ level, ii) significantly decreased tibial growth plate width, caused by a decrease in the width of the proliferative zone, iii) decreased the number of proliferating chondrocytes and increased the number of apoptotic chondrocytes and iv) decreased the number of IGF-I expressing chondrocytes in the growth plate. This study also allowed us to compare the localization of components of the IGF axis in prepubertal mice

(4-week-old), with our earlier study [13] in postpubertal mice (7-week-old). Likewise, a short-term GC treatment (1 week) could be compared with a longer GC treatment (4 weeks) as performed in our earlier study [13].

In the growth plates of prepubertal mice, we detected expression of IGF-I, IGF-II and IGFBP-2, while the other IGFBPs were not detected. This confirms our previous study, where we detected the same three components of the IGF system in the growth plates of postpubertal mice [13]. IGF-I, IGF-II and IGFBP-2 mRNAs were predominantly detected in the proliferative and prehypertrophic zones. In contrast, in our previous report on postpubertal mice, IGF-I and IGF-II were predominantly present in the hypertrophic zone and not in the proliferative zone [13]. The expression of IGFBP-2 also shifts from predominantly in the proliferative and prehypertrophic zones in prepubertal mice to hypertrophic chondrocytes in postpubertal mice [13]. In previous reports on prepubertal animals, also predominant expression of IGF-I [3] and IGF-II [9] was shown in the proliferative and prehypertrophic zones of the growth plate, whereas in rats of 9 weeks of age, IGF-I was predominantly present in the hypertrophic zone [10]. We are the first to describe expression of IGFBP-2 in the postnatal growth plate, previously only low levels of IGFBP-6 were shown in murine growth plates, using radioactive *in situ* hybridization [9]. The age-dependent expression pattern of the IGF system in the postnatal growth plate might indicate a shift in the function of the IGF system during chondrogenesis and postnatal development. Early in postnatal development, the local expression of the IGF system in the growth plate is probably important for the clonal expansion of chondrocyte columns in the proliferative zone, as postulated for IGF-I in the "dual effector theory" [32], as well as for the differentiation of proliferative chondrocytes into hypertrophic chondrocytes [3,4,13,33,34]. IGF-I has indeed been shown to stimulate growth plate chondrocytes at all stages of differentiation [35], which might also be true for the other components of the IGF system. It could well be that later on in postnatal development, after puberty, the main function of the IGF system is to augment chondrocyte hypertrophy [4,13] and in a much lesser extent to stimulate the proliferation of chondrocytes. This latter function might become less important as the growth rate at postpubertal age is much lower.

The GC treatment of the prepubertal mice for 1 week inhibited body length, weight and organ weight to a similar extent as observed after 4 weeks of treatment [17]. This suggests that most of the inhibiting effects of GCs on growth occur during the early period of the treatment. Longitudinal bone growth is also already decreased after 1 week of GC treatment. Differential effects on organ growth are observed, since the thymus and spleen are more severely affected than the liver and the kidneys by the GC treatment, as also shown after the long-term GC treatment [17].

GC-induced growth retardation is suggested to involve a mechanism intrinsic to the growth plate [18,19]. Therefore, we studied the effects of the short-term GC treatment on the growth plate. The short-term GC treatment significantly decreased the total tibial growth plate width, confirming previous studies in rats [19] and mice [36]. The decrease in total growth plate width was caused by a significant decrease in the width of the proliferative zone. Proliferating cell nuclear antigen (PCNA) staining decreased, indicating a decreased proliferation, which is likely to contribute to the decrease in the width of the proliferative zone. Several *in vitro* studies confirm the susceptibility of proliferative chondrocytes to GCs, in which GCs caused a decrease in proliferation [21,25,37]. The GC treatment also affected the hypertrophic zone, increasing the number of apoptotic chondrocytes 1.6-fold. This finding confirms previous reports, which showed a similar increase in apoptotic chondrocytes in GC-treated rats [19,38].

Both the decreased proliferation and increased apoptosis in the growth plate probably contribute to the observed GC-induced growth retardation.

The growth retarding effects on GCs are suggested to involve impaired action of the IGF system [15,20,21]. The short-term GC treatment decreased IGF-I serum levels, which confirms previous data in chickens [16]. However, in humans [22,23] and during long-term GC treatment of mice [17], GC treatment increased IGF-I serum levels. These discrepancies are probably due to species, age and/or treatment differences. The IGF-II serum levels are very low in mice and are not affected by GC treatment [17]. IGFBP serum levels were not affected by the DXM treatment, confirming previous reports [16,17]. Serum levels of the IGF system, however, are reported to provide little insight into the involvement of the IGF system in GC-induced growth retardation [23], indicating the importance to study the local effects of GCs on the growth plate and the IGF system.

The short-term GC treatment decreased IGF-I expression in the growth plate, whereas the number of IGF-II and IGFBP-2 expressing chondrocytes was not affected. The decrease in IGF-I expressing chondrocytes is probably related to the decrease in proliferation, as indicated by the decreased number of PCNA-positive cells. In IGF-I knock-out mice, proliferation in the growth plate is decreased [3] and also chondrocyte hypertrophy is inhibited [3,34]. This further strengthens our suggestion that a decrease in IGF-I expression in the growth plate, caused by the GC treatment, results in a decreased proliferation of chondrocytes. The differentiation of chondrocytes to the hypertrophic phenotype might also be inhibited, as IGF-I expression in the prehypertrophic zone is also decreased. The decreased proliferation, and possibly the decreased differentiation, would then result in a retardation of the endochondral ossification, causing the retardation of longitudinal bone growth.

The decrease in IGF-I due to the short-term GC treatment is in contrast with the increase of IGF-I in the growth plate after long-term GC treatment [13]. This difference could be linked to a decrease in severity of GC effects on growth and the growth plate over time. We showed in this study that the growth retarding effect of GCs after a short-term treatment is almost equal to the growth retardation after a long-term treatment [17]. This suggests that the different organs as well as the growth plate do not seem to respond to the GCs anymore. It has been shown that the growth plate indeed becomes less sensitive to GCs after a period of time, as the number of glucocorticoid receptors is decreased after a 3 week GC treatment in rats [19]. The observed increase of IGF-I levels in the growth plate and serum, after a long-term treatment, might then be part of a compensatory mechanism for the induced growth retardation [13]. Both in the short-term and the long-term GC treatment [13], IGF-II and IGFBP-2 mRNA levels in the growth plate were not affected. This suggests that, in contrast to IGF-I, IGF-II and IGFBP-2 do not seem to be involved in GC-induced growth retardation.

In conclusion, we suggest that the action of IGF-I is impaired by GC treatment during the early periods of treatment, contributing to the GC-induced growth retardation. Later on, IGF-I becomes part of a compensatory mechanism of the growth plate to diminish the induced growth retardation, which is however not sufficient to completely abolish the growth deficit.

ACKNOWLEDGEMENTS

Thanks are due to Mr. C.M. Hoogerbrugge for technical assistance.

REFERENCES

1. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
2. Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu J-L, Ooi GT, Setser J, Frystyk J, Boisclair YR, LeRoith D 2002 Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest,* **110:** 771-784.
3. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A 2001 Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol,* **229:** 141-162.
4. LeRoith D, Bondy CA, Yakar S, Liu J-N, Butler A 2001 The somatomedin hypothesis: 2001. *Endocr Rev,* **22:** 53-74.
5. Baker J, Liu JP, Robertson EJ, Efstratiadis A 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* **75:** 73-82.
6. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev,* **16:** 3-34.
7. Nilsson A, Carlsson B, Isgaard J, Isaksson OGP, Rymo L 1990 Regulation by GH of insulin-like growth factor-I mRNA expression in rat epiphyseal growth plate as studied with *in situ* hybridization. *Endocrinology,* **125:** 67-74.
8. Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M 1993 Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology,* **132:** 1158-1167.
9. Wang E, Wang J, Chin E, Zhou J, Bondy CA 1995 Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology,* **136:** 2741-2751.
10. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology,* **141:** 2847-2853.
11. de los Rios P, Hill DJ 1999 Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of the ovine fetus: possible functional implications. *Can J Physiol Pharmacol,* **77:** 235-249.
12. Olney RC, Mougey EB 1999 Expression of the components of the insulin-like growth factor axis across the growth plate. *Mol Cell Endocrinol,* **156:** 67-71.
13. Smink JJ, Koster JG, Gresnigt R, Rooman R, Koedam JA, Van Buul-Offers SC 2002 IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice. *J Endocrinol,* **175:** 143-153.
14. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
15. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
16. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
17. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
18. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
19. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
20. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
21. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
22. Miell JP, Taylor AM, Jones J, Holly JMP, Gaillard RC, Pralong FP, Ross RJM, Blum WF 1993 The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF-binding proteins in normal male volunteers. *J Endocrinol,* **136:** 525-533.
23. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.

24. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
25. Smink JJ, Koedam JA, Koster JG, Van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *J Endocrinol,* **174:** 343-352.
26. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Bloemen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
27. Melton D, Krieg P, Rebaglati M, Maniatis T, Zinn K, Green MR 1984 Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res,* **12:** 7035-7056.
28. Jansen M, Van Schaik FMA, Van Tol H, Van den Brande JL, Sussenbach JS 1985 Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. *FEBS Lett,* **179:** 243-246.
29. Jansen M, Van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabbay KH, Nussbaum AL, Sussenbach JS, Van den Brande JL 1983 Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature,* **306:** 609-611.
30. Schuller AGP, Zwarthoff EC, Drop SLS 1993 Gene expression of the six insulin-like growth factor binding proteins in the mouse conceptus during mid- and late gestation. *Endocrinology,* **132** 2544-2550.
31. Smink JJ, Koster JG, Hendriks-Stegeman BI, Van Buul-Offers SC 1999 Insulin-like growth factor (IGF) II induced changes in expression of IGF binding proteins in lymphoid tissues of hIGF-II transgenic mice. *Endocrinology,* **140:** 5876-5882.
32. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC 1998 Growth hormone and bone. *Endocr Rev,* **19:** 55-79.
33. Siebler T, Robson H, Shalet S, Williams GR 2001 Glucocorticoids, thyroid hormone and growth hormone interactions: implications for the growth plate. *Horm Res,* **56 (suppl 1):** 7-12.
34. Wang J, Zhou J, Bondy CA 1999 Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J,* **13:** 1985-1990.
35. Hunziker EB, Wagner J, Zapf J 1994 Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes *in vivo*. *J Clin Invest,* **93:** 1078-1086.
36. Altman A, Hochberg Z, Silbermann M 1992 Interactions between growth hormone and dexamethasone in skeletal growth and bone structure of young mice. *Calcif Tissue Int,* **51:** 298-304.
37. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.
38. Sanchez CP, He Y-Z 2002 Alterations in the growth plate cartilage of rats with renal failure receiving corticosteroid therapy. *Bone,* **30:** 692-698.

Chapter 5

Short-term glucocorticoid treatment of piglets causes changes in growth plate morphology and angiogenesis

J.J. Smink, I.M. Buchholz[*], N. Hamers, C.M. van Tilburg, C. Christis, R.J.B. Sakkers[*], K. de Meer[†], S.C. van Buul-Offers and J.A. Koedam

Department of Pediatric Endocrinology and [*]Pediatric Orthopedic Surgery, University Medical Center Utrecht, Utrecht, The Netherlands. [†] Departments of Clinical Chemistry and Pediatrics, VU University Medical Center, Amsterdam, The Netherlands

Submitted for publication

ABSTRACT

Glucocorticoid (GC) treatment of children often leads to growth retardation, and the precise target(s) in the growth plate responsible for this effect are unknown. We treated 6-week-old prepubertal piglets (10 kg) for 5 days with prednisolone (PRDL) and studied whether apoptosis and angiogenesis of the growth plate could be possible targets of GCs.

In the PRDL-treated animals, the total width of the growth plate decreased to 81% of controls ($P<0.02$), which was explained by a decrease of the width of the proliferative zone to 73% ($P<0.05$). The treatment had no effect on the orderly organization of the chondrocyte columns. In the growth plates of control animals, apoptosis was shown in 5.8% of the hypertrophic chondrocytes and was limited to the terminal hypertrophic chondrocytes. In PRDL-treated animals, 40.5% of the hypertrophic chondrocytes was apoptotic ($P<0.02$), with apoptotic chondrocytes also appearing higher in the hypertrophic zone.

CD31 immunohistochemistry showed fewer capillaries and loss of their parallel organization in the metaphysis in the PRDL-treated animals. The capillaries were shorter and chaotic in appearance. In contrast to controls, in PRDL-treated animals VEGF mRNA and protein could not be detected in the hypertrophic zone of the growth plate. Trabecular bone length in the primary spongiosa was also diminished by the treatment.

These results indicate that short-term GC treatment of growing piglets severely disturbs the width of the growth plate, apoptosis of chondrocytes, VEGF expression by hypertrophic chondrocytes, the normal invasion of blood vessels from the metaphysis to the growth plate and bone formation at the chondro-osseous junction. These effects could alter the dynamics of endochondral ossification and thus contribute to GC-induced growth retardation.

INTRODUCTION

Endochondral bone formation and longitudinal bone growth are the result of proliferation, differentiation, maturation and eventually apoptosis of chondrocytes within the growth plate [1]. Apoptosis of terminal hypertrophic chondrocytes (*i.e.*, adjacent to the ossification front) [2-4] is associated with extracellular matrix degradation and vascular invasion of the growth plate [1]. It results in a cartilaginous scaffold on which new bone will be formed by invading osteoblasts. To allow osteoblasts to invade the growth plate, vascularization (*i.e.*, angiogenesis) at the chondro-osseous junction between metaphyseal bone and the growth plate is required. It has been suggested that a mutual control exists between apoptosis and angiogenesis [5]. Endothelial cells express growth factors that promote the differentiation of chondrocytes to a hypertrophic phenotype [5]. Conversely, the growth plate secretes factors which promote angiogenesis [6]. One of these factors, vascular endothelial growth factor (VEGF), is a key regulator of angiogenesis [6]. VEGF inactivation in mice results in suppression of blood vessel invasion, impaired bone formation and expansion of the hypertrophic zone of the growth plate, demonstrating that VEGF is essential for attraction of capillaries to the growth plate and appears necessary for growth plate function [7]. Matrix metalloproteinases (MMPs) are among

the other factors, which are important for angiogenesis and bone formation. They degrade the cartilage extracellular matrix and release angiogenic factors such as VEGF from the growth plate matrix [8]. In MMP-9 knock-out mice, abnormal vascularization of the growth plate, delayed apoptosis of hypertrophic chondrocytes and expansion of the hypertrophic zone are observed [9]. The growth plates of these mice show similar abnormalities as the growth plates of mice in which VEGF is inactivated [7]. These results suggest a link between cartilage matrix degradation, apoptosis, angiogenesis, and VEGF and MMP-9 expression.

Agents which disturb longitudinal growth, could potentially act through interference with apoptosis and angiogenesis. Glucocorticoids (GCs) are effective drugs in anti-inflammatory and immuno-suppressive therapy, but are well known to result in growth retardation in children [10,11] and experimental animal models [12-16]. GCs act locally to inhibit longitudinal bone growth, suggesting a mechanism intrinsic to the growth plate [4,17].

Treatment with GCs can also result in osteoporosis [18]. One of the mechanisms involved is an induction of apoptosis in osteoblasts and osteocytes [18]. Treatment of rats for 3 weeks with GCs indeed resulted in an increase of apoptosis of osteoblasts and, in addition, of hypertrophic chondrocytes [4]. This indicates that GCs can also interfere with apoptosis in the growth plate. We have previously shown that GC treatment of cultured porcine growth plate chondrocytes resulted in a down-regulation of VEGF expression [19]. Given the pivotal role of growth plate-derived VEGF in vascularization, it is conceivable that, in addition to increasing apoptosis, GCs could target vascularization of the growth plate, thus contributing to the mechanisms of growth retardation.

We aimed to study whether apoptosis, angiogenesis and architecture in the growth plate are disturbed by short-term (5 days) GC treatment of growing piglets. The growing piglet is a good model for growing children [20]. When compared to the growth plates of rodents, the pig growth plate more closely resembles the human growth plate in terms of cellular numbers in the different zones, cell kinetics and patterns of closure [21,22].

MATERIALS AND METHODS

Animal studies

Twelve 6-week-old (prepubertal) female cross-bred (Landrace x Yorkshire) piglets [23] with an average weight of 10 kg were studied. The animals were fed 50 g/kg per day of a standard diet (De Heus Brokking Koudijs BV, Barneveld, The Netherlands). One group of 6 piglets received orally 5 mg/kg bodyweight prednisolone (PRDL) daily, for a period of 5 days. Piglets were terminated at the end of the experiment by injection of 1 g pentothal.

The experimental protocol was approved by the committee for Animal Experiments of the University Medical Center Utrecht, The Netherlands.

Tissue preparation

Tibiae were dissected and the proximal heads were cut sagitally and fixed in buffered 3.8% formalin for 3-6 days. They were subsequently decalcified in 0.45 M phosphate-buffered EDTA, pH 8.0 for 18-25 days, washed in PBS, dehydrated through a series of ethanol and embedded in paraffin.

Sections of 10 µm were cut on an ultramicrotome and mounted on 2% amino-propyl-triethoxy silane/3% glutaraldehyde coated glass slides. Sections were deparaffinized and hydrated prior to histochemical analyses (see below).

Morphometry

Growth plate sections were stained with haematoxylin and eosin and pictures of the growth plate sections were taken with a Zeiss Axiomat HRC camera equipped with the Axiovision software, version 3.0 (Zeiss, München-Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width.

Total width of the growth plate (distance between the epiphysis and the chondro-osseous junction) was determined from 4 images (magnification X50) per growth plate section, covering the entire transverse area within about 500 µm of the cortex. Of these images, measurements at 200 µm intervals were performed (about 50 measurements per growth plate) and averaged. The measuring lines were subsequently shortened to the first appearance of regular chondrocyte columns (the boundary between the resting and the proliferative zones) and to the first enlargement of the flattened cells (the boundary between the proliferative and the hypertrophic zones). From the lengths of these lines the widths of the three different zones of the growth plate were calculated.

In situ hybridization for VEGF

cDNA encoding human VEGF was kindly provided by M.F. Gebbink and E.E. Voest (University Medical Center Utrecht, the Netherlands) [24] and was used as a template for the synthesis of antisense and sense digoxigenin-labeled cRNA probes. Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP (Roche, Mannheim, Germany) as a substrate [25].

The *in situ* hybridization was performed as described previously [26]. As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals. Analysis of the different animals of both groups was performed in the same *in situ* hybridization.

To compare the control and PRDL-treated piglets with each other, the glass slides were independently scored by three investigators.

Immunohistochemistry

Sections were blocked with 10% serum (Vector Laboratories, Inc., Burlingame, CA, USA) from the species in which the secondary antibody was raised. The following primary antibodies directed against human proteins were used: rabbit anti-VEGF, goat-anti CD31/PECAM-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-MMP-9 (R&D Systems, Inc., Minneapolis, MN, USA). A blocking peptide for VEGF was from Santa Cruz Biotechnology. Antibodies were used at 1:100 dilution in 1.5% blocking serum in PBS and incubated for 1 h at room temperature. For negative controls, the first antibody was omitted from this diluent and for VEGF a blocking peptide was also used. Negative controls did not show any signal. Biotinylated secondary antibodies (Vector Laboratories) were used at 1:200 dilution and incubated for 30 min. For detection, the avidin-biotin peroxidase complex method (Vector Laboratories Vectastain ABC kit) in combination with nickel-enhanced 3,3'-Diaminobenzidine Tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) as substrate was used. Sections were counterstained with nuclear fast red, dehydrated and mounted with DPX (Klinipath, Duiven, The Netherlands).

Analysis of the different animals of both groups was performed in the same immunohistochemistry. To compare the control and PRDL-treated piglets with each other, the glass slides were independently scored by three investigators.

Determination of apoptosis

Apoptotic death was determined by the TUNEL reaction (Promega, Leiden, The Netherlands), which was performed according to the procedures of the manufacturer. For negative controls, the TdT enzyme was replaced by water, which resulted in the absence of any signal. After the DAB staining, sections were counterstained with 0.1% light green, dehydrated and mounted with DPX.

For quantitative evaluation of the number of TUNEL-positive chondrocytes in the hypertrophic zone, sections were coded and the number of positive cells in the hypertrophic zone were counted and expressed relative to the total number of cells in the hypertrophic zone, by two independent observers.

Statistical Analysis

Results from the TUNEL-staining and growth plate width are expressed as means ± SEM. Statistical differences between the control and the PRDL-treated group were determined by the unpaired t-test, using InStat version 3.00 (GraphPad Software, Inc., San Diego, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Growth plate morphology

A 5 day prednisolone (PRDL) treatment (50 mg/day) of prepubertal piglets significantly decreased the total width of the proximal tibial growth plate to 81% ± 6% of control values (P<0.02) (Fig. 1A, B), caused by a significant decrease of the proliferative zone to 73% ± 9% of control values (P<0.05) (Fig. 1C). The hypertrophic zone showed a small, non-significant, decrease, whereas the resting zone was not affected by the PRDL treatment. The PRDL treatment had no effect on the morphology of the growth plate, the chondrocytes being organized in the same orderly columns as in the control animals (Fig. 1A, B).

Trabecular bone length in the primary spongiosa of the PRDL-treated piglets was clearly diminished when compared to the untreated controls (Fig. 1D, E). At the chondro-osseous junction, many of the calcified longitudinal septae of the growth plate did not continue as bone trabeculae, but were in direct contact with the marrow cavity.

In the growth plates of the untreated piglets, only the row of terminal hypertrophic chondrocytes contained a low percentage (5.8% ± 1.7%) of apoptotic cells (Fig. 1F). PRDL treatment significantly increased the number of apoptotic chondrocytes in the hypertrophic zone to 40.5% ± 5.4% (6.9-fold increase, P<0.02) (Fig. 1G). In addition to the terminal hypertrophic zone, also the higher layers of the hypertrophic zone contained apoptotic chondrocytes in the PRDL-treated piglets (Fig. 1G).

Angiogenesis

In the control piglets, metaphyseal blood vessels ran parallel to the chondrocyte columns in the growth plate and ended at the osteochondral junction, as shown by immunohistochemistry for CD31 (Fig. 2A). PRDL treatment resulted in fewer, disorganized and short blood vessels, although they still penetrated the hypertrophic zone of the growth plate (Fig. 2B).

We studied whether this disturbed angiogenesis could be due to changes in expression of VEGF and/or MMP-9 protein. *In situ* hybridization analysis showed predominant expression of VEGF mRNA in the hypertrophic zone and in occasional chondrocytes of the proliferative and resting zones (Fig. 2C). Immunohistochemistry showed a similar expression pattern for the VEGF protein (Fig. 2E).

In the PRDL-treated piglets, VEGF mRNA and protein in the hypertrophic zone were not detectable. In the osteoblasts aligning the trabeculae, the levels of VEGF mRNA and protein did not appear to be affected by the PRDL treatment (Fig. 2D, F). In control animals, MMP-9 protein was detected in cells alining the bone trabeculae and at the transverse septae of the cartilage-bone junction (Fig. 2G). PRDL treatment did not alter this expression pattern (Fig. 2H).

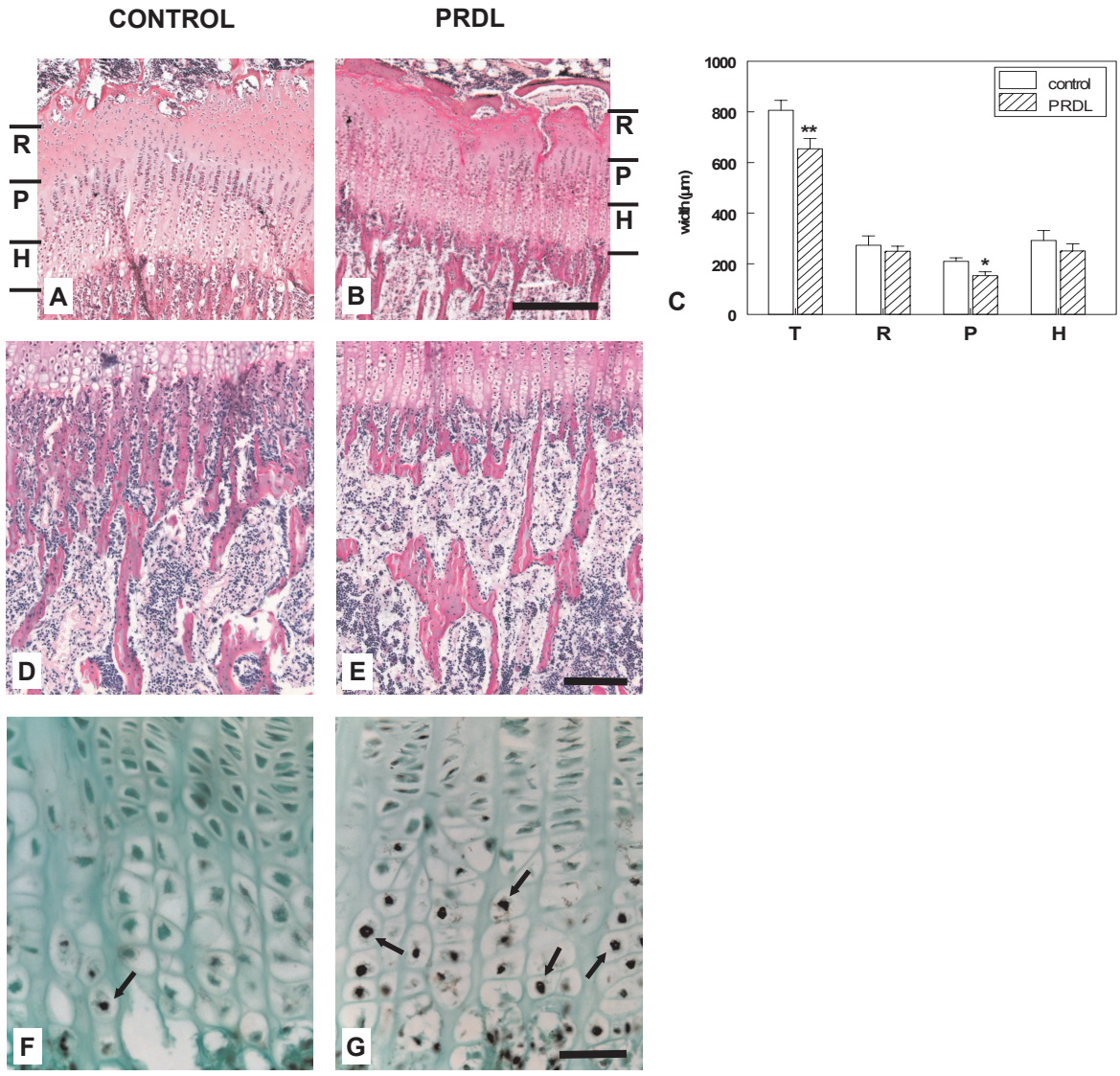

Figure 1 Haematoxylin and eosin staining of representative sections of proximal tibial growth plates of 6-week-old prepubertal control (**A, D**) and PRDL-treated piglets (**B, E**). (**C**) result of measurements of total growth plate width of control and PRDL-treated piglets and of the three different zones of the growth plate. The data represent the mean ± SEM. *P < 0.05, **P<0.02, PRDL relative to control. Bars represent 500 µm in (**A**, **B**). and 200 µm in (**D**, **E**). **T**, denotes the total growth plate; **R** resting zone; **P**, proliferative zone; **H**, hypertrophic zone.
(**F**,**G**) Apoptosis, as shown by TUNEL-staining, in representative sections of tibial growth plates. (**F**) control piglets; and (**G**) PRDL-treated piglets; TUNEL staining is shown as a dark precipitate. Arrows indicate examples of TUNEL-positive cells. The scale bar represents 50 µm.

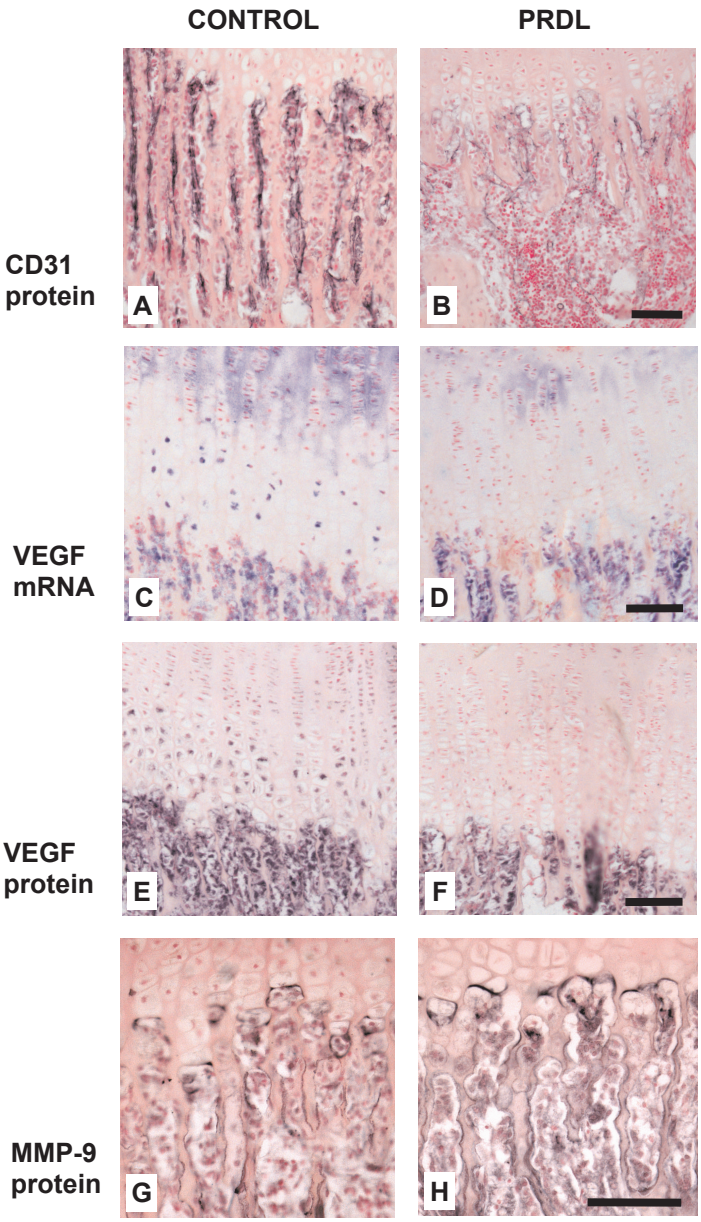

Figure 2 Angiogenesis in representative sections of tibial growth plates of control (**A**, **C**, **E**, **G**) and PRDL-treated piglets (**B**, **D**, **F**, **H**).
(**A**, **B**) CD31 immunohistochemistry for detection of blood capillaries;
(**C**, **D**) VEGF mRNA expression, as analyzed by *in situ* hybridization;
(**E**, **F**) VEGF protein expression, as analyzed by immunohistochemistry;
(**G**, **H**) MMP-9 protein expression, as analyzed by immunohistochemistry.
The mRNA signal is shown as a blue precipitate and the protein signal as a dark precipitate. Scale bars represent 100 μm.

DISCUSSION

In our study, piglets treated with GCs showed i) diminished proliferative zone and total growth plate widths, ii) changes in the morphology of the trabecular bone in the primary spongiosa, iii) increased apoptosis in hypertrophic chondrocytes and iv) decreased VEGF expression in the growth plate and disturbed blood vessel arrangement, but no changes in MMP-9.

GC treatment results in decreased growth in children [10,11], several experimental animal models [12-14] and also in piglets [15,16], which are a good model for growing children [20]. GC-induced growth retardation occurs at the local level of the growth plate [4,17] and as the pig growth plate more closely resembles the human growth plate [21,22], it is important to study the effects of GCs on the pig growth plate. We found a 27% reduction in the width of the proliferative zone in the piglet tibial growth plate due to GC treatment. In rats, GC treatment

suppressed the proliferation rate without changing the width of this zone [27,28] and in mice we found a 10% decrease in the proliferative zone, concomitant with dexamethasone-induced growth inhibition [12,26]. Also in rabbits, dexamethasone decreased bone growth together with the width of the proliferative zone [29]. In addition to these *in vivo* data, also *in vitro* experiments using cultured rodent and porcine chondrocytes have shown that GCs inhibit their proliferation [30-32].

In addition to the proliferative zone, the hypertrophic zone was also affected by the GC treatment: there was a 7-fold increase in apoptosis in the PRDL-treated piglets, with apoptotic chondrocytes also appearing higher in the hypertrophic zone. In rats, GC treatment resulted in a 2-fold increase in apoptosis of hypertrophic chondrocytes, only in terminal hypertrophic chondrocytes [4,28]. It is possible that these differences are due to different susceptibilities of growth plates between species, or due to differences in age and duration of the GC treatment. Interestingly, cell death of terminal chondrocytes has been found to inversely correlate with the growth rate of the bone [33]. GCs, by increasing apoptosis, thus may negatively regulate bone growth. In our study, the width of the hypertrophic zone was only marginally decreased. Possibly, an accelerated differentiation of proliferative chondrocytes into hypertrophic chondrocytes [34] compensates for the enhanced apoptosis, leaving the number of chondrocytes in the hypertrophic zone almost unchanged. GC-induced apoptosis could involve the Bcl-2 protein family, the balance between Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) supposedly determining the rate of apoptosis [35]. However, PRDL treatment of our piglets did not show an effect on the expression patterns of Bcl-2 and Bax (data not shown). In previous studies, the ratio Bcl-2 to Bax could only partly explain the increased apoptosis in the growth plate due to GC treatment [28,36], suggesting that the occurrence of apoptosis is not directly related to detection of altered Bax and Bcl-2 protein levels.

Besides the effects of the PRDL treatment on growth plate cartilage, the structure of primary trabeculae and trabecular bone formation of the primary spongiosa were also severely disturbed, already after this short-term GC treatment. GCs have also been shown to affect bone formation in the secondary spongiosa by decreasing bone formation and increasing bone resorption [37,38], both of which could contribute to GC-induced osteoporosis [18]. Our study demonstrates that the primary spongiosa is affected as well.

PRDL treatment also resulted in disorganized and short metaphyseal blood vessels in the tibia, which has not been shown before. Normal blood vessel invasion of the growth plate is required for endochondral ossification to occur [1,6]. Disturbance of angiogenesis has been shown to affect endochondral ossification and longitudinal bone growth [7].

Many genes have been described to be involved in angiogenesis, with VEGF being one of the main modulators [6]. In our control piglets, VEGF mRNA and protein are predominantly expressed in the hypertrophic zone, which is in accordance with other studies [7,39]. In the PRDL-treated animals, VEGF mRNA and protein were no longer detectable in the hypertrophic zone. In prepubertal rats, GC treatment also resulted in a down-regulation of VEGF in the growth plate [28], although this decrease was not as severe as shown in our piglets. We have previously shown a down-regulation of VEGF expression by GCs in piglet chondrocytes *in vitro* [19]. Together, our findings suggest that GCs decrease VEGF expression in the growth plates of growing piglets, which could be the cause of the disturbed angiogenesis, resulting in the observed defects in capillary architecture and metaphyseal bone. Besides the involvement of VEGF, possibly other angiogenic and/or anti-angiogenic factors could be involved in the disturbed angiogenesis in our PRDL-treated piglets. We examined MMP-9, which is known to make VEGF bioavailable and is important for angiogenesis to

occur [6,8], but found no effect of the GC treatment on the MMP-9 expression pattern. Still other factors could be postulated as intermediates in GC action. For instance, TGF-β inhibits angiogenesis [40] and stimulates apoptosis [41]. IGF-I, on the other hand, protects against GC-induced apoptosis [42], stimulates production of VEGF in growth plate chondrocytes [19] and is important for angiogenesis [43]. We have previously suggested that IGF-I in the growth plate could contribute to or counteract the adverse effects of GCs on growth [26].

In conclusion, within 5 days, GC treatment suppresses VEGF expression in the tibial growth plate of growing piglets, which could be responsible for the observed disturbed vascularization of the growth plate. In the growth plate, both proliferative and hypertrophic chondrocytes are affected by the GC treatment. These changes indicate a rapid onset in disturbance of endochondral ossification, with disturbed growth as a likely result.

REFERENCES

1. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
2. Hatori M, Klatte KJ, Teixeira CC, Shapiro IM 1995 End labeling studies of fragmented DNA in the avian growth plate: evidence of apoptosis in terminally differentiated chondrocytes. *J Bone Miner Res,* **10:** 1960-1968.
3. Zenmyo M, Komiya S, Kawabata R, Sasaguri Y, Inoue A, Morimatsu M 1996 Morphological and biochemical evidence for apoptosis in the terminal hypertrophic chondrocytes of the growth plate. *J Pathol,* **180:** 430-433.
4. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
5. Bittner K, Vischer P, Bartholmes P, Bruckner P 1998 Role of the subchondral vascular system in endochondral ossification: endothelial cells specifically derepress late differentiation in resting chondrocytes *in vitro*. *Exp Cell Res,* **238:** 491-497.
6. Gerber H-P, Ferrara N 2000 Angiogenesis and bone growth. *Trends Cardiovasc Med,* **10:** 223-228.
7. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N 1999 VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med,* **5:** 623-628.
8. Engsig MT, Chen Q-J, Vu TH, Pedersen A-C, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, Delaissé J-M 2000 Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing bones. *J Cell Biol,* **151:** 879-889.
9. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, and Werb Z 1998 MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell,* **93:** 411-422.
10. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
11. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.
12. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
13. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
14. Ortoft G, Gronbaek H, Oxlund H 1998 Growth hormone administration can improve growth in glucocorticoid-injected rats without affecting the lymphocytopenic effect of the glucocorticoid. *Growth Horm IGF Res,* **8:** 251-264.
15. Fritz PC, Ward WE, Atkinson SA, Tenenbaum HC 1998 Tamoxifen attenuates the effects of exogenous glucocorticoid on bone formation and growth in piglets. *Endocrinology,* **139:** 3399-3403.
16. Guo C-Y, Ward W, Cairns P, Atkinson S 2000 Comparative response in growth and bone status to three dexamethasone treatment regimens in infant piglets. *Pediatr Res,* **48:** 238-243.

17. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
18. Weinstein RS 2001 Glucocorticoid-induced osteoporosis. *Rev Endocr Metab Disord,* **2:** 65-73.
19. Koedam JA, Smink JJ, Van Buul-Offers SC 2002 Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. *Mol Cell Endocrinol,* **197:** 35-44.
20. Miller ER, Ullrey DE 1987 The pig as a model for human nutrition. *Annu Rev Nutr,* **7:** 361-382.
21. Thurston MN, Kember NF 1985 *In vitro* thymidine labeling in human and porcine growth plates. *Cell Tissue Kinet,* **18:** 575-582.
22. Reiland S 1978 Growth and skeletal development of the pig. *Acta Radiol,* **358 (suppl):** 15-22.
23. de Meer K, Smolders HC, Meesterburrie J, de Sain-van der Velden M, Voorbij HAM, Okken A, Reijngoud D-J, Kulik W 2001 A single food bolus stimulates albumin synthesis in growing piglets. *J Pediatr Gastroenter Nutr,* **31:** 251-257.
24. Weindel K, Marmé D, Weich HA 1992 AIDS associated Kapsoi's sarcoma cells in culture express vascular endothelial growth factor. *Biochem Biophys Res Commun,* **183:** 1167-1174.
25. Melton D, Krieg P, Rebaglati M, Maniatis T, Zinn K, Green MR 1984 Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res,* **12:** 7035-7056.
26. Smink JJ, Koster JG, Gresnigt R, Rooman R, Koedam JA, Van Buul-Offers SC 2002 IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice. *J Endocrinol,* **175:** 143-153.
27. Kember NF, Walker KVR 1971 Control of bone growth in rats. *Nature,* **229:** 428-429.
28. Sanchez CP, He Y-Z 2002 Alterations in the growth plate cartilage of rats with renal failure receiving corticosteroid therapy. *Bone,* **30:** 692-698.
29. Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S, Baron J 2001 Catch-up growth is associated with delayed senescence of the growth plate in rabbits. *Pediatr Res,* **50:** 618-623.
30. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
31. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.
32. Smink JJ, Koedam JA, Koster JG, Van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *J Endocrinol,* **174:** 343-352.
33. Farnum CE, Wilsman NJ 1989 Condensation of hypertrophic chondrocytes at the chondro-osseous junction of growth plate cartilage in yucatan swine: relationship to long bone growth. *Am J Anat,* **186:** 346-358.
34. Takano T, Takigawa M, Suzuki F 1985 Stimulation by glucocorticoids of the differentiated phenotype of chondrocytes and the proliferation of rabbit costal chondrocytes in culture. *J Biochem,* **97:** 1093-1100.
35. Basu A, Haldar S 1998 The relationship between Bcl-2, Bax and p53: consequences for cell cycle progression and cell death. *Mol Hum Reprod,* **4:** 1099-1109.
36. Mocetti P, Silvestrini G, Ballanti P, Patacchioli FR, Di Grezia R, Angelucci L, Bonucci E 2001 Bcl-2 and Bax expression in cartilage and bone cells after high-dose corticosterone treatment in rats. *Tissue and Cell,* **33:** 1-7.
37. Chappard D, Legrand E, Basle MF, Fromont P, Racineux JL, Rebel A, Audran M 1996 Altered trabecular architecture induced by corticosteroids: a bone histomorphometric study. *J Bone Miner Res,* **11:** 676-685.
38. Eberhardt AW, Yeager-Jones A, Blair HC 2001 Regional trabecular bone matrix degeneration and osteocyte death in femora of glucocorticoid-treated rabbits. *Endocrinology,* **142:** 1333-1340.
39. Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE 1999 Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. *J Anat,* **194:** 519-524.
40. Wing-hoi C, Kwong-man L, Kwok-pui F, Pauline LP-Y, Kwok-sui L 2001 TGF-β1 is the factor secreted by proliferative chondrocytes to inhibit neo-angiogenesis. *J Cell Biochem,* **36 (suppl):** 79-88.
41. Schuster N, Krieglstein K 2002 Mechanisms of TGF-β-mediated apoptosis. *Cell Tissue Res,* **307:** 1-14.
42. Distelhorst CW 2002 Recent insights into the mechanism of glucocorticoisteroid-induced apoptosis. *Cell Death Differ,* **9:** 6-19.
43. Hellstrom A, Carlsson B, Niklasson A, Segnestam K, Boguszewski M, De Lacerda L, Savage M, Svensson E, Smith L, Weinberger D, Albertsson WK, Laron Z 2002 IGF-I is critical for normal vascularization of the human retina. *J Clin Endocrinol Metab,* **87:** 3413-3416.

Chapter 6

Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes

J.A. Koedam, J.J. Smink and S.C. van Buul-Offers

Department of Pediatric Endocrinology, University Medical Center Utrecht, Utrecht, The Netherlands

Adapted from
Molecular and Cellular Endocrinology 2002, **197**: 35-44

ABSTRACT

Vascular endothelial growth factor (VEGF) plays an essential role in angiogenesis in the growth plate and ultimately in regulating endochondral ossification. Since longitudinal bone growth is often disturbed in children who are treated with glucocorticoids, we investigated the effects of dexamethasone (DXM) on VEGF expression by epiphyseal chondrocytes. Cells were cultured from tibial growth plates of neonatal piglets, which expressed the chondrocyte-specific genes aggrecan, collagen II and CD-RAP, as well as the glucocorticoid receptor (GR). VEGF protein secreted from these cells was examined by ELISA and Western immunoblotting. The $VEGF_{121}$ and $VEGF_{165}$ isoforms were detected in the supernatant. As determined by RT-PCR, all three major mRNA splice variants of VEGF were produced, including the species encoding $VEGF_{189}$.

DXM (100 nM) inhibited both VEGF protein and mRNA expression by approximately 45%. Hydrocortisone (cortisol) and prednisolone also inhibited VEGF secretion, but they were less active than DXM. The inhibitory actions of DXM were almost completely blocked by the GR antagonist Org34116, indicating that the GR mediates these actions. Degradation of the VEGF mRNA was not accelerated by DXM. Therefore, a transcriptional mechanism seems likely. Downregulation of VEGF could lead to disruption of the normal invasion of blood vessels in the growth plate, which could contribute to disturbed endochondral ossification and growth.

INTRODUCTION

Longitudinal bone growth depends on the proliferation, differentiation and subsequent ossification of growth plate cartilage. The rates of chondrocyte proliferation and differentiation to the hypertrophic phenotype are determined by a complex interplay between many signaling molecules. Also the ensuing process of endochondral ossification is tightly regulated, and involves the interrelated processes of extracellular matrix degradation, apoptosis of terminal hypertrophic chondrocytes and angiogenesis: the invasion of the growth plate by new blood vessels from the metaphysis. The molecular mechanisms involved in chondrocyte differentiation and endochondral bone formation have been reviewed by Stevens and Williams [1]. Signals that direct the polarity of the growth plate, angiogenesis and bone formation originate in the growth plate itself, as was elegantly demonstrated by excising, inverting and reimplanting rabbit growth plates [2]. While the polarity of the cartilage was maintained, the epiphyseal bone, which became adjacent to the hypertrophic zone of the growth plate, became the site of blood vessel and bone cell invasion and longitudinal bone growth.

Angiogenesis can be induced by basic fibroblast growth factor [3], transferrin [4] and other factors [5], but vascular endothelial growth factor (VEGF) is the quintessential angiogenic factor in this respect, as was demonstrated by treating growing mice with a soluble antagonist [6]. This treatment resulted in suppression of blood vessel invasion, accompanied by impaired resorption of terminal chondrocytes and formation of the primary spongiosa (trabeculae of the metaphyseal bone). VEGF is expressed by many tissues and cell types, including the growth plate cartilage [6-9]. Its expression can be induced by many factors, including hypoxia [10], estrogen [11], osteogenic protein-1/BMP-7 [12], PDGF [13] and IGF-I [9,14-17].

Given its pivotal role in maintaining the normal structure of the growth plate [6], VEGF could be a potential target for agents, which disturb longitudinal growth. Glucocorticoids (GCs) are well known to induce growth retardation [18-20] as well as osteoporosis [21]. Direct local actions of GCs on the growth plate have been demonstrated *in vivo* [22], which likely occur through interaction with the glucocorticoid receptor (GR) [23]. Although many of the inhibitory actions of pharmacological doses of GCs on skeletal growth appear to be mediated by interference with the GH/IGF-I axis [24,25], their effects on angiogenesis and in particular VEGF expression in the growth plate are worth investigating. In this study, we set out to characterize the effects of pharmacological doses of DXM and other GCs on VEGF expression by cultured porcine growth plate chondrocytes.

MATERIALS AND METHODS

Materials

Recombinant human IGF-I was kindly provided by Eli Lilly & Co. (Indianapolis, IN, USA). Cortisol (HC) (hydrocortisone, Solu-Cortef) was from Pharmacia & Upjohn B.V. (Woerden, The Netherlands). Prednisolone (PRDL) (Di-Adreson-F) was from N.V. Organon (Oss, The Netherlands). The GR antagonist Org34116 was kindly provided by Dr. M. de Gooyer (N.V. Organon, Oss, The Netherlands). DXM (as dexamethasone disodium phosphate) was from Merck Sharpe & Dohme (Haarlem, The Netherlands). Platelet-derived growth factor (PDGF)-AB, protease (P-6911), collagenase (C-9891), actinomycin D, BSA, salmon sperm DNA and Alcian Blue 8GX were from Sigma Chemical Co. (St, Louis, MO, USA). Rabbit polyclonal anti-VEGF antibody and blocking peptide were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated donkey anti-rabbit antibody, Oligo(dT)$_{12-18}$ primer, RediPrime Random Primer labeling mixture, [α-^{32}P]dCTP (10 mCi/ml), Nylon membrane (Hybond-N+, 0.2 μm) and Hyperfilm ECL film were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). Centricon-10 ultrafiltration membranes and PVDF membranes (Immobilon-P) were from Millipore Corp. (Bedford, MA, USA). Restriction enzymes, AmpliTaq DNA polymerase, modifying enzymes and TriPure isolation reagent were purchased from Roche Molecular Biochemicals (Mannheim, Germany). M-MLV Reverse Transcriptase was from Promega Corp. (Madison, WI, USA). The TOPO TA Cloning kit was from Invitrogen Corp. (Carlsbad, CA, USA).

Cell culture

The growth plates were dissected from the distal and proximal tibiae of neonatal piglets. The piglets were used and sacrificed for unrelated experiments by the Neonatology Department of the University Medical Center Utrecht. These experiments did not interfere with the normal physiology of the growth plate cartilage. Subsequently, the epiphyseal growth plate chondrocytes were isolated and grown as described previously [26]. Generally, second passage cells were used for experiments, but identical results were obtained with first and third passage cells. When cells were to be treated with growth factors and hormones followed by VEGF ELISA or Western blotting, they were seeded (40,000 or 80,000 cells per well) on 24-well plates

Conditioned medium was prepared by incubating the cells in medium containing 10% FCS, which had been stripped of steroids by adsorption with dextran-coated charcoal. In order to analyse the secreted VEGF protein by electrophoresis and Western immunoblotting, serum-free medium supplemented with 10 nM IGF-I and 0.005% BSA was used instead.

In a few cases, also the amount of VEGF secreted under these conditions was measured by ELISA, and no differences were found between both milieus for collecting conditioned medium, neither in the amount of VEGF secreted, nor in the inhibitory effect of DXM. Experiments in which RNA was to be isolated from the cells were always performed in the presence of 10% stripped FCS.

Analysis of VEGF protein

Chondrocyte-conditioned medium (1 ml) was collected after 48 h, centrifuged for 10 min at 3000 x g (4°C) and stored at –80°C. VEGF concentrations were measured by ELISA (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The adherent cells were washed three times with PBS and lysed in 100 μl 2% SDS, 125 mM Tris-HCl pH 6.8, followed by boiling for 5 min. Ten microliter was used to measure the protein content (using the BCA reagent, Pierce, Rockford, IL, USA). VEGF levels were normalized to the protein content of the cells.

For Western immunoblotting, the conditioned media were concentrated 10-fold on Centricon-10 ultrafiltration membranes and loaded on a 12% gel for reducing SDS-PAGE. VEGF was visualized by electrotransfer to PVDF membranes, followed by incubation with rabbit polyclonal antibody (1/200 dilution) and peroxidase-conjugated donkey anti-rabbit antibody (1/4000 dilution). The bands were visualized using the SuperSignal WestPico Chemiluminescent Substrate of Pierce (Rockford, IL, USA) and Hyperfilm ECL film. Molecular weights were calculated using BioRad (Hercules, CA, USA) broad range markers as standards.

RNA isolation and Northern blot analysis

Cells (1 x 10^6) were cultured in 25 cm^2 tissue culture flasks and treated with GCs for 24 h. Cells were directly lysed with Tripure solution reagent and total RNA was extracted according to the procedures of the manufacturer, based on the single-step acid guanidinium-thiocyanate method [27]. Total RNA was separated by electrophoresis in a 1% (w/v) agarose/2.2 M formaldehyde gel in 1x 3-(morpholino) propanesulphonic acid buffer, transferred to a 0.2 µm nylon membrane and crosslinked to the membrane by UV radiation.

The membranes were prehybridized for 2 h at 55ºC in a solution containing 0.1% SDS, 3xSSC, 5x Denhardt's solution, 10% dextran-sulfate and 50 µg/ml denaturated salmon sperm DNA. Hybridization was performed at 55ºC (65ºC for GAPDH) overnight in the same solution, containing the indicated [α-^{32}P]dCTP-labeled probe. Probes (see below) were labeled with [α-^{32}P]dCTP using the random hexamer RediPrime kit. The blots were washed up to a stringency of 0.5x SSC, 0.1% SDS at 55°C (0.1x SSC, 0.1% SDS at 65°C for GAPDH, collagen II).

The hybridization signals were analyzed by densitometry using the GS-363 Molecular Imager and the Molecular Analyst software program (version 1.5) (Biorad) and the relative abundance of the different mRNAs was subsequently normalized to the GAPDH values. In addition, the filters were exposed to Fuji Super RX film (Düsseldorf, Germany).

The cDNA probes for collagen types I and II, aggrecan and GAPDH were described before [25]. A probe for porcine CD-RAP (cartilage-derived retinoic acid sensitive protein) [28] was prepared by RT-PCR from total RNA isolated from the cultured chondrocytes as described below. The PCR product was ligated into the pCRII-TOPO TA Cloning vector as described by the manufacturer and sequenced using a capillary sequencer. The cDNA probe for human VEGF was a kind gift of Dr. M.F. Gebbink and Dr. E.E. Voest (University Medical Center Utrecht). The 520 bp insert encompassing the sequence encoding $VEGF_{121}$ [29] was excised using *NcoI* / *BglII* double digestion.

RT-PCR and Southern blotting

One microgram of total RNA was reverse transcribed using 1 µg of oligo(dT) as primer and 200 U of Reverse Transcriptase for 90 min at 42°C in a total volume of 25 µl. Two microliter of cDNA was amplified by PCR using the following primers: CD-RAP, forward 5'-ATGCCCAAGCTGGCTGAC-3' and reverse 5'-ATGCTACTGGGGAAATAGC-3' (240 bp product); GR, forward 5'-GTGAGTACCTCTGGAGGACA-3' and reverse 5'-CTTTGCCCATTTCACTGC-3' (761 bp product). The VEGF primers (forward 5'-AGTGTGTGCCCACTGAGGAG, corresponding to nt 278-297 in exon 3 and reverse 5'-ACCGCCTCGGCTTGTCACAT, corresponding to nt 627-648 in exon 8) were selected, because they are identical in human and porcine VEGF, and have identical melting temperatures. In each case, the PCR consisted of an initial denaturation step for 5 min at 94°C, followed by 30-35 cycles (1 min at 94°C, 1 min at 58°C, 1 min at 72°C) and a final extension step (10 min at 72°C) in a total volume of 50 µl containing 10 pmol of primers. PCR products were analyzed on a 1% agarose gel.

For Southern blotting of VEGF, 4 µl from the PCR reaction was loaded on a 1% agarose gel. Southern blotting and hybridization with ^{32}P-labeled VEGF cDNA probe was essentially carried out as described above for the Northern blot analysis.

Statistical analysis

Data from VEGF ELISA were normalized for total protein content and data from Northern blots were normalized for GAPDH content. Results are expressed as means ± SEM. Statistical differences between two groups were determined by two-tailed Student's t-test. Statistical differences between multiple treatments were determined by one-way ANOVA using InStat version 3.00 (GraphPad Software, Inc., San Diego, CA). The Tukey-Kramer post-hoc test was used to determine significance between treatment groups. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Characterization of growth plate chondrocytes

The cultured porcine growth plate chondrocytes maintained their chondrocyte phenotype, as shown by the mRNA expression of specific chondrocyte markers. Using Northern blot analysis, transcripts were detected for collagen II (5.4 kb), aggrecan (8.0 kb) and CD-RAP (0.8 kb) (Fig.1).

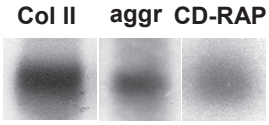

Figure 1 Characterization of cultured primary porcine growth plate chondrocytes. Total RNA was isolated and subject to Northern blot analysis for the expression of chondrocyte-specific markers. Representative Northern blot data are shown. Col II, collagen II; aggr, aggrecan; CD-RAP, cartilage-derived retinoic acid-sensitive protein.

Collagen I mRNA, specific for fibroblasts, osteoblasts and dedifferentiated chondrocytes, was expressed at very low levels, whereas in NIH 3T3 mouse fibroblasts a strong signal was observed (data not shown). Staining of sulfated proteoglycans in the extracellular matrix with Alcian blue also showed that the chondrocyte cultures were not contaminated with fibroblasts or osteoblasts and that the chondrocytes were not de-differentiated [30] (data not shown).

Secretion of VEGF protein from chondrocytes

Chondrocytes, when cultured in the presence of serum, secreted 400-1600 pg/ml (1.3-4.6 pg/µg protein) VEGF over a 48 h period, as measured with an ELISA assay. In the absence of serum, VEGF production was stimulated by PDGF (threefold at 5 nM) and by IGF-I (1.8 ± 0.2 -fold at 10 nM, n = 5 independent experiments, $P<0.02$). In the presence of 10 nM IGF-I, 600-1300 pg/ml (2.3-6.1 pg/µg protein) VEGF accumulated over a 48 h period. DXM caused a dose-dependent decrease of secreted VEGF protein (Fig. 2A). The GR antagonist Org34116 [31] largely reversed the inhibitory action of DXM, indicating the involvement of functional GRs in the action of DXM.

When compared with the inhibitory effect of DXM, hydrocortisone (HC; cortisol) and prednisolone (PRDL) were less potent, but at 1000 nM were also able to cause 80% inhibition of VEGF secretion (Fig. 2B).

On Western immunoblot, two species of secreted VEGF were detected at 20 and 23 kDa (Fig. 2C). When the antibody was first preabsorbed with VEGF blocking peptide, these bands were not detected (data not shown). Based on their relative weights and the fact that they were released from the cells, these forms most likely represent the $VEGF_{121}$ and $VEGF_{165}$ isoforms. $VEGF_{121}$ lacks both heparin-binding regions, making it highly diffusible, while $VEGF_{165}$ still retains some cell-binding properties. The intensities of both isoforms decreased in parallel in response to DXM treatment, while Org34116 blocked the action of DXM.

Thus, the results obtained from the immunoblot completely correspond to the ELISA measurements. Similarly, HC and PRDL inhibited the secretion of both VEGF isoforms (data not shown).

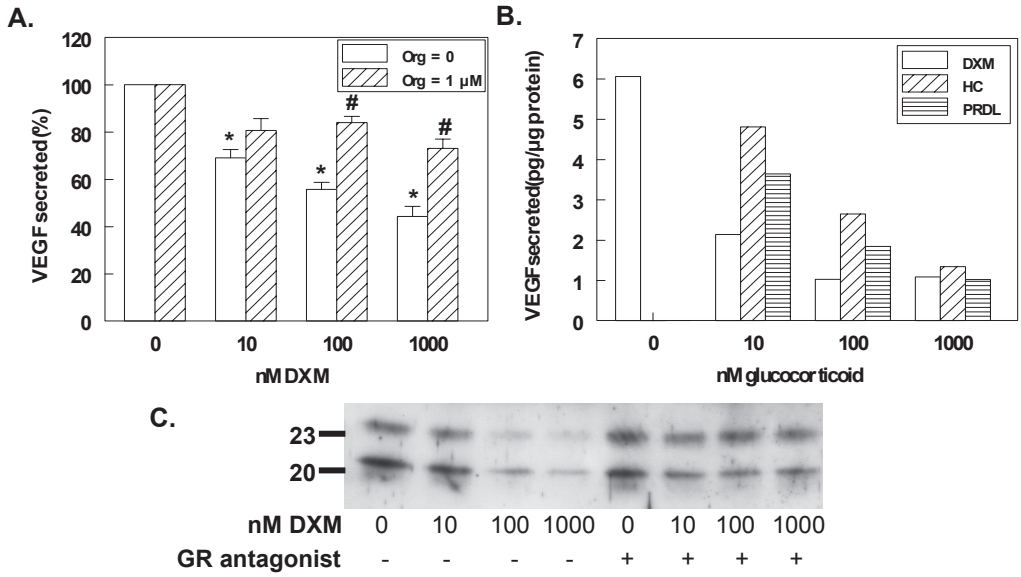

Figure 2 Inhibitory effect of GCs on VEGF secretion. Conditioned medium was collected 48 h after the addition of GCs and/or the GR antagonist Org34116 (Org) (1 µM) (as indicated). (**A**) VEGF was measured by ELISA assay. Values are the means ± SEM of four experiments, each performed in duplicate and normalized for protein content in each well. **P*<0.01, compared with controls without DXM; # *P*<0.01, compared with corresponding DXM concentration in the absence of Org34116. (**B**) Values (ELISA assay) are from one experiment (carried out in serum-free medium supplemented with 10 nM IGF-I), which was repeated with identical results. DXM, dexamethasone; HC, hydrocortisone; PRDL, prednisolone. (**C**) Western blot of VEGF secreted from chondrocytes treated with DXM (as indicated) in the absence (**-**) or presence (**+**) of the GR antagonist. VEGF protein was detected using a rabbit polyclonal antibody. Numbers on the left indicate the molecular masses (in kDa).

RT-PCR and Southern blotting

RT-PCR was performed on total RNA isolated from epiphyseal chondrocytes to characterize the splice variants of VEGF mRNA (Fig. 3A). Oligonucleotide primers were chosen to correspond with sequences in exons 3 and 8, allowing to distinguish between forms which contain both exons 6 and 7 ($VEGF_{189}$), or lacking exon 6 ($VEGF_{165}$) or lacking both exons 6 and 7 ($VEGF_{121}$) [32]. The major amplicon corresponded with the predicted size of 297 bp for the $VEGF_{165}$ species. Likewise, minor bands representing the $VEGF_{121}$ (165 bp) and $VEGF_{185}$ (369 bp) isoforms were detected.

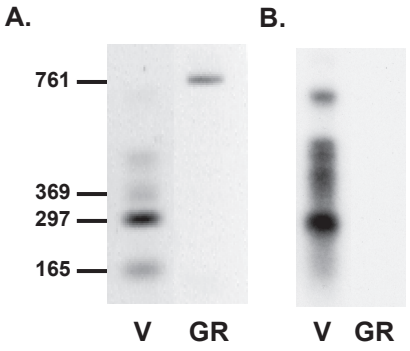

Figure 3 Expression of VEGF isoforms. Total RNA from chondrocytes was reverse-transcribed to cDNA, followed by PCR using primer pairs corresponding with exons 3 and 8. Samples were run in duplicate, and either stained with (**A**) ethidium bromide or (**B**) subjected to Southern blot analysis by hybridization with a ^{32}P-labeled VEGF cDNA probe. In addition to PCR for VEGF (V), PCR was performed for the GR (GR) as a negative control. Numbers on the left indicate the sizes of the PCR products in bp.

Also several larger products were weakly visible. None of these bands corresponded to the $VEGF_{206}$ isoform [33], which would contain an extra insertion of 51 bp between exons 6 and 7 and would thus be expected to yield a PCR product of 420 bp. On the corresponding Southern blot (Fig. 3B), all of the PCR-amplified products hybridized with the VEGF probe. As a negative control, the PCR product of the GR (761 bp product) was transferred to the filter, but did not hybridize with the VEGF probe. In conclusion, porcine epiphyseal chondrocytes produce all major splice variants of VEGF, with $VEGF_{165}$ being the most abundant.

DXM effect on VEGF mRNA

Northern blots of total RNA extracted from chondrocytes hybridized with a VEGF cDNA probe showed a major transcript of approximately 4.8 kb, together with minor transcripts of approximately 1.3 and 5.3 kb (Fig. 4). Expression of all transcripts was inhibited by DXM (100 nM), the abundance of the major transcript was decreased to 57.5 ± 5.2% (n = 4 independent experiments, $P<0.01$). As for the protein expression, the inhibitory effect of DXM was blocked by the GR antagonist (Fig. 4). A time-course experiment, in which RNA was isolated from cells at different times after addition of DXM, indicated that the maximum inhibition of VEGF mRNA expression was reached after 1 h (data not shown).

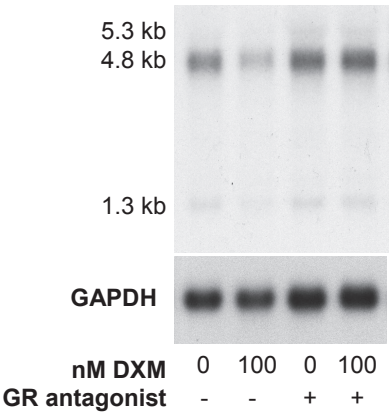

Figure 4 Expression of VEGF mRNA and effects of DXM treatment. Chondrocytes were treated with 100 nM DXM or 10 μM of the GR antagonist (Org34116) or the combination of both (as indicated) for 24 h. Total RNA was isolated and subject to Northern blot analysis. Re-hybridization with a GAPDH probe was performed to check for equal loading of the lanes. A representative experiment is shown. Sizes of the transcripts are indicated on the left.

To test whether DXM decreased the abundance of VEGF mRNA by accelerating its degradation, the transcription inhibitor actinomycin D was used. Actinomycin D was added to the cells 1 h after the addition of DXM, and the stability of the VEGF mRNA was determined. As shown in Fig. 5, the halflife was just under 2 h, and was not altered in the presence of DXM, indicating that the downregulation of VEGF by DXM is not caused by destabilization of its mRNA.

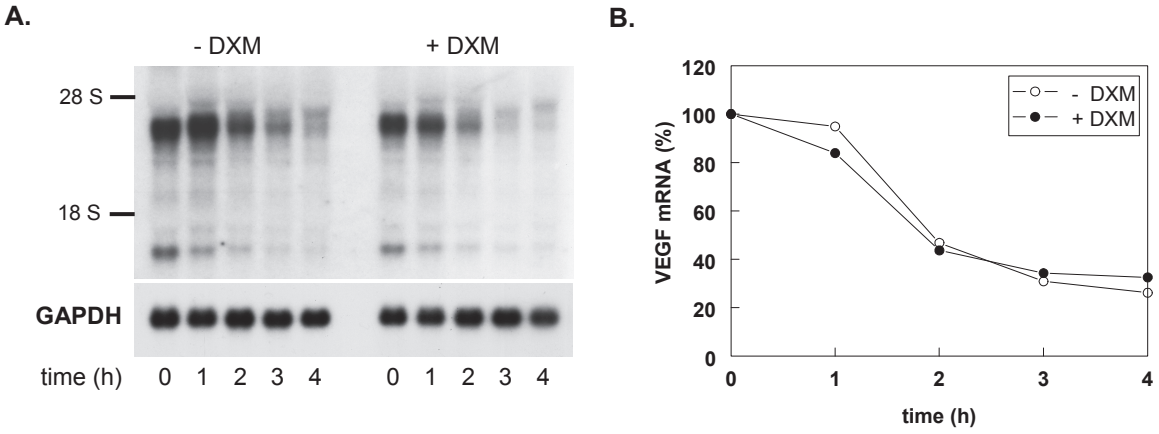

Figure 5 Stability of VEGF mRNA. (**A**) Chondrocytes were cultured in 25 cm^2 flasks in the presence of 10% FCS and treated with 100 nM DXM. After 1 h, actinomycin D (5 μg/ml) was added. RNA was isolated at 1, 2, 3 and 4 h after the addition of actinomycin D. VEGF mRNA was assayed by Northern blot analysis. (**B**) Quantification of the major VEGF transcript, corrected for GAPDH content. The graph shows the average result of two experiments.

DISCUSSION

In this study we have examined the effects of GCs, in particular DXM, on the expression of VEGF. We cultured primary chondrocytes from the epiphyseal growth plates of neonatal piglets. This model system was used earlier in the study of DXM effects on the expression of the components of the IGF system [26]. Although no attempts were made to separate the various layers of the growth plate, it is assumed that the *in vitro* phenotype most closely resembles that of the proliferative zone [24,34]. We found no evidence of de-differentiation during our experiments, as demonstrated by a lack of decreased collagen type II expression or increased collagen type I expression during passaging of the cells.

We demonstrate here that these chondrocytes synthesize and secrete VEGF, an important angiogenic factor, and that its production is regulated by IGF-I and PDGF. VEGF protein has been detected earlier by immunohistochemistry in growth plate cartilage of human neonates [7], chicken and mouse embryos [8] and human fetuses [9]. Similarly, VEGF mRNA expression was found by *in situ* hybridization in juvenile [6] and embryonic [35] mouse growth plates. In most of these cases, VEGF was exclusively found in the hypertrophic zone; only Garcia-Ramirez *et al.* [9] also detected VEGF protein in the resting and proliferative zones. In agreement with our result, they also found VEGF secreted by cultured proliferative chondrocytes.

The sizes of the VEGF transcripts cannot be precisely linked to the various splice variants. Since the 297 bp PCR product representing the $VEGF_{165}$ species is the most abundant species, it is tempting to speculate that the major 4.8 kb transcript represents this variant. Bermont *et al.* [16] came to the same conclusion for a 4.5 kb transcript. A 4.4 or 4.5 kb transcript has been reported for many cell types, *e.g.* human fetal vascular smooth muscle cells [32], endometrial adenocarcinoma cells [16] and human breast cancer cells [36]. In all of these cell types, also a 3.7 kb transcript was found, which was usually the predominant species. In addition, this 3.7 kb form was found in *e.g.*, human fetal epiphyseal chondrocytes [9] and mouse osteoblastic cells [37]. We did not, however, detect this species, a discrepancy for which we have no explanation. The minor 5.3 kb band was also reported by others, including Tischer *et al.* [32] and Bermont *et al.*[16]. The 1.3 kb VEGF transcript has not been described before, although it is weakly visible on a Northern blot in the paper by Garcia-Ramirez *et al.* [9].

Downregulation of VEGF expression by DXM has previously been demonstrated in rat glioma cells [39,40], human vascular smooth muscle cells [13], mouse and rat pituitary folliculostellate cells [41] and porcine brain endothelial cells [42]. Many mechanisms exist by which GCs can repress gene function [43]. Since the GR is present in our cells, and the specific GR antagonist blocked the inhibitory action of DXM on VEGF expression, the DXM-GR complex is most likely the mediator of VEGF repression. The 5' promoter region of the VEGF gene does not contain a glucocorticoid responsive element (GRE), but it does contain four AP-1 binding sites [32]. The transcription factor AP-1 is activated by growth factors via mitogen-activated protein (MAP) kinases. Since the induction of VEGF expression by IGF-I, at least in NIH3T3 fibroblasts, involves the MAP kinase signaling pathway [17], it is likely that in our experiments (in which IGF-I was present) AP-1 activity is involved. It is conceivable, therefore, that a mechanism operates by which the ligand-occupied GR prevents the AP-1 transactivator complex from stimulating the VEGF gene [44]. In this case, GR binding to DNA and GR-dependent gene transcription are not required.

It is not known which factors regulate VEGF expression in the growth plate. IGF-I is an obvious candidate, however, since others and we have detected expression of this growth factor in the growth plate [45,46]. IGF-I production in cultured rat epiphyseal chondrocytes, especially when stimulated by GH, is inhibited by DXM [24]. Similarly, DXM blocks the IGF-I-stimulated rise in type 1 IGF receptor molecules [24]. In addition to a direct effect on the AP-1 transactivator complex as described above, GCs could therefore also regulate VEGF expression indirectly through interactions with the somatotropic axis.

Another well established inducer of VEGF expression is hypoxia [10], mediated by the transcriptional activator hypoxia-inducible factor 1 (HIF-1) [38]. Because the cartilage is not vascularized, it has been widely assumed that chondrocytes, especially in the hypertrophic zone, are hypoxic. Using a hypoxia-sensing drug and immunohistochemistry, Shapiro *et al*. [47] found that hypertrophic cartilage, at least in the chicken, is actually not oxygen deficient. In contrast, Schipani *et al*. [48], using a similar technique, recently reported that hypoxia does occur in the growth plates of developing mice. VEGF expression in the growth plate appears to be regulated through both HIF-1-dependent and –independent mechanisms [48]. The possible role of GCs in regulating these mechanisms needs to be addressed, especially since hypoxia-induced VEGF expression seems to be much less susceptible to DXM inhibition than VEGF expression induced by serum and growth factors [39,40].

We also explored another possibility by which DXM could regulate VEGF expression, namely by destabilizing its mRNA. We found no evidence for such a post-transcriptional regulation. When transcription was interrupted by actinomycin D, the halflife of the VEGF mRNA was unaffected by the presence of DXM. GC-regulated mRNA turnover is thought to involve AUUUA sequences (AU response elements, or AREs) in the 3' untranslated region of mRNAs [49,50], and these sequences are present in the VEGF transcript [51]. The regulation of the stability of VEGF mRNA is very complex, however, and involves sequences in the 3' untranslated region as well as the 5' untranslated region and the coding region [52]. This complexity is also illustrated by the different results, which have been reported with respect to the ability of IGF-I to stabilize VEGF mRNA. While IGF-I increased VEGF mRNA halflife in colon carcinoma cells [15] and endometrial adenocarcinoma cells [16], it had no such effect in osteoblastic cells [14] and fibroblasts [17].

VEGF is of utmost importance for the vascular invasion of the growth plate cartilage and the resulting processes of apoptosis and bone formation [6]. It is therefore highly likely that the negative effect of GCs on VEGF expression by epiphyseal chondrocytes contributes to the mechanism by which these steroid hormones cause growth retardation.

ACKNOWLEDGEMENTS

We thank Ms. C.M.P.C.D. Peeters and Dr. F. Groenendaal for making the tibiae of neonatal piglets available to us.

REFERENCES

1. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
2. Abad V, Uyeda JA, Temple HT, De Luca F, Baron F 1999 Determinants of spatial polarity in the growth plate. *Endocrinology,* **140:** 958-962.
3. Baron J, Klein KO, Colli MJ, Yanovski JA, Novosad JA, Bacher JD, Bolander ME, Cutler GB 1994 Induction of growth plate cartilage ossification by basic fibroblast growth factor. *Endocrinology,* **35:** 2790-2793.

4. Carlevaro MF, Albini A, Ribatti D, Gentili C, Benelli R, Cernelli S, Cancedda R, Cancedda FD 1997 Transferrin promotes endothelial cell migration and invasion: implication in cartilage neovascularization. *J Cell Biol,* **136:** 1375-1384.
5. Alini M, Marriott A, Chen T, Abe S, Poole AR 1996 A novel angiogenic molecule produced at the time of chondrocyte hypertrophy during endochondral bone formation. *Dev Biol,* **176:** 124-132.
6. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N 1999 VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med,* **5:** 623-628.
7. Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE 1999 Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. *J Anat,* **194:** 519-524.
8. Carlevaro M, Cermelli S, Cancedda R, Descalzi CF 2000 Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci,* **113:** 59-69.
9. Garcia-Ramirez M, Toran N, Andaluz P, Carrascosa A, Audi L 2000 Vascular endothelial growth factor is expressed in human fetal growth cartilage. *J Bone Miner Res,* **15:** 534-540.
10. Shweiki D, Itin A, Soffer D, Keshet E 1992 Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature,* **359:** 843-845.
11. Cullinan-Bove K, Koos RD 1993 Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology,* **133:** 829-837.
12. Yeh L-CC, Lee JC 1999 Osteogenic protein-1 increases gene expression of vascular endothelial growth facotr in primary cultures of fetal rat calvaria cells. *Mol Cell Endocrinol,* **153:** 113-124.
13. Nauck M, Karakiulakis G, Perruchoud AP, Papakonstantinou E, Roth M 1998 Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. *Eur J Pharmacol,* **341:** 309-315.
14. Goad DL, Rubin J, Wang H, Tashjian AH Jr, Patterson C 1996 Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. *Endocrinology,* **137:** 2262-2268.
15. Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB 1996 Induction of vascular endothelial growth factor by insulin-like growth factor I in colorectal carcinoma. *J Biol Chem,* **271:** 29483-29488.
16. Bermont L, Lamielle F, Fauconnet S, Esumi H, Weisz A, Adessi GL 2000 Regulation of vascular endothelial growth factor expression by insulin-like growth factor-I in endometrial adenocarcinoma cells. *Int J Cancer,* **85:** 117-123.
17. Miele C, Rochford JJ, Filippa N, Giorgetti-Peraldi S, Van Obberghen E 2000 Insulin and insulin-like growth factor-I induced vascular endothelial growth factor mRNA expression via different signaling pathways. *J Biol Chem,* **275:** 21695-21702.
18. Blodgett FM, Burgin L, Iezzoni D, Gribetz D, Talbot NB 1956 Effects of prolonged cortisone therapy on the statural growth, skeletal maturation and metabolic status of children. *N Engl J Med,* **254:** 636-641.
19. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
20. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
21. Manelli F, Giustina A 2000 Glucocorticoid induced osteoporosis. *Trends Endocrinol Met,* **11:** 79-85.
22. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
23. Silvestrini G, Mocetti P, Ballanti P, Di Grezia R, Bonucci E 1999 Cytochemical demonstration of the glucocorticoid receptor in skeletal cells of the rat. *Endocrine Res,* **25:** 117-128.
24. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
25. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
26. Smink JJ, Koedam JA, Koster JG, Van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *J Endocrinol,* **174:** 343-352.
27. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* **162:** 156-159.
28. Dietz UH, Sandell LJ 1996 Cloning of a retinoic acid-sensitive mRNA expressed in cartilage and during chondrogenesis. *J Biol Chem,* **271:** 3311-3316.

29. Weindel K, Marmé D, Weich HA 1992 AIDS associated Kapsoi's sarcoma cells in culture express vascular endothelial growth factor. *Biochem Biophys Res Commun,* **183:** 1167-1174.
30. Sahni M, Ambrosetti D-C, Mansukhani A, Gertner R, Levy D, Basilico C 1999 FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev,* **13:** 1361-1366.
31. Karst H, De Kloet ER, Joël M 1997 Effect of Org 34116, a corticosteroid receptor antagonist, on hippocampal Ca^{2+} currents. *Eur J Pharmacol,* **339:** 17-26.
32. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA 1991 The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem,* **266:** 11947-11954.
33. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW 1991 The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol,* **5:** 1806-1814.
34. de los Rios P, Hill DJ 2000 Expression and release of insulin-like growth factor binding proteins in isolated epiphyseal growth plate chondrocytes from the ovine fetus. *J Cell Physiol,* **183:** 172-181.
35. Colnot CI, Helms JA 2001 A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mech Dev,* **100:** 245-250.
36. Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Härkönen PL 1999 Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. *Mol Cell Endocrinol,* **149:** 29-40.
37. Saadeh PB, Mehrara BJ, Steinbrech DS, Spector JA, Greenwald JA, Chin GS, Ueno H, Gittes GK, Longaker MT 2000 Mechanism of fibroblast growth factor-2 modulation of vascular endothelial growth factor expression by osteoblastic cells. *Endocrinology,* **141:** 2075-2083.
38. Semenza GL 2001 Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med,* **7:** 345-350.
39. Heiss JD, Papavassiliou E, Merrill MJ, Nieman L, Knightly JJ, Walbridge S, Edwards NA, Oldfield EH 1996 Mechanism of dexamethasone suppression of brain tumor-associated vascular permeability in rats. Involvement of the glucocorticoid receptor and vascular permeability factor. *J Clin Invest,* **98:** 1400-1408.
40. Machein MR, Kullmer J, Rönicke V, Machein U, Krieg M, Damert A, Breier G, Risau W, Plate KH 1999 Differential downregulation of vascular endothelial growth factor by dexamethasone in normoxic and hypoxic rat glioma cells. *Neurtopath Applied Neurobiol,* **25:** 104-112.
41. Gloddek J, Pagotto U, Paez Pereda M, Arzt A, Stalla GK, Renner U 1999 Pituitary adenylate cyclase-activating polypeptide, interleukin-6 and glucocorticoids regulate the release of vascular endothelial growth factor in pituitary folliculostellate cells. *J Endocrinol,* **160:** 483-490.
42. Fischer S, Renz D, Schaper W, Karliczek GF 2001 In vitro effects of dexamethasone on hypoxia-induced hyperpermeability and expression of vascular endothelial growth factor. *Eur J Pharmacol,* **411:** 231-243.
43. Webster JC, Cidlowski JA 1999 Mechanisms of glucocorticoid-receptor-mediated repression of gene expression. *Trends Endocrinol Met,* **10:** 396-402.
44. Schüle R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM 1990 Functional antagonism between oncoprotein c-Jun and glucocorticoid receptor. *Cell,* **62:** 1217-1226.
45. Smink JJ, Koster JG, Gresnigt R, Rooman R, Koedam JA, Van Buul-Offers SC 2002 IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice. *J Endocrinol,* **175:** 143-153.
46. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology,* **141:** 2847-2853.
47. Shapiro IM, Mansfield KD, Evans SM, Lord EM, Koch CJ 1997 Chondrocytes in the endochondral growth plate cartilage are not hypoxic. *Am J Physiol,* **272:** C1134-C1143.
48. Schipani E, Ryan HE, Didrickson s, Kobayashi T, Knight M, Johnson RS 2001 Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. *Genes Dev,* **15:** 2865-2876.
49. Malter JS 1989 Identification of an AUUUA-specific meesenger RNA binding protein. *Science,* **246:** 664-666.
50. Peppel K, Vinci JM, Baglioni C 1991 The AU-rich sequences in the 3' untranslated region mediate the increased turnover of interferon mRNA induced by glucocorticoids. *J Exp Med,* **173:** 349-355.
51. Claffey KP, Shih S-C, Mullen A, Dziennis S, Cusick JL, Abrams KR, Lee SW, Detmar M 1998 Identification of a human VPF/VEGF 3' untranslated region mediating hypoxia-induced mRNA stability. *Mol Biol Cell,* **9:** 469-481.
52. Dibbens JA, Miller DL, Damert A, Risau W, Vada MA, Goodall GJ 1999 Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA elements. *Mol Biol Cell,* **10:** 907-919.

Chapter 7

Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components

J. J. Smink, J. A. Koedam, J.G. Koster and S. C. van Buul-Offers

Department of Pediatric Endocrinology, University Medical Center Utrecht, Utrecht, The Netherlands

Journal of Endocrinology 2002, **174**: 343-352

ABSTRACT

High (pharmacological) doses of glucocorticoids inhibit the proliferation of growth plate chondrocytes, which leads to one of the side-effects of these steroids, namely suppression of longitudinal growth. Growth inhibition by glucocorticoids is thought to be mediated in part by impaired action of components of the IGF axis, which are important for chondrocyte regulation and hence for longitudinal growth. The aim of the present study was to determine whether glucocorticoid-induced growth retardation involves changes in IGF axis components. Chondrocytes were isolated from epiphyseal growth plates of neonatal piglets and treated with pharmacological doses of dexamethasone (DXM) for 24 h to study glucocorticoid-induced growth retardation. Under IGF-I-supplemented (10 nM) culture conditions, IGF-binding proteins (IGFBPs)-2, -4 and -5 were secreted by the growth plate chondrocytes and IGFBP-2 protein and mRNA levels were decreased by the DXM treatment, whereas IGFBP-4 and -5 were not affected.

Proliferation of the chondrocytes, as measured by [^{3}H]thymidine incorporation, was 3.5-fold higher in serum-supplemented medium in contrast to IGF-I-supplemented (10 nM) medium. In the presence of serum, DNA synthesis was significantly inhibited by 50 - 63% when treated with 100 nM DXM, which was prevented by the glucocorticoid receptor antagonist Org34116. mRNA levels of IGF axis components were determined using Northern blot analysis. IGFBP-2 to -6 were expressed in the chondrocytes, IGFBP-1 was absent and both IGF-I and IGF-II, and the type I and type II IGF receptors were expressed. Treatment with dexamethasone (100 nM) resulted in a 2-fold increase in mRNA levels of both IGFBP-5 and the type I IGF receptor, whereas IGFBP-2 mRNA levels decreased by 55%, in concert with the decrease in protein level observed under IGF-I-supplemented culture conditions. The changes in mRNA levels due to the dexamethasone treatment were prevented by the glucocorticoid receptor antagonist.

Our data show that exposure to pharmacological doses of DXM results in inhibition of proliferation and changes in components of the IGF axis, IGFBP-2 and -5 and the type I IGF receptor, suggesting a role for these components in glucocorticoid-induced growth retardation at the local level of the growth plate.

INTRODUCTION

Longitudinal bone growth results from the proliferation, differentiation, maturation and eventually apoptosis of chondrocytes within the growth plates of the long bones [1]. These processes must be correctly coordinated in order to maintain normal growth.

Glucocorticoids (GCs), although effective drugs in the treatment of several diseases, such as asthma, respiratory distress syndrome and rheumatic arthritis, may induce growth retardation in children as a side-effect [2]. Studies in experimental animal models have also shown that high levels of GCs have a growth suppressive effect on longitudinal bone growth [1,3-5]. Several *in vivo* studies showed that GCs act locally to restrain growth, suggesting a local mechanism which is intrinsic to the growth plate [6,7].

The inhibitory actions of GCs on longitudinal growth are suggested to be due to impaired action of the components of the insulin-like growth factor (IGF) axis [5,8,9]. IGF-I and IGF-II are important mediators of longitudinal growth, as shown by *in vitro* and *in vivo* experiments [10-12]. The IGFs are potent mitogenic and differentiation-promoting growth

factors [13]. They are produced by multiple tissues, and can act in both an endocrine and autocrine/paracine fashion. IGFs are bound to members of high-affinity IGF-binding proteins (IGFBPs), which modulate the IGF availability and bioactivity [14,15]. They act mainly as autocrine and/or paracrine factors at or close to their sites of synthesis and have different affinities for the IGFs [16]. Growth plate chondrocytes produce both IGFs and IGFBPs, which are presumed to be important for chondrocyte regulation and hence for longitudinal growth [5,17-19]. Both IGFs and several IGFBPs are regulated by GCs [20], both *in vitro*, *e.g.* in fibroblasts [21] and in osteoblasts [22], as well as *in vivo*, *i.e.* determined in serum [3,5,23,24] and in liver and lung tissue [5]. However, scarce data are available with respect to the regulation of IGFBPs by GCs on growth plate chondrocytes. We have reported that in rabbit costal chondrocytes, IGFBP-5 expression is downregulated and IGFBP-3 expression is induced by dexamethasone (DXM) [25]. In rat growth plate chondrocytes, GCs impair growth hormone (GH)-induced stimulation of local secretion and paracrine action of IGF-I, which would contribute to GC-induced growth retardation [8]. However, in patients treated with GCs, serum IGF-I levels are usually not altered, while its bioactivity is [26], indicating that the IGFBPs might be the IGF axis components that are involved in GC-induced growth retardation [5]. Since locally produced IGFs are suggested to have an autocrine/paracrine action on growth and GCs act locally to inhibit longitudinal growth, serum levels of IGF axis components provide little insight into the mechanisms by which GCs induce growth retardation [24].

The aim of this study was to investigate whether GC-induced growth retardation indeed is related to changes in expression of components of the IGF axis (both IGFs, the IGFBPs and the IGF receptors) in *in vitro* cultures of chondrocytes isolated from the neonatal porcine growth plate. The obtained data suggest a role for specific components of the IGF axis in the regulation of chondrocyte growth by GCs at a local level.

MATERIALS AND METHODS

Materials

Restriction enzymes, modifying enzymes, Tripure isolation reagent and Agarose Gel DNA extraction kit were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Nylon membranes (Hybond-N^+), RediPrime Random Primer labeling mixture, [α-^{32}P]dCTP (10 mCi/ml), [^{3}H]methyl-thymidine (1 mCi/ml), the Hyperfilm ECL film and the horseradisch peroxidase-conjugated secondary antibody were obtained from Amersham Pharmacia Biotech. Collagenase (C-9891) and protease (P-6911) enzymes were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin, streptomycin, fetal calf serum (FCS) and SuperScript II Reverse Transcriptase were obtained from Life Technologies. The TOPO TA Cloning kit was obtained from Invitrogen. Cell strainers were purchased from Beckton Dickingson. Centricon-10 ultrafiltration membranes and PVDF (Immobilon-P) were from Millipore Corp. BioMax MR X-ray films were obtained from Kodak. Supersignal chemiluminescent substrate was purchased from Pierce. Org34116 (a GC receptor (GR) antagonist) was provided by Organon (Oss, The Netherlands). Dexamethasone disodium phosphate (DXM) from Merck Sharp & Dohme was from our hospital pharmacy. Recombinant human IGF-I was kindly provided by Eli Lilly & Co. The human IGFBP-2 antibody (rabbit antibody directed against a peptide sequence from bovine IGFBP-2), the human IGFBP-5 antibody (rabbit antibody directed against a peptide sequence from human IGFBP-5) and the recombinant human IGFBP-2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Recombinant human IGFBP-5 was obtained from GroPep (Adelaide, Australia). Human IGFBP-1 to -6 cDNAs were kindly provided by Dr. S. Shimasaki (La Jolla, CA, USA) [27]. GAPDH cDNA was a gift from Dr. H. van Teeffelen (Utrecht, The Netherlands) [28]. Rat type I [29] and mouse type II IGF receptor cDNAs [30] were a gift from Dr. J.W. van Neck (Rotterdam, The Netherlands). Human IGF-I [31] and -II [32] cDNAs were obtained from Dr. M. Jansen (Utrecht, The Netherlands).

Cell culture

Epiphyseal growth plate chondrocytes were isolated from the distal and proximal tibial growth plate of neonatal piglets by splitting the growth plate transversally and dissecting the growth plate free under sterile conditions.

The growth plates were minced with a scalpel and digested with 1 mg/ml protease for 30 min at 37ºC with constant shaking in DMEM containing 4.5 g/l glucose, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, followed by digestion with 0.5 mg/ml collagenase for 6 h at 37ºC in the same medium supplemented with 10% FCS. Cells were filtered through a sterile 70 µm nylon cell strainer, centrifuged, counted and seeded in 75 cm^2 tissue culture flasks. The cells were grown as a monolayer culture at 37ºC in a 5% CO_2 and humid atmosphere. The chondrocyte phenotype of the cells was verified by Alcian blue staining for sulfated proteoglycans [33], expression of type II collagen [34] and cartilage-derived retinoic acid-sensitive protein (CD-RAP) [35] by Northern blot analysis [36].

In general, second and third passage cells were used at subconfluency. Cells were cultured in 10% FCS-supplemented medium. The amount of GCs contributed by 10% FCS was measured using a chemoluminescent immunoassay on the Nichols Advantage System (San Juan Capistrano, CA, USA) and was less than 1 nM. This concentration is negligible compared with the doses applied in our experiments. Proliferation of cells cultured in dextran-coated charcoal-stripped serum was only 20% of proliferation of cells cultured in 10% FCS (data not shown). Therefore, no experiments in dextran-coated charcoal-stripped serum were performed. Proliferation of cells in serum-free medium was only 20% of cells incubated with 10 nM IGF-I. Therefore, no experiments were performed in serum-free medium; instead, 10 nM IGF-I was added to the serum-free medium. In experiments with the GR antagonist (Org34116), cells were preincubated for 1 h with 10 µM of the GR antagonist or an equivalent volume of the solvent (ethanol) and subsequently treated with DXM (still in the presence of the GR antagonist).

Cell viability at the end of the incubations with DXM was determined by the trypan blue exclusion technique.

[^{3}H]thymidine incorporation

Cells were grown on 24-well plates and incubated with increasing concentrations of DXM for 24 h, with or without the GR antagonist, as described above and each experiment was conducted in triplicate, both in serum-free conditions supplemented with 10 nM IGF-I/ 0.005% BSA and in 10% FCS conditions. The rate of chondrocyte proliferation was assessed by incubating the cells in 0.5 µCi/ml of [^{3}H]-thymidine for the final 4 h of incubation. Cells were subsequently washed with PBS to remove unincorporated isotope and the DNA and protein were precipitated with three incubations of 1 ml 10% (w/v) ice-cold trichloroacetic acid for 15 min at 4ºC and solubilized by incubation at room temperature with 0.4 M NaOH for 1 h. Isotope incorporation was measured by liquid scintillation counting.

Western blotting

Cells were first preincubated with serum-free medium for 2 h, after which medium was changed for medium supplemented with 10 nM IGF-I and 0.005% BSA. Subsequently, cells were treated with increasing concentrations of DXM. Chondrocyte media were collected after 48 h (to assure enough accumulation of protein to be measured) and centrifuged for 10 min. at 3000 *g* (4°C) and stored at -80°C. The chondrocyte-conditioned medium (1 ml) was concentrated 10-fold on Centricon-10 ultrafiltration membranes and loaded on a 12% gel for non-reducing SDS-PAGE. IGFBPs were visualized by electrotransfer to PVDF membranes followed by incubation with ^{125}I-IGF-II (ligand blotting) [37]. Molecular masses were calculated using BioRad (Hercules, CA, USA) broad range markers as standard. Bands were quantified by densitometry using a GS-363 Molecular Imager and Molecular Analyst software program, version 1.5 (Biorad).

For immunobloting, 10 ml conditioned medium from 75 cm^2 tissue culture flasks was concentrated 10-fold on Centricon-10 ultrafilration membranes. IGFBP-2 was detected using a bovine IGFBP-2 antibody at 1/2500 dilution, followed by a horseradish peroxidase-conjugated secondary antibody at 1/7500 dilution. IGFBP-5 was detected using a human IGFBP-5 antibody at 1/2000 dilution, followed by a horseradish peroxidase-conjugated secondary antibody at 1/7500 dilution. The bands were visualized using the SuperSignal chemiluminescent substrate and Hyperfilm ECL film.

Northern blot analysis

RNA extraction

Cells (3 x 10^6) were cultured in 75 cm^2 tissue culture flasks and treated with DXM for 24 h. Cells were directly lysed with Tripure solution reagent and total RNA was extracted according to the procedures of the manufacturer, based on the single-step acid guanidinium-thiocyanate method [38].

Probes

Twenty nanograms of gel-purified inserts of plasmids containing human IGFBP-1 to -6, human IGF-I and -II, rat type I IGF receptor, mouse type II IGF receptor and rat GAPDH cDNA, were radiolabeled with 50 µCi [α-^{32}P]dCTP, using random primed DNA labeling as described by the manufacturer.

Northern blot hybridization

Twenty micrograms of total RNA was separated by electrophoresis in a 1% (w/v) agarose/2.2 M formaldehyde gel in 1x 3-(morpholino) propanesulphonic acid buffer, transferred to a 0.2 μm nylon membrane and crosslinked to the membrane by UV radiation. The membranes were prehybridized for 2 h at 60ºC in a solution containing 0.1% SDS, 3xSSC, 5xDenhardt's solution, 10% dextran-sulfate and 50 μg/ml denaturated salmon sperm DNA. Hybridization was performed at 60ºC overnight in the same solution, containing the [α-^{32}P]dCTP-labeled probe. Following hybridization, the membranes were washed to a stringency of 0.2xSSC, 0.1% (w/v) SDS at 60ºC. The hybridization signals were analyzed by densitometry using the GS-363 Molecular Imager and the Molecular Analyst software program (Biorad) and the relative abundance of the different mRNAs was subsequently normalized to the GAPDH values. The signals were also visualized by autoradiography on BioMax MR X-ray films.

GR RT-PCR

Total RNA was isolated as described above. First strand cDNA synthesis of 3.5 μg total RNA was performed using SuperScript II reverse transcriptase, as described by the manufacturer. After cDNA synthesis, the final volume was diluted to 100 μl. PCR amplification of the GR and GAPDH was performed using 5 μl template and 10 pmol forward and reverse primers. The following primer pair was used for the GR: forward primer, 5'-GTGAGTACCTCTGGAGGACA-3'; reverse primer, 5'-CTTTGCCCATTTCACTGC-3', which were expected to yield a 761 bp product. Following an initial denaturation step of 5 min 94ºC, amplification consisted of 34 cycles of 30 s at 92ºC, 45 s at 50ºC, 60 s at 72ºC, followed by a final extension step of 10 min at 72ºC. The following primer pair was used for GAPDH: forward primer, 5'-CTCAAGATTGTCAGCAATGC -3'; reverse primer, 5'-TTGCCCACAGCCTTGGCA-3', which were expected to yield a 226 bp product. Following an initial denaturation step of 5 min 94ºC, amplification consisted of 31 cycles of 30 s at 92ºC, 30 s at 53ºC, 60 s at 72ºC, followed by a final extension step of 10 min at 72ºC. PCR products were analyzed on a 1% agarose gel. Initial experiments established that the PCR reactions were in the log phase, allowing semi-quantitative determination of the mRNA levels. Densities of the bands were measured using digitized images coupled to the Molecular Analyst software and normalized for GAPDH expression.

Statistical Analysis

Data are expressed as means ± SEM. Statistical differences between two groups were determined by Student's *t*-test. Statistical differences between multiple treatments were determined by one-way ANOVA using InStat version 3.00 (GraphPad Software, Inc., San Diego, CA, USA). The Dunnett post-hoc test was used to determine significance between the control and the treatment groups. Significance of the difference between the various treatments with or without the GR antagonist in the [^{3}H]thymidine incorporation were determined using the Bonferroni post-hoc test. A *P* value of less than 0.05 was considered statistically significant. Growth plate cultures of at least three different animals were used per analyzed mRNA (both for the Northern Blot analysis and for the semi-quantitative RT-PCR) and for the [^{3}H]thymidine incorporation.

RESULTS

Characterization of secreted IGFBPs and regulation by DXM

The production of IGFBPs by the primary porcine growth plate chondrocytes was determined using Western ligand blot analysis. Under serum-free incubations, two IGFBPs of 34 and 24 kDa, were detected (Fig. 1A). When the medium was supplemented with 10 nM IGF-I the expression of a 31/32 kDa doublet was induced and the expression of the 24 kDa protein was increased 2.9-fold (Fig. 1A). At 100 nM DXM, the intensity of the 34 kDa IGFBP, in both the absence and presence of IGF-I, was decreased to respectively 35% and 50% of control levels. The 24 kDa band and the 31/32 kDa doublet were not influenced by DXM treatment in neither of the two incubations (Fig. 1A).

Immunoblotting identified the 34 kDa band as IGFBP-2 (Fig. 1B) and the 31/32 kDa doublet as IGFBP-5 (Fig. 1B). The band of 24 kDa probably represents IGFBP-4 [25]. Therefore, the DXM-sensitive protein was identified as IGFBP-2 and the major IGF-I-inducible IGFBP produced by growth plate chondrocytes was identified as IGFBP-5. IGFBP-4 was increased by IGF-I, but not affected by the DXM treatment.

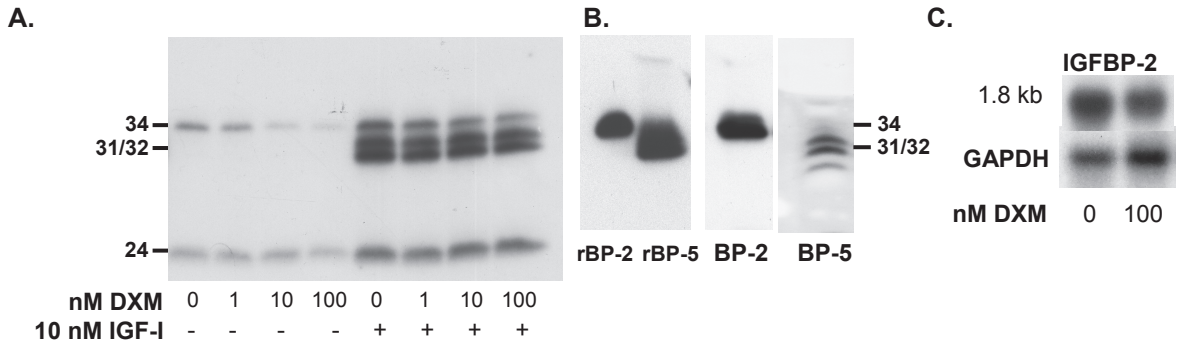

Figure 1 Characterization of secreted insulin-like growth factor-binding proteins (IGFBPs) and regulation by dexamethasone (DXM). (**A**) Growth plate chondrocytes were treated with increasing concentrations of DXM in serum-free medium in the absence (**-**) or presence (**+**) of 10 nM IGF-I. Conditioned media were collected, concentrated and IGFBPs were detected by Western ligand blot analysis, using ^{125}I-IGF-II as described in *Materials and Methods*. Numbers on the left and right are molecular masses (in kDa) of the detected IGFBPs. (**B**) Identification of the major IGFBP species produced by the porcine growth plate chondrocytes. Recombinant human IGFBP-2 (rBP-2) and IGFBP-5 (rBP-5)were used as positive controls, as indicated, and detected using Western ligand blot analysis. IGFBPs in the conditioned media of chondrocytes were detected using immunoblotting using an anti-IGFBP-2 (BP-2) and anti-IGFBP-5 (BP-5) antibody, as indicated. (**C**)**.** Expression of IGFBP-2 mRNA in chondrocytes in IGF-I-supplemented serum-free medium in the absence and presence of DXM (100 nM). Total RNA was isolated and subjected to Northern blot analysis (as described in *Materials and Methods)*. Subsequently, hybridization with a GAPDH probe was performed, which served as an internal control.

Northern blot analysis confirmed the expression of IGFBP-2, -4 and -5 at the mRNA level, when cultured in IGF-I-supplemented medium. In agreement with the protein levels, mRNA levels of IGFBP-2 (1.8 kb transcript) were also decreased by DXM treatment to 69% of control values (Fig. 1C). IGFBP-4 and -5 mRNA levels were not affected by the DXM treatment (data not shown), in concert with the protein levels.

DXM-induced growth retardation

To determine the effect of DXM on chondrocyte proliferation, [^{3}H]thymidine incorporation into DNA was measured. The [^{3}H]thymidine incorporation decreased significantly to 66% ± 3% of the control values when the chondrocytes were cultured in IGF-I-supplemented medium and subsequently treated with 100 nM DXM (Fig. 2). However, when growth plate chondrocytes were cultured in the presence of 10% FCS, basal proliferation (without any further treatment) increased 3.5-fold when compared with chondrocytes cultured in IGF-I-supplemented medium. Subsequent treatment with 100 nM DXM resulted in a 2.0 ± 0.1-fold reduction in proliferation (Fig. 2). Culturing in 10% FCS, therefore, resulted in higher cell proliferation and a larger decrease in proliferation when treated with DXM.

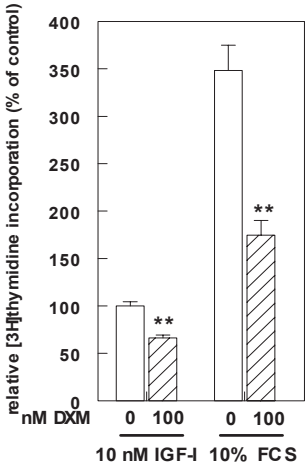

Figure 2 Modulation of [^{3}H]thymidine incorporation into growth plate chondrocytes by DXM. Epiphyseal growth plate chondrocytes of neonatal piglets were cultured in 10% FCS-supplemented medium. Treatment with 100 nM DXM was for 24 h in 10% FCS or in serum-free medium in the presence of 10 nM IGF-I, as indicated. For the final 4 h, [^{3}H]thymidine was added and the incorporation assessed as described in *Materials and Methods*. For the DXM-treated chondrocytes (bars with diagonal lines), means ± SEM are expressed relative to incorporation levels of untreated chondrocytes (open bars), ** P<0.01. The Figure shows the results of a typical experiment performed in triplicate. The experiment was performed another three times with different chondrocyte cultures, derived from three different piglets, each giving similar results.

Further experiments were conducted in the presence of 10% FCS, as this was shown to be the optimal condition to study GC-induced growth retardation. A dose-response study showed that a significant decrease in the [^{3}H]thymidine incorporation was observed at 50 nM and higher concentrations of DXM (Fig. 3). The [^{3}H]thymidine incorporation decreased to 37% ± 4% of the control values at 100 nM DXM and remained at 40% - 60% of control values at higher doses. The trypan blue exclusion technique showed that the viability of the cells was the same at the different concentrations used, indicating that the decrease in [^{3}H]thymidine incorporation was due to a decrease in proliferation.

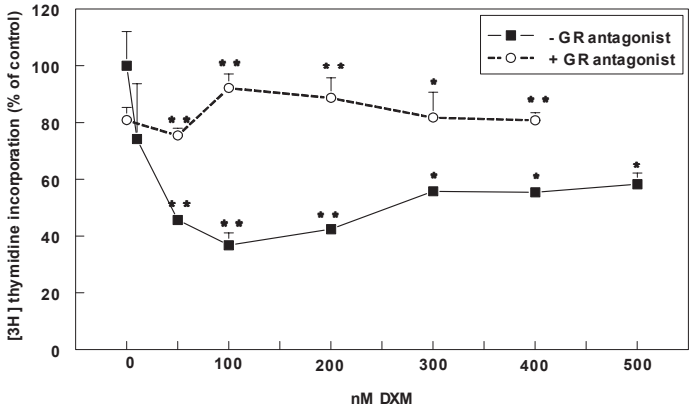

Figure 3 Modulation of [^{3}H]thymidine incorporation into growth plate chondrocytes cultured in 10% FCS-supplemented medium and subsequent treatment with increasing concentrations of DXM (- GR antagonist) for 24 h and with DXM in combination with the glucocorticoid receptor (GR) antagonist (+ GR antagonist). [^{3}H]thymidine incorporation was assessed as described in Fig. 2. For the DXM-treated chondrocytes, means ± SEM are expressed relative to incorporation levels of untreated chondrocytes (0 nM DXM), * P<0.05, ** P<0.01. For the co-incubation with the GR antagonist, means ± SEM are expressed relative to incorporation levels of the corresponding DXM-treated chondrocytes treated with the same dose of DXM, * P<0.05, ** P<0.01. The Figure shows the results of a typical experiment performed in triplicate. The experiment was performed another four times with different chondrocyte cultures, derived from four different piglets, each giving similar results.

Treatment with DXM in combination with the GR antagonist Org34116 abolished the DXM-induced growth inhibition, as shown in Fig. 3. The GR antagonist on its own resulted in a small, but not significant, decrease in proliferation as compared to control values.

To exclude the possibility that the GR was down-regulated by the treatment with DXM, we also performed RT-PCR of the GR on RNA extracted from our primary chondrocytes and found the expected 761 bp product, indicating that these chondrocytes expressed the GR (Fig.4). Increasing concentrations of DXM did not affect the mRNA levels of the GR as shown by semi-quantitative RT-PCR analysis. Although a decrease in mRNA levels at 500 nM DXM (74% ± 14% of control values; n=5) was visible, this was not statistically significant (Fig. 4).

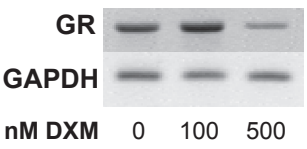

Figure 4 Expression of the GR. Cells were treated with the indicated concentrations DXM for 24 h and total RNA was isolated as described in *Materials and Methods* and analyzed using semi-quantitative RT-PCR analysis. GAPDH served as an internal control. The Figure shows the results of a typical experiment.

mRNA levels of IGF axis components and regulation by DXM

Northern blot analysis of primary porcine growth plate chondrocytes cultured in 10% FCS showed expression of the same IGFBP species as detected at the protein level (Fig. 1). The major transcripts detected (Fig. 5A) were: IGFBP-2 (1.8 kb), IGFBP-4 (2.0 kb) and IGFBP-5 (7.0 kb). IGFBP-6 mRNA was detected at low levels (2.0 kb). No IGFBP-1 and only very weak IGFBP-3 expression (5.0 kb, in one out of five conducted experiments) was found (not shown).

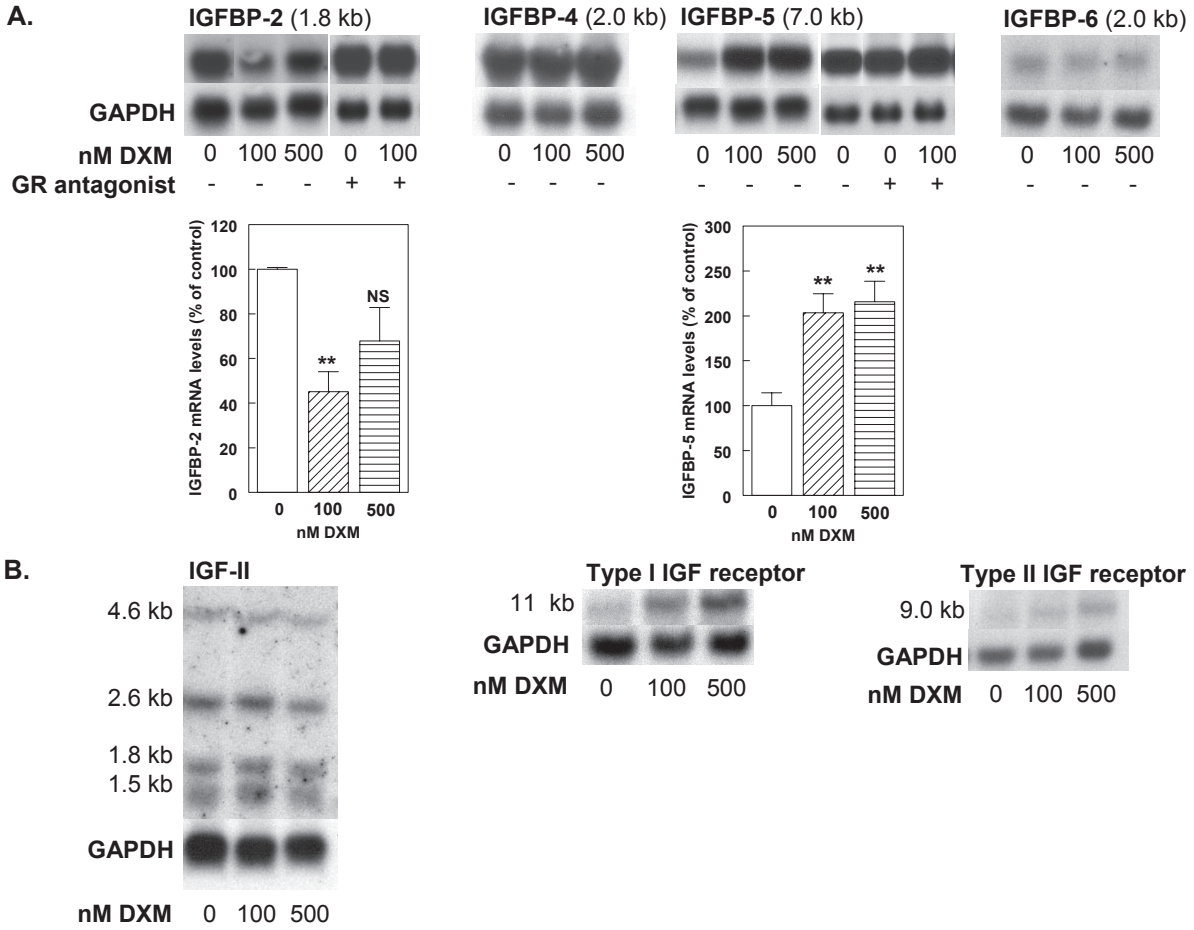

Figure 5 Expression of IGF axis components in porcine growth plate chondrocytes cultured in 10% FCS-supplemented medium and effects of DXM treatment. Growth plate chondrocytes were treated with DXM (100 and 500 nM) (as indicated) for 24 h and analyzed as described in Fig. 1C. (**A**) mRNAs of IGFB-2, -4, -5 and -6 (as indicated). The data are expressed relative to mRNA levels of control values (open bars). The bars with diagonal lines represent mRNA levels of chondrocytes treated with 100 nM DXM; the bars with horizontal lines, treatment with 500 nM DXM. mRNA levels of IGF axis components affected by DXM treatment were also co-incubated with the GR antagonist (Org34116), as indicated (**+**). (**B**) mRNA levels of IGF-II and the type I and II IGF receptor (as indicated). The relative abundance of the different mRNAs was quantified by densitometry (as described in *Materials and Methods*) and normalized to the GAPDH values. The data represent the means ± SEM of at least four different animals per analyzed transcript, the means ± SEM are expressed relative to expression levels of untreated chondrocytes, ** $P<0.01$, NS, non significant. Representative Northern blot data are shown.

We determined the effects of pharmacological doses of DXM on the expression of IGF axis components in growth plate chondrocytes cultured in 10% FCS, using Northern blot analysis. The IGFBP-2 mRNA levels significantly decreased to 45% ± 9% of the control values, when treated with 100 nM DXM (Fig. 5A). The 7.0 kb transcript of IGFBP-5 was significantly increased by 2.0 ± 0.2-fold at 100 nM DXM (Fig. 5A). The mRNA levels of IGFBP-4 and -6 were not affected by the DXM treatment (Fig. 5A). The levels of IGFBP-3 were too low to be quantified.

Besides the IGFBPs, we also studied the presence and effects of DXM treatment on the remaining IGF axis components. The growth plate chondrocytes of neonatal piglets expressed very low levels of a 5.0 kb IGF-I transcript (data not shown). Northern blots hybridized with a ^{32}P-labeled human IGF-II cDNA probe revealed four transcripts of IGF-II of 4.6, 2.6, 1.8 and 1.5 kb respectively (Fig. 5B). Low levels of a transcript of 11 kb of the type I IGF receptor were detected (Fig. 5B), and low levels of a transcript of 9.0 kb of the type II IGF receptor (mannose-6-phosphate receptor) (Fig. 5B).

DXM treatment did not affect the expression levels of the four detected transcripts of IGF-II (Fig. 5B). Also, the mRNA levels of the type II IGF receptor were not affected (Fig. 5B). The levels of IGF-I were too low to be quantified. In contrast, type I IGF receptor expression levels increased when treated with 100 nM DXM (approximately 2- to 3-fold) (Fig. 5B); due to the low expression level of the untreated chondrocytes, no precise quantification of the increase was possible.

Treatment with DXM in combination with the GR antagonist (Org34116) abolished the effects of DXM on the mRNA levels of IGFBP-2 and -5, as shown in Fig. 5A. Similarly, the GR antagonist prevented the DXM-induced increase of mRNA levels of the type I IGF receptor, although quantification was not possible due to the low expression levels as described in Fig. 5B (data not shown).

DISCUSSION

This is the first study to describe the possible involvement of all the IGF axis components, the IGFs, both IGF receptors and the IGFBPs, in GC-induced growth retardation at the local level of the growth plate chondrocytes. Epiphyseal tibial growth plate chondrocytes of neonatal piglets were used as a model system for postnatal growth. The chondrocytes were treated with high (pharmacological) doses of DXM to induce growth retardation and levels of the IGF axis components were determined at the protein and mRNA level.

First, we characterized the production and regulation of the IGFBPs by the primary porcine growth plate chondrocytes, as the IGFBPs could be the IGF axis components that are involved in GC-induced growth retardation [5,39]. We have previously reported that IGFBP-5 is the major IGF-I-inducible IGFBP produced by rabbit costal chondrocytes [25]. The present result in porcine epiphyseal growth plate chondrocytes confirmed this finding. In contrast to the costal chondrocytes, the growth plate chondrocytes expressed IGFBP-2, and showed upregulation of IGFBP-4 by IGF-I. Further differences are noted with respect to the regulation by DXM. While DXM inhibited the expression of IGFBP-5 in the costal chondrocytes [25], this binding protein was unaffected by DXM at the protein level. In addition, we reported an induction of IGFBP-3 in the costal chondrocytes, while IGFBP-3 remained undetected in the growth plate chondrocytes in the presence of DXM. As costal chondrocytes of the neonatal piglets showed similar results to the porcine tibial growth plate chondrocytes (data not shown), we suggest that species and/or age differences can account for these differences and not the difference in the origin of the tissue of the chondrocytes. The decrease in IGFBP-2 protein levels was paralleled by a decrease of IGFBP-2 mRNA levels, suggesting a regulation at the transcriptional level. GCs have indeed been shown to affect IGF axis components primarily at the level of gene expression [5,20,22,25].

GC treatment of our chondrocytes resulted in inhibition of proliferation without an increase in cell death, in accordance with other *in vitro* studies, *e.g.* in osteoblasts [22] and in tibial growth plate chondrocytes [8,40]. It has been previously reported that the decreased proliferation

of chondrocytes treated with GCs is due to an increase in cell doubling times with a reduction in the number of S phase cells; in addition, no signs of increasing cell death or increase in morphological signs of apoptosis were shown [8,40].

We found that basal proliferation in IGF-I-supplemented medium was much lower than proliferation in serum-supplemented medium. Subsequently, inhibition of proliferation due to GC treatment resulted in a larger decrease in proliferation. Therefore, further experiments to study GC-induced growth retardation were conducted in these conditions. In addition, treatment of the chondrocytes with a GR antagonist prevented the DXM-induced growth retardation, which provides evidence for both the specific effects of DXM on growth and the presence of an active GR.

As a consequence of the presence of serum in the experimental conditions, measurements of IGFBP protein production by the porcine tibial growth plate chondrocytes were unreliable. Further study of the effects of DXM on the IGF axis components (including also both IGFs and IGF receptors) on the growth plate chondrocytes was therefore only performed at the mRNA level. Northern blot analysis showed that not all components of the IGF axis were expressed in neonatal porcine growth plate chondrocytes. IGFBP-1 was not detectable, as described previously [41], and only very low levels of IGFBP-3 and low levels of IGFBP-6 were shown. IGFBP-2, -4 and -5 were the predominant IGFBPs expressed by the growth plate chondrocytes, which was in accordance with the IGFBPs detected at the protein level in IGF-I-supplemented serum-free culture conditions. This is in accordance with data from others, who also showed expression of IGFBP-2 to -6 in growth plate chondrocytes [18,41]. In accordance with others, we found IGF-II to be the most prominent IGF expressed in the growth plate [42]. Only very low levels of IGF-I were detected. Expression of the type I IGF receptor was also previously observed in rat growth plate chondrocytes [43]. This is the first study to describe expression of the type II IGF receptor in growth plate chondrocytes.

Pharmacological doses of DXM did not influence the expression of IGFBP-3, -4, and -6, of both IGFs and of the type II IGF receptor. This contrasts with the observed effects of GCs on other cell types as downregulation by GCs of the type II IGF receptor [44] and IGFBP-4 [22] in osteoblasts. IGFBP-6 was shown both to be stimulated in osteoblasts [45] and to be not affected by GCs in osteoblasts [22] and in fibroblasts [21]. IGFBP-3 expression was stimulated by GCs in costal chondrocytes [25]. Obviously, the regulation of expression of IGF axis components is cell type specific, and also depends on developmental stage and species used [20].

DXM treatment did influence the expression levels of the type I IGF receptor, IGFBP-5 and IGFBP-2. We showed that pharmacological doses of DXM increased the type I IGF receptor mRNA levels in the growth plate chondrocytes. This increase of type I IGF receptor mRNA by DXM has previously been shown *in vivo* [5] and might be a response to counteract the effect of the GC-induced growth retardation. However, this response is insufficient to compensate for the growth inhibition observed.

DXM treatment increased IGFBP-5 mRNA levels in the presence of 10% FCS, which has not been described previously. In contrast, reduction of IGFBP-5 levels was reported in costal chondrocytes [25], osteoblasts [22,46] and in fibroblasts [21], a discrepancy which is probably due to a difference in cell type and species used as discussed above [20]. In the rabbit costal chondrocytes we described before [25], a decrease in IGFBP-5 was shown due to DXM treatment, whereas an increase in IGFBP-3 was observed. In contrast, our porcine growth plate chondrocytes expressed almost no IGFBP-3, but expressed IGFBP-5 at a high level. It has been suggested that IGFBP-3 and IGFBP-5 could have similar functions [47-49]. An increase in IGFBP-5 due to DXM treatment in piglet growth plate chondrocytes could therefore

resemble the increase in IGFBP-3 due to DXM treatment in rabbit costal chondrocytes [25]. Furthermore, IGFBP-5 inhibits IGF-II-dependent DNA synthesis in growth plate chondrocytes [41]. An increase in IGFBP-5 thus can inhibit proliferation of growth plate chondrocytes by inhibiting IGF-II activity, which is the predominant IGF in our growth plate chondrocytes. IGFBP-5 could therefore account in part for the GC-induced growth retardation observed in our growth plate chondrocytes.

We observed a decrease in IGFBP-2 due to DXM treatment, both in IGF-I-supplemented experimental conditions as well as in the presence of 10% FCS. This observation is in accordance with data on osteoblasts [50] and *in vivo* experiments [51]. Treatment of chondrocytes with GH increased IGFBP-2 levels [52]. Together with our data, this implies that IGFBP-2 levels are correlated with growth in chondrocytes. IGFBP-2 can inhibit, but also stimulate IGF activity and it has a preference for binding IGF-II [16], the predominant IGF in the growth plate. IGFBP-2 was also suggested to facilitate targeting of the IGFs, in particular IGF-II prohormone, to skeletal tissues, resulting in stimulation of proliferation [53]. A decrease in IGFBP-2 could result in a decrease in IGF targeted to the chondrocytes, resulting in a decreased proliferation. Downregulation of IGFBP-2 expression in chondrocytes might therefore contribute to the inhibition of growth. This strengthens our conclusion that IGFBP-2, the only IGFBP which is affected by DXM in different culture conditions, could play a role in the regulation of growth in the growth plate, the mechanisms of which needs to be further elucidated.

In conclusion, all the IGF axis components (besides IGFBP-1) are present in neonatal porcine chondrocytes and some specific components, such as the type I IGF receptor, IGFBP-2 and -5, are regulated by GCs at the local level. These specific changes of IGF axis components by GCs indicate an impaired function of the IGF axis and might contribute to the GC-induced growth retardation

ACKNOWLEDGEMENTS

We are grateful to Ms. C.M.P.C.D. Peeters and Dr. F. Groenendaal for providing the tibiae of the neonatal piglets. Thanks are also due to Mr. C.M. Hoogerbrugge for technical assistance.

REFERENCES

1. Stevens DA, Williams GR 1999 Hormone regulation of chondrocyte differentiation and endochondral bone formation. *Mol Cell Endocrinol,* **151:** 195-204.
2. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
3. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
4. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
5. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
6. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
7. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
8. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.

9. Klaus G, Weber L, Rodriguez J, Fernandez P, Klein T, Grulich HJ, Hugel U, Ritz E, Mehls P 1998 Interaction of IGF-I and 1 alpha, 25(OH)2D3 on receptor expression and growth stimulation in rat growth plate chondrocytes. *Kidney Int,* **53:** 1152-1161.
10. Van Buul-Offers SC, Hoogerbrugge CM, Branger J, Feijlbrief M, Van den Brande JL 1988 Growth-stimulating effects of somatomedin-/insulin-like peptides in Snell dwarf mice. *Horm Res,* **29:** 229-236.
11. D'Ercole AJ 1996 Insulin-like growth factors and their receptors in growth. *Endocrinol Metab Clin North Am,* **25:** 573-590.
12. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC 1998 Growth hormone and bone. *Endocr Rev,* **19:** 55-79.
13. Cohick WS, Clemmons DR 1993 The insulin-like growth factors. *Annu Rev Physiol,* **55:** 131-153.
14. Clemmons DR 1997 Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev,* **8:** 45-62.
15. Rajaram S, Baylink DJ, Mohan S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev,* **18:** 801-831.
16. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev,* **16:** 3-34.
17. Bhaumick B 1993 Insulin-like growth factor (IGF) binding proteins and insulin-like growth factor secretion by cultured chondrocyte cells: identification, characterization and ontogeny during cell differentiation. *Regul Pept,* **48:** 113-122.
18. Olney RC, Mougey EB 1999 Expression of the components of the insulin-like growth factor axis across the growth plate. *Mol Cell Endocrinol,* **156:** 67-71.
19. Sunic D, Belford DA, McNeil JD, Wiebkin OW 1995 Insulin-like growth factor binding proteins (IGF-BPs) in bovine articular and ovine growth-plate chondrocyte cultures: their regulation by IGFs and modulation of proteoglycan synthesis. *Biochim Biophys Acta,* **1245:** 43-48.
20. Dell G, Ward A, Shokrai A, Madej A, Engstrom W 1999 Regulation of the IGF system by glucocorticoids. *Zool Science,* **16:** 377-385.
21. Conover CA, Clarkson JT, Bale LK 1995 Effect of glucocorticoid on insulin-like growth factor (IGF) regulation of IGF-binding protein expression in fibroblasts. *Endocrinology,* **136:** 1403-1410.
22. Okazaki R, Riggs BL, Conover CA 1994 Glucocorticoid regulation of insulin-like growth factor-binding protein expression in normal human osteoblast-like cells. *Endocrinology,* **134:** 126-132.
23. Miell JP, Taylor AM, Jones J, Holly JMP, Gaillard RC, Pralong FP, Ross RJM, Blum WF 1993 The effects of dexamethasone treatment on immunoreactive and bioactive insulin-like growth factors (IGFs) and IGF-binding proteins in normal male volunteers. *J Endocrinol,* **136:** 525-533.
24. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.
25. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
26. Caufriez A, Copinschi G 1986 Somatomedins and steroids. *Horm Res,* **24:** 185-188.
27. Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K 1996 IGF-binding protein mRNAs in the human fetus: tissue and cellular distribution of developmental expression. *Horm Res,* **45:** 160-166.
28. Dufourny B, Alblas J, Van Teeffelen HAAM, Van Schaik FMA, van der Burg B, Steenbergh PH, Sussenbach JS 1997 Mitogenic signalling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phophatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J Biol Chem,* **272:** 31163-31171.
29. Werner H, Woloschak M, Adamo M, Shen-Orr Z, Roberts CT Jr, LeRoith D 1989 Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc Natl Acad Sci U S A,* **86:** 7451-7455.
30. Ludwig T, Ruther U, Metzger R, Copeland NG, Jenkins NA, Lobel P, Hoflack B 1992 Gene and pseudogene of the mouse cation-dependent mannose 6-phosphate receptor. *J Biol Chem,* **267:** 12211-12219.
31. Jansen M, Van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabbay KH, Nussbaum AL, Sussenbach JS, Van den Brande JL 1983 Sequence of cDNA encoding human insulin-like growth factor I precursor. *Nature,* **306:** 609-611.
32. Jansen M, Van Schaik FMA, Van Tol H, Van den Brande JL, Sussenbach JS 1985 Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. *FEBS Lett,* **179:** 243-246.
33. Leonard C, Fuld HM, Frenz DA, Downie SA, Massague J, Newman SA 1991 Role of transforming growth factor beta in chondrogenic pattern formation in the embryonic limb. *Dev Biol,* **145:** 99-109.
34. Cancedda R, Descalzi CF, Castagnola P 1995 Chondrocyte differentiation. *Int Rev Cytol,* **159:** 265-358.
35. Dietz UH, Sandell LJ 1996 Cloning of a retinoic acid-sensitive mRNA expressed in cartilage and during chondrogenesis. *J Biol Chem,* **271:** 3311-3316.

36. Koedam JA, Smink JJ, Van Buul-Offers SC 2002 Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. *Mol Cell Endocrinol,* **197:** 35-44.
37. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Bloemen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
38. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* **162:** 156-159.
39. Unterman TG, Phillips LS 1985 Glucocorticoid effect on somatomedins and somatomedin inhibitors. *J Clin Endocrinol Metab,* **61:** 618-626.
40. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.
41. de los Rios P, Hill DJ 1999 Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of the ovine fetus: possible functional implications. *Can J Physiol Pharmacol,* **77:** 235-249.
42. Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M 1993 Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology,* **132:** 1158-1167.
43. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
44. Rydziel S, Canalis E 1995 Cortisol represses IGF II receptor transcritpion in skeletal cell cultures. *Endocrinology,* **136:** 4254-4260.
45. Gabbitas B, Canalis E 1996 Cortisol enhances the transcription of insulin-like growth factor-binding protein-6 in cultured osteoblasts. *Endocrinology,* **137:** 1687-1692.
46. Gabbitas B, Pash JM, Delany AM, Canalis E 1996 Cortisol inhibits the synthesis of insulin-like growth factor-binding protein-5 in bone cell cultures by transcriptional mechanisms. *J Biol Chem,* **271:** 9033-9038.
47. Twigg SM, Baxter RC 1998 Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *J Biol Chem,* **273:** 6074-6079.
48. Schedlich LJ, Young TF, Firth SM, Baxter RC 1998 Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *J Biol Chem,* **273:** 18347-18352.
49. Booth BA, Boes M, Andress DL, Dake BL, Kiefer MC, Maack C, Linhardt RJ, Bar K, Caldwell EE, Weiler J, Bar RS 1995 IGFBP-3 and IGFBP-5 association with endothelial cells: role of C-terminal heparin binding domain. *Growth Reg,* **5:** 1-17.
50. Chen TL, Chang LY, Bates RL, Perlman AJ 1991 Dexamethasone and 1,25-dihydroxyvitamin D_3 modulation of insulin-like growth factor-binding proteins in rat osteoblast-like cell cultures. *Endocrinology,* **128:** 73-80.
51. Orlowski CC, Brown AL, Ooi GT, Yang YW, Tseng LY, Rechler MM 1990 Tissue, developmental, and metabolic regulation of messenger ribonucleic acid encoding a rat insulin-like growth factor-binding protein. *Endocrinology,* **126:** 644-652.
52. Borromeo V, Bramani S, Holder AT, Carter C, Secchi C, Beattie J 1996 Growth hormone stimulates the secretion of insulin-like growth factor binding protein-2 (IGFBP-2) by monolayer cultures of sheep costal growth plate chondrocytes. *Mol Cell Biochem,* **162:** 145-151.
53. Khosla S, Hassoun AAK, Baker BK, Liu F, Zein NN, Whyte MP, Reasner CA, Nippoldt TB, Tiegs RD, Hintz RL, Conover CA 1998 Insulin-like growth factor system abnormalities in hepatitis C-associated osteosclerosis. *J Clin Invest,* **101:** 2165-2173.

addendum

Chapter 7

Regulation of the porcine IGFBP-2 promoter by glucocorticoids

A PRELIMINARY STUDY

J.J. Smink, P.E. Holthuizen[†], J.A. Koedam, R. Berger[*] and S.C. van Buul-Offers

Department of Pediatric Endocrinology, [†]Laboratory of Physiological Chemistry and [*]Metabolic Diseases, University Medical Center Utrecht, Utrecht, The Netherlands

ABSTRACT

Glucocorticoid (GC)-induced growth retardation is suggested to involve impaired action of the components of the IGF system. In a previous study, we have shown that GC treatment of primary porcine growth plate chondrocytes resulted in inhibition of cell proliferation, which was accompanied by an increase of IGFBP-5 and type I IGF receptor mRNA levels and a decrease of IGFBP-2 mRNA levels.

We have studied whether the GC-induced decrease of IGFBP-2 mRNA levels was due to a decrease in transcriptional activity of the IGFBP-2 promoter, using transient transfections of the porcine IGFBP-2 promoter in porcine growth plate chondrocytes. These preliminary studies indicate that GCs significantly decrease the porcine IGFBP-2 promoter transcriptional activity of both the full-length promoter construct (-1397 bp) as well as of a 5' deletion construct (-874 bp), to respectively 73% ± 10% and 54% ± 15%. This decrease in transcriptional activity explains, at least in part, the GC-induced decrease of IGFBP-2 mRNA levels in porcine growth plate chondrocytes.

INTRODUCTION

Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive drugs, but they cause growth retardation as a side-effect in both children [1,2] and experimental animal models [3-6]. GCs act locally to inhibit growth, suggesting a mechanism intrinsic to the growth plate [7,8].

Impaired action of the IGF system is suggested to be involved in GC-induced growth retardation [3,9-11]. However, few studies exist which have studied the effects of GCs on the IGF system in the growth plate. In rat growth plate chondrocytes, GCs impaired GH-induced stimulation of IGF-I expression [9] and in rabbit costal chondrocytes, we have shown that GCs decreased IGFBP-5 and increased IGFBP-3 expression [12]. In contrast, GC-induced inhibition of proliferation of porcine growth plate chondrocytes was concomitant with increased IGFBP-5 and type I IGF receptor mRNA levels and decreased IGFBP-2 mRNA levels [13] (chapter 7 of this thesis). GC treatment of fetal ovine growth plate chondrocytes showed a similar decrease in IGFBP-2, but also showed a decrease in IGFBP-5 levels [14]. Despite the discrepancies between these *in vitro* studies, the latter two both showed a down-regulation of IGFBP-2 due to GC treatment, whereas GH treatment was shown to increase IGFBP-2 levels in chondrocytes [15]. This suggests a positive association between IGFBP-2 levels and growth in chondrocytes. In addition, IGFBP-2 is the only IGFBP detected in the postnatal growth plate *in vivo* [11] (chapters 3 and 4 of this thesis). Together with the *in vitro* studies, this might indicate that IGFBP-2 plays an important role in growth plate chondrocytes.

To further study a possible involvement of IGFBP-2 in GC-induced growth retardation, we have performed an preliminary study on the transcriptional regulation of the porcine IGFBP-2 promoter by GCs in porcine growth plate chondrocytes.

MATERIALS AND METHODS

Cell culture

Epiphyseal growth plate chondrocytes were isolated from the distal and proximal tibial growth plates of neonatal piglets as described previously [13,16]. The chondrocytes were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM), containing 4.5 g/l glucose, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycine and 10% fetal calf serum (FCS) (Life Technologies Ltd, Paisley, UK), at 37°C in a 5% CO_2 and humid atmosphere.

Transient transfections

For transfection experiments, the following plasmids were used. The porcine IGFBP-2 promoter region (-1397/+73; relative to the first codon) was fused to the promoter-less pGL2-enhancer vector (Promega Corp., Madison, WI, USA), containing the firefly luciferase reporter gene [17]. Similarly, a 5' deletion fragment of the porcine IGFBP-2 promoter (-874/+73) was cloned in the same vector [17] (kind gifts of Dr. F.A. Simmen, Gainesville, FL, USA [17]). A schematic representation of both porcine IGFBP-2 promoter constructs is shown in Fig. 1. The promoter-less pGL2-enhancer (gift of Dr. F.A. Simmen) and the pGL2-control-luc plasmid controlled by a SV40 promoter (Promega Corp., Madison, WI, USA), served as transfection controls. The pRSV-LacZ [18] was used to monitor transfection efficiency.

Second and third passage cells were used for the transient transfection experiments and were plated on 6-well plates. One day after plating, the chondrocytes (60% confluency) were transiently transfected using FuGENE6 (Roche Molecular Biochemicals, Mannheim, Germany), according to the procedures of the manufacturer. Briefly, the cells were grown in antibiotic-free DMEM containing 10% FCS and incubated for 6 h at 37°C with the FuGENE6 solution, which contained 7.5 µl FuGENE6, 2 µg of the porcine IGFBP-2 promoter construct and 0.5 µg pRSV-LacZ. Subsequently, the cells received fresh antibiotic-free medium containing 10% FCS, in the absence or presence of 100 nM dexamethasone (DXM) (Merck Sharp & Dohm, Haarlem, The Netherlands) for 24 h. Subsequently, the cells were lysed and luciferase and β-galactosidase assays were performed as described in [19]. Reagents used for both assays were obtained from Roche Molecular Biochemicals (Mannheim, Germany).

Three transfection experiments were performed, each with growth plate cultures of a different animal and performed in triplicate. The time response curve was performed in duplicate, using the growth plate culture of 1 animal.

Statistical analysis

Data are expressed as the means ± SEM. The effects of the DXM treatment were statistically tested using Student's t-test. A *P* value of less than 0.05 was considered statistically significant.

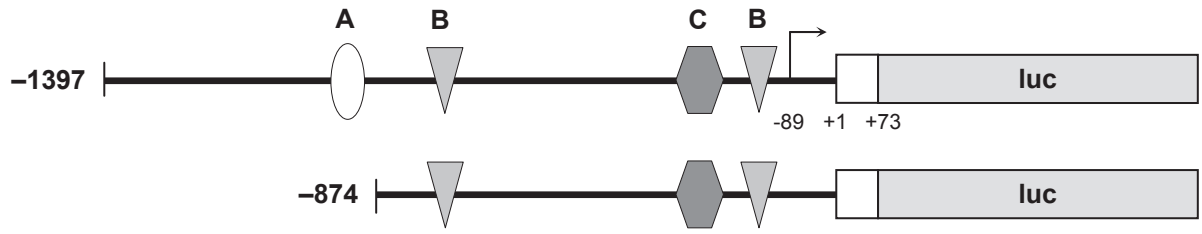

Figure 1 Schematic representation of the porcine IGFBP-2 promoter construct, containing the luciferase (luc) reporter gene. Both the full-length (-1397) and the 5' deletion (-874) constructs are shown. The arrow indicates the position of the transcription start site (-89, relative to the first codon), the translation start site position is indicated as +1. The luciferase gene is larger (1649 bp) than suggested in this figure. Putative binding sites for a number of transcription factors are indicated; site **A** is a binding site for AP-1 (-937), **B** for Sp1 (-147, -747) and **C** for NF-κB (-257).

RESULTS

We have previously shown that a 24 h treatment of primary porcine growth plate chondrocytes with 100 nM DXM [13], decreased the [^{3}H]thymidine incorporation of the chondrocytes to 37% ± 4% and using Northern blot analysis, we showed that the IGFBP-2 mRNA levels decreased to 45% ± 9% of control values (chapter 7 of this thesis). To determine whether this DXM-induced decrease of IGFBP-2 mRNA was caused by an

inhibition of the transcriptional activity of the IGFBP-2 promoter, we performed transient transfections in cultured porcine growth plate chondrocytes, using a construct containing the porcine IGFBP-2 promoter linked to the luciferase reporter gene.

We performed a time-response experiment from 2 to 24 h, to determine at which time-point the transcriptional activity of the porcine IGFBP-2 promoter was most affected by DXM (100 nM). We used the same concentration of DXM (100 nM) as previously used in the [^{3}H]thymidine and Northern blot experiments [13]. The transcriptional activity of both the full-length IGFBP-2 luciferase construct (-1397 bp) and the 5' deletion IGFBP-2 luciferase construct (-874 bp) was maximally inhibited after 24 h of DXM treatment (data not shown). Therefore, in subsequent experiments, cells were subjected to 24 h DXM treatment (100 nM) (data from one representative experiment are shown, Table 1). Three independent experiments showed that basal luciferase activities of both the full-length (-1397 bp) and the 5' deletion IGFBP-2 promoter construct (-874 bp) were similar. The region between nt -1397 and nt -874 seems therefore not important for basal IGFBP-2 promoter activity in these growth plate chondrocytes. DXM treatment (100 nM) for 24 h significantly decreased the transcriptional activity of the full-length IGFBP-2 construct (-1397 bp) to 73% ± 10% of control levels. DXM also decreased the transcriptional activity of the 5' deletion construct (-874 bp), to 54%± 15% of control levels (Table 1). These inhibitions do not significantly differ from each other, and were similar to those found in the time-response experiment.

Table 1 Transcriptional activity of the porcine IGFBP-2 promoter in primary porcine growth plate chondrocytes and the effect of DXM treatment.

promoter construct	DXM treatment (100 nM)	luciferase activity ± SEM	% ± % SEM
–1397 bp	-	40850 ± 1925	100 ± 5.8
	+	30000 ± 3401	73 ± 10.2 *
–874 bp	-	39668 ± 3865	100 ± 11.9
	+	21504 ± 4870	54 ± 15 *

The effect of DXM (100 nM; 24 h) on the transcriptional activity of the porcine IGFBP-2 promoter was examined. The luciferase activity corrected for the transfection efficiency is indicated. The representative data from one experiment conducted in triplicate are shown. Data are expressed as means ± SEM. *P<0.05 compared to untreated porcine chondrocytes.

DISCUSSION

We have previously shown that GCs decrease the proliferation of porcine growth plate chondrocytes, concomitant with decreased IGFBP-2 mRNA levels [13], which was also shown by others in fetal ovine growth plate chondrocytes [14].

In the present study, we showed that the GC-induced decrease of IGFBP-2 mRNA levels in porcine growth plate chondrocytes is, at least partially, caused by a decrease in the transcriptional activity of the porcine IGFBP-2 promoter by GCs. The full-length and the 5' deletion IGFBP-2 promoter construct showed similar basal luciferase activities, indicating that the region between nt -1397 and nt -874 of the IGFBP-2 promoter does not contain sequences which are important for IGFBP-2 gene transcription in growth plate chondrocytes, in serum-supplemented culture conditions. In contrast, in HepG2 liver cells, deletion of this same region resulted in a 2-fold decrease of basal luciferase activity, indicating that this region is important for IGFBP-2 gene regulation in liver cells [17]. In these HepG2 liver cells,

the region between nt -874 and nt -765 of the porcine IGFBP-2 promoter was shown to be essential for IGFBP-2 gene regulation. Using deletion and mutation analysis of the IGFBP-2 promoter, two AP-4 binding sites were identified in this region, which were required for the basal activation of the porcine IGFBP-2 promoter [17].

To determine whether GC-responsive elements were present in the region between nt -1397 and nt -874, we treated chondrocytes transfected with these constructs with DXM. Treatment with DXM resulted in a similar decrease in luciferase activity of both IGFBP-2 promoter constructs, indicating that the region between nt -1397 and nt -874 does not significantly contribute to the inhibition of the transcriptional activity of the IGFBP-2 promoter by GCs.

Activation of genes by GCs requires DNA consensus sequences, such as glucocorticoid responsive elements (GRE) [20], whereas inhibition of transcription by GCs does not involve GREs. Several other mechanisms have been proposed for the inhibition of gene transcription by GCs, such as the involvement of other DNA consensus sequences (the negative GRE, nGRE) or the interaction with other transcription factors such as AP-1 and NF-κB [21]. Although the presence of a nGRE in the IGFBP-2 promoter cannot be excluded, sequence analysis did not show the presence of a possible nGRE as proposed by others [22]. However, using a motif searching program (*http://motif.genome.ad.jp/*), putative DNA binding sites for AP-1 (nt -937) and NF-κB (nt -257) were identified, which could be involved in the inhibition of the transcriptional activity of IGFBP-2 by GCs. Although the 5' deletion construct of IGFBP-2 did no longer contain the proposed AP-1 binding site (Fig. 1), its transcriptional activity was similarly decreased by DXM as for the full-length construct. Possibly, NF-κB or other transcription factors (including possible non-identified AP-1 sites) might be involved in the decrease of IGFBP-2 in our chondrocytes.

In contrast, in lung tissue of the rat, GCs increased IGFBP-2 mRNA levels both *in vivo* [3], and *in vitro* at the transcriptional level [23]. The difference with our porcine growth plate chondrocytes could be caused by the cell-type and species specific regulation of components of the IGF system by GCs [24].

Several studies have shown that IGFBP-2 is an important mediator of cell growth [25]. In growth plate chondrocytes, growth-inhibiting conditions decrease IGFBP-2 levels [13,14], whereas growth-stimulating conditions increase IGFBP-2 levels [15], suggesting a positive association between IGFBP-2 levels and growth. In addition, in the rat IGFBP-2 promoter a silencer element has been identified, using deletion analysis of the promoter, which activity depends on cell growth [26]. Therefore, the inhibition of IGFBP-2 transcriptional activity in growth plate chondrocytes by GCs might play a role in GC-induced growth retardation.

REFERENCES

1. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
2. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.
3. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
4. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
5. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.

6. Fritz PC, Ward WE, Atkinson SA, Tenenbaum HC 1998 Tamoxifen attenuates the effects of exogenous glucocorticoid on bone formation and growth in piglets. *Endocrinology,* **139:** 3399-3403.
7. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
8. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.
9. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
10. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
11. Smink JJ, Koster JG, Gresnigt R, Rooman R, Koedam JA, Van Buul-Offers SC 2002 IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice. *J Endocrinol,* **175:** 143-153.
12. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
13. Smink JJ, Koedam JA, Koster JG, Van Buul-Offers SC 2002 Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *J Endocrinol,* **174:** 343-352.
14. de los Rios P, Hill DJ 2000 Expression and release of insulin-like growth factor binding proteins in isolated epiphyseal growth plate chondrocytes from the ovine fetus. *J Cell Physiol,* **183:** 172-181.
15. Borromeo V, Bramani S, Holder AT, Carter C, Secchi C, Beattie J 1996 Growth hormone stimulates the secretion of insulin-like growth factor binding protein-2 (IGFBP-2) by monolayer cultures of sheep costal growth plate chondrocytes. *Mol Cell Biochem,* **162:** 145-151.
16. Koedam JA, Smink JJ, Van Buul-Offers SC 2002 Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. *Mol Cell Endocrinol,* **197:** 35-44.
17. Badinga L, Song S, Simmen RC, Simmen FA 1998 A distal regulatory region of the insulin-like growth factor binding protein-2 (IGFBP-2) gene interacts with the basic helix-loop-helix transcription factor, AP-4. *Endocrine,* **8:** 281-289.
18. Hendriks-Stegeman BI, Augustijn KD, Bakker B, Holthuizen PE, van der Vliet PC, Jansen M 2001 Combined pituitary hormone deficiency caused by compound heterozygosity for two novel mutations in the POU-domain of the PIT1/POU1F1 gene. *J Clin Endocrinol Metab,* **86:** 1545-1550.
19. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning (a laboratory manual). Cold Spring Harbour, NY: Cold Spring Harbor Laboratory Press, 1989.
20. Beato M, Herrlich P, Schütz G 1995 Steroid hormone receptors: many actors in search of a plot. *Cell,* **83:** 851-857.
21. Webster JC, Cidlowski JA 1999 Mechanisms of glucocorticoid-receptor-mediated repression of gene expression. *Trends Endocrinol Met,* **10:** 396-402.
22. Beato M 1989 Gene regulation by steroid hormones. *Cell,* **56:** 335-344.
23. Mouhieddine OB, Cazals V, Kuto E, Le BY, Clement A 1996 Glucocorticoid-induced growth arrest of lung alveolar epithelial cells is associated with increased production of insulin-like growth factor binding protein-2. *Endocrinology,* **137:** 287-295.
24. Dell G, Ward A, Shokrai A, Madej A, Engstrom W 1999 Regulation of the IGF system by glucocorticoids. *Zool Science,* **16:** 377-385.
25. Hoeflich A, Reisinger R, Lahm H, Kiess W, Blum WF, Kolb HJ, Weber MM, Wolf E 2001 Insulin-like growth factor-binding protein 2 in tumorigenesis: protector or promoter. *Cancer Res,* **61:** 8601-8610.
26. Kutoh E, Margot J, Schwander J 1998 Cell-density (cycle) dependent silencer of the rat insulin-like growth factor binding protein-2 (IGFBP-2) gene. *Growth Factors,* **16:** 217-223.

Chapter 8

General discussion

Introduction

Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive drugs in various diseases [1]. The use of these potent drugs, however, is associated with several severe side-effects such as suppression of the hypothalamus-pituitary axis, osteoporosis and growth retardation in children [2]. In this thesis, we attempted to elucidate some of the mechanisms involved in GC-induced growth retardation. GCs act locally to inhibit growth, suggesting a mechanism intrinsic to the growth plate [3,4]. Growth plate chondrocytes of the different zones express the glucocorticoid receptor (GR), suggesting they are indeed target cells for GCs [5-7].

It has been suggested, for many years already, that GC-induced growth retardation involves impaired action of the components of the IGF system [8-11]. However, very few studies have analyzed the effects of GCs on the IGF system at the local level of the growth plate, and these studies were limited to cell culture systems [12,13]. The primary growth plate chondrocyte cultures used in these *in vitro* studies predominantly represent proliferative chondrocytes [12,14]. Possible effects of GCs on resting and hypertrophic chondrocytes are therefore not included in these kinds of studies. Furthermore, the complex architecture of the growth plate cannot be mimicked *in vitro*. This indicates the importance to study also the effects of GCs on the growth plate *in vivo*. Naturally, the *in vitro* studies remain important to study processes in detail. Therefore, we have performed both *in vitro* and *in vivo* studies to analyze the effects of GCs on the IGF system at the level of the growth plate, as described in this thesis.

The IGF system in the postnatal growth plate

IGF-I and IGF-II

For several years, there have been conflicting reports whether both IGF-I and IGF-II are expressed in growth plate chondrocytes. In fetal growth plates, both IGF-I and -II are present [15,16]. In cultured growth plate chondrocytes, we also showed expression of both IGFs (chapter 7), as shown previously [17]. *In vivo* in the postnatal growth plates of rodents, Wang *et al.* [18] and Shinar *et al.* [19], could only detect IGF-II, whereas others did show expression of IGF-I, but did not study IGF-II [20-23]. We demonstrated, for the first time, the presence of both IGF-I and IGF-II *in vivo* in the postnatal growth plate of mice (chapters 3 and 4). The type I IGF receptor is expressed in all zones of the growth plate [15,24] and we are the first to describe the expression of the type II IGF receptor in the growth plate, *in vitro* (chapter 7).

We studied the growth plates of prepubertal (4-week-old) (chapter 4) and postpubertal (7-week-old) (chapter 3) mice and demonstrated an age-dependent shift in expression pattern of both IGF-I and -II (Fig. 1), which could indicate an age-dependent autocrine/paracrine function of both IGFs at various stages of chondrogenesis and postnatal development. Expression of IGF-I and -II in the proliferative and prehypertrophic zones in prepubertal animals (chapter 4) [18,20,23] indicates that they are probably important in the clonal expansion of proliferative chondrocytes, as postulated for IGF-I in the "dual effector theory" [25] (see the General introduction). They are probably also important in the differentiation of proliferative chondrocytes toward a hypertrophic state.

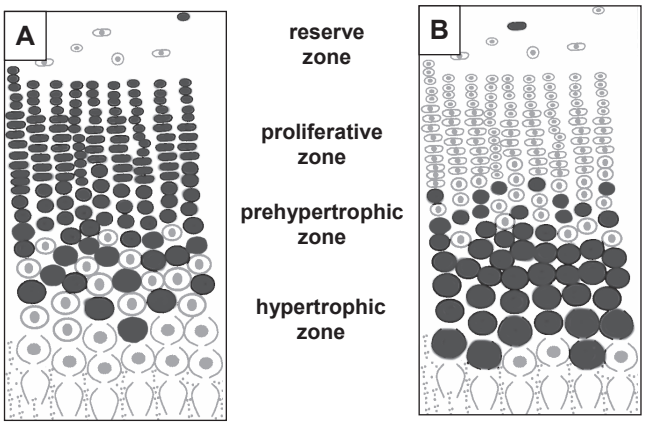

Figure 1 Age-dependent expression pattern of IGF-I and IGF-II in the postnatal growth plate. A schematic representation of the expression of both IGFs in the growth plates of (**A**) prepubertal and (**B**) postpubertal mice, as shown by *in situ* hybridization. IGF-I and IGF-II co-localize in the different growth plate zones. The age-dependent shift in expression pattern might indicate an age-dependent function of both IGF-I and -II in the growth plate. In prepubertal mice, they might be important in stimulating proliferation and differentiation. In postpubertal mice, the main function might be to stimulate differentiation.

In postpubertal mice, both IGFs are predominantly expressed in the pre- and hypertrophic zones (chapter 3) [21,22]. At this age, the main function of both IGFs might be to augment chondrocyte hypertrophy and to a lesser extent to stimulate proliferation, as the proliferation might become less important as the growth velocity decreases in postpubertal mice. IGF-I has indeed previously been shown *in vivo* to be involved in stimulating proliferation and augmenting hypertrophy of chondrocytes [23,26].

IGF-I and IGF-II are suggested to each have a unique and complementary role in the growth plate [27]. However, the IGFs have similar expression patterns in the growth plate (chapters 3 and 4). Treatment of chondrocytes with either IGF-I or -II has similar effects, on IGFBP expression [14] and on clonal expansion of chondrocytes [28]. However, concerning clonal expansion, IGF-I was more effective in adult chondrocytes and IGF-II in fetal chondrocytes [28]. This suggests that IGF-I might play a more prominent role in the postnatal growth plate and IGF-II in the fetal growth plate. IGF-II knock-out mice indeed indicated a predominant role for IGF-II in fetal growth [29]. IGF-II does, however, stimulate postnatal longitudinal growth [30,31] and clonal expansion of postnatally derived chondrocytes [28], and is present in the postnatal growth plate (chapters 3 and 4). This indicates that IGF-II could still play a role in the postnatal growth plate, which however, has to be established. Our GC treatment studies (see below) did show that IGF-I and -II did not respond in the same way to GCs, indicating that IGF-I and -II still may have both a unique function in the postnatal growth plate (chapters 3 and 4).

IGFBPs

In chapters 3 and 4, we demonstrated for the first time the presence of only IGFBP-2 in the postnatal growth plate *in vivo*. Using radioactive *in situ* hybridization, Wang *et al.* detected only low levels of IGFBP-6 in postnatal murine growth plates *in vivo*, which rapidly declined with age [18]. In many cases, a wider array of IGFBPs has been found in cultured chondrocytes [16,17,32]. In porcine growth plate chondrocytes we demonstrated remarkable expression of IGFBP-2, -4 and -5, which is in accordance with several other *in vitro* studies [14,17,32,33]. The high expression of IGFBP-2, -4, and -5 in chondrocytes (chapter 7) [14,32] might be related to the important roles these IGFBPs play in bone formation and resorption [34-38]. IGFBP-4 and -5 are also known to affect the proliferation of chondrocytes [39,40].

IGFBP-2 is the only IGFBP which we detected both *in vitro* (chapter 7) and *in vivo* (chapters 3 and 4) in postnatal growth plate chondrocytes, in co-localization with IGF-I and -II *in vivo*. This suggests that IGFBP-2 might play a specific modulatory role of IGF action in

growth plate chondrocytes. However, IGFBP-2 knock-out mice did not shown an effect on longitudinal growth, which was suggested to be caused by compensation of the other IGFBPs [41]. In contrast, IGFBP-2 was shown to inhibit GH-induced growth *in vivo* [42]. Also, IGFBP-2 transgenic mice, which displayed a reduced body weight gain caused by a reduction in carcass weight, showed that IGFBP-2 is a negative regulator of bone growth and modeling [34,35].

GC-induced growth retardation and the growth plate

Growth retardation as a side-effect of GC treatment is observed in both children [2,43] and in animal models [44-48]. In children, already short-term GC treatment can result in growth retardation [43,49] and especially just before puberty, children are susceptible to growth suppression by GCs [2]. We confirmed this in prepubertal mice, where short-term GC treatment resulted in growth inhibition (chapter 4). Comparison of the effects of short-term (chapter 4) and long-term (chapter 3) [44] GC treatments indicated that the growth inhibition is already established, for the large part, during the initial phase of the treatment. Prolonged treatment did not seem to have much additional effects on general growth (chapter 4) [44].

GCs act locally to inhibit growth [3,50], which is confirmed in several studies showing that GCs decreased the total width of the growth plate [4,51,52]. In chapters 4 and 5, we showed that the total growth plate width already decreased after a short-term GC treatment, which was mainly caused by a diminished width of the proliferative zone. This was due to a reduced proliferation, resulting in a decreased number of chondrocytes in the proliferative zone.

The susceptibility of proliferative chondrocytes to GCs was already shown in chondrocyte cultures from rodents [9,12], where GCs decreased cellular turnover and reduced the number of cells in S phase [53]. In chapter 7, we confirmed these findings and demonstrated that the decreased proliferation was a direct effect of the GCs, as a GR antagonist prevented the reduced proliferation. The disturbed chondrocyte proliferation likely contributes to a disturbed chondrogenesis. Several other stages in endochondral ossification are also affected by GCs, as described below.

Disturbed apoptosis and angiogenesis by GC treatment

Vascular invasion (*i.e.* angiogenesis) of the growth plate is associated with apoptosis of hypertrophic chondrocytes [54]. In mice in which hypertrophic chondrocyte differentiation is inhibited, no vascular invasion of the growth plate occurs [55,56]. Vice versa, inhibition of angiogenesis delays chondrocyte apoptosis [54]. An essential growth factor for angiogenesis is VEGF [57], which is expressed by hypertrophic chondrocytes [54,58,59], as we confirmed in chapter 5. In chapter 6, we demonstrated that cultured porcine growth plate chondrocytes also express and secrete VEGF.

We showed that GC treatment increased apoptosis in hypertrophic chondrocytes in prepubertal mice (chapter 4) and piglets (chapter 5). The same result was previously shown in rats [4,60]. Apoptosis of chondrocytes is inversely correlated with the growth rate of the long bones [61]. Increased apoptosis could therefore contribute to the decreased growth, which is observed as a result of the GC treatment.

In chapter 5, we demonstrated that GCs severely disturb the architecture of the blood vessels in the primary spongiosa, with disorganized and short blood vessels as a consequence. VEGF, important for the vascularization, was decreased by GCs in the porcine growth plate,

both *in vivo* and *in vitro* (chapters 5 and 6), which was also shown *in vivo* in rats [60]. The decrease of VEGF *in vitro* was mediated by the GR, as a GR antagonist almost completely blocked the decrease of VEGF expression. The decrease of VEGF expression in the growth plate by GCs could be involved in the disturbed angiogenesis, which we observed in our GC-treated piglets (chapter 5).

In conclusion, several stages in endochondral ossification are affected by GCs, *e.g.* decreased proliferation, increased apoptosis and disturbed angiogenesis. This can all contribute to GC-induced growth retardation.

The IGF system in GC-induced growth retardation

The possible involvement of the IGF system in GC-induced growth retardation has mainly been studied at the endocrine level. These studies, however, resulted in conflicting data and serum levels of the IGF system were suggested to provide little insight into the mechanisms of GC-induced growth retardation [62]. Although changes in endocrine levels of the IGF system may still be involved in GC-induced growth retardation, the local effects on the growth plate are probably more important [10].

In chapter 7, we addressed the question which components of the IGF system are regulated by GCs in neonatal porcine growth plate chondrocytes, concomitant with the decreased chondrocyte proliferation. GCs increased type I IGF receptor mRNA expression, confirming *in vivo* data [11]. However, decreased type I IGF receptor mRNA expression in the rat growth plate has also been reported [60]. Although seemingly contradictory, the increase of type I IGF receptor levels might be a response to counteract the adverse effects of GCs on growth. The GC-induced increase in IGFBP-5 mRNA expression is in contrast with observed reduced levels in rabbit costal chondrocytes [13] and fetal ovine growth plate chondrocytes [14]. GC-regulated expression of IGF components is species- and cell-type specific and depends on age and developmental stage [63], possibly explaining the observed differences.

IGFBP-2

IGFBP-2 was the only component which was both decreased at the mRNA and protein level by GCs *in vitro*. This decrease was mediated by the GR (chapter 7). A similar GC-induced decrease of IGFBP-2 was shown in fetal ovine growth plate chondrocytes [14]. The observed decrease of IGFBP-2 under growth inhibiting conditions is in concert with the observed increase of IGFBP-2 under growth stimulating conditions in chondrocytes [64]. This suggests that the proliferation of chondrocytes *in vitro*, is positively associated with IGFBP-2 levels. In preliminary promoter studies of IGFBP-2, the regulation of IGFBP-2 by GCs appeared to occur at the transcriptional level (addendum of chapter 7). However, *in vivo* GC treatment had no effect on IGFBP-2 mRNA expression in the growth plate (chapters 3 and 4), indicating a different regulation of IGFBP-2 by GCs in these different model systems.

In chapter 2, we showed that growth stimulation *in vivo* of lymphoid tissues, by the presence of a hIGF-II transgene, is also associated with increased mRNA expression of IGFBP-2. This suggests that not only in chondrocytes *in vitro*, but also in other systems, IGFBP-2 mRNA expression is positively associated with growth. In turn, this association could affect the outcome of the growth modulating condition. In the same way, the GC-induced decrease of IGFBP-2 *in vitro* might play a growth modulating role in the GC-induced growth retardation.

A dual action of IGF-I in GC-induced growth retardation

We are the first to describe the *in vivo* effects of GCs on the expression of the IGF system in the growth plate. Both the short-term and the long-term *in vivo* GC treatment appeared to have no effect on IGFBP-2 mRNA expression. In addition, IGF-II did not change either, however, both treatments did affect IGF-I mRNA expression (chapters 3 and 4). This contrasts with the *in vitro* situation in the porcine growth plate chondrocytes, where IGF-I was not affected by GCs, whereas IGFBP-2 decreased and IGF-II was also not affected (chapter 7).

Following 1 week of GC treatment, IGF-I levels decreased both in serum and in the growth plate (chapter 4). The decreased IGF-I mRNA expression in the proliferative zone of the growth plate probably contributed to the observed decrease in proliferation. In addition, the reduced IGF-I mRNA expression in the prehypertrophic zone, might result in inhibition of the differentiation of chondrocytes to the hypertrophic phenotype [23,24,26]. Also in rat growth plate chondrocytes, GCs were shown to inhibit GH-stimulated IGF-I secretion [12]. The inhibition of IGF-I by GCs was demonstrated to occur at the transcriptional level, in rat osteoblasts [65]. Although we could not demonstrate a direct relationship between decreased IGF-I levels and disturbed endochondral ossification, our data do suggest that the action of IGF-I in the growth plate is impaired upon GC treatment.

During long-term GC treatment of rats for 3 weeks, GR levels decreased in the growth plate [4], indicating that the growth plate becomes less susceptible to GCs in time. GCs can then no longer or to a lesser extent exert their actions. Comparing our short-term and long-term GC experiment, the same conclusion can be drawn: the organism becomes less susceptible as most of the damage seems to occur in the early phase of the treatment (chapters 3 and 4) [44]. This is supported by the finding in humans that the growth inhibiting effect of GCs is also maximal during the initial period of the treatment [66]. After 4 weeks of GC treatment, IGF-I levels increased, both in the growth plate and serum in our mice (chapter 3). Since IGF-I is suggested to be able to counteract the inhibiting effects of GCs [9], the increased IGF-I levels in the growth plate might be part of a compensatory mechanism to diminish the growth inhibiting effect of GCs, together with the decreased GR levels [4]. This suggestion is strengthened by the notion that in hIGF-II transgenic mice, which are partially protected against the adverse effects of GCs [44], also increased IGF-I mRNA levels are observed in the growth plate (chapter 3). Our observations indicate that IGF-I could have a dual action in GC-induced growth retardation. During the initial phase of GC treatment, IGF-I action would be impaired by GCs, resulting in disturbed chondrogenesis and subsequent longitudinal bone growth (in Fig. 2 proliferation is used as an example of the effects of IGF-I). In a later phase of the treatment, GCs might not be able to impair IGF-I action anymore, due to the decreased GR levels. IGF-I expression is no longer inhibited and IGF-I levels increase, which would be part of a mechanism to counteract the adverse effects of GCs (Fig. 2).

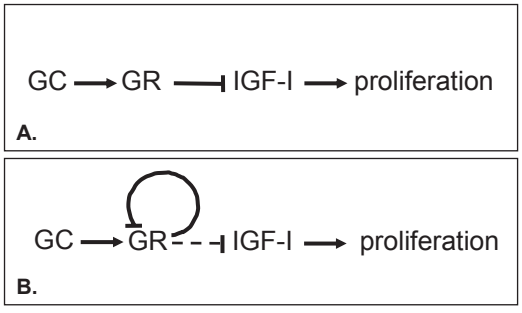

Figure 2 A dual action of IGF-I in GC-induced growth retardation A proposed model for the actions of IGF-I in GC-induced growth retardation. (**A**) In the intial phase of the GC treatment, GCs decrease IGF-I levels, resulting *e.g.* in a decreased proliferation of chondrocytes (and affecting several other stages in endochondral ossification). (**B**) In a later phase of the GC treatment, GR levels decrease, resulting in a decreased sensitivity for GCs. GCs are no longer able to impair IGF-I action. IGF-I levels rise, resulting in a compensatory mechanism to counteract the adverse effects of GC by increasing *e.g.* proliferation of the chondrocytes.

A central role for IGF-I in GC-disturbed endochondral ossification

The possible involvement of IGF-I in GC-induced growth retardation is probably not limited to chondrocyte proliferation and differentiation, but may involve more stages in endochondral ossification (Fig. 3). IGF-I is known to protect cells against GC-induced apoptosis [67]. Decreased IGF-I levels in the growth plate could therefore contribute to increased levels of chondrocyte apoptosis (chapters 4 and 5). Vascularization also depends on IGF-I. It requires minimal levels of IGF-I, as shown by inhibited vascularization in the retina of IGF-I knock-out mice [68] and humans with low IGF-I levels [69]. IGF-I might mediate its effects on vascularization through VEGF, as IGF-I stimulates VEGF production in both chondrocytes (chapter 6) and osteoblasts [70]. Moreover, minimal levels of IGF-I seem to be required to promote maximal function of VEGF [69]. Disturbed angiogenesis caused by GCs (chapter 6), could therefore be caused by decreased levels of IGF-I in the growth plate, with VEGF as a possible intermediate. However, direct effects of GCs on VEGF or other factors influencing vascularization can also be involved.

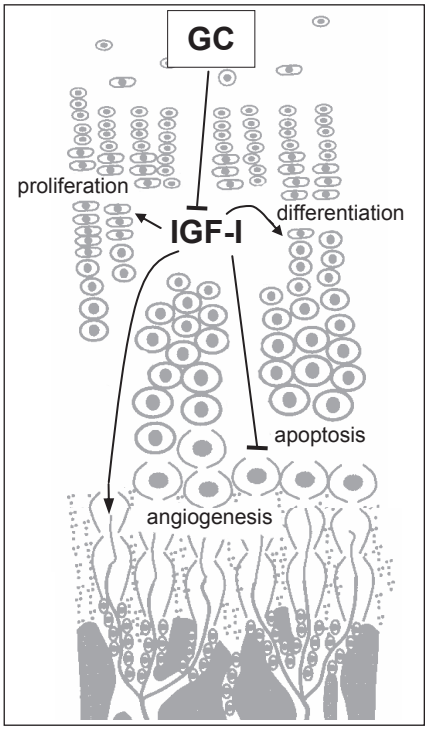

Figure 3. Possible central role for IGF-I in endochondral ossification. IGF-I could play a central role in different stages in endochondral ossification. GCs disturb many of these different stages, which could be mediated by IGF-I, as indicated. The effects on the ECM are not indicated.

Another important aspect of endochondral ossification, ECM synthesis, also involves IGF-I. IGF-I stimulates the production of proteoglycans [33,71,72]. GC-induced down-regulation of IGF-I in the growth plate might result in a decreased matrix production by chondrocytes. A decreased matrix production is suggested to contribute to GC-induced growth retardation [73]. Disturbed matrix production might also have an effect on the function of IGFBPs, as their biological activity can depend on ECM association [74].

Taking into account the different aspects of GC-induced growth retardation, IGF-I is likely to play a central role in the different disturbed stages in endochondral ossification caused by GCs, at the local level of the growth plate.

Therapeutic implications

The suggested involvement of impaired action of the GH-IGF axis in GC-induced growth retardation has already resulted in some studies on the use of these components as therapeutic agents. GH therapy of children treated with GCs has been shown to attenuate the GC-induced growth retardation [75]. It resulted in increased serum IGF-I levels, which was suggested to mediate the effects of GH [2]. Our data on the involvement of the IGF system in GC-induced growth retardation indicate that IGF-I might indeed be an important player. IGF-I has been shown to restore growth retardation in diabetes-induced growth retardation in rats [76] and also partially in GC-induced growth retardation in rats [77]. However, whether treatment with IGF-I would be beneficial remains to be solved. Another important aspect is that most damage caused by GC treatment is suggested to occur in the initial phase of the GC treatment [66]. Both the search for prevention therapy as well as the development of GCs with less side-effects [78] is therefore important.

Concluding remarks

The observed effects of GCs on the growth plate suggests that local IGF-I plays a central role in GC-induced growth retardation. Although we have been the first to show an effect of GCs on the IGF system in the growth plate *in vivo*, we did not prove a direct relationship between changed local IGF-I expression levels, the disturbed stages in endochondral ossification, and the subsequent growth retardation. Studies have already been performed to analyze the systemic effects of IGF-I on longitudinal bone growth, using the liver-specific IGF-I and ALS knock-out mice (described in the Introduction). However, the effects of a total IGF-I knock-out on postnatal longitudinal bone growth is more severe. This indicates the importance of local IGF-I. For this reason, one should first study the function of local IGF-I in the growth plate, which could indicate if there is indeed a direct relationship between IGF-I and the different stages in endochondral ossification. Generating a growth-plate specific IGF-I knock-out mouse, using the Cre/LoxP system, would be a useful tool for this kind of study. To delineate in more detail the function of IGF-I during chondrogenesis, IGF-I expression could be suppressed at only particular stages in chondrogenesis, using the mouse chondrogenic cell line ATDC5 and antisense oligo's or RNA interference experiments. This chondrogenic cell line undergoes a reproducible multistep chondrogenic differentiation program in a tightly regulated time-span [79,80], providing an accurate model of the growth plate. Using the above mentioned experimental models, one can study whether the main target of GCs is indeed IGF-I, as suggested in this thesis and whether other IGF system components, as IGFBP-2, or other factors might also be involved.

REFERENCES

1. Greenspan FS, Strewler G. Basic and clinical endocrinology. 5th ed. New Jersey: Prentice-Hall International Inc., 1997.
2. Allen DB 1996 Growth suppression by glucocorticoid therapy. *Endocrinol Metab Clin North Am,* **25:** 699-717.
3. Baron J, Huang Z, Oerter KE, Bacher JD, Cutler GC 1992 Dexamethasone acts locally to inhibit longitudinal bone growth in rabbits. *Am J Physiol,* **263:** E489-E492.
4. Silvestrini G, Ballanti P, Patacchioli FR, Mocetti P, Di Grezia R, Martin Wedard B, Angelucci L, Bonucci E 2000 Evaluation of apoptosis and the glucocorticoid receptor in the cartilage growth plate and metaphyseal bone cells of rats after high-dose treatment with corticosterone. *Bone,* **26:** 33-42.

5. Silvestrini G, Mocetti P, Ballanti P, Di Grezia R, Bonucci E 1999 Cytochemical demonstration of the glucocorticoid receptor in skeletal cells of the rat. *Endocrine Res,* **25:** 117-128.
6. Siebler T, Robson H, Shalet SM, Williams GR 2002 Dexamethasone inhibits and thyroid hormone promotes differentiation of mouse chondrogenic ATDC5 cells. *Bone,* **31:** 457-464.
7. Abu EO, Horner A, Kusec V, Triffitt JT, Compston JE 2000 The localization of the functional glucocorticoid receptor α in human bone. *J Clin Endocrinol Metab,* **88:** 883-889.
8. Robson H, Siebler T, Shalet S, Williams GR 2002 Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. *Pediatr Res,* **52:** 137-147.
9. Klaus G, Jux C, Fernandez P, Rodriguez J, Himmele R, Mehls O 2000 Suppression of growth plate chondrocyte proliferation by corticosteroids. *Pediatr Nephrol,* **14:** 612-615.
10. Mehls O, Himmele R, Hömme M, Kiepe D, Klaus G 2001 The interaction of glucocorticoids with the growth hormone-insulin-like growth factor axis and its effects on growth plate chondrocytes and bone cells. *J Pediatr Endocrinol Met,* **14:** 1475-1482.
11. Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ 1992 Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology,* **130:** 1424-1432.
12. Jux C, Leiber K, Hugel U, Blum W, Ohlsson C, Klaus G, Mehls O 1998 Dexamethasone impairs growth hormone (GH)-stimulated growth by suppression of local insulin-like growth factor (IGF)-I production and expression of GH- and IGF-I-receptor in cultured rat chondrocytes. *Endocrinology,* **139:** 3296-3305.
13. Koedam JA, Hoogerbrugge CM, Van Buul-Offers SC 2000 Differential regulation of IGF-binding proteins in rabbit costal chondrocytes by IGF-I and dexamethasone. *J Endocrinol,* **165:** 557-567.
14. de los Rios P, Hill DJ 2000 Expression and release of insulin-like growth factor binding proteins in isolated epiphyseal growth plate chondrocytes from the ovine fetus. *J Cell Physiol,* **183:** 172-181.
15. Olney RC, Mougey EB 1999 Expression of the components of the insulin-like growth factor axis across the growth plate. *Mol Cell Endocrinol,* **156:** 67-71.
16. de los Rios P, Hill DJ 1999 Cellular localization and expression of insulin-like growth factors (IGFs) and IGF binding proteins within the epiphyseal growth plate of the ovine fetus: possible functional implications. *Can J Physiol Pharmacol,* **77:** 235-249.
17. Bhaumick B 1993 Insulin-like growth factor (IGF) binding proteins and insulin-like growth factor secretion by cultured chondrocyte cells: identification, characterization and ontogeny during cell differentiation. *Regul Pept,* **48:** 113-122.
18. Wang E, Wang J, Chin E, Zhou J, Bondy CA 1995 Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis. *Endocrinology,* **136:** 2741-2751.
19. Shinar DM, Endo N, Halperin D, Rodan GA, Weinreb M 1993 Differential expression of insulin-like growth factor-I (IGF-I) and IGF-II messenger ribonucleic acid in growing rat bone. *Endocrinology,* **132:** 1158-1167.
20. Nilsson A, Carlsson B, Isgaard J, Isaksson OGP, Rymo L 1990 Regulation by GH of insulin-like growth factor-I mRNA expression in rat epiphyseal growth plate as studied with *in situ* hybridization. *Endocrinology,* **125:** 67-74.
21. Reinecke M, Schmid AC, Heyberger-Meyer B, Hunziker EB, Zapf J 2000 Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes *in vivo*. *Endocrinology,* **141:** 2847-2853.
22. Lazowski DA, Fraher LJ, Hodsman A, Steer B, Modrowski D, Han VK 1994 Regional variation of insulin-like growth factor-I gene expression in mature rat bone and cartilage. *Bone,* **15:** 563-576.
23. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A 2001 Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol,* **229:** 141-162.
24. Hunziker EB, Wagner J, Zapf J 1994 Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes in vivo. *J Clin Invest,* **93:** 1078-1086.
25. Green H, Morikawa M, Nixon T 1985 A dual effector theory of growth-hormone action. *Differentiation,* **29:** 195-198.
26. Wang J, Zhou J, Bondy CA 1999 IGF1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J,* **13:** 1985-1990.
27. LeRoith D, Bondy CA, Yakar S, Liu J-N, Butler A 2001 The somatomedin hypothesis: 2001. *Endocr Rev,* **22:** 53-74.
28. Vetter U, Zapf J, Heit W, Helbing G, Heinze E, Froesch ER, Teller WM 1986 Human fetal and adult chondrocytes. Effect of insulin like growth factors I and II, insulin, and growth hormone on clonal growth. *J Clin Invest,* **77:** 1903-1908.
29. DeChiara TM, Efstratiadis A, Robertson EJ 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature,* **345:** 78-80.

30. Van Buul-Offers SC, Reijnen-Gresnigt MG, Hoogerbrugge CM, Bloemen RJ, Kuper CF, Van den Brande JL 1994 Recombinant insulin-like growth factor-II inhibits the growth-stimulating effect of growth hormone on the liver of Snell dwarf mice. *Endocrinology,* **135:** 977-985.
31. Van Buul-Offers SC, Hoogerbrugge CM, Branger J, Feijlbrief M, Van den Brande JL 1988 Growth-stimulating effects of somatomedin-/insulin-like peptides in Snell dwarf mice. *Horm Res,* **29:** 229-236.
32. Olney RC, Smith RL, Kee Y, Wilson DM 1993 Production and hormonal regulation of insulin-like growth factor binding proteins in bovine chondrocytes. *Endocrinology,* **133:** 563-570.
33. Sunic D, Belford DA, McNeil JD, Wiebkin OW 1995 Insulin-like growth factor binding proteins (IGF-BPs) in bovine articular and ovine growth-plate chondrocyte cultures: their regulation by IGFs and modulation of proteoglycan synthesis. *Biochim Biophys Acta,* **1245:** 43-48.
34. Hoeflich A, Wu M, Mohan S, Foll JL, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb H, Wolf E 1999 Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. *Endocrinology,* **140:** 5488-5496.
35. Hoeflich A, Pavicic T, Nedbal S, Schmidt C, Weher U, Rambeck W, Wolf E, Eckstein F 2002 Effects of IGFBP-2 and GH overexpression on bone growth in transgenic mice (abstract). *GH and IGF Res,* **12:** 224-225.
36. Mohan S, Baylink DJ 1996 Insulin-like growth factor system components and the coupling of bone formation to resorption. *Horm Res,* **45 (suppl 1):** 59-62.
37. Mohan S, Farley JR, Baylink DJ 1995 Age-related changes in IGFBP-4 and IGFBP-5 levels in human serum and bone: implications for bone loss with aging. *Prog Growth Factor Res,* **6:** 465-473.
38. Miyakoshi N, Richman C, Kasukawa Y, Linkhart TA, Baylink DJ, Mohan S 2001 Evidence that IGF-binding protein-5 functions as a growth factor. *J Clin Invest,* **107:** 73-81.
39. Matsumoto T, Tsurumoto T, Goldring MB, Shindo H 2000 Differential effects of IGF-binding proteins, IGFBP-3 and IGFBP-5, on IGF-I action and binding to cell membranes of immortalized human chondrocytes. *J Endocrinol,* **166:** 29-37.
40. Kiepe D, Andress DL, Mohan S, Ulinski T, Himmele R, Mehls O, Tönshoff B 2001 Intact IGF-binding protein-4 and -5 and their respective fragments isolated from chronic renal failure serum differentially modulate IGF-I actions in cultured growth plate chondrocytes. *J Am Soc Nephrol,* **12:** 2400-2410.
41. Pintar JE, Schuller A, Ceroo JA, Czick M, Grewal A, Green B 1995 Genetic ablation of IGFBP-2 suggests functional redundancy in the IGFBP family. *Prog Growth Factor Res,* **6:** 437-445.
42. Hoeflich A, Nedbal S, Blum WF, Erhard M, Lahm H, Brem G, Kolb HJ, Wanke R, Wolf E 2001 Growth inhibition in giant growth hormone transgenic mice by overexpression of insulin-like growth factor-binding protein-2. *Endocrinology,* **142:** 1889-1898.
43. Ahmed SF, Tucker P, Mushtaq T, Wallace AM, Williams DM, Hughes IA 2002 Short-term effects on linear growth and bone turnover in children randomized to receive prednisolone or dexamethasone. *Clin Endocrinol,* **57:** 185-191.
44. Rooman R, Koster JG, Bloemen RJ, Gresnigt R, Van Buul-Offers SC 1999 The effect of dexamethasone on body and organ growth of normal and IGF-II transgenic mice. *J Endocrinol,* **163:** 543-552.
45. Leili S, Scanes CG 1998 The effects of glucocorticoids (dexamethasone) on insulin-like growth factor-I, IGF-binding proteins, and growth in chickens. *Proc Soc Exp Biol Med,* **218:** 329-333.
46. Ortoft G, Gronbaek H, Oxlund H 1998 Growth hormone administration can improve growth in glucocorticoid-injected rats without affecting the lymphocytopenic effect of the glucocorticoid. *Growth Horm IGF Res,* **8:** 251-264.
47. Sheagren JN, Jowsey J, Bird DC, Gurton ME, Jacobs JB 1977 Effect on bone growth of daily versus alternate-day corticosteroid administration: an experimental study. *J Lab Clin Med,* **89:** 120-130.
48. Ward WE, Donovan SM, Atkinson SA 1998 Dexamethasone-induced abnormalitites in growth and bone metabolism in piglets are partially attenuated by growth hormone with no synergistic effect on insulin-like growth factor-I. *Pediatr Res,* **44:** 215-221.
49. Ahmed SF, Wallace WHB, Crofton PM, Wardhaugh B 1999 Short-term changes in lower leg length in children treated for acute lymphoblastic leukaemia. *J Pediatr Endocrinol Met,* **12:** 75-80.
50. Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De-Levi S, Baron J 2001 Catch-up growth is associated with delayed senescence of the growth plate in rabbits. *Pediatr Res,* **50:** 618-623.
51. Altman A, Hochberg Z, Silbermann M 1992 Interactions between growth hormone and dexamethasone in skeletal growth and bone structure of young mouse. *Calcif Tissue Int,* **51:** 298-304.
52. Annefeld M 1992 Changes in rat epiphyseal cartilage after treatment with dexamethasone and glycosaminoglycan-peptide complex. *Pathol Res Pract,* **188:** 649-652.
53. Robson H, Anderson E, Eden OB, Isaksson O, Shalet S 1998 Chemotherapeutic agents used in the treatment of childhood malignancies have direct effects on growth plate chondrocyte proliferation. *J Endocrinol,* **157:** 225-235.

54. Gerber H-P, Ferrara N 2000 Angiogenesis and bone growth. *Trends Cardiovasc Med,* **10:** 223-228.
55. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T 1997 Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell,* **89:** 755-764.
56. Kim IS, Otto F, Zabel B, Mundlos S 1999 Regulation of chondrocyte differentiation by *Cbfa1*. *Mech Dev,* **80:** 159-170.
57. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N 1999 VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med,* **5:** 623-628.
58. Garcia-Ramirez M, Toran N, Andaluz P, Carrascosa A, Audi L 2000 Vascular endothelial growth factor is expressed in human fetal growth cartilage. *J Bone Miner Res,* **15:** 534-540.
59. Horner A, Bishop NJ, Bord S, Beeton C, Kelsall AW, Coleman N, Compston JE 1999 Immunolocalisation of vascular endothelial growth factor (VEGF) in human neonatal growth plate cartilage. *J Anat,* **194:** 519-524.
60. Sanchez CP, He Y-Z 2002 Alterations in the growth plate cartilage of rats with renal failure receiving corticosteroid therapy. *Bone,* **30:** 692-698.
61. Farnum CE, Wilsman NJ 1989 Condensation of hypertrophic chondrocytes at the chondro-osseous junction of growth plate cartilage in yucatan swine: relationship to long bone growth. *Am J Anat,* **186:** 346-358.
62. Ward WE, Atkinson SA, Donovan SM, Paes B 1999 Bone metabolism and circulating IGF-I and IGFBPs in dexamethasone-treated preterm infants. *Early Hum Dev,* **56:** 127-141.
63. Dell G, Ward A, Shokrai A, Madej A, Engstrom W 1999 Regulation of the IGF system by glucocorticoids. *Zool Science,* **16:** 377-385.
64. Borromeo V, Bramani S, Holder AT, Carter C, Secchi C, Beattie J 1996 Growth hormone stimulates the secretion of insulin-like growth factor binding protein-2 (IGFBP-2) by monolayer cultures of sheep costal growth plate chondrocytes. *Mol Cell Biochem,* **162:** 145-151.
65. Delany AM, Canalis E 1995 Transcriptional repression of insulin-like growth factor I by glucocorticoids in rat bone cells. *Endocrinology,* **136:** 4776-4781.
66. Hochberg Z 2002 Mechanisms of steroid impairment of growth. *Horm Res,* **58 (suppl 1):** 33-38.
67. Distelhorst CW 2002 Recent insights into the mechanism of glucocorticoidsteroid-induced apoptosis. *Cell Death Differ,* **9:** 6-19.
68. Hellstrom A, Perruzzi C, Ju M, Engström E, Hård A-L, Liu J-L, Albertsson-Wikland K, Carlsson B, Niklasson A, Sjödell L, LeRoith D, Senger DR, Smith LEH 2001 Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinophathy of prematurity. *Proc Natl Acad Sci U S A,* **98:** 5804-5808.
69. Hellstrom A, Carlsson B, Niklasson A, Segnestam K, Boguszewski M, De Lacerda L, Savage M, Svensson E, Smith L, Weinberger D, Albertsson WK, Laron Z 2002 IGF-I is critical for normal vascularization of the human retina. *J Clin Endocrinol Metab,* **87:** 3413-3416.
70. Akeno N, Robins J, Zhang M, Czyzyk-Krzeska MF, Clemens TL 2002 Induction of vascular endothelial growth factor by IGF-I is osteoblast-like cellsis mediated by the PI3K pathway through the hypoxia-inducible factor-2α. *Endocrinology,* **143:** 420-425.
71. Ohlsson C, Nilsson A, Isaksson OGP, Lindahl A 1992 Effect of growth hormone and insulin-like growth factor-I on DNA synthesis and matrix production in rat epiphyseal chondrocytes in monolayer culture. *J Endocrinol,* **133:** 291-300.
72. Harvey AK, Yu XP, Frolik CA, Chandrasekhar S 1999 Parathyroid hormone (1-34) enhances aggrecan synthesis via an insulin-like growth factor-I pathway. *J Biol Chem,* **274:** 23249-23255.
73. Silbermann M, Maor G 1978 Mechanisms of glucocorticoid-induced growth retardation: impairment of cartilage mineralization. *Acta Anat,* **101:** 140-149.
74. Jones JI, Clemmons DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev,* **16:** 3-34.
75. Mauras N 2001 Growth hormone therapy in the glucocorticoidsteroid-dependent child: metabolic and linear defects. *Horm Res,* **56 (suppl 1):** 13-18.
76. Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL, Ballard FJ 1993 Insulin-like growth factor-I and more potent variants restore growth of diabetic rats without inducing all characteristic insulin effects. *Biochem J,* **291:** 781-786.
77. Tomas FM, Knowles SE, Owens PC, Chandler CS, Francis GL, Read LC, Ballard FJ 1992 Insulin-like growth factor-I (IGF-I) and especially IGF-I variants are anabolic in dexamethasone-treated rats. *Biochem J,* **282:** 91-97.

78. Rao R, Gregson RK, Jones AC, Miles EA, Campbell MJ, Warner JO 1999 Systemic effects of inhaled corticosteroids on growth and bone turnover in childhood asthma: a comparison of fluticasone and beclomethasone. *Eur Respir J,* **13:** 87-94.
79. Atsumi T, Miwa Y, Kimata K, Ikawa Y 1990 A chondrogenic cell line derived from a differentiation culture of AT805 teratocarcinoma cells. *Cell Differen Dev,* **30:** 109-116.
80. Shukunami C, Shigeno C, Atsumi T, Ishizeki K, Suzuki F, Hiraki Y 1996 Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. *J Cell Biol,* **133:** 457-468.

Summary

Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive drugs. The use of these potent drugs, however, often results in side-effects, such as growth retardation in children. For already many years, this GC-induced growth retardation is suggested to involve impaired action of the components of the growth hormone (GH) - insulin-like growth factor (IGF) system. The components of this system are among the main regulators of postnatal longitudinal bone growth. Longitudinal bone growth is regulated by the growth plates, layers of cartilage (containing cartilage cells, chondrocytes) located at the proximal and distal ends of the long bones. In the growth plates, the chondrocytes follow a tightly controlled program of differentiation (resulting in the different zones of the growth plate), which is part of the process of endochondral ossification, resulting in the formation of new bone. This bone formation is responsible for longitudinal bone growth. GC-induced growth retardation involves a mechanism intrinsic to the growth plate. However, not much is known about the effects of GCs on the components of the IGF system at the level of the growth plate. In this thesis, we studied the involvement of the IGF system in GC-induced growth retardation at the level of the growth plate, using several model systems.

In **chapter 2**, we used non-radioactive *in situ* hybridization to study mRNA expression of the components of the IGF system in lymphoid tissues of human (h) IGF-II transgenic mice. In these mice, high transgene expression was observed in the spleen and the thymus, resulting in increased growth of the thymus, but not of the spleen. The high IGF-II expression resulted in increased mRNA expression of several of the IGF binding proteins (IGFBPs), which modulate the bioactivity of both IGF-I and IGF-II. The increased expression of the IGFBPs suggest an important role for these proteins in the growth regulation of these lymphoid tissues.

To study the involvement of the IGF system in GC-induced growth retardation at the level of the growth plate, we treated 3-week-old mice with dexamethasone (DXM), a synthetic GC, for four weeks, which resulted in growth retardation. Subsequently, we studied the effects on the mRNA expression of the IGF system in the growth plate, as described in **chapter 3**. IGF-I, IGF-II and IGFBP-2 were present in the growth plates of control mice, predominantly in the pre- and hypertrophic zones. DXM treatment increased the number of IGF-I positive cells, whereas the number of chondrocytes expressing IGF-II and IGFBP-2 were not affected. In hIGF-II transgenic mice, which are partially protected against the adverse effects of GCs, the number of IGF-I positive chondrocytes was elevated, whereas IGF-II and IGFBP-2 expression was not affected. Subsequent DXM treatment of the hIGF-II transgenic mice further increased the number of IGF-I positive chondrocytes, to a similar extent as in the DXM-treated normal mice. These data suggest a possible role for IGF-I in GC-induced growth retardation. IGF-I could contribute to the GC-induced growth retardation by accelerating the endochondral ossification or it could be part of a compensatory mechanism to minimize the GC-induced growth retardation. We showed in **chapter 4** that already a short-term DXM treatment (one week) of 3-week-old mice resulted in growth retardation and defects of the growth plate. Total growth plate width decreased, mainly due to a decreased width of the proliferative zone. Moreover, the number of proliferating cell nuclear antigen (PCNA) immunopositive chondrocytes decreased. In the hypertrophic zone the number of

apoptotic chondrocytes increased. In these 4-week-old mice, IGF-I, IGF-II and IGFBP-2 were predominantly present in the proliferative and prehypertrophic zones. DXM treatment again only affected IGF-I. The number of IGF-I positive chondrocytes decreased, which could contribute to the observed GC-induced growth retardation.

We confirmed the short-term effects of GCs on the growth plates described in chapter 4, in the growth plates of piglets in **chapter 5**. Treatment with prednisolone (PRDL), another synthetic GC, of 6-week-old piglets for 5 days also resulted in a decrease of the total width of the growth plate, due to a decreased width of the proliferative zone. Also, in the hypertrophic zone, apoptosis increased. Apoptosis is closely linked to angiogenesis. This process of vascularization is important to allow the formation of new bone. PRDL treatment resulted in decreased VEGF expression in the growth plate, an important angiogenic growth factor. The decreased VEGF expression could be involved in the observed disturbed vascularization. Disturbed apoptosis and angiogenesis could contribute to GC-induced growth retardation. The regulation of VEGF by GCs was studied in more detail in **chapter 6**, using primary growth plate chondrocytes derived from neonatal piglets. VEGF expression and secretion were inhibited by GCs, which was mediated by the glucocorticoid receptor (GR).

In **chapter 7**, the same primary porcine growth plate chondrocytes were used to study the components of the IGF system in more detail. In contrast to the *in vivo* model, the chondrocytes expressed, besides IGF-I, IGF-II and IGFBP-2, also IGFBP-4, -5 and -6 as well as the type I and type II IGF receptor (not studied *in vivo*). DXM treatment inhibited the proliferation of the chondrocytes, which was mediated by the GR. Concomitant with the decreased proliferation, IGFBP-5 and type I IGF receptor mRNA levels increased, whereas IGFBP-2 mRNA levels decreased. In addition, DXM treatment also decreased IGFBP-2 mRNA and protein levels in serum-free culture conditions, whereas the other components were not affected. The decreased IGFBP-2 expression is possibly caused by inhibition of transcriptional activity of the IGFBP-2 promoter, as shown in the IGFBP-2 promoter studies in the **addendum of chapter 7**. The decreased IGFBP-2 levels could contribute to GC-induced growth retardation observed in this model system.

The data in this thesis suggest that the IGF system is indeed impaired at the local level, which could contribute to GC-induced growth retardation, as discussed in **chapter 8**. IGF-I might play a role in several GC-disturbed processes, as apoptosis and angiogenesis. In addition, IGF-I could have a dual action in GC-induced growth retardation. In the initial phase of the GC treatment, decreased IGF-I levels could contribute to GC-induced growth retardation, whereas later on in the treatment, increased IGF-I levels might become part of a compensatory mechanism.

Samenvatting

Glucocorticoïden (GCs) worden veel gebruikt als ontstekingsremmers en voor het onderdrukken van de immuunreactie. Het gebruik van deze medicijnen geeft vaak bijeffecten, zoals groeivertraging bij kinderen. Deze GC-geïnduceerde groeivertraging wordt al sinds enige jaren toegeschreven aan een verstoorde werking van de componenten van het groeihormoon (GH) en insuline-achtige groeifactor (IGF) systeem. De componenten van dit systeem zijn een van de belangrijkste regulatoren van de postnatale lengtegroei. Lengtegroei van de botten wordt gereguleerd door de groeischijven, kraakbeenplaten (bestaande uit kraakbeencellen, de chondrocyten), die zich aan de uiteinden van de pijpbeenderen bevinden. In deze groeischijven ondergaan de chondrocyten een differentiatie programma, wat leidt tot de verschillende zones die aanwezig zijn in de groeischijf. Dit differentiatie programma is een onderdeel van het proces endochondrale ossificatie, dat resulteert in de vorming van nieuw bot. Deze botvorming is verantwoordelijk voor de lengtegroei van het bot. Er is aangetoond dat de GC-geïnduceerde groeivertraging plaatsvindt in de groeischijf, wat dus wijst op een lokaal mechanisme. Er is echter nog weinig bekend van de effecten van GCs op de componenten van het IGF systeem in de groeischijf. In dit proefschrift hebben we de mogelijke rol van de componenten van het IGF systeem in GC-geïnduceerde groeivertraging op het niveau van de groeischijf bestudeerd, waarbij we gebruik hebben gemaakt van verschillende modelsystemen.

In **hoofdstuk 2** hebben we gebruik gemaakt van niet-radioactieve *in situ* hybridisatie voor het analyseren van de mRNA expressie van de componenten van het IGF systeem, in de milt en de thymus van humaan (h) IGF-II transgene muizen. In deze hIGF-II transgene muizen kwam het hIGF-II transgen hoog tot expressie in de milt en de thymus, wat bij de thymus, maar niet bij de milt, leidde tot versnelde groei. De aanwezigheid van het transgen in deze lymfoïde organen leidde tot een toename in mRNA expressie van een aantal IGF bindende eiwitten (IGFBPs), in de milt meer dan in de thymus. Deze IGFBPs moduleren de bioactiviteit van de IGFs door te binden aan de IGFs. De toename in expressie van deze IGFBPs suggereert dat ze een belangrijke rol spelen in de regulatie van de groei van deze lymfoïde organen.

Om de betrokkenheid van het IGF systeem in GC-geïnduceerde groeivertraging op het niveau van de groeischijf te bestuderen, hebben we muizen van 3 weken oud, 4 weken lang behandeld met dexamethason (DXM) (een synthetisch GC), wat leidde tot groeivertraging. Vervolgens hebben we gekeken naar de effecten van deze GC behandeling op de mRNA expressie van de componenten van het IGF systeem in de groeischijf, zoals beschreven in **hoofdstuk 3**. IGF-I, IGF-II en IGFBP-2 waren de 3 componenten die aanwezig waren, voornamelijk in de prehypertrofe en de hypertrofe zone van de groeischijf. DXM behandeling resulteerde in een toename van het aantal IGF-I positieve chondrocyten, terwijl IGF-II en IGFBP-2 mRNA expressie niet beïnvloed werd. In hIGF-II transgene muizen, die gedeeltelijk beschermd zijn tegen de negatieve effecten van GCs, waren dezelfde 3 componenten aanwezig in de groeischijf, maar er was een toename in het aantal IGF-I positieve chondrocyten. DXM behandeling van deze hIGF-II transgene muizen resulteerde vervolgens in een verdere toename van het aantal IGF-I positieve cellen, tot hetzelfde niveau als in de normale DXM behandelde muizen. Deze gegevens suggereren een mogelijke rol voor IGF-I

in GC-geïnduceerde groeivertraging. De toename van IGF-I zou kunnen bijdragen aan de groeivertraging door het versnellen van de endochondrale ossificatie, of juist onderdeel kunnen zijn van een compensatie mechanisme waarbij een toename van IGF-I de groeivertraging zou verminderen. In **hoofdstuk 4** hebben we laten zien dat een korte DXM behandeling (1 week) van muizen van 3 weken oud al leidde tot groeivertraging en ook al een effect had op de groeischijf. De dikte van de groeischijf nam af, doordat de dikte van de proliferatieve zone afnam. De afname van de proliferatieve zone correleerde met de afname in het aantal chondrocyten positief voor proliferating cell nuclear antigen (PCNA), een marker voor prolifererende cellen. In de terminale hypertrofe zone nam het aantal apoptotische chondrocyten toe als gevolg van de DXM behandeling. IGF-I, IGF-II en IGFBP-2 mRNAs kwamen nu met name tot expressie in de proliferatieve en prehypertrofe zone van de groeischijf. Weer was IGF-I de enige component die beïnvloed werd door DXM, de mRNA expressie nam af. De afgenomen IGF-I mRNA expressie zou kunnen bijdragen aan de waargenomen GC-geïnduceerde groeivertraging.

De waargenomen korte termijn effecten in hoofdstuk 4, hebben we bevestigd in de groeischijven van biggen van 6 weken oud in **hoofdstuk 5**. Behandeling met prednisolon (PRDL) (een ander synthetisch GC) gedurende 5 dagen, leidde tot een soortgelijke afname in de dikte van de groeischijf als waargenomen bij de muis, ook hier als gevolg van een afname in de dikte van de proliferatieve zone. Ook hier nam het aantal apoptotische cellen toe in de hypertrofe zone van de groeischijf. Het proces van apoptose in de groeischijf is nauw verbonden met angiogenese, het proces van bloedvatvorming nodig voor de vorming van nieuw bot. Een groeifactor die erg belangrijk is voor angiogenese, VEGF (vasculaire endotheel groeifactor), nam af in mRNA en eiwit expressie in de groeischijf door de PRDL behandeling. Deze afname in VEGF expressie zou betrokken kunnen zijn bij de waargenomen verstoorde bloedvatvorming in het bot onder de groeischijf van de PRDL behandelde biggen. De verstoorde angiogenese en apoptose zouden kunnen bijdragen aan GC-geïnduceerde groeivertraging. De regulatie van VEGF door GC hebben we nader bestudeerd in **hoofdstuk 6**. In dit hoofdstuk hebben we gebruik gemaakt van primaire groeischijf chondrocyten, afkomstig van de groeischijven van neonatale biggen. De VEGF expressie en secretie door chondrocyten werd geremd door verschillende GCs, hetgeen gemedieerd werd door de glucocorticoïd receptor (GR).

In **hoofdstuk 7** hebben we dezelfde primaire biggenchondrocyten gebruikt om ook de regulatie van de componenten van het IGF systeem door GCs meer in detail te kunnen bestuderen. In tegenstelling tot ons muizen *in vivo* model, brachten deze primaire biggenchondrocyten naast IGF-I, IGF-II en IGFBP-2, ook andere IGFBPs zoals IGFBP-4, -5 en -6, als ook beide type IGF receptoren (hebben we niet bestudeerd in het *in vivo* model) tot expressie. DXM behandeling van deze chondrocyten resulteerde in remming van de proliferatie, wat gemedieerd werd door de GR. Samen met de afname in proliferatie, hebben we een toename in IGFBP-5 en type I IGF receptor mRNA expressie aangetoond, terwijl IGFBP-2 mRNA expressie afnam. IGFBP-2 was de enige component die ook nog veranderde op eiwit niveau onder serum-vrije condities. De afname van IGFBP-2 expressie kon, in ieder geval gedeeltelijk, worden verklaard door een afname in transcriptionele activiteit van de IGFBP-2 promoter, zoals aangetoond in de promoterstudies van IGFBP-2 in het **addendum bij hoofdstuk 7**. De afname van IGFBP-2 zou kunnen bijdragen aan de waargenomen GC-geïnduceerde groeivertraging in dit model systeem.

De beschreven data in dit proefschrift tonen aan dat de expressie van het IGF systeem inderdaad verstoord is door GCs op het niveau van de groeischijf, wat zou kunnen bijdragen

aan de GC-geïnduceerde groeivertraging, zoals we bediscussieren in **hoofdstuk 8**. IGF-I zou hierbij een rol kunnen spelen in diverse processen die verstoord zijn door de GC behandeling, zoals in apoptose en angiogenese. Daarnaast zou IGF-I op 2 manieren betrokken kunnen zijn bij de GC-geïnduceerde groeivertraging. Aanvankelijk zou een afname van IGF-I, zoals waargenomen in het begin van de GC behandeling, kunnen bijdragen aan de groeivertraging. Later in de behandeling, als IGF-I toeneemt, zou IGF-I betrokken kunnen zijn bij een compensatie mechanisme om de groeivertraging te minimaliseren.

Dankwoord

Alleen nog deze 2 laatste pagina's en dan is ook mijn proefschrift afgerond. Reden om te beginnen met waar het eindigt. Ik wil iedereen bedanken die, op welke manier dan ook, betrokken is geweest bij het tot stand komen van dit proefschrift. En dan natuurlijk niet alleen diegenen die hebben bijgedragen aan het ontstaan van de 55.000 woorden die de 8 hoofdstukken van dit boekje vormen, maar ook de mensen die ervoor hebben gezorgd dat mijn "wereld" de afgelopen 4½ jaar verder reikte dan de groeischijf.

Daarnaast zijn er ook een aantal mensen die ik nog speciaal wil noemen. Als eerste wil ik mijn co-promotor Dr. Sylvia van Buul-Offers bedanken. Sylvia, jij hebt de afgelopen 4½ jaar, en ook daarvoor tijdens mijn stage bij het WKZ, altijd veel interesse getoond in zowel mijn labwerk als alles wat daarnaast speelde. Ook de mogelijkheid die ik van jou kreeg om elk jaar op congres te gaan zorgde voor gezellige en leerzame momenten, bedankt. Mijn andere co-promoter, Dr. Joost Koedam wil ik ook bedanken. Joost, ik heb met jou altijd plezierig kunnen samenwerken op het lab, vooral als er weer eens varkenspootjes gehaald moesten worden. Ik wil je ook bedanken voor jouw hulp bij het schrijven van artikelen. Prof. Dr. Ruud Berger wil ik bedanken voor het mogelijk maken van mijn promotie. Ruud, ook al werd je pas 2 jaar geleden betrokken bij mijn project, ik heb jouw inbreng altijd erg gewaardeerd. Na elke bespreking met jou, ging ik altijd weer een stuk opgewekter aan de slag, bedankt.

Ik wil ook een aantal mensen bedanken die gezorgd hebben voor het "biggen" materiaal en zonder wie dit proefschrift wel erg "muizerig" was geworden. Voor de neonatale biggenpootjes, Dr. Cacha Peeters en Dr. Floris Groenendaal en in het laatste jaar Jan van Vliet, Jan Berends & Jan van Mourik van de Tolakker en voor de prepuberale biggenpoten: Ivo Buchholz, Dr. Ralph Sakkers en Dr. Kees de Meer. Dr. Marcel Karperien (LUMC) wil ik bedanken voor alle tips en de experimenten die we gezamenlijk hebben uitgevoerd, als ook Dr. Bram van der Eerden voor alle "groeischijf-tips" en voor alle metingen die we in Leiden achter de microscoop hebben kunnen uitvoeren. Prof. Dr. Dick de Rooij en Dr. Elly Holthuizen wil ik bedanken voor het deelnemen in mijn AIO commissie de afgelopen 2 jaar. Elly, ook nog bedankt voor alle hulp en tips bij het opzetten en uitvoeren van de IGFBP-2 promoterstudies.

Ria, dankzij jouw snijkunst van coupes en ook later bij de in situ's, zijn er heel wat mooie plaatjes van groeischijven gecreëerd. Bedankt voor de gezelligheid en alles wat ik van je geleerd heb. Nicole, samen met Ria heb je de afgelopen zomer voor een hoogtepunt gezorgd, terug te vinden in hoofdstuk 4. Bedankt voor jouw (eigenzinnige) inbreng het afgelopen jaar. Robert-Jan, de ATDC5 cellen zal je niet snel vergeten en zij jou ook niet, bedankt voor je pionierswerk met de mineralisatie experimenten. Cok, bedankt voor al het eiwit werk. De studenten, Korneel, Chantal en Liselotte en de "scriptie-studenten" Astrid en Suzanne, bedankt voor jullie bijdragen aan mijn promotie onderzoek en voor de gezelligheid, het was leuk en leerzaam om met jullie samen te werken. Natuurlijk ook alle anderen van het moleculaire lab: Mariëtte, Jet, Truus, Brenda, Gitte, Hugo, Krista, Dennis en ook Jaap, Maria, Monique, Maarten en iedereen van de diagnostiek, bedankt voor de gezelligheid. De "borrel-unit": Gitte, Annemieke, Wendy en Harry, het was altijd erg gezellig. Bij het lab Metabole Ziekten was het altijd gezellig buurten en ook erg handig als er bij ons weer eens iets op was, dames bedankt.

Dan zijn er ook nog de lotgenoten van het eerste uur: Harry, François en Joost, ik heb heel wat lief en leed mogen delen met deze “heren”. Harry, we kwamen dezelfde dag (in januari 1997), al snel hadden we samen de boel overzien en binnen “no time” hadden we het lab veroverd! Waar jij was, daar volgde ik, het liefst met een spuitwaterfles in de hand of anders wat ijs. Waar ik was, volgde jij, wat vervolgens resulteerde in een zooitje op het lab. We hebben samen altijd erg veel plezier gehad (en nog steeds), maar ook in het serieuzere deel van het “AIO-zijn” konden we elkaar vinden, wat ondertussen is uitgegroeid tot een vriendschap buiten het lab. Bedankt dat je mijn paranimf bent en vooral voor al het plezier dat jij hebt weten te brengen in het “AIO-zijn”. François (kalmte zal je redden), jij zorgde voor de rust, kalmte en relativering in de AIO kamer (wat af en toe ook wel nodig was, in ieder geval voor mij). Aan “peptalk” heb ik dankzij jou nooit een tekort gehad en ook je niet aflatende humor heeft daar aan bijgedragen. Bedankt dat je mijn paranimf bent en ook voor alle gezellige uurtjes buiten het lab. Joost, ik wil ook jou bedanken voor de gezellige tijd in ons “AIO-hokje”, en nog bedankt dat je de boel niet heb laten “uitbranden” voordat Harry en ik ook helemaal klaar waren.

Ik wil ook alle vrienden bedanken voor de ontspannende en gezellige momenten die er altijd waren en hopelijk altijd zullen zijn. “Meiden van Banyuls”: Arzu, Brechje, Giulietta, Machteld en Saskia, of het nu Sinterklaas in april is of koninginnedag, Rotterdam of Utrecht, een avond met jullie zorgt altijd voor het nodige plezier en mijn verhaal kan ik ook altijd bij jullie kwijt, bedankt. Hanne, Esther en Bonnie, het begon allemaal op de atletiekbaan, maar is ondertussen verplaatst naar Catan. De weekenden samen weg zijn altijd erg gezellig (net als de etentjes), en zorgen voor de nodige ontspanning, bedankt.

Tenslotte wil ik mijn ouders bedanken, pap en mam, bedankt voor alles wat ik van jullie heb meegekregen (inclusief de boerenkool.....), de mogelijkheid om dit te kunnen bereiken en alle steun in de afgelopen jaren. Gerlinde en Magrèt, ook bij jullie kon ik vaak een luisterend oor vinden als ook de gezelligheid, bedankt (Manus en Irène natuurlijk ook bedankt). En Fleur, jij zorgde het afgelopen jaar voor een glimlach op mijn gezicht als het allemaal wat tegenzat. Lieve Giulietta, samen ’s ochtends op de fiets naar de Uithof, ’s avonds weer samen laat terug, en altijd maar weer het geduld om naar mijn “verhalen” te luisteren. Van Banyuls-sur-Mer tot Lunetten, van mijn proefschrift naar jouw proefschrift, samen zullen we nog vele reizen maken.

Jeske

Curriculum Vitae

Jeske J. Smink werd geboren op 22 februari 1975, te Amersfoort. Na het behalen van het atheneum diploma op 't Hooghe Landt College in 1993 in Amersfoort, begon zij in datzelfde jaar met de studie Biologie aan de Universiteit Utrecht. Tijdens haar studie, doorliep zij in 1996 een stage bij de afdeling Experimentele Embryologie van de faculteit Biologie, te Utrecht (Dr. A.E. van Loon). Hierop volgde in 1997 een stage bij de afdeling Pediatrische Endocrinologie van het Wilhelmina Kinder Ziekenhuis te Utrecht (Dr. J.G. Koster en Dr. S.C. van Buul-Offers). Deze stage werd in 1998 gevolgd door een stage aan de Vrije Universiteit Brussel (Brussel, België) bij de afdeling Farmacologie van de Medische School (Dr. R. Kooijman), waarna zij in augustus 1998 het doctoraal diploma Biologie behaalde (cum laude).

Vanaf september 1998 werd zij aangesteld als assistent in opleiding bij de afdeling Pediatrische Endocrinologie van het Wilhelmina Kinder Ziekenhuis, Universitair Medisch Centrum Utrecht, onder leiding van Dr. S.C. van Buul-Offers, Dr. J.A. Koedam en Prof. Dr. R. Berger (afdeling Metabole Ziekten). De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

Jeske J. Smink was born on February 22nd 1975 in Amersfoort, the Netherlands. She attended " 't Hooghe Landt College" secondary school in Amersfoort from 1987 to 1993. In 1993 she started to study Biology at the University of Utrecht. During her university training she performed several research projects. In 1996 she performed a research project at the department of Experimental Embryology of the University of Utrecht (Dr. A.E. van Loon). This was followed by a research project at the department of Pediatric Endocrinology of the Wilhelmina Children's Hospital (Utrecht) in 1997 (Dr. J.G. Koster and Dr. S.C. van Buul-Offers). At the Free University of Brussels (Belgium) she performed a research project in 1998 at the laboratory of Pharmacology (Faculty of Medicine and Pharmacy) (Dr. R. Kooijman). In august 1998 she graduated as a MSc in Biology (cum laude).

In september 1998 she started as a PhD student at the department of Pediatric Endocrinology of the Wilhelmina Children's Hospital, University Medical Center Utrecht under the supervision of Dr. S.C. van Buul-Offers, Dr. J.A. Koedam and Prof. Dr. R. Berger (department of Metabolic Diseases) on the project entitled "Glucocorticoid-induced effects on the growth plate and the IGF system". The results of the research she performed as a PhD student are described in this thesis.

List of publications

J.J. Smink, J.G. Koster, B.I. Hendriks-Stegeman and S.C. van Buul-Offers 1999. Insulin-like growth factor (IGF) II induced changes in expression of IGF binding proteins in lymphoid tissues of hIGF-II transgenic mice. *Endocrinology*, **140**: 5876-5882.

J.J. Smink, J.A. Koedam, J.G. Koster and S.C. van Buul-Offers 2002. Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied by changes in levels of IGF axis components. *Journal of Endocrinology*, **174**: 343-352.

J.J. Smink, J.G. Koster, M.G. Gresnigt, R. Rooman, J.A. Koedam and S.C. van Buul-Offers 2002. IGF and IGFBP expression in the growth plate of normal, dexamethasone-treated, and hIGF-II transgenic mice. *Journal of Endocrinology*, **175**: 143-153.

J.A. Koedam, **J.J. Smink** and S.C. van Buul-Offers 2002. Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. *Molecular and Cellular Endocrinology*, **197**: 35-44.

J.J. Smink, M.G. Gresnigt, N. Hamers, J.A. Koedam, R. Berger and S.C. van Buul-Offers 2003. Short-term glucocorticoid treatment of prepubertal mice decreases growth and IGF-I expression in the growth plate. *Journal of Endocrinology* (conditionally accepted).

J.J. Smink, I.M. Buchholz, N. Hamers, C.M. van Tilburg, C. Christis, R.J.B. Sakkers, K. de Meer, S.C. van Buul-Offers and J.A. Koedam 2003. Short-term glucocorticoid treatment of piglets causes changes in growth plate morphology and angiogenesis. *Submitted for publication*.