TRANSLATIONAL STUDIES IN GROWTH PLATE RESEARCH

THE EFFECT OF GLUCOCORTICOIDS AND GROWTH FACTORS ON THE GROWTH PLATE

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DEDICATION

This thesis is dedicated to my wife, Zarrin, my parents and my children, Hamzah, Sara and Haris who have provided me with the motivation and support to see this project to completion.

DECLARATION

No part of this thesis has been submitted in support of an application for another degree or qualification of this or any other University.

I helped to collate the results and write the initial clinical paper of these studies. This was the stimulus to embark on the thesis and subsequently all the work, other than that acknowledged below was performed by myself (Talat Mushtaq).

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ABBREVIATIONS

6MP	6-Mercaptopurine (6MP)
6TG	6-Thioguanine (6TG)
11ß-HSD	11ß-Hydroxysteroid Dehydrogenase
ACTH	Adrenocorticotropic hormone
AF	N-terminal Activation Function Domain
Ara-C	Cytarabine
ALL	Acute Lymphoblastic Leukaemia
ALP	Alkaline Phosphatase
ALS	Acid Labile subunit
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
bALP	Bone Alkaline Phosphatase (bALP)
BrdU	5-Bromo-2-deoxyuridine
CAH	Congenital Adrenal Hyperplasia
CRH	Corticotrophin Releasing Hormone
CRL	Crown Rump Length
CUG	Catch up Growth
Dex	Dexamethasone
DBD	Deoxypyridinoline
GC	Glucocorticoid
GCs	Glucocorticoids
GCR	Glucocorticoid Receptor
GCRA	Glucocorticoid Receptor Antagonist
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor

FGFR	Fibroblast Growth Factor Receptor
GH	Growth Hormone
GHR	Growth Hormone Receptor
HPA	Hypothalamic-Pituitary-Adrenal
HZ	Hypertrophic Zone
hsp 90	heat shock protein 90
Ihh	Indian Hedgehog
IGF-I	Insulin-like Growth Factor-1
IGF-II	Insulin-like Growth Factor-II
IGFBP	Insulin-like Growth Factor Binding Protein
IGFBP-rP	Insulin-like Growth Factor related Peptide
IGFs	Insulin-like Growth Factors
IT	Intrathecal
iv	Intravenous
IUGR	Intrauterine Growth Retardation
Jak2	Janus Kinase 2
JIA	Juvenile Idiopathic Arthritis
LBD	Ligand Binding Domain
LLL	Lower Leg Length
LLLV	Lower Leg Length Velocity
MZ	Mineralising Zone
MTX	Methotrexate
pNPP	p-nitrophenyl phosphate
PBS	Phosphate Buffered Saline
PZ	Proliferating Zone
PTH	Parathyroid Hormone

PTHrP	Parathyroid Hormone related Peptide
Pred	Prednisolone
sem	standard error of the mean
SD	Standard Deviation
SDS	Standard Deviation Scores
SGA	Small for Gestational Age
SGRM	Selective Glucocorticoid Receptor Modulators
SI	International System of Units
TE	Technical Error
TGFß	Transforming Growth Factor ß

ABSTRACT

Glucocortiocids (GCs) regulate many physiological systems in the body and have potent immunosuppressive and anti-inflammatory properties. In children prolonged administration causes a reduction in growth, which is in part due to the direct effects they have on the growth plate. This effect is dependent on the dose, duration and type of GCs used.

This thesis consists of four major types of studies each utilising different models of growth and chondrocyte biology, which in combination strengthens the understanding of the effects of GC and growth factors on the growing skeleton.

The initial *in vivo* study showed that in children treated with Dexamethasone (Dex) or Prednisolone (Pred) for Acute Lymphoblastic Leukaemia, the effects of Dex on body composition were more apparent in that it was up to 18 times more potent at reducing short term linear growth than Pred.

The ATDC5 chondrocyte cell line was fully characterised, which allowed a unique opportunity to study GC effects on a homogeneous population of chondrocytes at the chondrogenesis and terminal differentiation phases. The GCs caused a reduction in cell number, cell proliferation and proteoglycan content whilst stimulating chondrocyte differentiation. These effects were dose dependent and only observed during the chondrogenesis phase when the cells are rapidly dividing. Furthermore these negative effects could be partially reversed with the use of a GC receptor antagonist and completely reversed with IGF-I.

These observations were further translated into increasingly physiological models of bone growth. Foetal mouse metatarsal organ explants, where the three dimensional structure and cell connections of the growing bone remain intact, again demonstrated that Dex and IGF-I had opposite effects on bone growth. The length of the metatarsals at day 10 from harvesting day length in the control, Dex and IGF-I bones was 50% ± 3, 42% ± 2,

($p<0.05$) 99.3% ± 5($p<0.05$) respectively. In contrast to Dex the effects of IGF-I were immediate. Most importantly, it was demonstrated for the first time *in vitro* that IGF-I increased the size of the hypertrophic zone, as occurs *in vivo*, and this accounted for most of the increase in metatarsal length.

Prenatal administration of Dex caused a reduction in birth weight and length and this difference was greater in the female mice. The growth restriction was associated with elevated IGF-I and IGFBP-2 levels raising the possibility of a state of IGF-I insensitivity, which may explain subsequent growth failure.

In conclusion, this thesis translated the clinical observation that Dex is more potent than Pred at inhibiting linear growth and these effects are dependent on the dose and duration of the GC exposure as well as the chondrocyte phenotype. These negative effects of GC can be reversed by IGF-I administration.

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Best Poster ($500): for Best Experimental Study:- The Effects Of Growth Factors, Sex Steroids and Dexamethasone On Chondrocyte Proliferation. *33rd International Symposium, GH and Growth Factors in Endocrinology and Metabolism. Barcelona, **2002**.*

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND TO THESIS

Glucocorticoids (GC) are used extensively in many childhood diseases, including autoimmune and inflammatory conditions. It is estimated that 10% of children may require some form of GC treatment during childhood (Warner, 1995). Their use has undoubtedly led to improved survival of children with a number of chronic and life threatening illnesses, albeit at the expense of adverse growth and skeletal development. Children are prone to all the systemic effects of GC but additionally also show a retardation in linear growth attributed to the effect of GC on the chondrocytes within the growth plate. This is likely to be due to systemic, autocrine and paracrine effects.

1.2 BONE BIOLOGY

1.2.1 Bone

The word skeleton derives from the Greek word meaning 'dried up body', it is composed of bones, cartilages, joints and ligaments accounting for 20% of the body mass. As well as performing a vital role in body support, which is essential for locomotion it protects vital organs such as the heart, lungs and brain and acts as a reservoir for minerals such as calcium and phosphate that are made available for physiological requirements or pathological disturbances. Bone is a complex tissue made up of living cells enmeshed in a mineralised collagenous rich matrix. The inorganic mineral provides strength and resists compression whereas the organic collagen fibres withstand tension and torsion (Farquharson, 2003) such that the tensile strength of bone approaches that of cast iron, and its capacity to absorb and release energy is twice that of oak, yet the weight of bone is only one third that of steel (Martin & Burr, 1989). The bone marrow that lies within the long bones provides an environment for haematopoiesis during postnatal life (Beresford, 1989).

Most long bones have the same general structure. The diaphysis or shaft constitutes the long axis of bone. This is constructed of a thick collar of compact bone surrounding the medullary cavity. The ends of the diaphysis are expanded and termed the metaphysis and in turn these are flanked by the proximal and distal epiphyses, which are the cartilaginous portions of bone that ossify at puberty.

1.2.2 Bone Cells

There are three distinct type of cells found within bone: 1) the **Osteoblasts** which synthesise and regulate the deposition and mineralisation of the extracellular matrix of bone. These cells have a life span of up to 8 weeks in humans, during which time they lay down osteoid; this includes proteoglycans, glycoproteins and collagen fibres. Eventually they become trapped in their own calcified matrix, changing their phenotype and developing into osteocytes. 2) **Osteocytes**, account for 90% of all cells in the adult skeleton and although derived from osteoblasts are distinctly different in function. They are regularly placed within the mineralised matrix and are connected with each other via long slender cell processes, which provide much of the support network for the bones. 3) **Osteoclasts** are derived from haematopoietic stem cells and their main feature is the ability to absorb mineralised bone and cartilage. Osteoblasts and osteoclasts are involved in the complex process of bone remodelling whereby old bone is replaced by newly formed bone, thus allowing the bones to respond to and adapt to mechanical stresses and repair any microdamage. It is estimated that it may take 4 to 5 years for bones to remodel completely (Ott, 1996).

1.2.3 Bone Growth

Bones grow in two directions; through a cartilage template to increase their length (**longitudinal or endochondral growth**), and through the formation of new bone on the

outer surfaces of existing bone to increase their width (**appositional growth**). This work will focus on the endochondral process as it provides for the elongation of most of the skeletal mass during growth and is ultimately connected with overall body growth.

The mechanisms of long bone growth are similar across many animal species. There are, however, major variations in the growth rate between similar bones of different species, bones of an individual animal and of the two growth plates within the same bone.

1.2.4 Embryonic Bone

Embryonic bone formation occurs through two distinct processes. Intramembranous growth results in the formation of flat bones such as the cranium, mandible and scapula whereas the process of endochondral growth accounts for the formation of long bones, such as the tibia, femur and humerus.

Long bones of the skeleton first appear as limb buds and the earliest observable morphological event in this process (between 10.5 and 12.5 days post-coitum in the embryonic mouse) is the aggregation of committed, undifferentiated mesenchymal cells into structures known as precartilage condensations. These cells differentiate into chondrocytes and secrete extracellular matrix resulting in the formation of a cartilaginous template of the future bone. Concomitant with this, other mesenchymal cells at the periphery of the template differentiate to form a perichondrial sheath (Fig 1.1). This primary bone collar is penetrated by blood vessels that gain access to the underlying cartilage template, bringing elements that will form the bone marrow together with osteoclasts that erode the internal calcified cartilage (Howell & Dean, 1992). Osteoclastic resorption continues towards both ends of the template forming the primary ossification centre with osteoblasts replacing the eroded cartilage with new lamellar bone. Around birth in mammals a secondary ossification centre develops in the cartilage of the epiphyseal region and a transverse flat disc of cartilage situated between the two centres of

ossification forms the epiphyseal growth plate and assumes the specialised function of elongation and growth during postnatal bone formation (Farquharson, 2003) (Fig 1.1).

1.2.5 Endochondral ossification

Longitudinal bone growth is a multistep process whereby the chondrocyte stem cells at the ends of the long bones undergo an orderly series of events resulting in proliferation, differentiation, hypertrophy and finally mineralisation, leading to the replacement of the cartilage by bone. Undifferentiated progenitors within the reserve stem cell zone differentiate into chondrocytes and progress through a proliferative phase. In the proliferative zone, the cells have a flattened, oblate shape (Fig 1.1f). Immediately after the cessation of cell division the cells change to a spherical prolate form and undergo terminal differentiation into hypertrophic chondrocytes, (Breur *et al*, 1994) where the chondrocytes become more voluminous with increases in rough endoplasmic reticulum and Golgi apparatus, reflecting increased matrix production (Buckwalter *et al*, 1986) (Fig 1.1f). Associated with this hypertrophic phenotype are increased membrane alkaline phosphatase activity (ALP) and expression of collagen type X, chondrocalcin, osteonectin and osteopontin as well as the down regulation of collagen type II expression. The volume of hypertrophic chondrocytes is approximately 10 times larger than the volume of proliferative chondrocytes, and chondrocyte height increases up to fivefold (in the direction of growth) between the proliferative and hypertrophic zones (Hunziker *et al,* 1987). During terminal differentiation, mineralisation of the matrix surrounding the hypertrophic chondrocytes occurs. Functionally the matrix changes to an environment allowing vascular invasion (Buckwalter *et al*, 1983) and the hypertrophic chondrocytes undergo apoptosis to leave lacunae separated by cartilaginous septae that become calcified and form a scaffold for new bone formation. Histologically, the chondrocytes are arranged in columns that parallel the longitudinal axis of the bone. Each column and each

chondrocyte within a column are respectively separated by longitudinal and transverse septa made up of a collagenous and proteoglycan rich extracellular matrix (Fig 1.1) Consequently, the growth plate can be divided into several distinct zones containing resting, proliferating, maturing and terminally differentiated hypertrophic chondrocytes (Fig 1.1g).

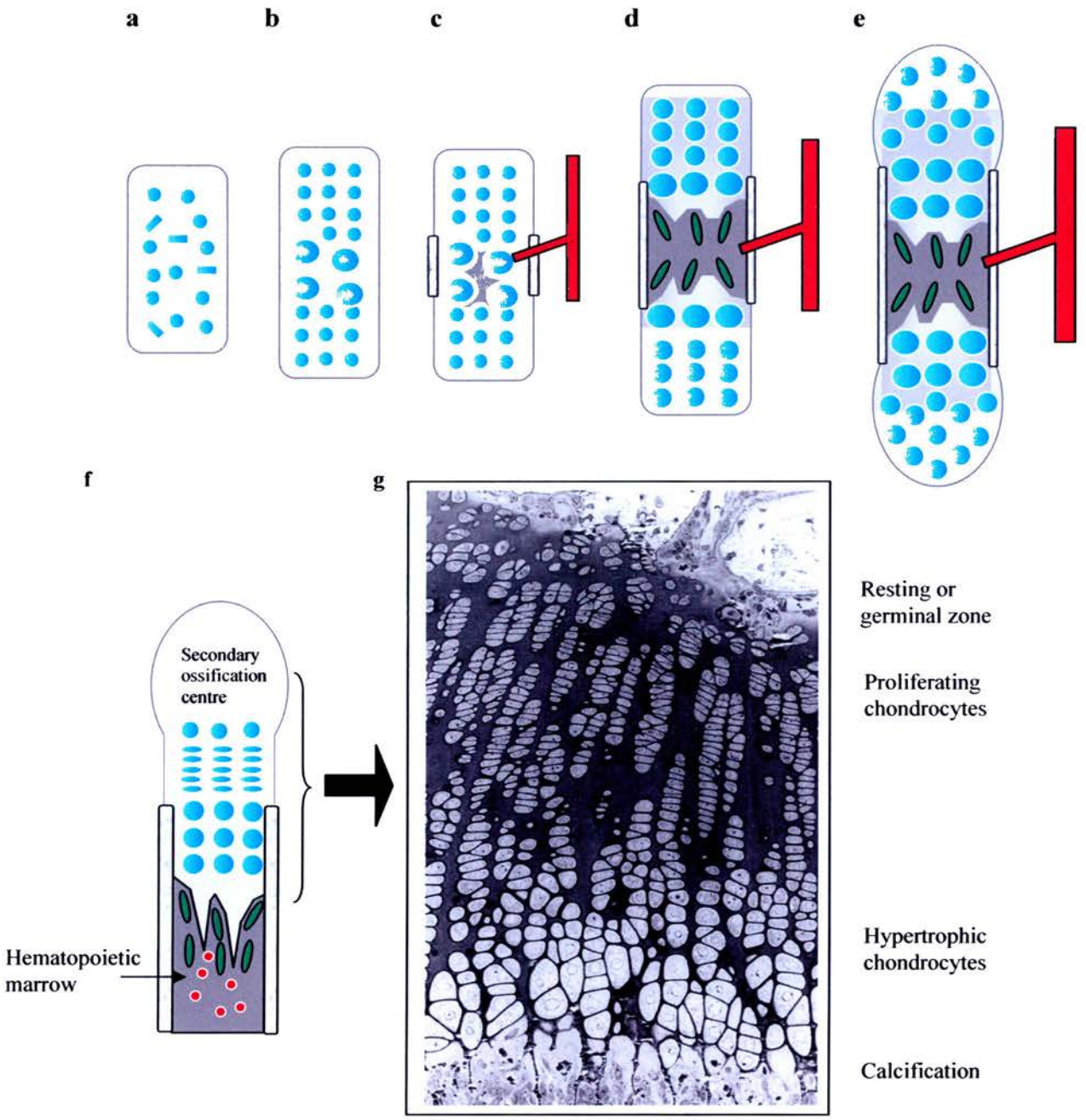
The contribution of individual growth plates to the growth rates of long bones varies enormously. Human bones grow extremely slowly and the growth rate of the distal femur has been estimated at 0.04 mm/day (Farquharson, 2003) to 0.22 mm/day in the rat and 0.39mm/day in the rabbit (Kember *et al,* 1976, 1983). The rate of bone growth attributed to a specific growth plate is determined by a complex interplay of proliferative kinetics, matrix production and hypertrophic chondrocyte enlargement (Breur *et al*, 1991). The chondrocyte proliferation rate and the size of the proliferative pool correlate positively with growth rates; cell duplication is more significant at faster rates of growth whereas matrix synthesis is more significant at slower rates of growth. The final hypertrophic volume and the rate of bone growth also correlate closely and have been shown to have a positive linear relationship (Breur *et al,* 1991). This variation in hypertrophic cell volume accounts for the different growth rates between species and also the different growth rates that occur at the proximal and distal ends within the same long bone (Hunziker *et al*, 1987).

1.2.6 Matrix proteins

Collagen fibres are constructed primarily of fibrous proteins, which are a triple helix of three polypeptide chains. These form strong rope like linear structures, that are insoluble in water and very stable, thus ideally suited for providing mechanical support and tensile strength. Whereas type I collagen is the most abundant protein in bone, collagen type II is the principal structural protein of the growth plate cartilage. It interacts with collagen

types IX and XI to form heterotypic fibrils that are distributed throughout the cartilage matrix (Mwale *et al*, 2002). During chondrocyte maturation, collagen type II gene expression decreases and the hypertrophic chondrocytes initiate the synthesis of collagen type X – a protein unique to this cell type (Schmid & Linsenmayer, 1985). This collagen type is a non-fibrillar, short chain collagen and it is thought to provide a structural role in maintaining the organization and mechanical properties of the matrix (Chan & Jacenko, 1998). There are also a number of proteoglycans, of which aggrecan predominates, that form a network that fills the space between the collagen fibrils in the matrix and non-collagenous proteins such as osteopontin and osteonectin within the growth plate matrix (Pacifici *et al*, 1990; Byers *et al*, 1992). As chondrocytes differentiate and hypertrophy, simultaneous changes also occur within the extracellular matrix, furthermore the chondrocytes must provide the correct extracellular network and establish cell-matrix interactions to allow progressive differentiation. This observation is consistent with a change from aggrecan to decorin and biglycan synthesis during normal chondrocyte maturation (Bianco *et al*, 1990).

Figure 1.1. Endochondral Bone Formation



Endochondrol bone formation: **a)** mesenchymal cells condense to become chondrocytes; **b)** the chondrocytes proliferate and the central ones become hypertrophic **c)** the perichondrial cells adjacent to the hypertrophic chondrocytes become osteoblasts and form a bone collar. These then direct the formation of mineralised matrix and attract blood vessels; **d)** formation of primary spongiosa; **e)** osteoblasts of the bone collar become cortical bone, whereas those in the primary spongiosa are the precursors of trabecular bone. Chondrocytes continue to proliferate, lengthening the bone; **f)** secondary ossification centre forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. The proliferating chondrocytes arrange themselves into orderly columns and the haematopoetic marrow expands into the marrow space; **g)** photomicrograph of a growth plate illustrating the columnar appearance of the proliferating chondrocytes and the prolate morphology of the hypertrophic chondrocytes.

1.2.7 Programmed cell death

The fate of the terminally differentiated hypertrophic chondrocyte is unclear. In growing rats it has been calculated that 8 hypertrophic chondrocytes (including their associated matrix) are eliminated from each column of cells every day (Hunziker *et al*, 1987). Evidence suggests that terminally differentiated chondrocytes either re-differentiate into bone cells (Cancedda *et al*, 1995), proliferate with one daughter chondrocyte dying and the other becoming an osteoblast (Roach *et al*, 1995) or undergo the widely accepted route of programmed cell death (Farnum & Wilsman, 1987; Gibson *et al,* 1995).

1.3 REGULATION OF GROWTH

Longitudinal bone growth is controlled by an intricate complex of *systemic* and *local* mediators that interact to regulate the activities of the growth plate chondrocytes. Receptors for growth hormone (GH), insulin-like-growth factor-I (IGF-I), thyroid hormones, GCs, oestrogens and androgens have all been detected in growth plates in various species, indicating that they may have direct effects on growth after birth. In addition these systemic hormones may exert their effects on growth plate chondrocytes by influencing the expression and or activity of locally acting growth factors such as Indian hedgehog (Ihh), parathyroid hormone related peptide (PTHrP), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). Some of the important systems are discussed in more detail below.

1.3.1 Regulation of Growth Via The GH and IGF-I axis.

a) The original somatomedin hypothesis

In 1957 Salmon & Daughaday demonstrated that GH may have indirect effects on linear growth and proposed the 'original somatomedin hypothesis', which postulated that GH controls growth by stimulating the liver production of a circulating substance

(somatomedin), which reaches it target tissues of bone and cartilage to convey the growth signals (Fig 1.2a). At this point somatomedin was an unknown substance, only discovered later during studies looking at the effects of pituitary hormones on somatic growth. They demonstrated that the hypophysectomised rats had markedly reduced radioactivity uptake into the epiphyseal cartilage (Murphy *et al*, 1956) but this was reversed by *in vivo* injections of purified bovine GH. However this positive effect of GH could not be demonstrated *in vitro* and it was postulated that an intermediary growth factor or endocrine hormone might be involved. This was given further credit when serum from normal rats increased the radioactivity uptake whereas there were minimal effects from the serum of hypophysectomised rats. Again GH treatment of hypophysectomised rats restored the normal growth promoting activities. The term 'somatomedin' was coined to reflect the ability of the substance to mediate the effects of GH (Daughaday *et al*, 1972) and finally to IGF-I when its structure was established (Rinderknecht & Humbel, 1978).

b) The dual effector theory

The discovery that IGF-I is expressed in almost all tissues (D'Ercole *et al*,1980) led to the theory that IGF-I also had autocrine/paracrine effects. It was shown that GH administration to hypophysectomised rats increased IGF-I expression in numerous non-hepatic tissues and it was thought that its expression may be regulated by GH (Low *et al*, 1987 & 1988). Furthermore Isaksson *et al* (1982) demonstrated that a direct injection of GH into the growth plates of hypophysectomised rats resulted in a significant increase in longitudinal bone growth but no effect in the contralateral limb, thus also indicating a local effect of GH. This was supported by the observation that GH was found to increase IGF-I mRNA expression in growth plate chondrocytes and that the growth promoting effects of locally administered GH was eliminated when an IGF-I antiserum was co-infused with GH (Schlechter *et al*, 1986).

This led to the proposition of the 'dual effector theory' in 1985 by Green *et al* who postulated that GH had direct effects on the growth plate germinal zone to promote chondrocyte differentiation and that GH also induces local IGF-I synthesis, which is thought to stimulate the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (Fig 1.2b)

c) Current Theories

Recent studies have revealed a greater complexity to the endocrine GH/IGF-I system. Up to six Insulin-like Growth Factor Binding Proteins (IGFBPs) have been identified, which bind IGFs in the circulation, thus prolonging their half lives (Firth & Baxter, 2002). By removing IGF-I from the circulation, they can modulate the function of IGFs in either an inhibitory or a stimulatory manner. The endocrine role of IGF-I is still unclear as liver specific IGF-I deletion in mice show normal growth (Yakar *et al*, 1999). Additionally the relative contribution of both direct and indirect GH action on bone growth remains uncertain. Current theories indicate that GH stimulates locally produced IGF-I to induce growth and regulates the bound and free concentrations of IGF-I in the circulation which then provides the negative feedback on pituitary derived GH (Le-Roith *et al*, 2001) (Fig 1.2c).

Figure 1.2. The Somatomedin Hypothesis

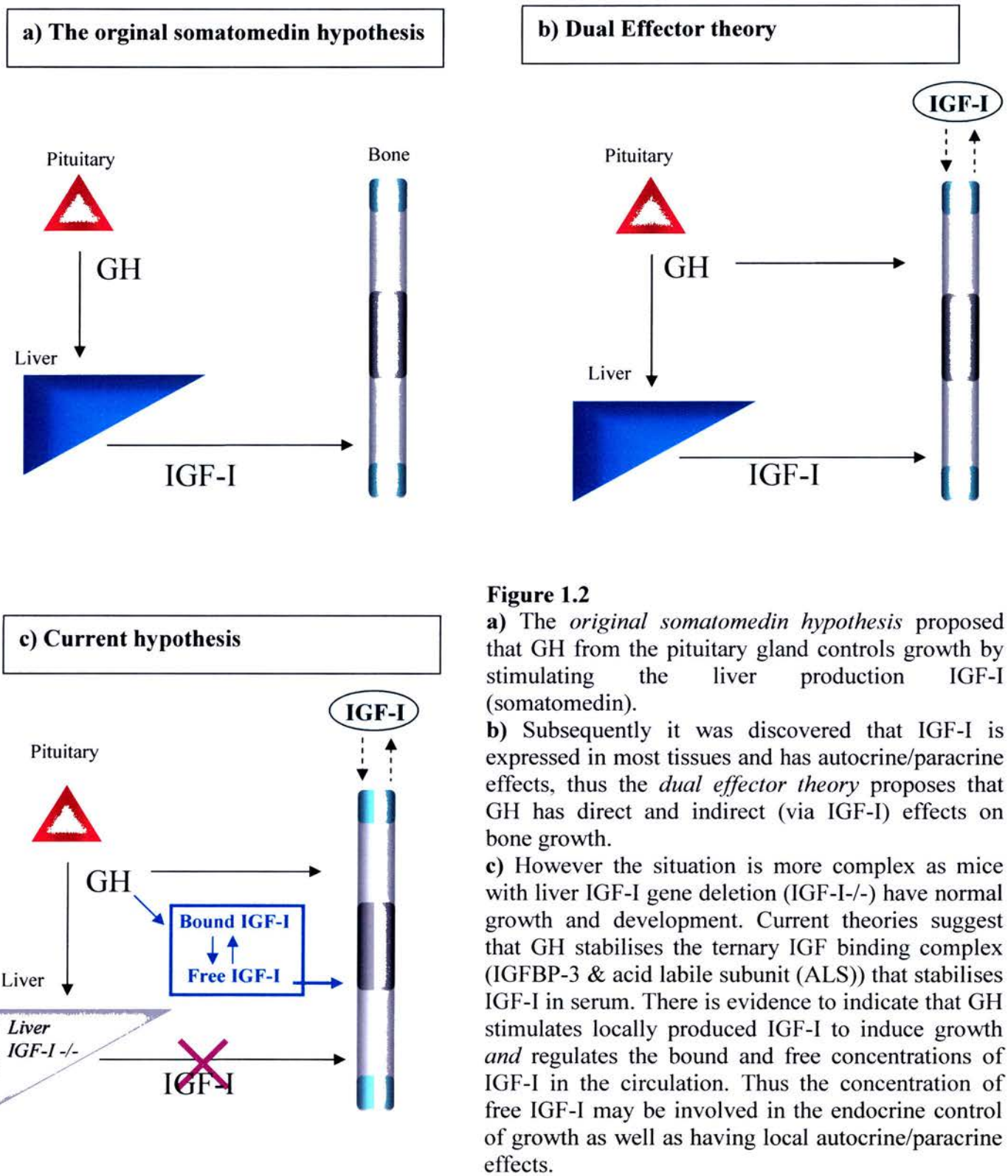


Figure 1.2

a) The *original somatomedin hypothesis* proposed that GH from the pituitary gland controls growth by stimulating the liver production IGF-I (somatomedin).

b) Subsequently it was discovered that IGF-I is expressed in most tissues and has autocrine/paracrine effects, thus the *dual effector theory* proposes that GH has direct and indirect (via IGF-I) effects on bone growth.

c) However the situation is more complex as mice with liver IGF-I gene deletion (IGF-I-/-) have normal growth and development. Current theories suggest that GH stabilises the ternary IGF binding complex (IGFBP-3 & acid labile subunit (ALS)) that stabilises IGF-I in serum. There is evidence to indicate that GH stimulates locally produced IGF-I to induce growth *and* regulates the bound and free concentrations of IGF-I in the circulation. Thus the concentration of free IGF-I may be involved in the endocrine control of growth as well as having local autocrine/paracrine effects.

d) IGF-II

IGF-II is a single chain 67 amino acid peptide that like IGF-I shares a significant homology with insulin (Blundell & Humbel, 1980). As well as a paracrine role it is secreted by the liver and has mitogenic and metabolic properties. Its major role appears to be in underpinning foetal growth, with subsequent postnatal effects being less clearly defined. All of its actions are mediated through the IGF-I receptor, with no signal transduction through the IGF-II receptor (Czech, 1989). However the IGF-II receptor plays an important role in the degradation of IGF-II. Inactivation of IGF-II-R expression in mice by gene targeting results in foetal overgrowth, skeletal abnormalities and perinatal death due to the overexposure of foetuses to IGF-II (Lau *et al*, 1994; Ludwig *et al*, 1996).

e) IGFBPs

Although IGF-I and IGF-II are important regulators of growth and metabolism their action is positively or negatively regulated by up to six IGFBPs. The IGFBPs comprise a family of 6 related proteins that interact with high affinity with IGF-I and compete with IGF-I receptors for their binding and thus influence mitogenesis, differentiation and cell survival (Firth *et al*, 2002). They were initially identified as carrier proteins for IGF-I and IGF-II activity. Their presumed function was to protect IGF peptides from degradation and clearance, increase the half life of the IGFs and deliver them to appropriate tissue receptors. However it is now apparent that they have IGF-I dependent and independent actions on cell growth (Firth *et al*, 2002; Ferry *et al*, 1999).

In the circulation the IGFs are present in a complex of 150kDa, composed of one molecule of IGF-I or II, a 85kDa acid-labile subunit (ALS), and IGFBP-3. A smaller proportion of the IGFs are associated with other IGFBPs and less than 1% is found in free form (Rajaram *et al*, 1997).

The precise role of individual IGFBPs is still unknown due to their complexity of actions and their regulation but they are expressed in a tissue specific manner and have different affinities for the IGFs. IGFBP-1 in particular inhibits IGF-I dependent cell growth and differentiation and is involved in reproduction, foetal growth and brain development. IGFBP-2 is the second most abundant binding protein in the circulation and also appears to inhibit the action of IGF-I. Overexpression of IGFBP-3 leads to organomegaly, while IGFBP-4 is most abundant in bones. IGFBP-6 is distinct as it has a preferential affinity for IGF-II. (Schnieder *et al*, 2000). In reality the full interactions of these high affinity IGFBPs and their modulation of the IGF dependent and independent effects remains to be fully unravelled. An additional layer of complexity exists with the reporting of at least four low affinity IGF binders – termed IGFBP related proteins (IGFBP-rP) (Baxter *et al*, 1998).

f) Prenatal and postnatal growth

Variation in human foetal growth occurs from around 16 weeks gestation. Excluding chromosomal and genetic disorders, the dominant cause of growth retardation in mid and late gestation relates to a diminished supply of nutrients or oxygen whereas the genetic differences in body size are almost entirely related to maternal factors (Gluckman & Pinal 2003; Gluckman, 1986; Robson, 1978).

IGF-I and II levels increase longitudinally throughout pregnancy (Gohlke *et al*, 2004). IGF-II is more important for embryonic growth, while IGF-I is the dominant foetal growth regulator in late gestation. In contrast to the postnatal situation, GH has little effects on IGF-I regulation as the GH receptors (GHR) are only expressed at very low levels in the foetal tissues (Oliver *et al*, 1996). Insulin has a major effect on growth and size at birth, predominantly during the third trimester when it stimulates foetal lipogenic activity, including a rapid accumulation of adipose tissue (Lifshitz & Botero, 2003). None the less

its somatogenic actions are also mediated through stimulating IGF-I release (Fowden *et al*, 1989). The IGF-I gene appears to be more responsive to nutritional change and thus the foetal IGF-I system is more sensitive to nutrient restriction than IGF-II. (Fowden *et al*, 1989) Similarly asphyxia also leads to a cessation of foetal growth and is associated with a fall in foetal IGF-I levels and altered IGFBPs (Bennet *et al*, 2001).

After birth the infant shifts from a growth rate that is predominantly determined by maternal factors to one that is increasingly related to his or her own genetic background. During the period of early infantile growth the growth velocity is independent of the endogenous GH, but continues to be dependent on nutrition and thyroid status. By early childhood growth becomes dependent on GH, and at puberty the sex steroids provide the acceleration in height velocity. However even the gonadal steroids require an intact GH, IGF-I action for their growth promoting effects to be most effective (Grimberg & De Leon 2005).

1.3.2 Local Regulation of Growth

a) Parathyroid hormone and Indian Hedgehog

Parathyroid hormone (PTH) is an essential regulator of the circulating levels of calcium and phosphate. It modulates the activities of cells in the intestine, kidney and bone. Both PTH and a second member of the PTH family, parathyroid hormone related peptide (PTHrP) bind and activate the common PTH/PTHrP receptor. They both cause hypercalcaemia and hypophosphatemia but as PTHrP circulating levels are significantly lower than PTH levels it is thought unlikely that PTHrP has any major role in maintaining calcium homeostasis. The physiological roles for PTHrP are recognised to be numerous as it is expressed by a wide variety of embryonic and adult tissues, and it is thought mainly to act as an autocrine/paracrine factor. PTHrP alongside indian Hedgehog (Ihh) a paracrine factor produced by prehypertrophic chondrocytes, promotes chondrocyte proliferation

while inhibiting hypertrophic differentiation, a function performed as part of a negative feedback loop. Ihh produced by prehypertrophic chondrocytes increases the expression of PTHrP in the periarticular region. PTHrP then binds to PTH/PTHrP receptors expressed on prehypertrophic chondrocytes (before their conversion to Ihh expressing cells) and blocks their further differentiation. As the population of committed cells progresses to the hypertrophic phenotype, they stop expressing Ihh, thereby attenuating the negative feedback loop and allowing the further differentiation of uncommitted prehypertrophic cells (Fig 1.3) (Farquharson, 2003). Mice missing the PTH/PTHrP receptor gene have a growth plate morphology similar to that of mice that are homozygous for the ablation of the PTHrP gene (Lanske *et al,* 1996) in that they show widespread accelerated differentiation of chondrocytes and premature mineralisation resulting in a narrow growth plate. In contrast, the phenotype of mice in which the PTHrP gene is over expressed is characterised by a dramatic slowing down of the differentiation of chondrocytes and a wider growth plate (Weir *et al,* 1996).

b) Fibroblast Growth Factors

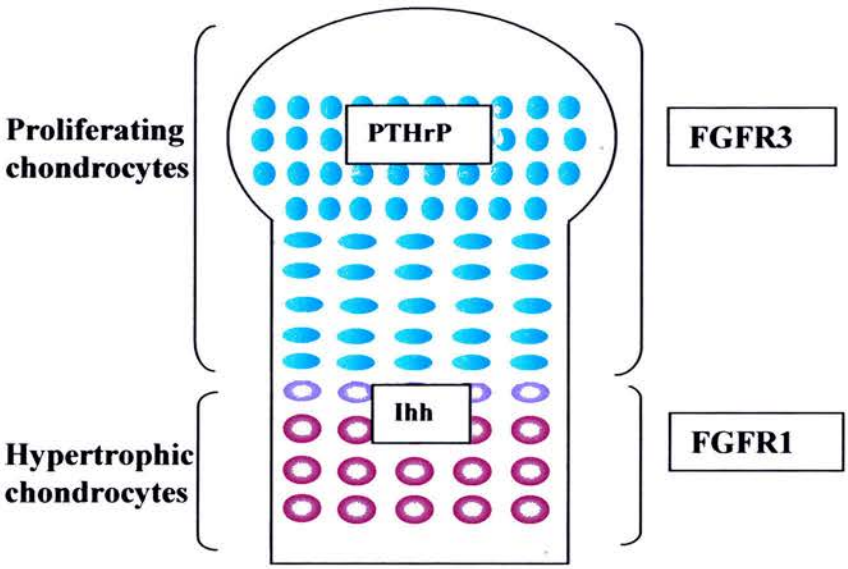
The family of Fibroblast Growth Factors (FGFs) constitute at least 22 members that interact with at least four FGF receptors (FGFR) and are the major regulators of embryonic bone development (Szebenyi & Fallon 1999). FGF signalling is critical for chondrocyte maturation and skeletal development during post-natal bone growth. They have similar biological effects and interact with members of the FGF receptor family of transmembrane tyrosine kinases to elicit their biological response. Both FGF-1 & 2 as well as FGFR1, -2, and -3 are widely expressed in chondrocytes (Fig 1.3)(Jingushi *et al*, 1995, Peters *et al* 1992). Upregulation of FGF receptor signalling results in bone abnormalities during endochondral growth, and is the basis of several genetic forms of human dwarfism including achondroplasia, which is caused by an activating mutation of

FGFR3 (Shiang *et al*, 1994). Conversely mice with an inactivating mutation of FGFR3 gene demonstrate increased longitudinal growth (Colvin *et al,* 1996).

c) Bone morphogenetic proteins / Transforming Growth Factor ß

The family of Bone morphogenetic proteins (BMPs) is comprised of at least 15 members, which are all part of the Transforming Growth Factor ß (TGFß) superfamily. BMPs were originally identified as important stimulators of bone formation but are now recognised as important regulators of growth, differentiation and morphogenesis during embryology (Reddi, 2001). The BMPs/TGFß and their receptors act as a signalling system both dependently and independently of the Ihh/PTHrP feedback loop at different levels during embryonic bone formation (van der Eerden *et al,* 2003). However the hallmark of BMPs is their ability to induce *de novo* bone formation in non-skeletal tissue of which the initial stages are characterized by the stimulation of collagen type II and the formation of a cartilaginous matrix (Urist, 1965). Data indicates that BMPs can regulate the complete cascade of events in cartilage formation, which includes the differentiation of the committed mesenchymal stem cells to the chondrocyte phenotype, their terminal differentiation and the mineralisation of the cartilage matrix. More specifically, BMP4 and BMP6 have been implicated in mediating the effects of PTHrP in regulating the pace of chondrocyte differentiation (Grimsrud et al, 1999; Farquharson *et al*, 2001).

Figure 1.3. PTH/PTHrP & Ihh and FGF signalling.



PTHrP is secreted from the perichondrial cells and proliferating chondrocytes, whilst Ihh is released from the pre-hypertrophic chondrocytes. Together they stimulate proliferaton and delay hypertrophic differentiation. Ihh also acts on perichondrial cells to convert these into the osteoblasts of the bone collar. Growth plates of gene ablated mice show a thinner proliferating layer and normal hypertrophic layer, suggesting that in the absence of PTHrP differentiation is accelerated so chondrocytes undergo premature hypertrophy and apoptosis. The FGFs are also involved in growth plate signalling and act to decrease chondrocyte proliferation and to accelerate the differentiation of hypertrophic chondrocytes into terminally differentiated chondrocytes. FGFR3 is predominantly expressed in proliferating chondrocytes, whereas FGFR1 is restricted to the hypertrophic and perichondrial chondrocytes.

1.4 GH AND IGF-I RECEPTORS

1.4.1 GH Receptor

The GHR was first identified in 1987 by Leung *et al*, and is part of the class I cytokine superfamily of receptors including erythropoietin, leptin and numerous interleukins (Finidori, 2000).

Most of the pathways initiated by GH binding appear to require as a first step, the activation of Janus Kinase 2 (Jak2), this then phosphorylates certain tyrosine residues of the cytoplasmic domain of the receptor, and other molecules that are in the region of the receptor-Jak2 complex. Signal transducers and activators of transcription-5 (Stat-5) play a crucial part as an intracellular signalling molecule. In addition proliferation and gene transcription signalling occurs via several other pathways including Ras/MAP Kinase (mitogen activated protein), Insulin receptor substrates - IRS-1/IRS-2 and PI3-Kinase pathways.

Growth hormone receptor knockout mice have reduced bone growth and reduction in chondrocyte proliferation after 2 weeks of age, however this is almost completely reversed by restoring the circulating IGF-I levels to normal indicating that GH induced postnatal growth is probably due to an increase in circulating IGF-I levels (Sims *et al*, 2000).

1.4.2 IGF-I Receptor

The cellular actions of IGF-I are mediated by a receptor tyrosine kinase (IGF-IR), which are expressed in a diverse range of tissues including chondrocytes and osteoblasts. Binding of IGF-I to its receptor utilises a family of soluble receptors, known as insulin receptor substrates (IRSs) to initiate a series of autophosphorylation events. Activation of the type I IGF-I receptor (IGF-IR) on the surface of cells (De Meyts *et al*, 1994) leads to intracellular signalling through two distinct signalling pathways, phosphatidylinositol 3-

kinase (**PI-3K**) and p42/p44 mitogen-activated protein kinase (**MAPK**), leading to proliferative and antiapoptotic effects.

Activation of **PI-3K** activity results in a series of intracellular downstream events, and recruitment of other downstream signalling molecules such as Akt to the plasma membrane. Signalling via this pathway is responsible for the cells resistance to programmed cell death (Franke *et al*, 1997).

The **MAPK** pathway is strongly dependent on tyrosine phosphorylation steps. Phosphorylated MAPK translocates to the nucleus where it further activates the genes necessary for cell cycle progression and DNA replication. The activation of the ribosomal kinase p70s6 can be a consequence of activation of both PI-3K and MAPK signalling and represents a potential convergence point of IGF-I dependent mitogenic and antiapoptotic signalling.

In neonatal mice IGF-IR deletion is lethal as they die of respiratory failure as well as exhibiting severe growth retardation, delayed ossification and generalised organ hypoplasia (Liu *et al* 1993).

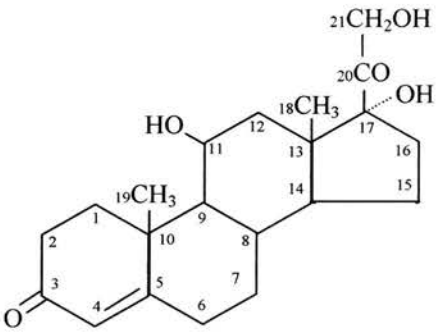
1.5 GLUCOCORTICOIDS

1.5.1 Physiology

The adrenal cortex synthesises two classes of steroids; the corticosteroids (glucocorticoids and mineralocorticoids) and the androgens. Glucocorticoids (GCs) influence most systems in the body and are essential for normal function. They are secreted from the adrenal cortex and in physiological doses, they help the body adapt to intermittent food intake by regulating blood sugar and electrolytes, promoting gluconeogenesis, mobilising fats for energy metabolism and depressing inflammatory and immune responses.

Physiological GCs include cortisol (hydorcortisone) which is the most predominant as well as cortisone and corticosterone. Its secretion is regulated by ACTH from the pituitary, which in turn is under the control of hypothalamic Corticotrophin Releasing Hormone (CRH). Cortisol secretion displays a diurnal rhythm with highest levels in the morning. Negative feedback by GC inhibits Adrenocorticotropic hormone (ACTH) secretion via direct and indirect actions on the CRH neurons, but this feedback can be overridden in response to stressful settings (Goodman & Gilman's, 2001).

Cortisol (Hydrocortisone) is the principal naturally occurring steroid and its structure is illustrated below.



From this structure various groups can be substituted to form synthetic compounds with different degrees of GC or mineralocorticoid activity. Such that hydroxylation at the C16 position reduces mineralocorticoid activity, while alpha-fluoro-substitution at the C9 position increases mineralocorticoid activity.

The normal daily secretion of cortisol is 10 – 30mg and the exogenous daily dose that completely suppresses the cortex is 40-80mg of Hydrocortisone, or 10-20mg of Prednisolone in adults. The relative potencies of the major steroid compounds are given below using cortisol as the reference: (Goodman & Gilman's, 2001).

Cortisone	0.8
Hydrocortisone	1
Prednisolone	4
Methylprednisolone	5
Betamethasone	25
Dexamethasone	25

1.5.2 Glucocorticoid receptor

The Glucocorticoid Receptor (GCR) is a steroid hormone-activated transcriptional factor that regulates, directly or indirectly, target genes involved in glucose homeostasis, bone turnover, cell differentiation, lung maturation and inflammation (Reichardt *et al*, 2000). It belongs to the nuclear receptor superfamily, which includes receptors for the mineralocorticoids, oestrogens, progestins and androgens as well receptors for peroxisome proliferators, Vitamin D and thyroid hormones.

The actions of GC are mediated at least in part via specific GCRs, which are ligand dependent DNA-binding nuclear receptors and belong to the superfamily of steroid/thyroid/retinoid/orphan receptors (Lazar, 1993). In 1985 the GCR was cloned by Hollenberg *et al*, who described 2 forms of the human GCR, the active GCRα (777 amino acids) and GCRß (742 amino acids). The α isoform of the GCR is expressed in appreciable levels in all nucleated normal cells, consistent with the widespread effects of GC on metabolism, differentiation and development. After ligand binding it is functionally active. This is in contrast to GCRß which does not bind ligand and indeed may act as a ligand-independent negative regulator of activated GCRα (Bamberger *et al*, 1995 & 1996).

Like most nuclear receptors, GCR is a modular protein that is organised into three major domains: an N-terminal activation function-1 domain (AF-1), which plays an important part in gene regulation (Giguere *et al*, 1986), a central DNA binding domain, and a C-terminal ligand binding domain (LBD). In addition to its role in ligand recognition, the LBD contains a ligand-dependent activation function (AF-2) that is tightly regulated by hormone binding.

In the absence of ligand, GCR is predominantly maintained in the cytoplasm as an inactive multi-protein complex. This consists of two heat shock proteins 90 (hsp90) molecules plus a number of other proteins including immunophilins p59 and calreticulin (Beato *et al*, 1996). Entry of GC into the cell and subsequent binding to the LBD of GCR leads to a conformational change in the receptor. These associated proteins are shed on steroid binding, allowing dimerisation and translocation of the receptor into the nucleus. Once within the nucleus, the GCR binds DNA promoter elements known as GC response elements to activate or repress a complex set of transcription factors depending on the context of the target promoters (Jantzen *et al*, 1987; Beato *et al*, 1996 & 1989). In addition GCR can also cross talk with other transcriptional factors such as nuclear factor-kB and activator protein-1 (AP-1) to repress their gene activation activities. (McKay & Cidlowski 1999). Both the ligand-dependent activation and repression by GCR require the intact function of the LBD. This GC mediated repression has been postulated to be a molecular basis for the anti-inflammatory and immunosuppressive activities of GC.

The presence of GCR has been demonstrated in human bone at different stages of life in humans (Abu *et al*, 2000) as well as in mice (Masuyama *et al*, 1992). Mutations in the GCR are associated with Cushings syndrome, autoimmune diseases and cancers (Werner & Bronnegard 1996). Meanwhile GCR gene knockout studies have shown that mice homozygous for a targeted disruption of the GC gene die within a few hours of birth due to respiratory failure as a result of severely impaired lung development (Cole *et al*, 1995),

while the few survivors have markedly elevated levels of ACTH and plasma corticosterone.

1.5.3 Systemic Side Effects

GCs affect most systems within the body and these include; endocrine effects – Cushing syndrome and obesity; musculoskeletal – myopathy and osteoporosis. This is partially due to hypogonadism as the sex steroids normally contribute to maintenance of both muscle and bone mass. Additionally steroids reduce the quality of trabecular bone resulting in an increase fracture rate (Ralston, 1999); immune – immunosuppression; gastrointestinal – peptic ulcer and haemorrhage; central nervous system – depression, raised intra-cranial pressure; cataracts and delayed tissue healing, the latter due to dermal atrophy and reduced remodelling of the skin. These systemic effects are dependent on the dose and duration of GCs as well as the type of GC used such that Dex causes greater immunosuppression and adrenal suppression (Hansen & Loriaux 1976; Hughes & Reid GF 1982, Kaspers *et al*, 1996). Unwanted effects with steroids are common with prolonged administration. These effects may be minimised by locally acting agents such as inhalers, intra-articular injection and topical preparations. The effects of GCs on growth and chronic disease are discussed in more detail below.

1.5.4 Clinical uses for GC and Effect on Growth

GCs are used extensively in paediatric practice, often in the treatment of a number of chronic inflammatory, autoimmune and neoplastic diseases. It is estimated that 10% of children may require some form of GC at some point in their childhood (Warner 1995). Impairment of childhood growth with an approximate cortisone dose of 1.5mg/kg/day was first described over 40 years ago (Blodget *et al*, 1956). Osteopenia in children receiving a Pred dose of less than 0.16mg/kg/day has also been reported (Avioli, 1993). The

maintenance of growth and bone health is a complex process that can be influenced not only by drugs, but also by the nutritional status of the patient and the underlying disease processes.

The common clinical indications for GC and their contribution to growth problems is summarised below.

a) Asthma, Eczema & Hayfever

The increasing incidence and prevalence of childhood atopy and the more widespread use of inhaled steroid therapy for asthma prophylaxis probably account for the largest group of children who are chronically exposed to steroids. Oral GC therapy in asthma is associated with a delay in growth and puberty and there is some evidence to suggest that final height may also be compromised (Allen *et al*, 1994). Systemic exposure to inhaled steroids may be higher with metered dose inhalers and dry powder devices where 80% of the drug is deposited in the oropharynx. Although earlier studies did not show a relationship between inhaled steroids and growth, there is now good evidence that inhaled steroids can temporarily slow growth and alter bone and collagen turnover. The magnitude of this effect may be influenced by the dose delivery system as well as the systemic bioavailability of the inhaled steroid used (Shaw *et al*, 1997). This effect may be most pronounced over the first few weeks of treatment (Doull *et al*, 1998). Long term studies are difficult due to a number of confounding factors including the plethora of drugs, delivery systems, compliance and disease severity but there is no clear evidence that final height is compromised following inhaled GC therapy in children with asthma. Some intranasal GCs such as budesonide have a very high level of systemic absorption when applied directly to the nasal mucosa and short and intermediate term studies of children on intranasal steroids such as budesonide and beclomethasone also show a deterioration in growth velocity (Edsbacker *et al*, 1985). Like asthma, there is some suggestion that the

effects of these intranasal steroids may be dose dependent and that the newer forms of intranasal steroids may not have these adverse growth effects (Pedersen, 2001). Assessment of short-term growth during topical steroid treatment for eczema has also been studied but the results have been up till now, inconclusive (Heuck *et al*, 1998).

b) Inflammatory Bowel Disease

Longitudinal studies show that the growth velocities of children in the year preceding diagnosis are reduced and growth retardation frequently complicates the clinical course in children (Markowitz *et al*, 1993). In children with inflammatory bowel disease, retardation of growth and skeletal maturation are widely reported and may be related to disease activity as well as to its treatment (Savage *et al*, 1999). Vertebral fractures have also been described in children with Crohn's disease with a short or absent history of steroid usage (Cowan *et al*, 1995, Semeao *et al*, 1997). A cross-sectional study of bone mineralisation using dual energy x-ray absorptiometry showed evidence of osteopenia even when corrected for sex, height, weight and puberty (Cowan *et al*, 1997). In this study, the bone status was related to steroid usage but had no relationship to disease activity. In a longitudinal study of 55 children, uncorrected total body bone mineral density SDS correlated negatively to cumulative steroid dosage and positively to body mass index (Boot *et al*, 1998). A reduction in bone mineral density of the lumbar spine, femoral neck and radius may be more prominent in children with Crohn's disease and those children who are of a pubertal or post pubertal age. The introduction of budesonide enemas for treatment of distal colitis has also been reported to be associated with suppression of markers of bone formation (Robinson *et al*, 1997).

c) Renal Disease

Impaired linear growth is one of the major complications of childhood-onset chronic renal failure and its treatment. Final height may be less than the third percentile in 50% of children who enter end stage renal failure in childhood. Children with a history of renal insufficiency who receive GC may grow more slowly, have a poorer bone mineralisation status and may not respond satisfactorily to Vitamin D replacement compared to those who do not receive GC (Chesney *et al*, 1998). The prolonged use of GC is also associated with growth failure and reduced bone mineral density in other chronic renal disease such as nephrotic syndrome. Here it is not clear whether intermittent GC therapy over a number of years has an adverse effect on growth and final height (Lettgen *et al*, 1994; Saha *et al*, 1998). Post transplant, the cumulative GC dosage may be inversely related to the change in relative height, however the GC also have less inhibitory effects on growth velocity, without compromising graft function, when given on alternate days (Schaefer *et al*, 1990; Jabs *et al*, 1996).

d) Arthritis

GC are widely used for treating chronic connective tissue diseases in children and as with other inflammatory conditions there is considerable overlap between the inflammatory process and steroid induced effects on bone health. A failure to develop adequate bone mineralisation is virtually universal in children with Juvenile Idiopathic Arthritis (JIA) and is characterised by a failure of bone formation, with a subsequent failure to undergo the normal increase in bone mass during puberty. These negative effects on bone may be increased if the child was on steroids (Cassidy & Hillman 1997; Kotaniemi *et al* 1998). Other studies have not shown any statistical significance of the cumulative dose of corticosteroids on growth although they did note a reduction in the growth velocity during the first year of treatment which was more apparent in the polyarticular group (Saha *et al*,

1999). It does appear that good control of disease activity in systemic onset JIA can be achieved by high dose alternate day Pred with minimal side effects and that GC effects can also be reversed after lowering the dosage in other autoimmune conditions (Kimura *et al*, 2000; Conti *et al*, 1996).

e) Acute Lymphoblastic Leukaemia

GC have been a mainstay of the therapy for children with acute lymphoblastic leukaemia (ALL). Dex is now replacing Pred as the drug of choice because it is reported to have a greater lymphocytotoxicity and higher CNS penetration. Recent studies have shown that bone mineralisation status as assessed by bone mineral density, corrected as well as uncorrected for body size, may be adversely affected at completion of treatment (Halton *et al*, 1996; Arikoski *et al*, 1999). The fracture incidence during ALL treatment was reported to be as high as 39% and this was confirmed by Strauss *et al* (2001) who have shown a 5-year cumulative incidence of 28% for fractures and 7% for osteonecrosis with a median follow-up of over 7 years (Halton *et al*, 1996; Strauss *et al*, 2001). Older, pubertal stage, the male sex and Dex have been shown to be independent risk factors for fractures and reduced bone mineralisation. Previous studies have shown alterations in bone turnover and short-term growth of children during ALL treatment and that these changes were most marked during periods of intensive chemotherapy and high dose systemic GC administration (Crofton *et al*, 1998; Ahmed *et al*, 1999).

f) Prenatal GC treatment

Newborns born small for gestational age (SGA) are at increased risk of significant health issues in later life as it not only affects infant mortality and morbidity, but may also predispose individuals to coronary heart disease, diabetes, hypertension and stroke in adults (Barker *et al*, 1989). Traditionally Intrauterine Growth Retardation (IUGR) has

been defined as a birthweight less than 2500g which ignores gestational age and population factors. Therefore the term SGA corrects for gestational age and various criteria based on centiles or standard deviations.

The foetus may be exposed to elevated levels of GC most commonly to decrease the incidence of respiratory distress syndrome and thus improve survival in infants born prematurely (Liggins & Howie, 1972). However there is controversy over the risk-benefit ratio of prenatal GCs for various re-treatment strategies if preterm delivery does not occur within one week after the initial dose. Although early postnatal GCs decrease the incidence of bronchopulmonary dysplasia and facilitate extubation, they do have short-term adverse effects such as gastrointestinal bleeding and perforation, hyperglycaemia, hypertension, growth failure in the long term and an increased incidence of cerebral palsy (Halliday *et al*, 2003). Prenatal GC have also been used in the deliberate suppression of the adrenal gland in the prenatal treatment of pregnancies with a female foetus affected with congenital adrenal hyperplasia (CAH), however there remains the potential for long term effects (Matthews, 2000). Furthermore women with CAH on maintenance treatment with hydrocortisone, Dex or Pred during their pregnancies may have infants with a lower birth weight (Krone *et al*, 2001).

Foetal GC levels are lower than maternal levels (Beitens *et al*, 1973) and the foetus is normally protected to some degree from maternal GC by the presence of the placental barrier enzyme, 11ß-HSD type 2, which catalyses the rapid metabolism of active cortisol to inactive cortisone (Brown *et al*, 1996). However this enzyme is not a complete barrier to maternal GC (Benediktsson *et al*, 1997) and deficiency of this enzyme such as with protein restriction leads to a greater exposure of maternal GCs to the developing rat (Langley-Evans *et al*, 1996). Similarly mutations of the 11ß-HSD gene in humans are also associated with a very low birth weight (Dave-Sharma *et al*, 1998).

1.5.5 Pathophysiology of Glucocorticoid induced growth retardation

a) Systemic Effects on growth

GCs interfere with several regulatory mechanisms all of which lead to growth retardation (Fig 1.4). They impact on the GH/IGF-I axis; whereas short term administration of GCs stimulate GH and IGF-I secretion (Veldhuis *et al*, 1992) high dose GC therapy can attenuate the physiological GH secretion by down-regulating the ghrelin receptors leading to decreased GH releasing hormone and thus an increase in somatostatin tone (Kaji *et al*, 2001). Additionally GCs reversibly impairs the GH response in stimulation tests (Pantelakis *et al*, 1972; Hughes *et al*, 1999) and prevent the induction of GHR and IGF-IR expression by GH and IGF-I in chondrocytes (Jux *et al*, 1998), although it has been shown that GC per se increase the GHR mRNA expression in liver, growth plate and osteoblasts (Canalis, 1998; Heinrichs *et al*, 1994).

The sex hormones also exert significant effects on skeletal development. Testosterone stimulates growth by increasing the amplitude of GH pulses as well as a direct action on cartilage. Oestrogens have a biphasic response; at low levels they stimulate GH secretion, IGF-I and growth and at high levels play a critical role in bone maturation and epiphyseal fusion at puberty. GC may impair the attainment of peak bone mass and delay growth through alterations in gonadal function at the level of the pituitary and through direct effects on the gonads. Studies in adults show that GC therapy may be associated with testosterone deficiency as well as reversible gonadotrophin deficiency (Kamischke *et al*, 1998; Sakakura *et al*, 1975). *In vitro* evidence suggests that GC impair the action of Follicle Stimulating Hormone, thus reducing oestrogen secretion (Hsueh & Erickson, 1978). Adrenal inactivity during chronic GC exposure may also lead to reduced levels of other sex steroids such as androstenedione and oestrogen (Crilly *et al*, 1978) (Fig 1.4).

Figure 1.4. Mechanisms of GC induced bone loss and growth retardation.

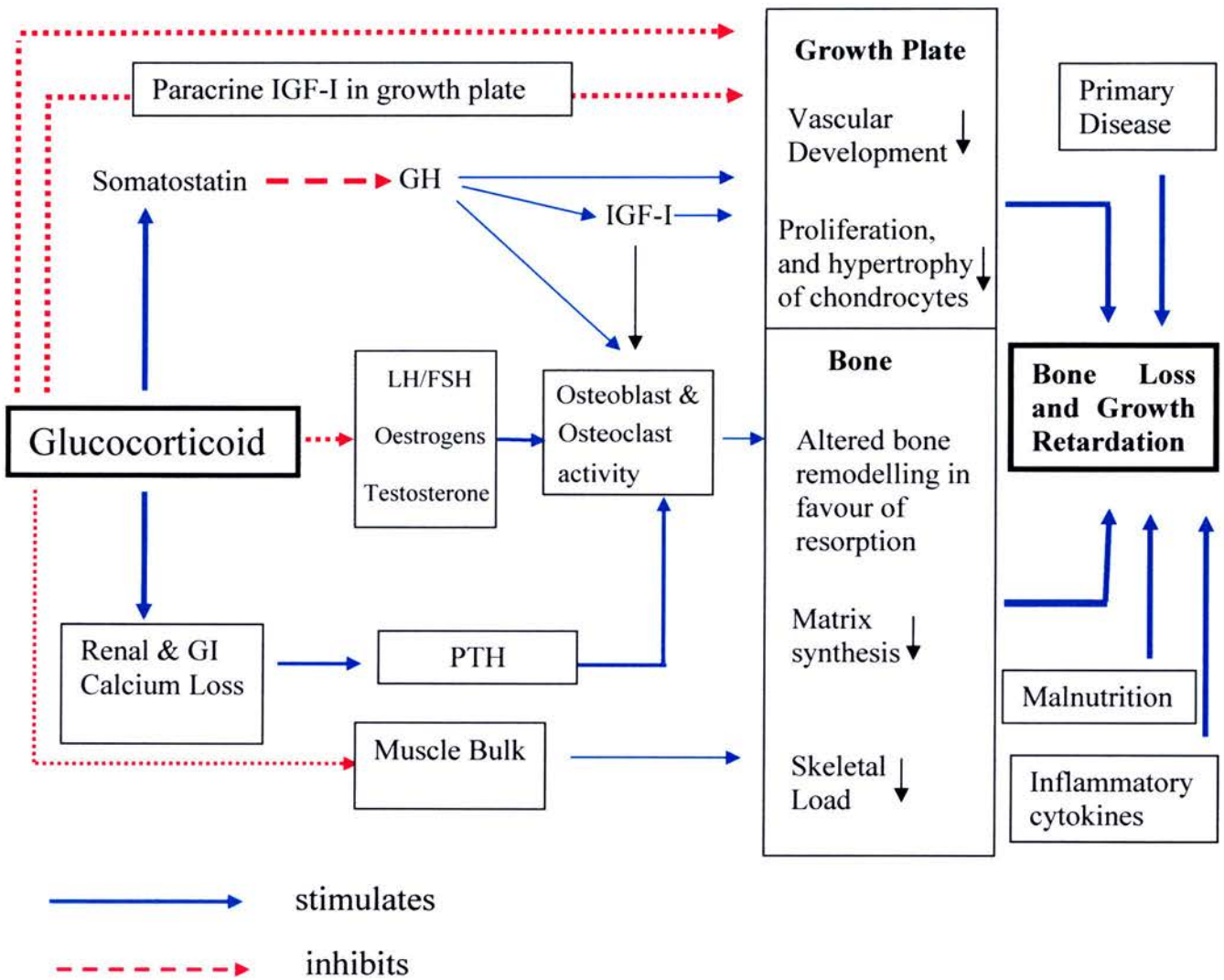
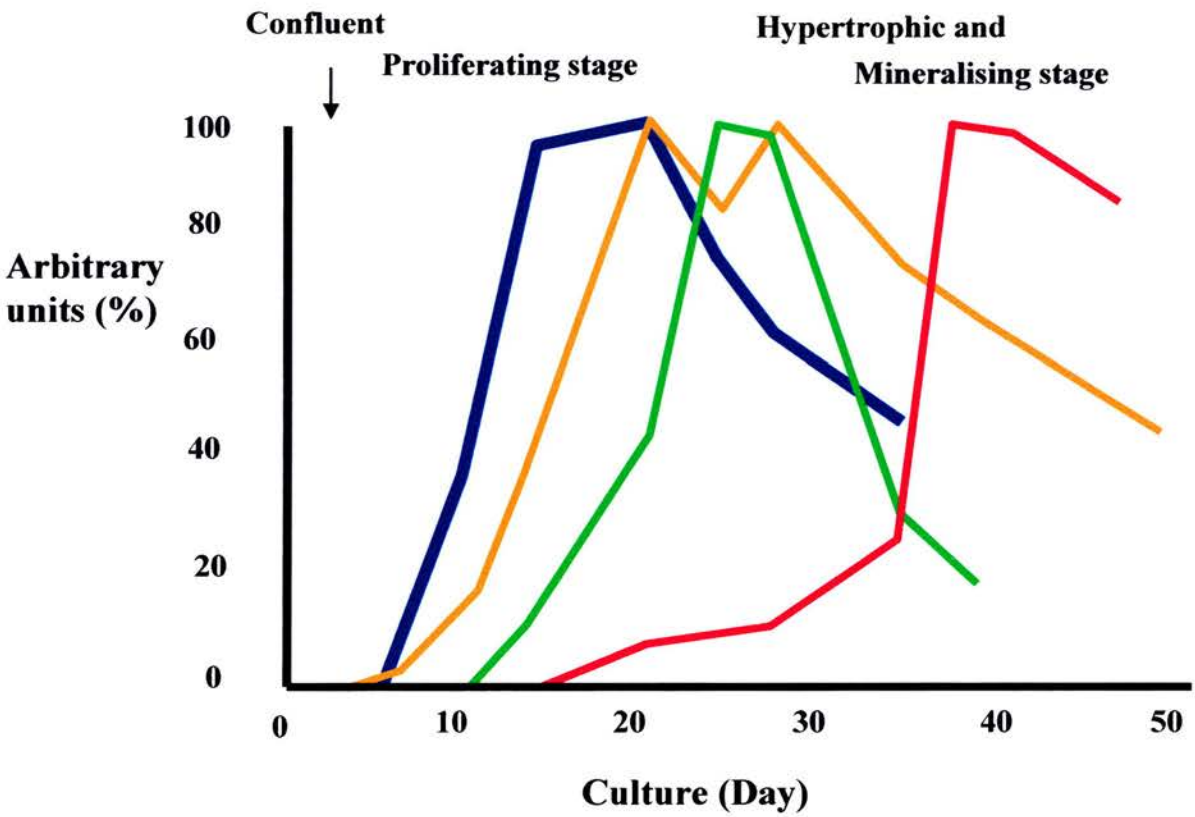


Figure 1.5. Time course marker gene expression during differentiation of ATDC5 cells. Total RNA was isolated on the indicated day in culture and analysed by slot blot. Values expressed as a percent of the highest hybridisation intensity for each mRNA.

Rat type II collagen cDNA (—) Rat PTH/PTHrP receptor (—)
Human aggrecan cDNA (—) Mouse type X collagen cDNA (—)

Initial elevation of type II collagen mRNA followed by an increase in the aggrecan mRNA. Expression of the PTH/PTHrP receptor gene occurs in close association with early chondrogenesis. Thereafter these three mRNA levels decline as the cells proceed to the hypertrophic stage which is associated with an elevation of type X collagen; which is not detected in the proliferating or undifferentiated ATDC5 cell.

(Figure adapted from Shukunami *et al,* 1997)



Robson *et al,* 1998; Gabrielsson *et al,* 1995; Unterman & Philips, 1985). *In vitro* studies indicate that Dex reduces basal chondrocyte proliferation in a dose dependent manner as well as inhibiting the proliferative effects of GH and IGF-I (Jux *et al,* 1998). While low dose GCs are required to maintain basal GHR expression and the differentiation of cells (Pal *et al*, 1992; Salles *et al,* 1994), high dose GC impair the actions of GH and IGF-I on the proliferative chondrocytes. Furthermore, the GCs cause a dose dependent decrease in GH stimulated IGF-I secretion. Jux *et al* showed that Dex decreases GHR mRNA content in chondrocytes and inhibits the homologous upregulation of GHR expression (Jux *et al,* 1998).

Although GHR and IGF-IR expression by GH and IGF-I in chondrocytes is restricted by GC, it has also been shown previously that GC increase GHR mRNA expression in the liver and growth plate (Jux *et al,* 1998, Heinrichs *et al,* 1994). However, in patients treated with GCs, serum IGF-I levels may not be altered while the bioactivity is (Caufriez & Copinschi, 1986). This would indicate another level of control involving the IGFBPs that may be disrupted by GC (Price *et al,* 1992; Smink *et al,* 2002).

1.6 CATCH UP GROWTH

Catch up growth (CUG) is defined as growth velocity (cm/yr) greater than the median for chronologic age and gender (Reiter & Rosenfeld, 1998). CUG, which was first reported by Prader *et al* in 1963, and is typically an early post natal process that occurs in most infants born SGA during the first 6 months and is complete by 2 years of age (Karlberg *et al,* 1997). However within this group infants born premature may take longer to catch up than the full term SGA (Hokken-Koelega *et al,* 1995). Approximately 10% of children born SGA will remain <-2SD for height throughout childhood and into adulthood (Karlberg *et al,* 1997).

Height is also an indicator of chronic disease in children as shown in a decreased growth rate in children diagnosed with chronic illnesses (Markowitz *et al,* 1993). Frequently GCs are used in the treatment of many childhood illnesses, which along with the inflammatory process compounds the reduction in growth. However upon cessation of the insult, including discontinuation of GC, this group of children also undergo a period of accelerated linear growth.

Two methods for the mechanism underlying CUG have been proposed. Initially Tanner (1963) proposed a neuroendocrine model, which adjusts the growth rate to an age appropriate set point. He suggested that the CNS contained a 'sizostat', which would sense a mismatch in growth and adjust the release of specific molecules accordingly. The evidence for this theory appears to be weakening and it is now apparent that CUG is also partially regulated by a mechanism intrinsic to the growth plate. This was demonstrated by Baron *et al,* (1994) who administered Dex directly into the growth plate of rabbits. A 77% reduction in the growth rate of 5-week rabbit limbs was noted in the proximal tibial growth plates infused with Dex. Following cessation of Dex, catch up growth was observed in the affected growth plate only and not in the contralateral tibia. Ultimately this corrected approximately half the deficit indicating that CUG cannot be explained by central mechanisms alone. It is postulated that this is due partly to a delay in growth plate senescence by Dex (Gafni *et al,* 2001). After the removal of the GC, the growth plate may be less senescent and therefore proliferate more rapidly than expected for age leading to catch up growth.

1.7 CHONDROCYTE MODELS

Human chondrocytes would be an ideal method to standardise the study of chondrocyte growth and regulation, however they are difficult to obtain, vary according to the condition and age of the patients and it is often difficult to isolate a sufficient number of

cells. To avoid this problem human immortalized chondrocyte cell lines were established to permit investigations in a standardized manner. Some human chondrocyte cell lines include the retroviral-mediated transfection of primary rib chondrocytes – T/C-28a, from which the C-28/12 and T/C-28a4 cell lines were derived (Goldring *et al,* 1994). However even though they express genes for chondrocyte differentiation and undergo proliferation they show less evidence for matrix synthesis and differentiation (Finger *et al,* 2003). This lack of a multistep differentiation capacity is also an issue for animal derived chondrocyte cell lines (Grigoriadis *et al,* 1988).

Other investigators have continued to utilise primary chondrocytes from various sources including rats and chicks, but as well as creating difficulty in comparing results across species, the chondrocytes are also at different stages of differentiation. Thus these heterogeneous population of cells may behave differently from a homogenous population of cells (Robson *et al,* 1998; Jux *et al,* 1998; Farquharson *et al,* 2001).

To circumvent these problems, the experiments in this thesis started primarily with a newer chondrocyte cell line which undergoes the complete chondrocyte cell differentiation process and worked towards utilising increasingly physiological models to study the effects of GC on growth plate chondrocytes, thus incorporating organ culture and *in vivo* models.

1.7.1 ATDC5 chondrocyte cell line

Studies to date have explored the effect of GC and growth factors on a heterogeneous population of chondrocytes comprising a mixture of maturational phenotypes (Robson *et al,* 1998; Koedam *et al,* 2000; Jux *et al,* 1998). The disadvantage with this approach is that it is not clear which population of chondrocytes are most affected by the GC effects. Although a few cell lines are known to be chondrogenic (Grigoriadis *et al,* 1990; Bernier & Goltzman *et al,* 1993), there is no report of any cell line that undergoes the complete

differentiation process as it has been difficult to generate stable cell lines that express the phenotype of chondrocytes that is uniformly maintained.

More recently the murine ATDC5 chondrocyte cell line has been shown to undergo the temporal sequence of events that occur during longitudinal bone growth *in vivo* and thereby provide a good model to study the molecular mechanisms underlying regulation of endochondral bone formation (Atsumi *et al,* 1990; Shukunami *et al,* 1997) (Fig 1.5). This ATDC5 cell line was isolated from a teratocarcinoma stem cell line AT805 on the basis of chondrogenic potentials in the presence of insulin (Atsumi *et al*, 1990). Subsequently it was shown that in the presence of insulin, the line undergoes the early phase of differentiation to chondrocytes to form cartilage nodules that increase in size due to proliferation. The late phase differentiation of the cell line is characterised by an increase in cell volume as well as a marked increase in ALP activity. Here the hypertrophic cells appeared in the centre of cartilage nodules in association with type X collagen gene expression and a dramatic elevation in ALP activity in culture, (Shukanami *et al,* 1997) both of which are implicated in the mineralisation of cartilage (McLean *et al,* 1987; Wu *et al,* 1989). With increasing time in culture these hypertrophic ATDC5 chondrocytes undergo the process of mineralisation which is visible macroscopically; at this point ALP activity starts to decline. Mineralisation appears to be a prerequisite for vascular invasion into cartilage during endochondral bone formation. Although not essential, the addition of ascorbic acid resulted in increased levels of type X collagen expression which facilitated a higher level of gene expression and maintenance of the hypertrophic phenotype (Shukunami *et al,* 1997).

A time course marker of gene expression during ATDC5 proliferation and differentiation, demonstrate an initial elevation in type II collagen, followed by an increase in aggrecan mRNA expression, however these then decrease during the phenotypic conversion of ATDC5 cells into hypertrophic cells which is associated with the induction of type X

collagen gene expression (Fig 1.5). The type X collagen could not be detected in undifferentiated cells or proliferating chondrocytes. This observation is compatible with findings during differentiation of chondrocytes *in vivo* (Iyama *et al,* 1991 & 1994).

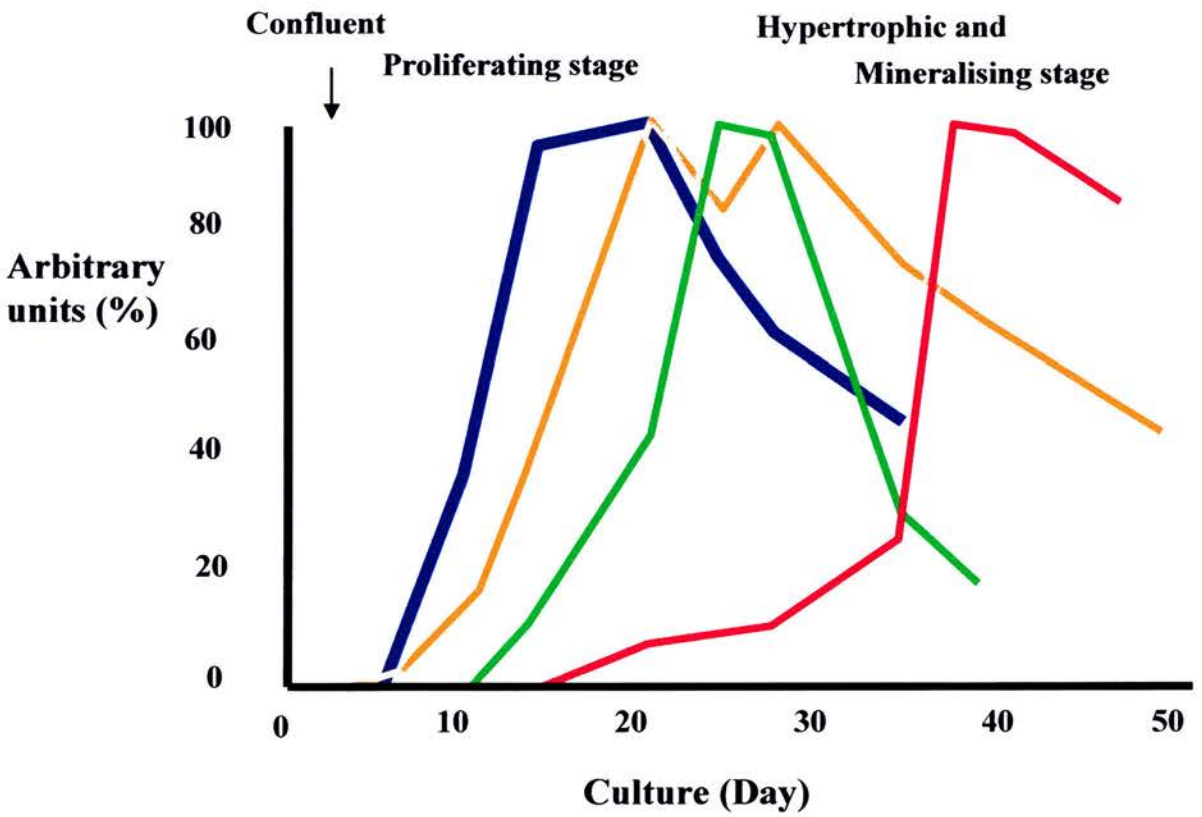
Thus, it has been established that the ATDC5 cells reproduce the orderly transition of all differentiation stages; from a prechondrogenic stem cell line it undergoes proliferation, early and late phase differentiation followed by mineralisation as observed during endochondral bone formation (Shukunami *et al,* 1997). As the effects of GC and growth factors could now be studied on a homogenous population of chondrocyte phenotypes it was felt that the ATDC5 cell line would be a valuable model for elucidating the molecular mechanisms that are disrupted during growth retardation.

Figure 1.5. Time course marker gene expression during differentiation of ATDC5 cells. Total RNA was isolated on the indicated day in culture and analysed by slot blot. Values expressed as a percent of the highest hybridisation intensity for each mRNA.

Rat type II collagen cDNA (—) Rat PTH/PTHrP receptor (—)
Human aggrecan cDNA (—) Mouse type X collagen cDNA (—)

Initial elevation of type II collagen mRNA followed by an increase in the aggrecan mRNA. Expression of the PTH/PTHrP receptor gene occurs in close association with early chondrogenesis. Thereafter these three mRNA levels decline as the cells proceed to the hypertrophic stage which is associated with an elevation of type X collagen; which is not detected in the proliferating or undifferentiated ATDC5 cell.

(Figure adapted from Shukunami *et al,* 1997)



1.7.2 Foetal Mouse Metatarsal Culture.

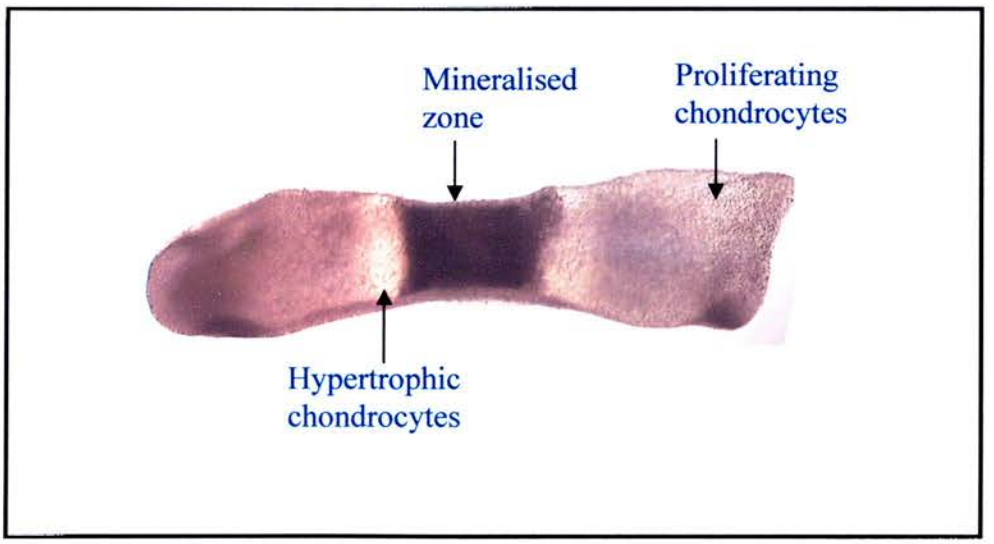
The complex mechanisms of GC effects on growth plate chondrocytes may be studied more closely by utilising more physiological and established chondrocyte models such as the foetal mouse metatarsal model. Here the normal histological architecture of the bone and the cell-cell interactions are maintained and therefore represent an excellent model to gain a greater understanding of the effects of GC on linear bone growth. The metatarsals are dissected at 18 days - 3 days prior to full gestation; as the foetal metatarsal is grown in serum free culture, direct measurements of total length, mineralising zone length and width can be taken.

The foetal mouse metatarsal explant culture is a highly physiological model for studying growth as the growth rate of foetal bones in culture is similar to that found *in vivo* whereas bones harvested postnatally from 2-day-old rats arrest in culture after 2-days *in vitro* (Scheven & Hamilton 1991). Recent studies indicate that mouse metatarsals may not show this growth arrest if harvested postnatally (Macrae *et al,* 2006). In 15-day-old mouse metatarsals cultured in serum free conditions, endochondral ossification, proceeded in a normal, though slower manner. After 6 days in culture, the developmental stage of the 15-day-old metatarsals resembled that of day 17 *in vivo* (Haaijman *et al,* 1997). Thus rapid development occurs in the metatarsal rudiments between days 15 and 17. At 15 days gestation the metatarsal rudiments consist of a core of weakly metachromatic cartilage surrounded by mesenchymal cells of the early perichondrium. Chondrocyte hypertrophy and matrix mineralisation are not yet present. In foetal mouse metatarsals mRNA expression patterns have shown that type I collagen is expressed by cells of the connective tissue and osteogenic lineage. Type II collagen is a general marker for the chondrogenic lineage (Devlin *et al,* 1988; Hayashi *et al,* 1986) and type X collagen is a marker of the hypertrophic chondrocyte differentiation (Gibson & Flint, 1985). At day 17, endochondral ossification has reached a stage characterised by a zone of mineralised hypertrophic

cartilage in the centre of the rudiment. A thin bone collar lines the mineralised centre where type II collagen was expressed. Type X collagen mRNA was not yet expressed in day 15 metatarsals, but by day 17 a large area of type X mRNA expression was present, demarcating the hypertrophic chondrocytes in the centre of the rudiment. Type X collagen disappeared again in the area of calcified cartilage (Haaijman *et al*, 1997). All this data suggests that cultured metatarsals possess an intrinsic differentiation capacity to undergo endochondral ossification in a normal albeit a slower manner in the absence of serum (Haaijman *et al*, 1997).

Postnatally, the ratio of the mineralising zone to the cartilaginous proliferating and hypertrophic zones increases and thus there is reduced potential for overall growth. At 18 days the metatarsals display a small central core of mineralised cartilage juxtaposed on both sides to a translucent area representing the hypertrophic chondrocytes. The proliferating chondrocytes are situated at both the proximal and distal ends of the metatarsals (Fig 1.6).

Figure 1.6. Foetal Metatarsal Culture: A metatarsal cultured for 4 days is shown. This displays a central core of mineralised cartilage, flanked with the lucent zones of hypertrophic chondrocytes and the proliferating chondrocytes at either end.



A few studies have utilised the foetal organ explants to investigate the actions of GC, GH and IGF-I in this model system (Picherit *et al,* 2000, Scheven & Hamilton 1991, Coxam *et al,* 1996) but none has looked at the interactions of these drugs and hormones at the molecular and histological level.

Thus in contrast to cell culture studies, the metatarsal explant model in serum free medium allows GC and hormonal effects on growth and cartilage differentiation to be studied in greater detail, as the heterogeneous population of cells allows interactions to occur between cells and the extracellular matrix in the morphogenetically patterned way that replicates the *in vivo* process.

1.7.3 Prenatal GC exposure

Impaired foetal growth can be caused by a number of foetal, maternal, placental, and demographic factors but in up to 40% of SGA cases, no cause for growth restriction can be identified (Wollmann, 1998). Prenatal exposure to GC is known to cause growth restriction in most mammalian species including humans. (Mosier *et al,* 1982; Reinisch *et al,* 1978). Indeed, human IUGR is associated with elevated maternal and foetal levels of endogenous GC (Goland *et al,* 1993), and these levels are more likely to be elevated in those who fail to display catch-up growth (Economides *et al,* 1988; Clark *et al,* 1996; Cianfarani *et al,* 2003). It is unclear whether the raised GC levels in the foetus per se have a direct effect at the level of the growth plate chondrocyte.

SGA mice can be produced by the administration of prenatal Dex by subcutaneous injections of 100μg/kg Dex for the last 6 days (14-20) of pregnancy (Nyirenda *et al,* 1998). This can cause a 15-20% IUGR without foetal loss or alterations in the length of gestation. The extent of reduction in birth weight is similar to that observed by investigators using other rodent models of IUGR such as bilateral uterine artery ligation and protein restriction (Mosier *et al,* 1982; Harrel *et al,* 1995). In pregnant sheep,

Betamethasone administered in single or multiple doses causes a decrease in body weight in the offspring with increasing effects after multiple doses (Jobe *et al,* 1998). Although this model of SGA offspring has been utilised to demonstrate that prenatal GC exposure is linked to conditions such as hypertension, hyperglycaemia and hyperinsulinaemia (Seckl, 2001) there is little work looking at the effect of prenatal GCs on developing bones.

1.8 AIMS OF THE THESIS

This thesis was designed to discover the mechanisms by which GCs induce growth retardation in children, and subsequently to explore ways in which to ameliorate these negative effects. This was undertaken by utilising and refining novel models of longitudinal bone growth and to gain further insights into the molecular pathways that may be disrupted by GCs.

The principal aims of this thesis are to:

1. Fully characterise the ATDC5 chondrocyte cell line to understand the key time points in the chondrocyte life cycle that are affected by GCs.

2. Identify the cellular and molecular mechanisms underlying the adverse effects of GCs on growth plate chondrocytes.

3. Determine the effects of GH and IGF-I and their ability to ameliorate the GC effects in different growth plate models.

4. Study the effects of prenatal exposure to Dex on neonatal weight and length.

CHAPTER 2

SHORT-TERM EFFECTS ON LINEAR GROWTH AND BONE TURNOVER IN CHILDREN RANDOMIZED TO RECEIVE PRED OR DEX.

2. Short-term Effects On Linear Growth and Bone Turnover In Children Randomized To Receive Pred or Dex.

2.1 Introduction

The functional effects of steroids on target tissues is difficult to predict and their use is hampered in some individuals more than others because of side-effects such as growth retardation, osteoporosis, hypertension, altered body composition and altered blood glucose homeostasis. Alterations in growth and bone turnover, as assessed by knemometry and markers of GH secretion and bone turnover, can also occur during relatively short periods of GC therapy (Ahmed *et al,* 1999, Crofton *et a*l, 1998). The onset and severity of these GC induced effects may be dependent on the duration of therapy and the nature of the steroid compound, and the comparative biological potency of the GC such as Dex and Pred may be tissue specific (Orth & Kovacs, 1998). For instance, in children and young adults with congenital adrenal hyperplasia, Dex may be about 25 and 80 times more adrenal suppressive than Pred and hydrocortisone, respectively (Hansen & Loriaux, 1976, Hughes & Read, 1982). Dex is also reported to be 4 times more potent at suppressing the hypothalamo-pituitary-adrenal axis than Pred but it may be 16 times more lymphocytotoxic than Pred (Kaspers *et a*l, 1996). Dex also displays better CNS penetration (Balis *et* al, 1987), and this feature has led to a randomised trial to look at its efficacy in the treatment of ALL (Gaynon & Carel, 1999).

The relative potency of different GC on growth and bone turnover is unclear as there are no *in vivo* studies in children. The current study was performed on children entering a national trial of ALL therapy in which they were randomised to receive Pred or Dex as part of induction of remission and continuing treatment. By accounting for possible confounding factors such as other concurrent chemotherapy and the effects of the disease, the randomisation process provided a suitable opportunity to compare the short-term

effects of Pred and Dex on growth and bone turnover by the same methods as employed in earlier studies (Ahmed *et al,* 1999, Crofton *et a*l, 1998).

2.2 Subjects & Methods

a) Patients

All children presenting to a paediatric oncology centre with a diagnosis of ALL were eligible for the study. Out of a total of 22 eligible children, one child was excluded because of CNS disease at presentation and 2 children and their families declined participation in the study. Nineteen children (eight boys, eleven girls) with a median age of 5.9yrs (range, 2.6, 13yrs) were recruited.

b) Design

The children were entered into the national UK trial of ALL – MRC ALL97/99 and randomized to receive Pred (40mg/m^2, daily) or Dex (6.5mg/m^2, daily) as induction chemotherapy for 5 weeks (Table 2.1). The comparative doses of the GC were chosen based on previous lymphocytotoxic data (Kaspers *et al* 1996). GC were also administered as 5-day blocks in weeks 9 and 13. Brief details of the chemotherapy regimens are outlined in Fig.2.1. Seventeen out of 19 children received either Group A or B chemotherapy, which was determined by their age and white cell count at presentation. The remaining 2 children received a more intensive regimen of chemotherapy as they failed to remit over the first 4 weeks of induction therapy. Data from the first 4 weeks was analysed up to 5 weeks in these children. The study was approved by the local research ethics committee and informed consent was given by all parents and their children, where appropriate.

c) Samples and Anthropometric Measurements

Collection of blood samples coincided with vascular access for clinical management and were performed every one to two weeks at approximately the same time in late morning (Fig.2.1). Lower leg length was measured by knemometry at weekly intervals using the random zero method (Ahmed *et al*, 1995). The precision of the measurement was assessed by calculating the technical error (TE), ie 1SD from the mean of a set of triplicate measurements. The overall mean TE (+/- 1SD) was 0.15mm (0.13). Knemometry was performed in 13 (6 Dex, 7 Pred) out of 19 children. The remainder of the children had a median age of 3.1 years and were too young to cooperate with the measurements. Body weight with undergarments was measured by an electronic scale.

d) IGF-1

IGF-1 concentrations were measured using a two-site immunoenzymometric (IEMA) assay incorporating a sample pre-treatment to inactivate binding proteins (Immunodiagnostic Systems Limited, Tyne and Wear, United Kingdom). The intra-assay and inter-assay coefficients of variaton were <5% and <8%, respectively, over the sample concentration range. The lower limit detection of the assay was 10 ng/ml.

e) Bone Markers

All samples were analysed in duplicate and samples from each patient were analysed in a single run to minimise analytical variation. Bone Alkaline Phosphatase (bALP) was measured in plasma by ELISA (Alkphase-B, Metra Biosystems Inc, Mountain View, CA, USA). The sensitivity of the assay was 0.7U/l and within-run and between-runs coefficients of variation were <5% and <8%, respectively. Deoxypyridinoline crosslinks (DPD) were measured in urine by ELISA (Pyrilink-D, Metra Biosystems Inc, Mountain View, CA, USA). Assay sensitivity was 1.1nmol/l and within-run and between-runs

coefficients of variation were <6% and <11%, respectively. The results were expressed in relation to creatinine measured on the same urine sample.

2.3 Statistical Analyses

For the knemometry data, lower leg length velocity (LLLV) was calculated for each time point by subtracting the LLL at that time point from that measured at the previous time point, and dividing by the time interval (in weeks) between the two measurements. LLLV was expressed as millimetres per week (mm/wk). Change in LLL was also expressed as a percentage of the previous LLL. Body weight was expressed as a percentage change in body weight from the pre-treatment body weight. Serum IGF-I, Bone ALP and urinary DPD were expressed as absolute values as well as percentage change in IGF-I, bALP and DPD (%IGF-I, %bALP, %DPD). The data were expressed as medians and ranges and analysed using non-parametric tests. Comparison between groups was performed using the Mann-Whitney U test and Spearman rank correlations were used to compare any association between variables at each time point. Data were analyzed using SPSS software v9.0.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 97 SR-2 (Microsoft Corp, Redmond, WA, USA).

2.4 Results

Lower Leg Length Velocity (LLLV)

At Wk 2 of therapy, median LLLV in the Dex group was -1.5mm/wk (range, -2.1, 0.7) and significantly lower than the LLLV in the Pred group which was -0.1mm/wk (range -0.28, 0.2) ($p<0.05$). During GC therapy, LLLV rose temporarily before falling by week 6 when GC therapy ended. In the Dex group, LLLV remained lower at week 8 (med LLLV, -0.3mm/wk, r -1.3, 0) compared to LLLV in the Pred group at 0.3mm/wk (r, 0.2, 1.0) ($p<0.05$). By weeks 12 and 16 of the chemotherapy protocol, LLLV was similar in both

groups (Fig.2.2a). Mean LLLV between week 2 & 8 in the Dex and Pred groups were -0.27mm/wk and 0.18mm/wk respectively. Compared to previous studies of healthy children where the mean LLLV was 0.39mm/wk (1SD,0.12) (Ahmed *et al*, 1995), the LLLV in the Dex and Pred groups were 5.7SD and 1.8SD below the mean.

Body Weight

During GC therapy, body weight showed an increase after week 2 and reached a peak in both groups of children at week 6 (Fig. 2.2b). Although, the increase in weight from baseline was generally greater in the Dex group, the difference did not reach statistical significance until week 5 when the median change in weight from baseline in the Dex group was twice that of the Pred groups at 17.5% (range 5 - 25) and 8.7% (range –3 - 18) respectively ($p<0.05$). Children in the Dex group continued to remain at a higher level of weight gain from baseline until week 16 when there weight gain became similar to that of the Pred group (Fig. 2.2b). At Wks 2, 3, 4, 6 and 8, the percentage gain in weight was 1, 9.5, 1.6, 2.0 and 2.2 times higher in the Dex group than the Pred group, respectively. Therefore, on average, percentage gain in weight was 3.1 times higher in the Dex group.

IGF-I

At presentation, median IGF-I level for the whole group was 83.5 μg/l (31.8, 293); median IGF-I level in the Pred and Dex groups were 69.3 μg/l (33.8, 175) and 166 μg/l (39, 293), respectively ($p=0.12$). During the study period median IGF-I levels remained between 100 μg/l and 150 μg/l in the whole group and the absolute values were similar in the Pred and Dex groups. However, in the Dex group, IGF-I levels fell much more markedly during the period of steroid therapy and continued to remain lower than baseline (Fig.2.2c) At weeks 4, 6 and 8, median change in IGF-I from baseline was lower in the Dex group than

the Pred group at -16% (-45, -9) vs 19% (-50, 195), -3% (-58,43) vs 56% (-43, 146) and -42% (-74, 3) vs 44% (-73, 415), respectively ($p<0.05$).

Bone Alkaline Phosphatase

At presentation median bALP concentration was low but similar in the Pred and Dex groups at 37U/L (17, 159) and 46U/L (23, 69). From week 1 to week 3, change in bALP, as median %bALP, was 72% (-8, 304) in the Pred group, whereas in the Dex group %bALP was –1% (-28, 23) ($p<0.005$). By Wk 3 of therapy, median bALP concentration was higher in the Pred group at 65U/L (36, 187) than in the Dex group at 39U/L (26, 60) ($p<0.05$). By the end of Pred therapy at week 6, median bALP concentration had fallen to a similar level in both groups (Fig 2.2d). By week 12 and 16, pooled bALP concentrations had risen to a median value of 80U/L (36, 123) compared to a median pooled value of 44U/L (17, 187) between weeks 1 and 8 ($p<0.005$). At weeks 2, 3, 4, 6 and 8, bALP levels were 1, 1.7, 1.4, 1.3 and 1.2 times lower in the Dex group than the Pred group, respectively. Therefore, on average, bALP was 1.3 times lower in the Dex group than the Pred group.

Deoxypyridinoline

At presentation, median DPD excretion was similar in the Pred and Dex groups at 22nmol/l (17, 38) and 20nmol/l (12, 26). DPD excretion fell in both groups reaching a nadir between week 3 and 6 (Fig.2.2e). The percentage change of DPD in the Pred and Dex group from week 1 to week 3 was –34% (-7, 14) and – 53% (-69, -6), respectively (NS). By week 8, DPD excretion had started to rise more dramatically in the Pred group such that the median DPD was 35nmol/l (10, 53) in the Pred group and 22 (9, 30) in the Dex group ($p<0.05$). Subsequently, DPD excretion continued to rise but there was wide variation during this recovery period (Fig.2.2e). At Wks 2, 3, 4, 6 and 8, DPD excretion

was 1.4, 1.6, 1.4, 1.2 and 2.2 times lower in the Dex group than the Pred group, respectively. Therefore, on average, DPD was 1.5 times lower in the Dex group than the Pred group.

2.5 Discussion

Dex and Pred are two GCs that are commonly used in immunosuppressive therapy. As Dex has a longer half-life, higher lymphocytoxicity and penetrates better into the CSF it may be better suited for treatment in ALL (Gaynon & Carrel, 1999, Kaspers *et al* 1996, Veerman *et al,* 1990). The improved CNS penetration may explain the finding of an increased risk of neurocognitive late effects in those children who received Dex rather than Pred during ALL treatment (Waber *et a*l, 2000). Besides ALL, there may be other conditions where Dex may be a more efficacious than Pred but a lack of data on relative efficacy as well as adverse effects of the two drugs has hindered an objective choice. By evaluating a number of short-term physical and biochemical changes, this study attempted to quantify the effects of Pred and Dex in children.

The changes documented in short-term growth and bone turnover in this study were generally similar to a previous study of children undergoing treatment for ALL using Pred during induction of remission (Ahmed *et a*l, 1999, Crofton *et a*l, 1998). By the end of the induction period there was a reduction in short-term growth and suppression of markers of bone turnover.

The current study shows that at the dose used, Dex had a more profound suppressive effect on bone turnover and short-term growth than Pred. Short-term growth as assessed by knemometry and bone formation as assessed by bALP levels was less in the Dex group. Similar changes were also observed in urinary DPD excretion, a marker of bone resorption (Calvo *et a*l, 1996). Not only did they fall further, bone formation and resorption remained suppressed for longer in the Dex group. In addition, the rise in bALP seen over the first

two weeks of Pred was not observed in the group who received Dex. This paradoxical rise in bALP has been attributed to premature maturation of osteoblasts and our data suggests that this effect may be specific to Pred (Canalis, 1996, Stein *et al*, 1990). The rise and subsequent fall in bALP emphasizes the importance of sequential measurements of bALP during monitoring of GC effects on bone formation. These results showing an increased potency of Dex are in keeping with the observation of an increased cumulative incidence of fractures in those children who received Dex compared to Pred during ALL therapy (Strauss *et al*, 2001).

GC induced changes in growth may be due to a combination of factors such as a disruption in the GH-IGF-I axis and direct effects on the growth plate (Robson, 1999). High dose GC therapy alters pulsatility of GH secretion through an elevation of somatostatin tone and may alter GH binding protein activity (Tonshoff *et al*, 1996, Gabrielsson *et al*, 1995). Previous studies have suggested that a state of GH resistance may exist in children undergoing ALL treatment (Crofton *et* al, 1998) and the current study shows that Dex is more potent at depressing IGF-I levels than Pred. Absolute IGF-I levels are age dependent and may be easier to assess following standardization for age as well as standard deviation scores. However this standardization was not employed in this study as the age range of children in the two randomized groups was similar and the aim of the study was to assess the change in IGF-I levels over a short period of a few weeks.

GCs promote food consumption both directly and through stimulation of neuropeptide Y and inhibition of corticotrophin releasing hormone release (Tataranni *et* al, 1996, Tempel *et* al, 1994). Short-term changes in energy intake secondary to GC administration during maintenance treatment in children with ALL have recently been reported by Reilly *et al* (2001). This group did not show any significant differences in energy intake or weight gain between Dex and Pred and this may have been due to the short, 5-day period of steroid administration. Our study shows that the changes in weight gain were more

marked in those children who were randomised to receive Dex for a longer period of 4 weeks.

Previous studies of short-term growth by knemometry have shown that changes in body weight may independently influence lower leg length due to the action of gravity on the soft tissues of the lower leg (Hermanussen *et a*l, 1988, Ahmed *et a*l, 1996). Previous studies in a pregnant adult subject showed that an increase in body weight was associated with a reduction in LLL until the subject developed dependent oedema at which point LLL started to increase (Ahmed *et al,* 1996). Although changes in general body weight may explain the negative growth or actual shrinkage that was observed in some children, weight changes cannot solely account for the observed LLLV differences between the two groups as some of the major differences in LLLV between the Pred & Dex groups preceded the major differences in body weight in the current study. Some of the early shrinkage may be explained by ultrasound studies that show that systemic steroids therapy may have an early water depleting effect on connective tissues and may lead to a reduction in subcutis thickness (Schou *et al,* 2003). This water depleting effect may similarly affect the growth plate which is susceptible to the effects of GCs.

The Mann-Whitney U test was used to display the difference and thus the trend between the medians for each group over the time. However there can be limitations to this approach as the curve joining the means may not be a good descriptor for an individual as it does not take into account the fact that different measurements at different time points are from the same subject – thus each time point is analysed separately. Also successive results are likely to be correlated to the significance level of the previous measurement. A possible alternative approach would have been to utilise summary measures where the summary of a response in the individual is identified and calculated for each subject prior to undertaking statistical analysis (Matthews *et al*, 1990).

Nineteen children were recruited into the study, and after randomisation 7 were left in the Dex group. Although differences were detected in between Dex and Pred groups it is possible that a type 1 error has occurred (that is a difference was detected between the GCs when one did not exist). This could be circumvented by increasing the number of subjects in each group. Likewise the chances of a type 2 error could also be minimised by increasing the numbers in the groups, but this is often difficult if the condition is not common in the population.

The dose of Pred (in milligrammes) was approximately 6 times that used of Dex and the study shows that LLLV was about 3 times lower in the Dex group over the period of treatment and the subsequent few weeks. Therefore as an estimate, between weeks 1 and 8 of the 5-week period of GC therapy, Dex was 18 times more potent at suppressing short-term growth. Similar calculations estimate that Dex was 19 times more potent at raising body weight and about 8 to 9 times more potent at suppressing bone turnover as assessed by serum bALP concentration and urinary DPD excretion. After 8 weeks all the above parameters, except change in body weight, were similar in the two groups. Thus although GC dosing is based on anti-inflammatory activity, they can have variable potencies in different tissues. Indeed blood samples from untreated ALL children also show a variation in antileukaemic activity after exposure to GCs, with Dex displaying a 16 fold greater effect on *in vitro* antileukaemic activity than Pred (Kaspers *et al*, 1996).

In summary, this study has attempted to quantify the relative effect of the two corticosteroids, Dex and Pred on short-term growth and bone turnover. Compared to Pred, Dex may be almost 20 times more potent at suppressing short-term growth and at raising body weight and almost 10 times more potent at suppressing bone turnover. GC have a variable effect on different parameters of growth and bone turnover and the intensity may depend on the steroid used. Subsequent studies in this thesis have progressed to explore the basic mechanisms by which GC impact growth.

Table 2.1 ALL Randomisation

Details of Patients who were randomised to receive Prednisolone (Pred) or Dexamethasone (Dex) as steroid therapy.

	Pred	Dex
N	12	7
Age (yrs)	5.6	6.3
(range)	(2.6,12.3)	(4.3,13)
Prepubertal	11/12	6/7
Knemometry	7	6
Group A, B, C	7, 4, 1	4, 2, 1

Figure 2.1

A flow diagram of chemotherapy schedule over the first 16 wks of MRC-ALL97/99 in children receiving regimen A or B. Children entering the trial were randomized to receive Dex or Pred. Drugs in the yellow area were only administered to Group A, drugs in the grey shaded area were only administered to Group B and drugs in the open area were administered to both groups. Drug doses and route of administration: Prednisolone (Pred), 40mg/m^2/day, oral; Dexamethasone (Dex), 6.5mg/m^2/day, oral; Vincristine, 1.5mg/m^2, iv; L-Asparaginase, 6000U/m^2; Cytarabine Intrathecal (IT), <2yrs-30mg, 2yrs-50mg, >3yrs-70mg; Methotrexate Intrathecal (IT), <2yrs-8mg, 2yrs-10mg, >3yrs-12mg; 6-Mercaptopurine (6MP), 75mg/m^2/day, oral; 6-Thioguanine (6TG), 40mg/m^2/day, oral; Daunorubicin, 25mg/m^2, iv; Cytarabine (Ara-C), 75mg/m^2/day, iv; Cyclophosphamide 1000mg/m^2, iv.

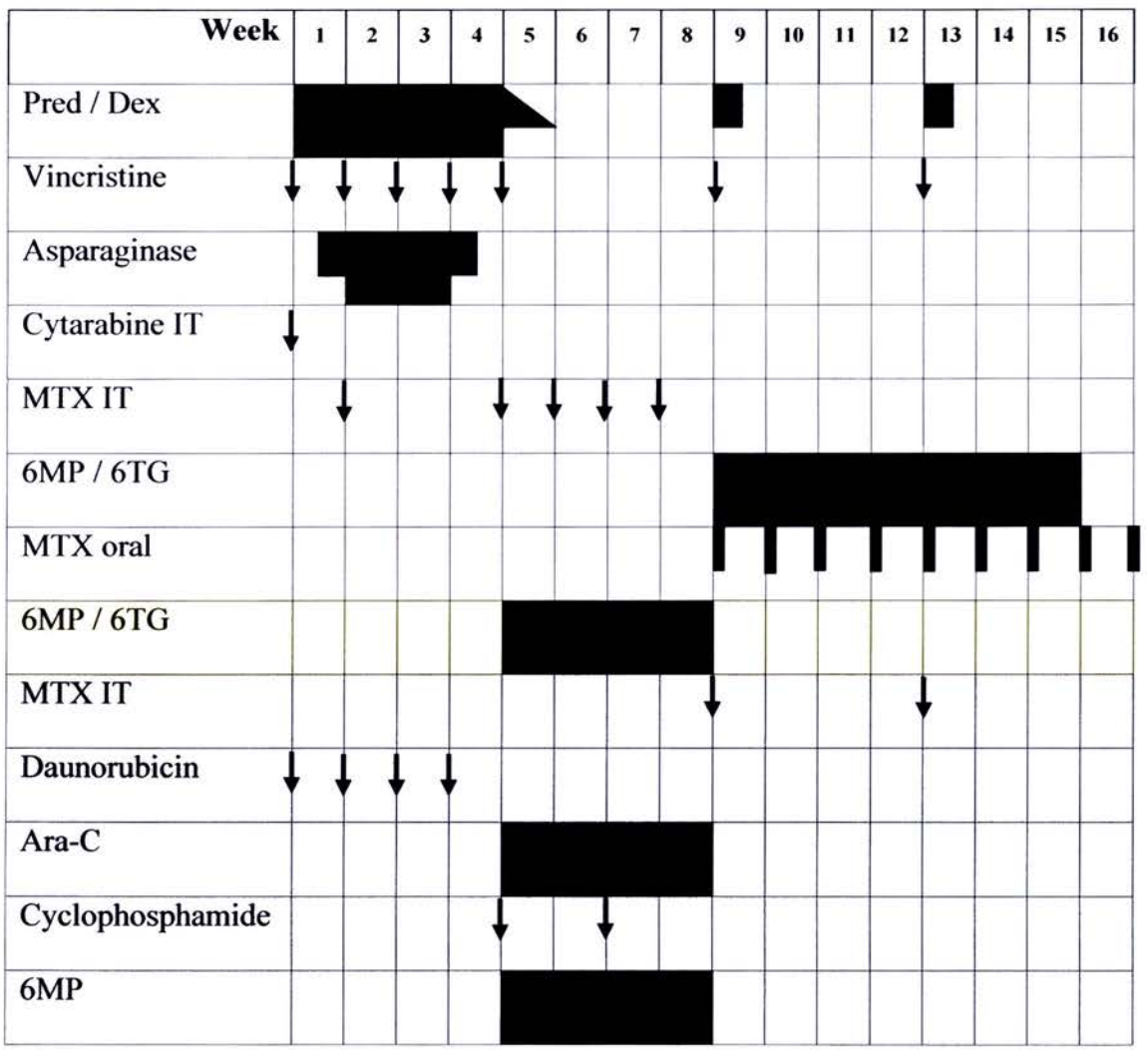


Figure 2.2

The effect of Pred (solid line) and Dex (broken line) on markers of bone growth and turnover. **(a)** Lower Leg Length Velocity (LLLV, mm/wk), **(b)** Percentage change in body weight from baseline, **(c)** Serum IGF-I concentration (IGF-I μg/l), **(d)** Serum bone ALP concentration (bALP, U/l) and **(e)** Urinary DPD excretion corrected for creatinine excretion (DPD, nmol/l/creat, nmol/l) over the first 16 weeks of ALL97. GC therapy was administered as induction of remission therapy for 4 weeks at full dose and for an additional week as a tapering dose. GCs were also administered as 5-day blocks in Wk 9 and 13. The results are presented as median and 25th and 75th centile values and measurements at any one time point in the two groups are clustered in pairs. * $p < 0.05$.

a

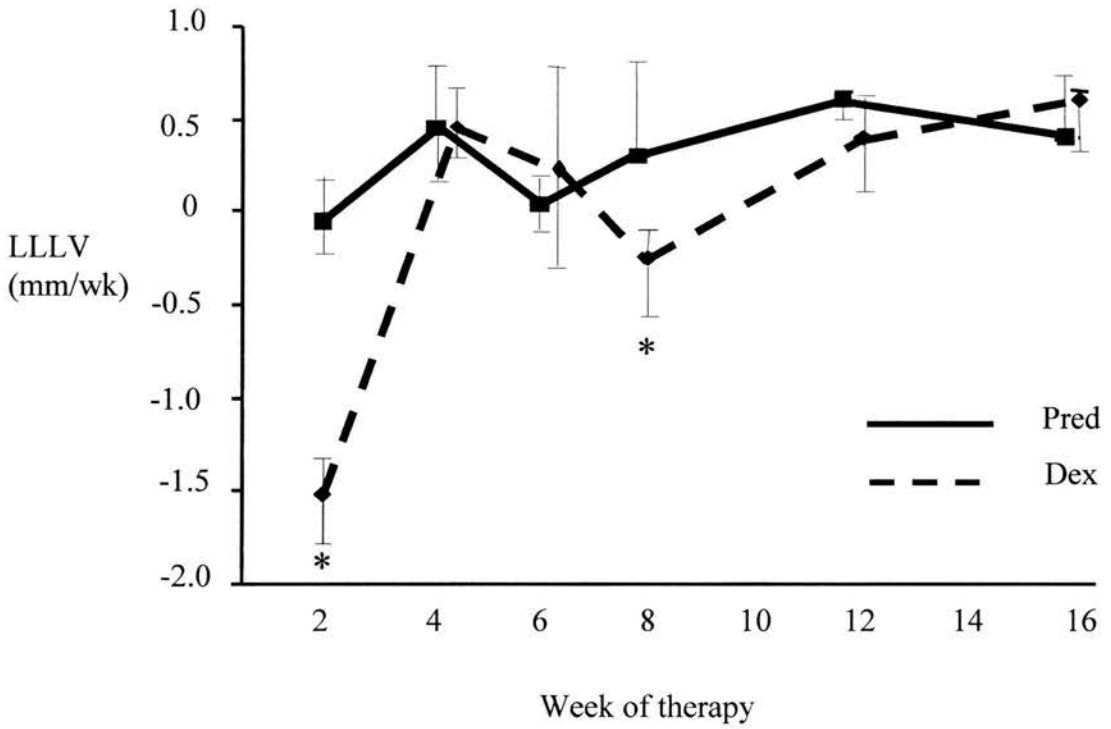
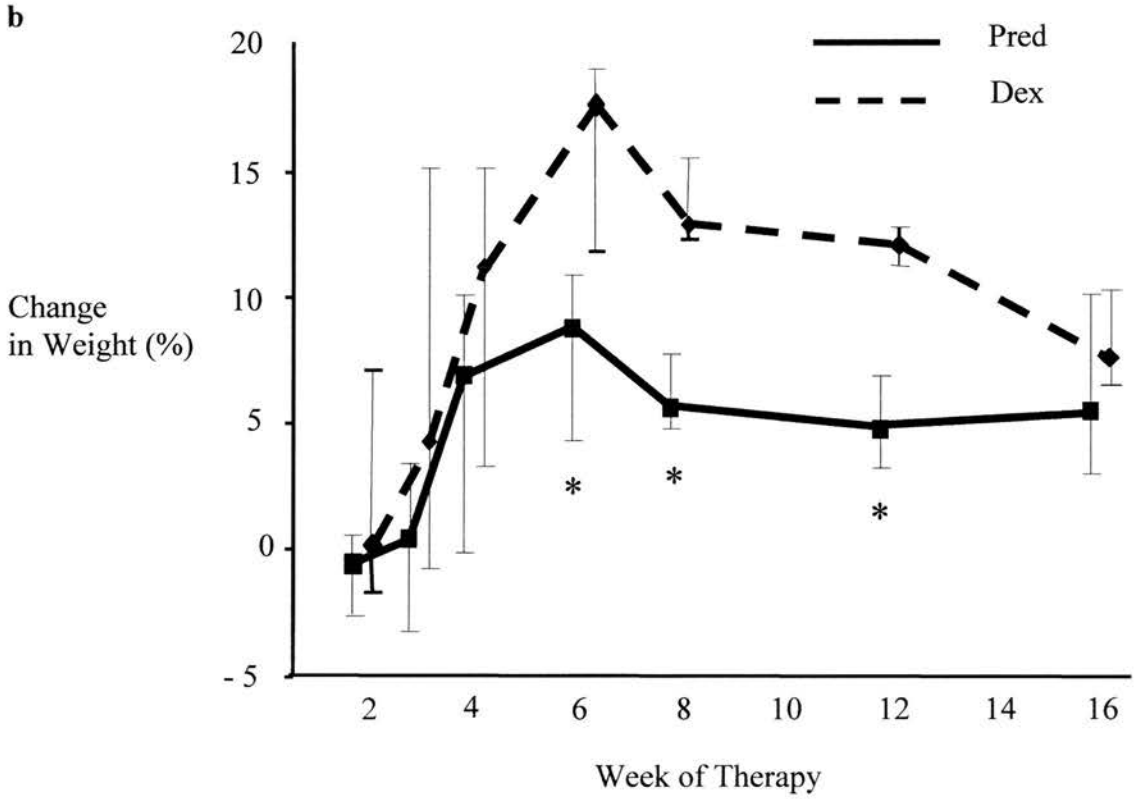


Figure 2.2 (cont)

b



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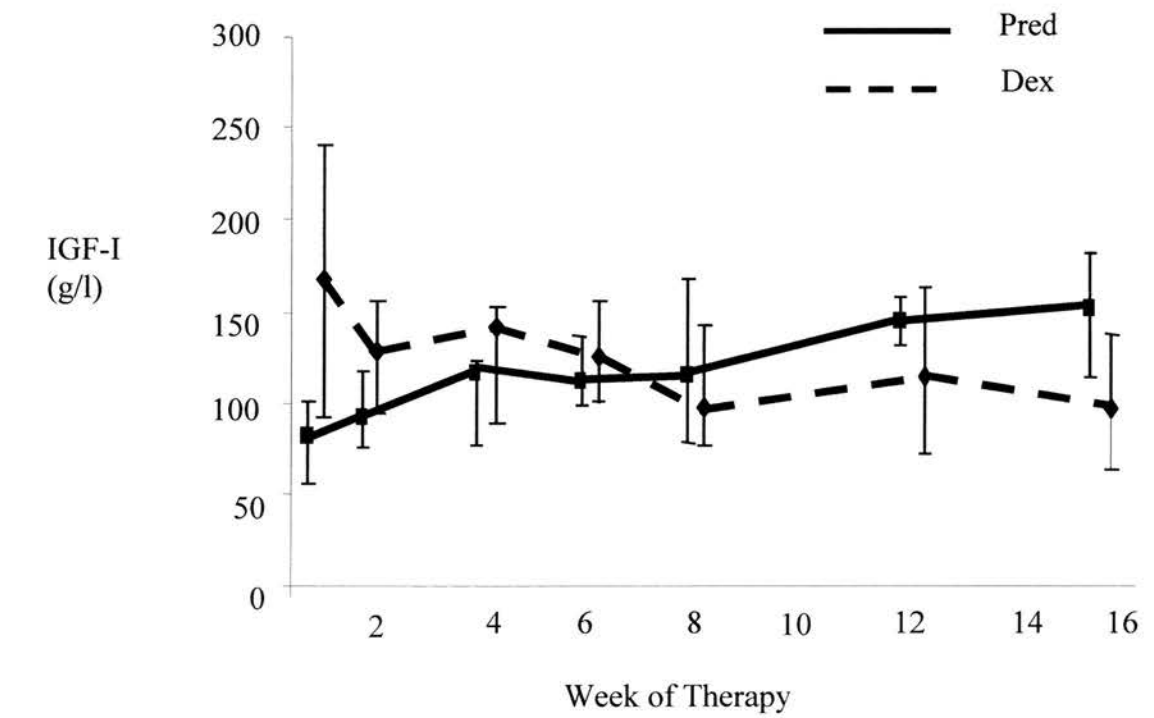

Figure 2.2 (cont)

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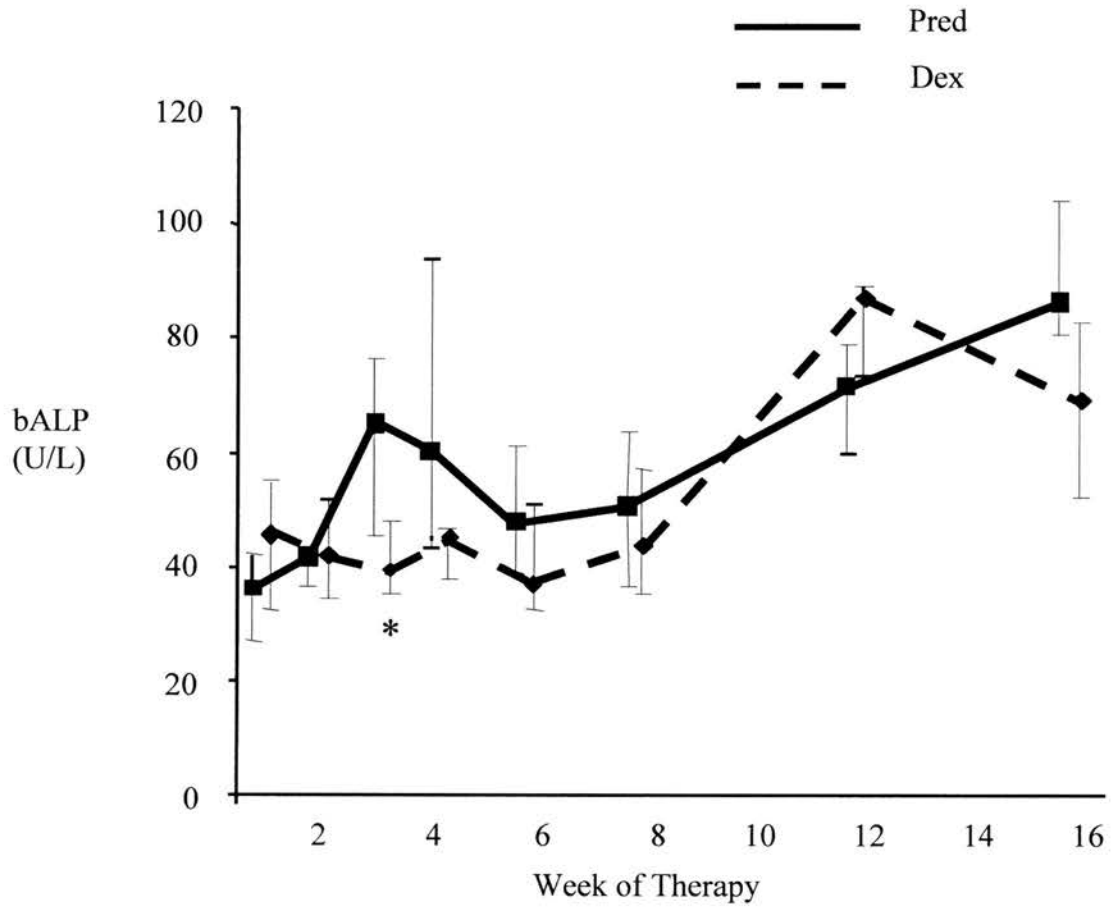
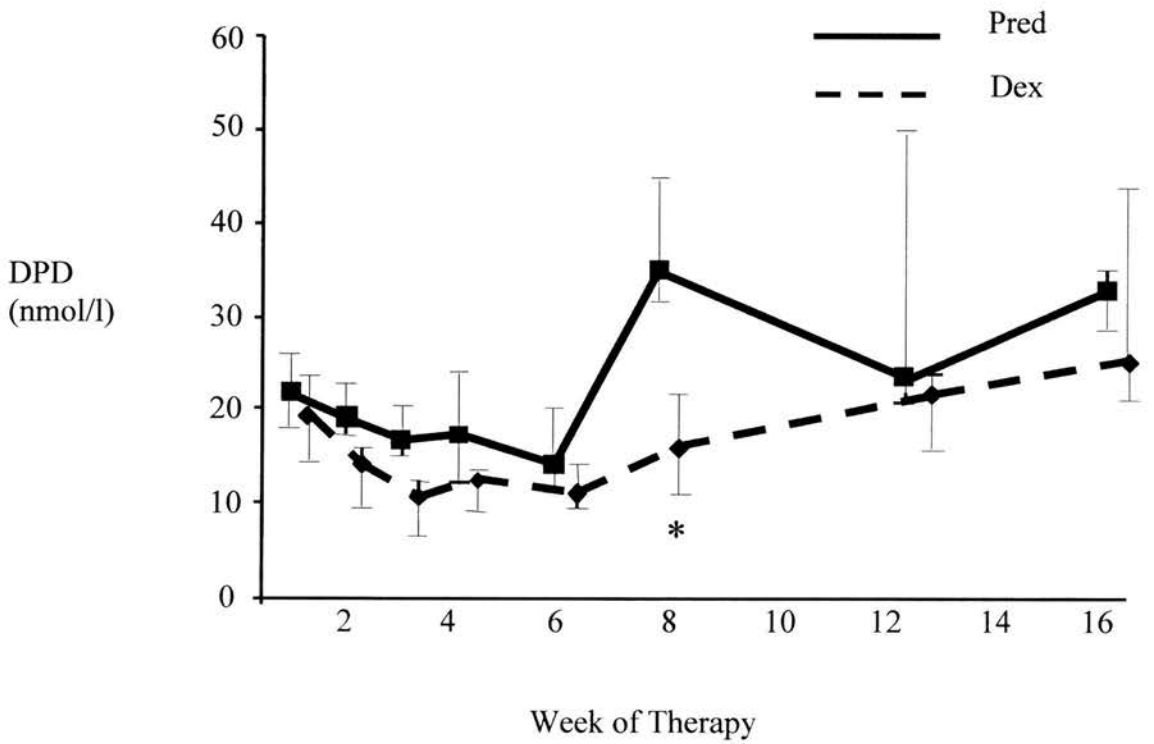


e



CHAPTER 3

CELL CULTURE STUDIES

3.1 ATDC5 Characterisation

3.1.1 Introduction

The direct effects of GC on chondrocytes are not clearly understood and previous studies that have examined the effect of GC on primary growth plate chondrocytes have been unable to elucidate the effect of GC on the different stages of the chondrocyte life cycle due to the heterogeneous mixture of maturational phenotypes (Robson *et al,* 1998, Koedam *et al,* 2000).

This ATDC5 cell line was isolated from a teratocarcinoma stem cell line on the basis of its chondrogenic potential. In the presence of insulin it was shown that the line undergoes the early phase of differentiation and proliferation to chondrocytes (Atsumi *et al,* 1990). This is associated with an initial elevation in type II collagen, which heralds the onset of a chondrogenic lineage (Hayashi *et al,* 1986). The late phase differentiation of the cell line is characterised by an increase in cell volume as well as a marked increase in ALP activity; this was associated with type X collagen expression (Shukanami *et al,* 1996) both of which are implicated in the subsequent mineralisation of cartilage (McLean *et al,* 1987; Wu *et al,* 1989). With increasing time in culture these hypertrophic ATDC5 chondrocytes undergo the process of mineralisation, which is visible macroscopically.

The first phase of the study was to establish the temporal sequence of events that occur in the maturational phenotypes of the ATDC5 chondrocyte cell line. Therefore to fully characterise the cell line, the expression of markers of the phenotypes over time were elucidated.

3.1.2 Materials and Methods

a) Chondrocyte cell culture

The ATDC5 chondrocyte line was obtained from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al* (1990). Cells were cultured at a density of 12000 cells per cm^2 in multi-well plates (Costar, High Wycombe, UK) in maintenance medium (DMEM/Hams' F12 (Invitrogen, Paisley, UK) supplemented with 5% Foetal Calf Serum (FCS) (Invitrogen), 10ug/ml human transferrin and 3 x 10^{-8}M sodium selenite (Sigma, Poole, UK) until confluent (Day 6). Thereafter, differentiation was induced by the addition of insulin (10ug/ml,Sigma) and ascorbic acid (20ug/ml) to the maintenance medium (differentiation medium). Incubation was at 37°C in a humidified atmosphere of 95% air/5% CO_2 and the medium was changed every second day.

b) Gene expression

For the determination of chondrocyte phenotype, cells were grown for up to 20 days as above and RNA was extracted, reverse transcribed and analysed for collagen type II and type X expression at Day 6, 8, 10, 13, 15, 17 and 20 by semi-quantitative RT-PCR. 18S was utilized as the house keeping gene as it is not influenced by any treatments and it gives an equal PCR band intensity in all preparations and time points.

c) *RNA extraction*

Total RNA was extracted from chondrocytes by repeated aspiration through a 25-gauge syringe needle in 1.5 ml Ultraspec (Biotecx, Houston, TX). After extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack resin (Biotecx) following the manufacturer's protocol. After washing with 75% ethanol, the RNA was eluted in 100µl ribonuclease-free water (Houston *et al,* 1999). In

each case the 260/280 ratio was 1.9–2.0, confirming the purity of the RNA. All preparations were diluted to a concentration of 50 ng/μl and stored at -70 C.

d) Semiquantative RT-PCR

Gene expression was analyzed by semiquantitative RT-PCR (Houston *et al,* 1999, Farquharson *et al,* 1999, Jefferies *et al,* 2000). Aliquots of 500 ng RNA (or an equivalent volume of water as a control) were reverse transcribed in 20-μl reactions with 200 ng random hexamers and 200 U Superscript II reverse transcriptase using the Superscript preamplification protocol (Invitrogen). PCR was performed in 20-μl reactions containing cDNA equivalent to 10 ng RNA and 200 nM gene-specific primers in 11.1 x PCR buffer (Jefferies *et al,* 1998) (Table 3.1). The cycling profile was 1 min at 92 C (first cycle, 2 min), 1 min at 55 C, and 1 min at 70 C. The number of cycles performed was carefully titrated to ensure that the reactions were in the exponential phase. Reaction products were analyzed on 1.5% agarose gels in the presence of ethidium bromide (250 μg/litre), and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK).

3.1.3 Results

Temporal Expression of chondrocyte phenotype specific markers.

Using gene specific primers (Table 3.1), collagen type II expression by the ATDC5 cells was first noted after 10 days in culture indicating that the differentiation of mesenchymal cells to the chondrocyte phenotype (chondrogenesis) had occurred. Similarly, collagen type X expression was noted from Day 15 onwards indicating that terminal differentiation of the chondrocytes occurred from Day 10 to Day 15 (Fig 3.1). As anticipated 18S gave similar band intensities at all time points confirming that the expression of the target genes were real changes (Fig 3.1).

The ATDC5 cells were in a confluent monolayer by day 6. In the presence of insulin the cells underwent chondrogenic differentiation to form cartilage nodules. These cartilage nodules continued to increase in size due to proliferation of the chondrocytes. A typical nodule at day 12 is shown in Fig 3.2.

3.1.4 Discussion

Characterisation of the ATDC5 cell line revealed that type II collagen is expressed at 10 days, which is a general marker for chondrocyte lineage and indicates the presence of a differentiated chondrocyte. Further differentiation and terminal differentiation is characterised by expression of collagen type X by 15 days, indicating the presence of the hypertrophic chondrocyte phenotype. If left longer in culture these chondrocytes would then undergo mineralisation as seen *in vivo* (Shukunami *et al,* 1997). This is similar to findings in chondrocyte populations *in vivo* where the type X collagen could not be detected in undifferentiated cells or proliferating chondrocytes. (Iyama *et al*, 1994).

Subsequent experiments studied the effects of Dex and Pred during the periods leading up to the expression of these maturation markers. This information is crucial as it allows the study of two critical events during cartilage formation: the early differentiation of committed mesenchymal cells into chondrocytes (**chondrogenesis**) and the **terminal differentiation** of proliferating to hypertrophic chondrocytes (Cancedda *et al,* 1995).

Thus it was possible to study for the first time the effects of GC on key points of cartilage formation in a homogenous population of chondrocytes rather than previous studies which have contained a phenotypically diverse population of primary chondrocytes (Robson *et al,* 1998; Koedam *et al,* 2000).

Table 3.1. Primer pairs used for specific gene analysis

Gene	Primer Sequence	Cycles	Product Size (bp)
18S	Unknown, purchased commercially from Ambion	15	488
Collagen Type II	TTAGAAAGGGGAGCACAGTCC(F) TACACTGCCATGAAGCATGG(R)	35	323
Collagen Type X	CAGAGGAAGCCAGGAAAGC (F) GGTGTCCAGGACTTCCATAGC(R)	32	330

Figure 3.1. Semiquantative RT-PCR analysis of the expression of chondrocyte marker genes. Collagen type II is expressed from day 10 and collagen type X from day 15. 18S is expressed at all time points with similar intensity.

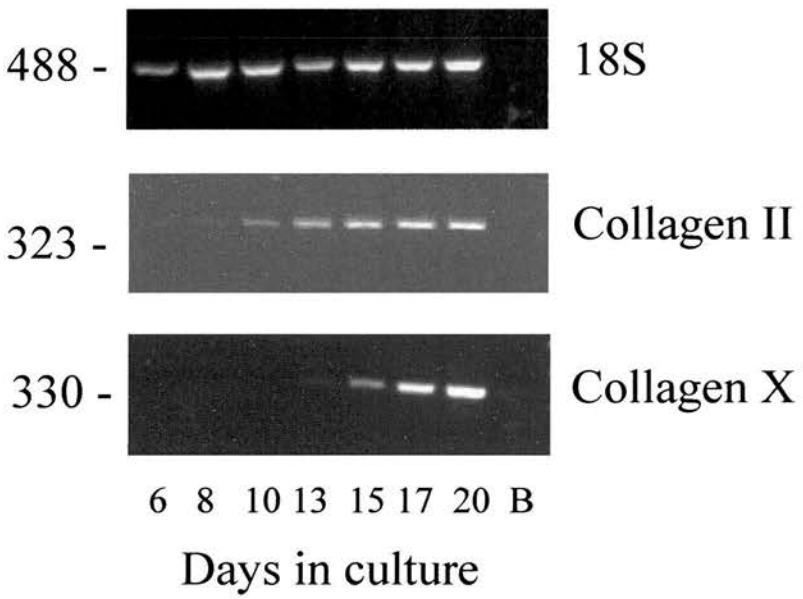
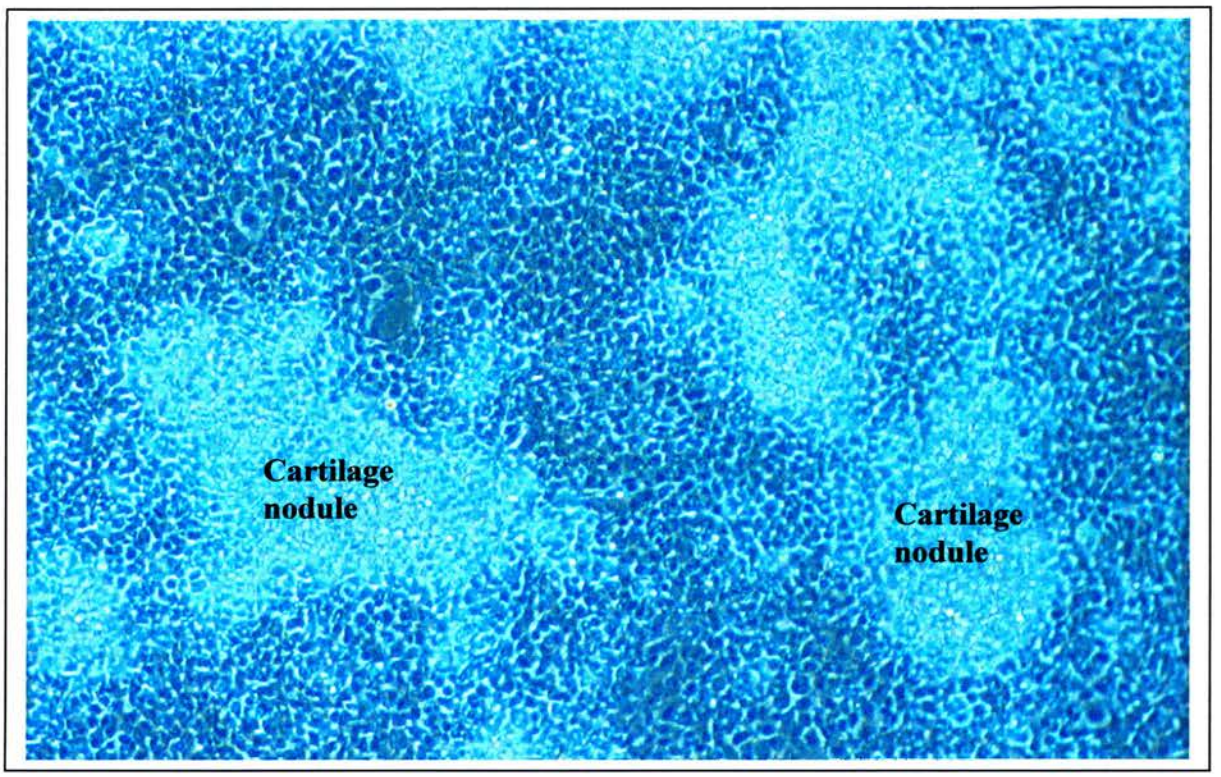


Figure 3.2. Phase contrast micrograph of ATDC5 cells. Nodule formation was visualised from day 10.



3.2 GC effects on Chondrogenesis and Terminal Differentiation

3.2.1 Introduction

The onset and severity of GC induced effects may be dependent on the duration of therapy, the nature of the steroid compound, and the comparative biological potency of GC such as Dex and Pred may be tissue specific (Orth & Kovacs, 1998). The initial study in this thesis was the first to show the varying effects of GC on bone growth and turnover, demonstrating that Dex is more potent at suppressing short term linear growth than Pred in children receiving treatment for Acute Lymphoblastic Leukaemia (Chapter 2).

This has been confirmed by *in vitro* studies looking at the joint effects of Dex and Pred on bone and cartilage cells. In human osteoblasts cultured from bone biopsies of healthy adults, Dex is more potent than Pred at inhibiting DNA synthesis (Kasperk *et al,* 1995) as well as in animal studies where Dex was ten times more potent at reducing the chondrocyte cloning ability than Pred (Robson *et al,* 1998).

GCs induce growth failure by a number of systemic effects, however it is apparent that they also have direct effects on the growth plate (Baron *et al,* 1992). The aim of this study was to ascertain the potencies of Dex and Pred on a number of parameters representing chondrocyte growth; including proliferation, differentiation and proteoglycan synthesis. These were done on a homogenous population of chondrocytes as characterised (Exp 3.1) to assess GC effects on different maturational phenotypes; either during the proliferative phase (**chondrogenesis**) or during the late differentiation phase (**terminal differentiation**).

3.2.2 Materials and Methods

a) Chondrocyte cell culture

The ATDC5 chondrocyte line was maintained and cultured as described (3.1.2a).

b) Chondrocyte number, proliferation, differentiation and matrix production.

Dex and Pred (Sigma) were added to the cells at a final concentration of 10^{-8}M, 10^{-7}M and 10^{-6}M, in 0.01% ethanol and compared with control cultures which contained 0.01% ethanol only. Collagen type II and collagen type X expression was first noted at 10 and 15 days respectively. The GC were added from Day 6 (**chondrogenesis**) or Day 11 (**terminal differentiation**) for the 4 days leading up to the expression of these two chondrocyte phenotypic markers.

The rate of chondrocyte proliferation was assessed by incubating the chondrocytes with 0.2uCi/ml of [^{3}H]-thymidine (37MBq/ml; Amersham Pharmacia Biotech, Little Chalfont, UK) for the last 18 hours of culture period and the amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates measured (Farquharson *et al,* 1999).

Cell layers were rinsed with phosphate buffered saline (PBS) and lysed with 0.9% NaCl and 0.2% Triton X-100 and centrifuged at 12000g for 15 min at 4°C. The supernatant was assayed for protein content and ALP activity as a measure of cell number and chondrocyte differentiation respectively. The protein content of the supernatant was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel-Hemstead, UK) based on the Bradford dye binding procedure and gamma globulin was used as standard (Farquharson *et al,* 1995). Enzyme activity was determined by measuring the cleavage of 10mM p-nitrophenyl phosphate (pNPP) at 410nm. Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein (Farquharson *et al,* 1999).

Proteoglycan synthesis was evaluated by staining with Alcian Blue as previously described (Shukanami *et al,* 1997). In brief, cells were washed twice with PBS, fixed in 95% methanol for 20 minutes and, stained with 1% Alcian Blue 8GX (Sigma) in 0.1 HCL overnight and rinsed with distilled water. Alcian blue stained cultures were extracted with 1ml of 6M guanidine-HCL for 6 hours at room temperature and the optical density was measured at 630 nm using a Jenway 6105 spectrophotometer.

3.2.3 Statistical analysis

All experiments were performed at least twice. Data was analysed by analysis of variance. All data are expressed as the mean ± sem of four observations within each experiment and statistical analysis was performed using Statview (version 5.0.1). A p value of <0.05 was considered to be significant.

3.2.4 Results

Effects of GC on cell number and proliferation.

In comparison to control cultures the addition of Dex and Pred to cells during the chondrogenic period (Days 6-10) caused a significant reduction in cell number (Table 3. 2). The reduction in cell number from control values for the Dex concentrations tested were 10^{-8}M (18.2%) 10^{-7}M (33.3%) and 10^{-6}M (31.8%) ($p<0.05$). The apparent plateau noted at 10^{-7}M for Dex was not seen with Pred, where a dose dependent reduction was observed over the three concentrations tested: 10^{-8}M (10.6%:NS), 10^{-7}M (21.2%:$p<0.05$) and 10^{-6}M (30.3%:$p<0.05$). The mean reduction in cell number over the 3 concentrations was 28% with Dex and 20% with Pred.

The effect of Dex and Pred on [^{3}H]-thymidine uptake during the chondrogenesis period is shown in Fig 3.3. Both GC caused a significant concentration dependent decrease in cell proliferation from control values, Dex: 10^{-8}M (11.7%) 10^{-7}M (33.8%) and 10^{-6}M (36.6%); Pred: 10^{-8}M (9.6%:) 10^{-7}M (24.7%) and 10^{-6}M (37%); ($p<0.05$). As was noted for cell number, the apparent plateau noted at 10^{-7}M for Dex was not seen with Pred, where a dose dependent decrease was observed over the three concentrations tested. The mean reduction over the three concentrations for Dex and Pred were 27% and 24% respectively. Dex at 10^{-7}M was significantly more antiproliferative than Pred 10^{-7}M. ($p<0.05$).

During the terminal differentiation phase (Days 10-15) Dex did not significantly alter cell numbers when compared to control values (table 3.2), whereas Pred caused a significant

reduction ($p<0.05$) at both 10^{-7}M (14.1%) and 10^{-6}M (10.9%). Cell proliferation rate in control cultures was six fold less during the terminal differentiation phase than the chondrogenic stage and the addition of GC led to a significant suppression of proliferation with Dex 10^{-8}M (40.9%), 10^{-7}M (24.1%) and 10^{-6}M (40.3%) whereas a reduction in proliferation by Pred was noted at 10^{-8}M (26.3%), with a rise in proliferation at 10^{-6}M ($p<0.05$) (Fig 3.3).

Effects of GC on proteoglycan production.

In comparison to control cultures during the chondrogenesis period, there was a concentration dependent reduction in proteoglycan synthesis ranging from 42 to 50% with Dex and 35 to 54% with Pred (Table 3.2). An apparent plateau was noted at 10^{-7}M for Dex which was not seen with Pred, where a dose dependent reduction was observed over the three concentrations tested. Comparing Dex and Pred at equivalent concentrations, Dex at 10^{-7}M caused a significantly greater fall in proteoglycans than Pred at 10^{-7}M ($p<0.05$) (Table 3.2). Over the three concentrations, Dex caused a mean reduction in proteoglycan synthesis of 47% compared to 43% with Pred. No significant differences were noted during terminal differentiation.

Effect on GC on chondrocyte differentiation.

The effect of GC on terminal chondrocyte differentiation as assessed by ALP activity is shown in table 3.2. During chondrogenesis, enzyme activity in comparison to control values was significantly increased with both Dex: 10^{-8}M (83%) 10^{-7}M (118%) and 10^{-6}M (116%) and Pred: (39%) 10^{-7}M (77%) and 10^{-6}M (77%) ($p<0.05$). The mean elevation in ALP with all concentrations of Dex and Pred are 106% and 62% respectively and at equimolar concentrations of GC, Dex caused significantly larger increases in ALP than

Pred. No significant differences in ALP activity were noted during the terminal differentiation phase.

3.2.5 Discussion

Studies using rat chondrocyte cultures show that Dex and Pred both reduced cell proliferation and colony formation and also that Dex was more potent than Pred at equimolar concentrations (Dearden *et al,* 1986; Robson *et al,* 1998). This culture data is in accord with the earlier *in vivo* observations where Dex appears to be more potent than Pred at causing impairment of normal bone growth (Strauss *et al,* 2001).

This experiment used the ATDC5 chondrocyte cell line, as it displays less phenotypic diversity than cultures containing a heterogeneous population of primary chondrocytes. Furthermore, it allows the study of two critical events during cartilage formation: the early differentiation of committed mesenchymal cells into chondrocytes (chondrogenesis) and the terminal differentiation of proliferating to hypertrophic chondrocytes (Cancedda *et al,* 1995). Cell numbers were reduced by both Dex and Pred during the chondrogenesis period, but little effect of both GC was noted during the terminal differentiation period. GC may reduce cell numbers by mechanisms such as loss of proliferative activity, increased apoptosis and cytostasis. This data strongly suggests that loss of proliferative activity is, at least in part, responsible for the decrease in chondrocyte numbers by GC treatment.

Cell proliferation rates and cell numbers were more greatly affected by Dex and Pred during the chondrogenesis period when the chondrocytes were rapidly proliferating. These results extend the data from cultures containing chondrocytes of various maturational phenotypes (Robson *et al,* 1998) and are also in agreement with studies on other bone cell types which indicate that Dex was more potent than Pred in reducing osteoblast cell number and DNA synthesis (Kasperk *et al,* 1995, Davies *et al,* 2002).

Davies and colleagues (2002) also reported that osteoblast precursor cells (HCC1) were more chemosensitive to Dex than fully differentiated osteoblasts and together with our present data, suggest that in bone cells, GCs exert their maximum effect at the cell precursor stage. Over the three concentrations, Dex was also more potent than Pred as it caused a 44% greater increase in ALP activity and greater reductions in proteoglycan synthesis, cell number and cell proliferation. Annfeld (1992) also showed that Dex treatment in rats, results in inhibition of both chondrocyte proliferation and cartilage matrix production. The pro-differentiating effects of Dex are in agreement with studies using costochondral cultures, in which Dex promoted ALP activity (Schwartz *et al,* 1995). However the results are at variance with other studies where Dex lowered enzyme activity in prehypertrophic chondrocytes but had minimal effects on hypertrophic or mineralising chondrocytes (Robson *et al,* 2001). Although these results are in contrast to those presented here, they do substantiate these and other observations that the response of chondrocytes to Dex is dependent on their stage of differentiation (Yasuda *et al,* 1995).

This and subsequent chapters in the thesis use GC concentrations expressed in moles rather than weight as in chapter 2. Historically pharmacological compounds have been manufactured and tested according to the weight of the drugs, whereas biological experimentation requires the knowledge of the amount of a substance present and is referred to as moles. Thus *Molarity* (M) denotes the number of moles of a given substance per litre of solution. The mole is useful in biological experiments because it allows different substances to be measured in a comparable way as the number of particles is a more useful unit in chemistry than mass or weight. Despite the variability in the measurement units adopted worldwide, the International System of Units (SI) are now the standard in Europe. However SI units are only available if an international standard is present at that time. Such that in clinical practice measured cortisol values are expressed in SI units of mmol/l (while Hydrocortisone is administered in weight units (mg)), whereas

IGF-I levels are still given in weight units of ug/ml. It is obvious that there is a divergence in measurement units between pharmacological and biological measurements. With knowledge of the molecular weights of the GCs it is possible to convert between millimoles and milligrams, however these figures then become unconventional and less easy to interpret and compare in the light of published literature.

In conclusion, Dex and Pred reduce cell number, cell proliferation and proteoglycan content whilst stimulating chondrocyte differentiation. The GCs have maximal effects during chondrogenesis with minimal effects during terminal differentiation. Thus these findings in the ATDC5 cell line may allow a more focused approach towards studying the mechanisms underlying GC induced growth retardation.

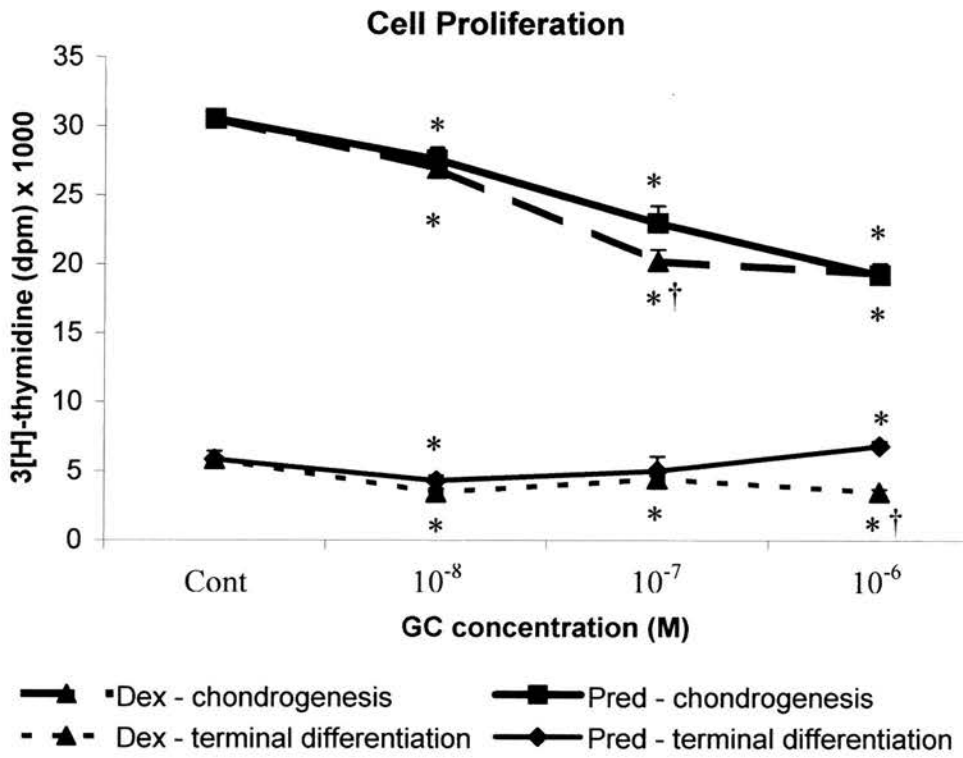
Table 3.2. GC effects on Chondrogenesis and Terminal Differentiation

Effect of Dex and Pred on cellular protein (cell number), proteoglycans and alkaline phosphatase activity during chondrogenesis and terminal differentiation. All data expressed as the mean ± sem, * = significantly different from control cultures (* $p<0.05$). † = Dex cultures significantly different from equivalent dose of Pred († $p<0.05$).

Chondrogenesis	Control	10^{-8}M		10^{-7}M		10^{-6}M	
		Dex	Pred	Dex	Pred	Dex	Pred
Cellular protein (mg)	0.66 ± 0.01	0.54 ± 0.05*	0.59 ± 0.04	0.44 ± 0.01*	0.52 ± 0.01*	0.45 ± 0.03*	0.46 ± 0.02*
Proteoglycan (O.D.)	0.26 ± 0.02	0.15 ± 0.01*	0.17 ± 0.005*	0.13 ±0.003*†	0.15 ± 0.01*	0.13 ± 0.003*	0.12 ± 0.002*
ALP(nmoles/hydrol/ min/mg/protein)	331 ± 8	660 ± 32*†	440 ± 15*	723 ± 27*†	585 ± 10*	716 ± 36*†	586 ± 19*

Terminal Differentiation	Control	10^{-8}M		10^{-7}M		10^{-6}M	
		Dex	Pred	Dex	Pred	Dex	Pred
Cellular protein (mg)	0.92 ± 0.01	0.91 ± 0.02	0.85 ± 0.05	0.84 ± 0.03	0.79 ± 0.03*	0.92 ± 0.02†	0.82 ± 0.04*
Proteoglycan (O.D.)	0.82 ± 0.07	0.89 ± 0.05	0.82 ± 0.03	0.7 ± 0.02	0.77 ± 0.04	0.73 ± 0.05	0.72 ± 0.04
ALP(nmoles/hydrol/ min/mg/protein)	262 ± 11	265 ± 17	244 ± 34	292 ± 21	270 ± 9	245 ± 10	253 ± 23

Figure 3.3. Effect of Dex and Pred on cell proliferation as assessed by [^{3}H]-thymidine uptake during chondrogenesis and terminal differentiation phases. All data expressed as the mean ± sem, * = significance compared to control (* $p<0.05$). † = significance level between dex and equivalent dose of pred († $p<0.05$).



3.3 Apoptosis

3.3.1 Introduction

Programmed cell death, or apoptosis was a term first coined by Kerr and colleagues in 1972, who observed that many dying cell types share the same characteristics; and postulated that the process may be the result of an endogenous cell death programme (Wyllie *et al,* 1980).

The human body is composed of approximately 10^{14} cells, each of which is capable of undergoing apoptosis (Nicholson, 2000). Of these approximately 10 billion a day will die to counteract the new cells that arise during mitosis (Hengartner, 2000). One of the mechanisms central to the apoptotic process is via the caspases which are a group proteins found in all species and elimination of these elements can slow or prevent apoptosis (Vaux & Korsmeyer 1999; Kerr *et al*, 1972, Earnshaw *et al,* 1999).

Growth retardation after GC treatment could occur via several mechanism; primarily by decreased proliferation of growth plate chondrocytes (Kember & Walker, 1971; Annefield, 1992), delayed senescence of the growth plate chondrocytes, having undergone fewer replications due to the inhibitory effect of Dex on cell proliferation (Gafni *et al,* 2001) or due to apoptosis.

Chrysis *et al* (2003) demonstrated that in rats treated for 7 days with Dex there was an increase in apoptosis in both the proliferative and the hypertrophic chondrocytes, accompanied by increased immunoreactivity for caspase-3. They observed an increased number of apoptotic resting/early proliferative chondrocytes after Dex treatment, which were rare in control animals but 18 fold higher after Dex treatment. Premature loss of resting or early proliferative cells could diminish the growth potential in spite of increased numbers of remaining replications of the chondrocytes (Chrysis *et al*, 2003). This experiment tried to elucidate whether the inhibitory effects of Dex and Pred on

chondrocyte proliferation in the previous experiment (Fig 3.3) were associated with changes in the rate of apoptosis on the two chondrocyte maturational phenotypes.

3.3.2 Materials and Methods

a) Chondrocyte cell culture

The ATDC5 chondrocyte line was maintained and cultured as described (3.1.2a).

b) Apoptosis

Apoptosis of the cells was measured by APOPercentage Apoptosis Assay, (Biocolor Ltd, Belfast, N.Ireland), which quantifies dye uptake by apoptotic cells only after the translocation of phosphatidylserine to the outer surface of the cell membrane (Fadok *et al,* 1992). This kit were used according to the manufacturers instructions. Dex and Pred at concentrations of 10^{-8}M, 10^{-7}M and 10^{-6}M were added to the cell cultures on Day 6 or Day 13 for a period of 24 hours. As a positive control, additional cells were incubated as above with 5% ethanol.

3.3.3 Statistical analysis

Data was analysed by analysis of variance. All data are expressed as the mean ± sem of four observations within each experiment and statistical analysis was performed using Statview (version 5.0.1). A p value of <0.05 was considered to be significant.

3.3.4 Results

Using the APOPercentage Apoptosis Assay the number of apoptotic cells was higher in the terminally differentiating chondrocytes in comparison to cultures in the chondrogenesis phase. No evidence was detected for an effect of Dex and Pred on apoptosis during the chondrogenesis phase (Fig 3.4a), however during terminal

differentiation all Dex concentrations and Pred at 10^{-6}M caused a significant decrease in apoptotic cell numbers ($p<0.05$)(Fig 3.4b). Ethanol acted as a positive control and caused an elevation in apoptosis ($p<0.05$).

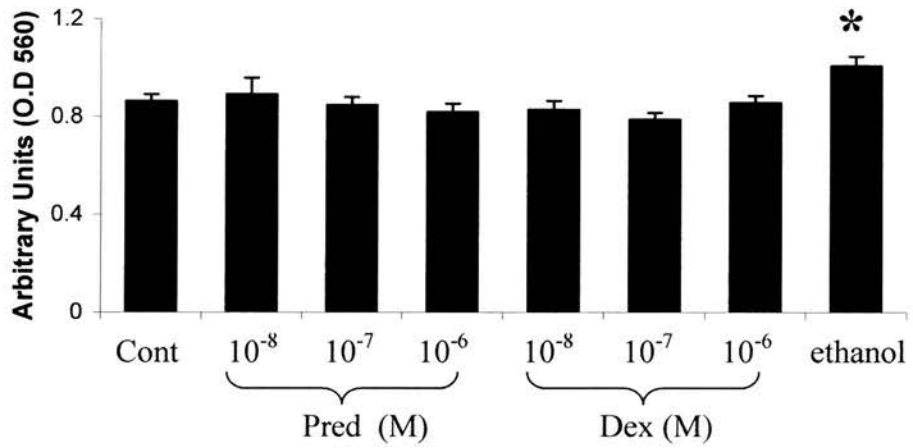
3.3.5 Discussion

GC may impact on cell numbers by a combination of methods including loss of proliferative activity, increased apoptosis and cytostasis. The previous data indicates that an anti-proliferative effect, is at least in part, responsible for the decrease in chondrocyte numbers by GC treatment. However no evidence of increased apoptosis was found, in this cell line; this is in accordance with Jux et al (1998) whose studies entailed the use of cultured rat chondrocytes primarily in the proliferative stage but is at variance with Silvestrini *et al* (2000) who did show that hypertrophic chondrocytes of rats have an increased apoptosis rate after high dose corticosterone exposure. Similarly in other bone cells Pred causes an increase in osteoblast and osteocyte apoptosis, thus contributing to the loss of mature osteoblasts (Weinstein *et al,* 1998).

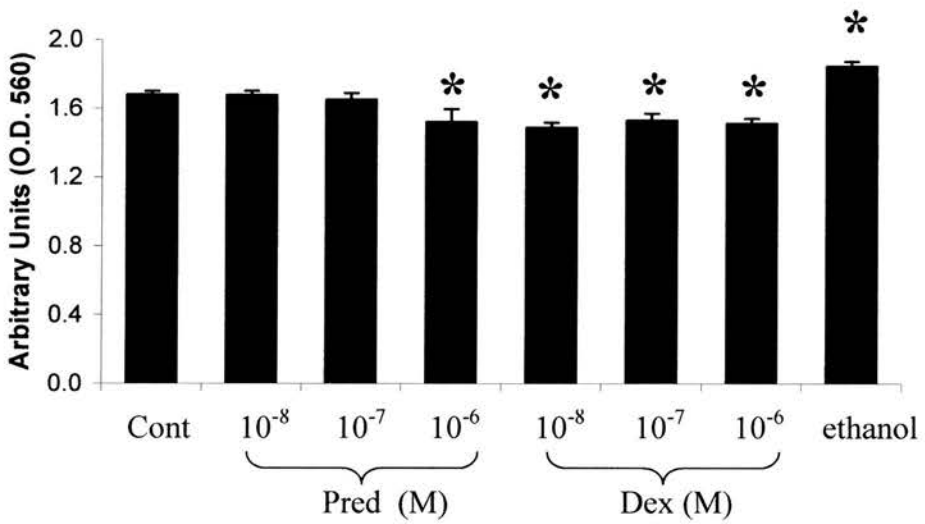
In the growth plate it is well recognised that apoptotic chondrocytes are most prevalent in the terminally differentiated zone (Ohyama *et al* 1997; Chrysis *et al* 2003) and this is also reflected in the ATDC5 cell line in this present study, which shows twice the apoptotic activity during terminal differentiation compared to the chondrogenic phase. Interestingly, Dex reduced apoptosis in the terminally differentiated cells whilst having no effect on the chondrogenic phenotype, suggesting that GC control of chondrocyte apoptosis is phenotype dependent. Alternatively it is possible that in this model, Dex and the highest dose of Pred have caused an initial reduction in cell proliferation to such an extent that the numbers of cells available for apoptosis are diminished. However this observation requires further study.

Figure 3.4. Effect of Dex and Pred treatment for 24 hours on the incidence of apoptosis during **a**) chondrogenesis and **b**) terminal differentiation. No effects of the GC were observed during the chondrogenesis phase, whereas all concentrations of Dex and Pred at 10^{-6}M, caused a significant reduction in apoptosis during terminal differentiation ($p<0.05$). Ethanol (5%) caused a significant elevation in apoptosis at both developmental time points ($p<0.05$).

a) Chondrogenesis



b) Terminal Differentiation



3.4 Glucocorticoid Receptor Antagonist

3.4.1 Introduction

Glucocorticoid hormones regulate physiological activity of almost all cell types in mammals via the soluble GC receptor, which becomes activated in the presence of an appropriate ligand.

These GC effects can be blocked by the addition of RU486 (Mifepristone), which was the first active antagonist to progesterone and GC that could be used in humans (Cadepond *et al,* 1997). The main structural characteristic is the phenyl-aminodimethyl group grafted onto the 11ß-position of the steroidal skeleton, so that it has high affinity interactions with a specific region of the receptor-binding pocket in the ligand-binding domain. RU486 binds with a high affinity to both the progesterone and GCR, but not the mineralocorticoid receptor.

Following steroid binding, the receptors undergo a conformational change that is crucial for receptor interaction with cellular targets. The antagonist binding seems to trigger a transconformation of the hormone-binding domain that differs from that observed with agonist binding. *In vitro* RU486 stabilises the hsp90 containing heteroligomeric complex with the GCR (Lefebvre *et al*, 1988), thus impeding or slowing down the formation of the activated receptor form and impairs nuclear transfer of the GCR in intact thymocytes (Lefebvre *et al,* 1988). However these *in vitro* actions are still controversial (Pekki *et al,* 1994).

The aim in this experiment was to ascertain if the negative GC effects on the ATDC5 cell line were mediated through the GC receptor. The chondrocyte proliferation rate was assessed with GCR antagonist – RU486, Dex and both compounds in combination to assess possible reversal of the antiproliferative effects of Dex.

3.4.2 Materials and Methods

a) Chondrocyte cell culture

The ATDC5 chondrocyte line was maintained and cultured as described (3.1.2a). The GCR antagonist (GCRA) was incubated at 3 concentrations (10^{-6}, 10^{-8} and 10^{-10}M) from day 6 to ascertain independent effects on the ATDC5 cell line. Additionally these 3 concentrations of the GCRA were also co-incubated with Dex 10^{-6}M to assess the effects of chondrocyte proliferation.

b) Chondrocyte proliferation

The rate of chondrocyte proliferation was assessed by incubating the chondrocytes with 0.2uCi/ml of [^{3}H]-thymidine for the last 18 hours of the culture period as described (3.2.2b). The amount of radioactivity incorporated was measured during the chondrogenic phase on day 9.

3.4.3 Statistical analysis

Data was analysed by analysis of variance. All data are expressed as the mean ± sem of four observations within each experiment and statistical analysis was performed using Statview (version 5.0.1). A p value of <0.05 was considered to be significant.

3.4.4 Results

RU486 had no effect on chondrocyte proliferation at the 10^{-6}M, 10^{-8}M or 10^{-10}M (Fig 3.5). Similar to earlier experiments Dex 10^{-6}M caused a 57% significant reduction in cell proliferation compared to the control cultures ($p<0.05$). Dex combined with the two lower doses of RU486 (10^{-8} and 10^{-10}M) did not reduce the anti-proliferative capability of Dex; showing a significant 57% and 58% reduction in cell proliferation as compared to the control group ($p<0.05$)

However co-incubation of Dex with the higher concentration of RU486 at 10^{-6}M, did significantly reverse the Dex effects ($p<0.05$) towards but not back to control levels ending in a 23% reduction from control levels (Fig 3.5)

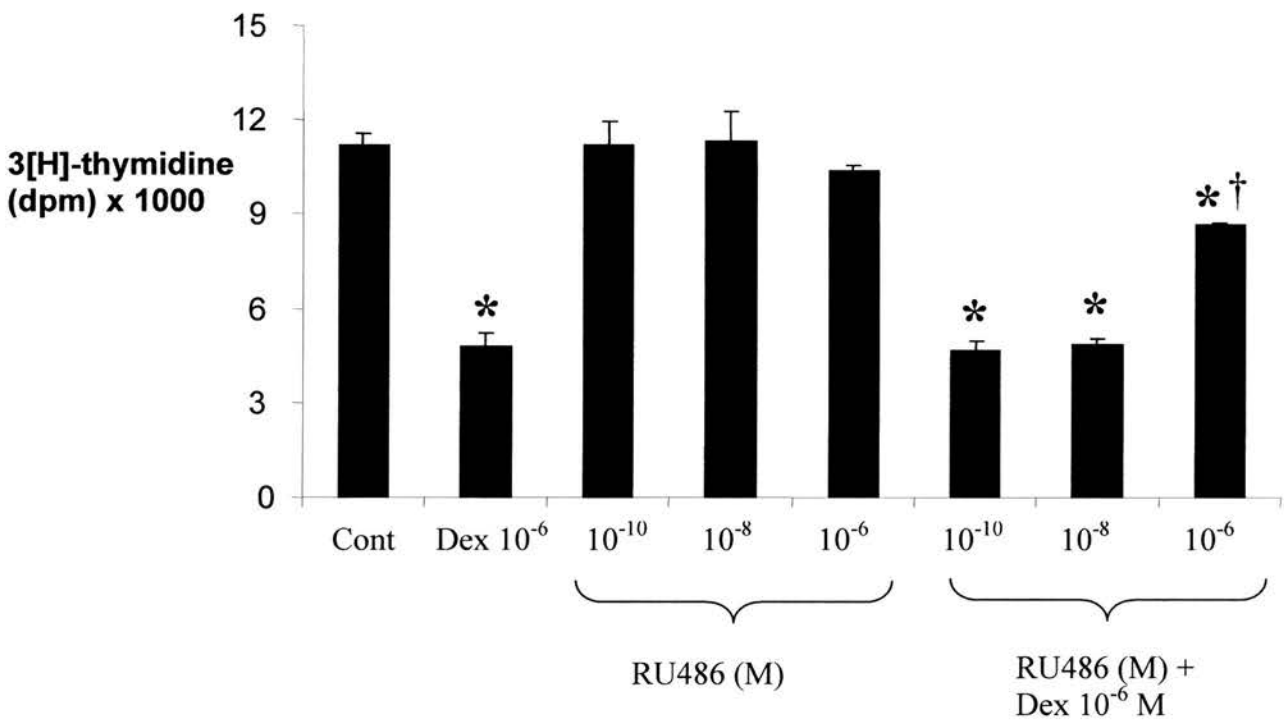
3.4.5 Discussion

This experiment provides evidence that the effects of the GC are mediated through the GC receptor. Dex as in the previous experiments reduces proliferation; this is partially reversed by the higher concentration of GCRA and it is possible that complete reversal may be achieved by higher doses of the GCRA. *In vivo* it has been shown that there is discrepancy between the doses of the two compounds as 400mg of RU486 antagonises 1mg of Dex (Raux-Demay *et al,* 1995). The antiprogesterone activity *in vivo* is utilised in obstretrics for voluntary early pregnancy termination (Cadepond *et al,* 1997) and the antiglucocorticoid activity (Bertagna *et al,* 1984), eliminates the negative feedback of cortisol on ACTH, leading to increased ACTH and cortisol secretion (Hermus *et al,* 1987). But so far the only efficient treatment utilising the antiglucocorticoid effect of RU486 is that of Cushings syndrome secondary to ectopic ACTH secretion or to adrenal carcinoma. RU486 has been shown to have effects in many body systems, including reversal of Dex induced muscle atrophy in rats (Konagaya *et al,* 1986) and in bone marrow it reversed B-cell apoptosis induced by GC (Garvy *et al,* 1993). It has similar GC reversing actions in osteoclasts (Wada *et al,* 1994). However there is scant data on chondrocyte effects, although Di Battista *et al,* 1991, did demonstrate a complete reversal of Dex induced suppression of metalloprotease synthesis in human chondrocytes.

This is the first documented study showing that chondrocyte proliferation *in vitro* can be partly reversed by a GCRA, however *in vivo* it is possible that RU486 may have weak agonist activity explaining why no clinical symptom of cortisol deficiency is observed after RU486 administration (Kling *et al,* 1993).

Figure 3.5. GC and GCR Antagonist effects on cell proliferation.

Effect of Dex 10^{-6}M and GCR Antagonist (RU486) on cell proliferation as assessed by [^{3}H]-thymidine uptake during chondrogenesis. Dex decreased cell proliferation ($p<0.05$), whereas the GCRA had no significant effects on proliferation. During co-incubation of Dex 10^{-6}M with increasing concentrations of GCRA, the negative effects of Dex were partially reversed towards control levels by GCRA at 10^{-6}M. All data expressed as the mean ± SEM, * = significance compared to control (* $p<0.05$). † = significance level between Dex 10^{-6}M and combined RU486 10^{-6}M + Dex 10^{-6}M († $p<0.05$).



3.5 Receptor Expression and Reversal of GC effects

3.5.1 Introduction

GCs cause a decrease in cell proliferation and cell number and reduce matrix synthesis while increasing differentiation. Although GC effects appear to be mediated through the GCR the ATDC5 cell line was further characterised to ascertain the temporal expression of the GCR. In addition IGF-IR receptor and GHR expression was also determined prior to the treatment of the cell line with IGF-I and GH.

Initial data revealed that in the presence of insulin, IGF-I or GH did not have effects on chondrocyte proliferation. Insulin is an important regulator of chondrocyte growth and the ATDC5 cell line undergoes the full extent of chondrocyte proliferation, differentiation and mineralisation in the presence of insulin (Atsumi *et al,* 1990). The insulin receptor and the IGF-IR belong to the same subfamily of receptor tyrosine kinases and thus share a high similarity of structure and intracellular signalling events. (Dupont & LeRoith, 2001). Thus subsequent experiments were carried out in the absence of insulin in the culture medium and restricted to the chondrogenic phase, as this is where the majority of the GC effects were centred.

3.5.2 Materials and Methods

a) Gene expression

To determinate the onset of the expression of the GC, GH and IGF-I receptors, the ATDC5 cells were grown for up to 16 days as described (3.1.2b). RNA was extracted, reverse transcribed and analysed for receptor expression at Day 6, 9, 11, 13 and 16 by semi-quantitative RT-PCR.

b) RNA extraction and Semiquantative RT-PCR

Total RNA, extracted from chondrocytes by repeated aspiration and gene expression was analyzed by semiquantitative RT-PCR as described (3.1.2 c & d). PCR was performed in 20-µl reactions containing cDNA equivalent to 10ng RNA and 200nM gene-specific primers in 11.1 x PCR buffer (Jefferies *et al* 1998) (Table 3.3).

c) Chondrocyte cell culture

The ATDC5 chondrocyte line was maintained and cultured until confluent on day 6 (3.1.2a). Insulin (10ug/ml) was added into the culture medium at day 6 as before but in subsequent experiments, insulin was not added into the standard culture medium when the cell layer became confluent (Day 6). At day 6, Dex 10^{-6}M was added to all wells and Insulin (Bacham, St Helens, UK) and IGF-I (Bacham) was added to the Dex containing wells at final concentrations of 50ng/ml, 100ng/ml and 500ng/ ml to obtain a dose response. Further experiments were undertaken to ascertain the effects of IGF-I and GH (Bacham), both at 100ng/ml on chondrocyte proliferation in the presence of Dex 10^{-6}M. All experiments were undertaken in triplicate.

d) Chondrocyte proliferation

At day 8 the rate of chondrocyte proliferation was assessed by incubating the chondrocytes with 0.2uCi/ml of [^{3}H]-thymidine for the last 18 hours of the culture period and the amount of radioactivity measured (3.2.2 b).

3.5.3 Statistical analysis

Data was analysed by analysis of variance. All data are expressed as the mean ± sem of four observations within each experiment and statistical analysis was performed using Statview (version 5.0.1). A p value of <0.05 was considered to be significant.

3.5.4 Results

Temporal Expression of GC, GH and IGF-I receptors.

Using gene specific primers (Table 3.3), the GC and IGF-I receptors are both expressed at the first time point of 6 days. The GH receptor is expressed from day 9 onwards (Fig 3.6).

Chondrocyte Proliferation

Initial experiments in the presence of insulin containing medium failed to show any difference in the rate of proliferation, with both IGF-I and GH at concentrations of 50ng/ml and 500ng/ml (Fig 3.7a,b).

In further experiments when insulin was omitted from the culture medium, Dex 10^{-6}M caused a significant decrease in proliferation ($p<0.05$). Dex cultured with insulin at concentrations of 50, 100 and 500ng/ml showed there was no reversal of the Dex effects on proliferation which remained significantly below control levels ($p<0.05$) (Fig 3.8). However IGF-I at all three concentrations reversed the negative Dex effects with a peak proliferation at a concentration of 100ng/ml (Fig 3.8). All three doses of IGF-I resulted in proliferation rates greater than the control cultures ($p<0.05$).

The IGF-I dose response data was utilised to study the effects of IGF-I and GH on chondrocyte proliferation (Fig 3.9). Dex and GH caused a decrease in proliferation ($p<0.05$), whereas IGF-I alone or in combination with Dex, GH or both causes a significant elevation in cell proliferation above control values ($p<0.05$).

3.5.5 Discussion

The ATDC5 cell line expresses both the GC and IGF-I receptors from the onset of the chondrogenic phase whereas GHR expression appears towards the onset of terminal differentiation. This may explain the lack of GH effects as these experiments were carried out at day 8, alternatively it could have been a suboptimal GH concentration. Subsequent

studies by another group have also confirmed that this cell line expresses the GCR (Siebler *et al,* 2002).

The dose response data was very important for future experiments as it established that IGF-I effects are maximal at 100ng/ml, with no additional benefit from increasing the concentration. Although IGF-I reversed the antiproliferative effect of Dex and indeed superseded the control values, increasing the dose of IGF-I to 500ng/ml did not result in a further increase in cell proliferation, but caused a small reduction compared to IGF-I at 100ng/ml. It is possible that there could be a downregulation in IGF-IR expression with increasing doses of IGF-I, but this requires further study.

Insulin plays an essential part in the regulation of cartilage and bone metabolism (Fitzsimmons *et al*, 2004; Torres *et al,* 2003). Both Insulin and IGF-I share similar signalling pathways, therefore the standard insulin concentration (10ug/ml) used in the culture of the ATDC5 cells may mask the IGF-I effects even at high doses of 500ng/ml. This would then be the reverse of the *in vivo* situation where circulating IGF-I levels are approximately 1000 times higher than insulin (Simpson *et al,* 1998). It could also be argued that IGF-I is merely replicating the insulin effects, which was used as a standard addition in earlier experiments; although this is possible we would then have expected to see some reversal of the Dex effects with insulin. Similarly *in vivo* experiments indicate that IGF-I is more important for growth as mice lacking insulin receptors show only slight growth retardation at birth (Louvi *et al,* 1997), whereas IGF-IR null mice are extremely growth retarded (Liu *et al,* 1993).

Thus it is apparent that GH and IGF-I effects in this experiment cannot be fully compared to the initial Dex and Pred data as the lack of insulin in these experiments is a confounding variable, but it is apparent that the proliferative properties of IGF-I are much greater than Insulin at equivalent concentrations and that Dex has antiproliferative effects with or without insulin and these are completely reversed by IGF-I.

Table 3.3. Primer pairs used to study expression of GC, GH and IGF-I receptors.

Gene	Primer Sequence	Product Size (bp)
18S	Unknown, purchased commercially from Ambion	488
GCR	ACTGTCCAGCATGCCGC(F) CTGCTCATTATTATTCAGATC(R)	478
GHR	CATTGGCCTCAACTGGACTT (F) GACTTCGCTGAACTCGCTGT (R)	283
IGF-IR	CACCGAGAACAACGAGTGCT (F) GTCACCGAATCGATGGTTTT (R)	366

Figure 3.6. Semiquantative RT-PCR analysis of the expression of the GC, GH and IGF-I receptors. GC and IGF-I receptors are expressed from day 6, whereas the GH receptor is expressed from 9 days.

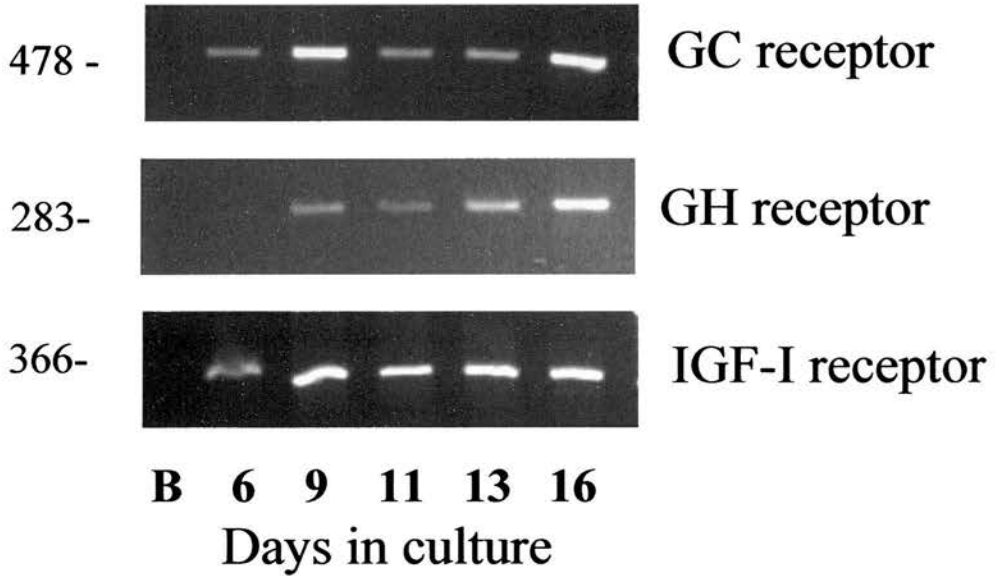


Figure 3.7. Chondrocyte proliferation with GH and IGF-I in the presence of insulin containing medium as assessed by [^{3}H]-thymidine at day 8 in the presence of **a)** GH and IGF-I both at 50ng/ml, and **b)** GH and IGF-I both at 500ng/ml. All data expressed as the mean ± sem. No significant differences in proliferation were observed.

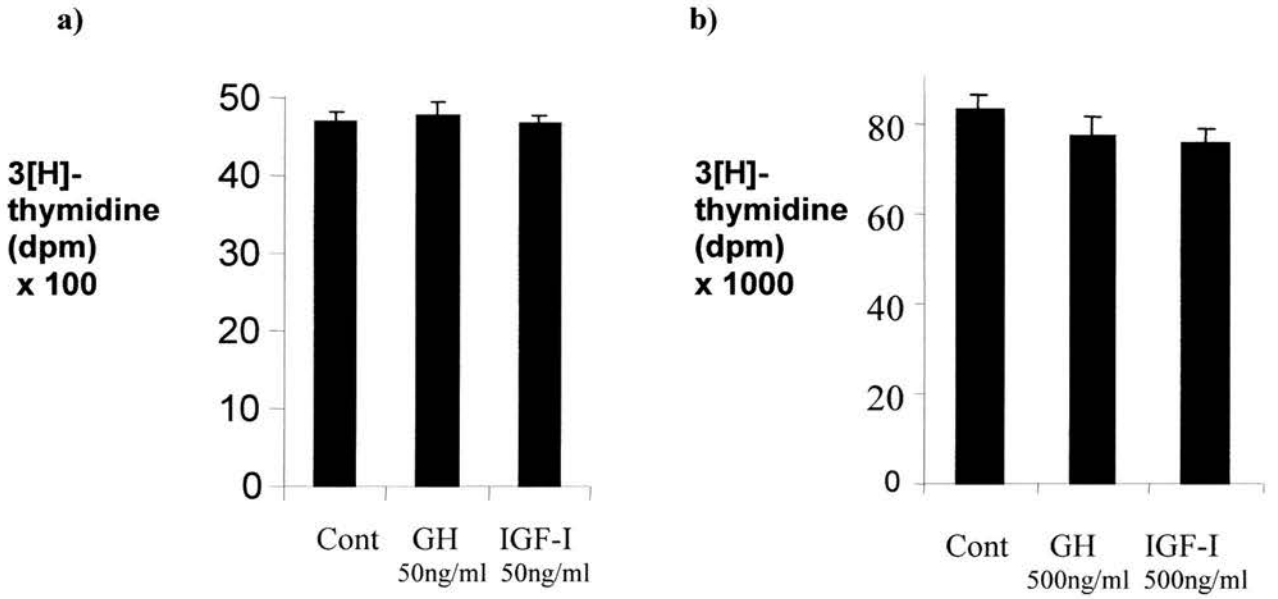


Figure 3.8. Effects of Dex 10^{-6}M, Insulin and IGF-I (both at 50, 100 and 500ng/ml) on chondrocyte proliferation as assessed by [^{3}H]-thymidine uptake at day 8 in the chondrogenesis period. Insulin was not a standard addition to the culture fluid. All data expressed as the mean ± sem, * = significance compared to control (* $p<0.05$).

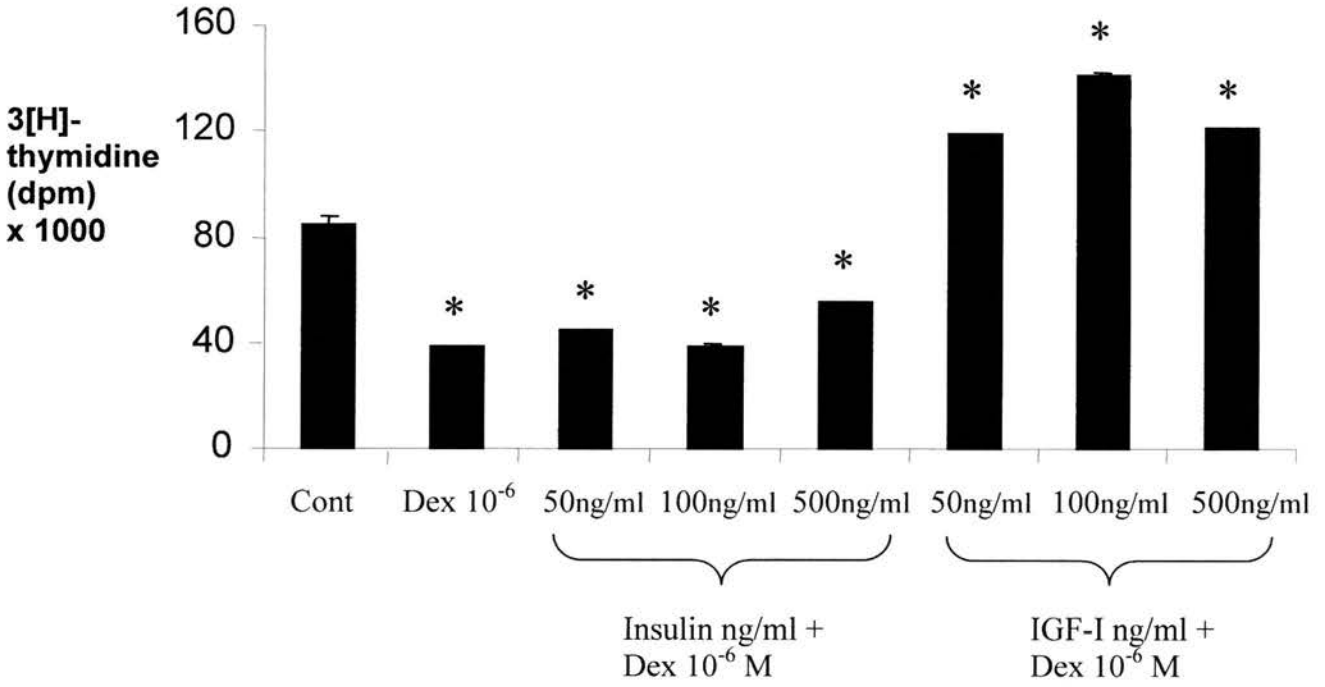
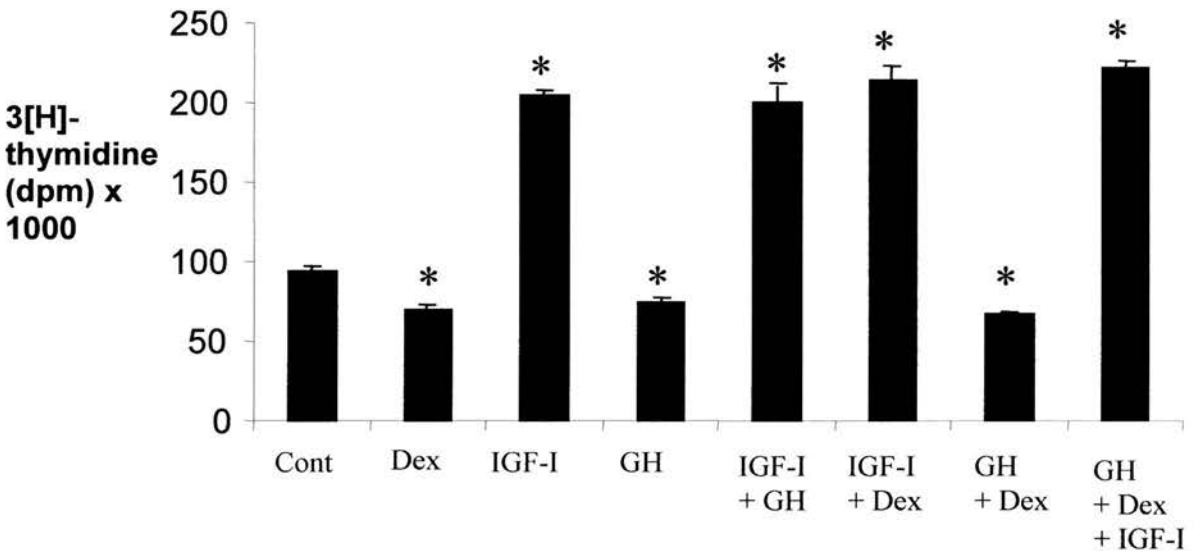


Figure 3.9. Effect of Dex 10^{-6}M, GH and IGF-I (both at 100ng/ml) on cell proliferation as assessed by [^{3}H]-thymidine uptake at day 8 in the chondrogenesis period. Dex and GH caused a significant reduction ($p<0.05$), whereas all IGF-I treatments cause a significant elevation in proliferation ($p<0.05$). All data expressed as the mean ± SEM, * = significance compared to control (* $p<0.05$).



CHAPTER 4

ORGAN EXPLANT STUDIES

4.1 The influence of Glucocorticoids and Growth Factors on Metatarsal Growth: Direct Linear Growth and Histomorphometry.

4.2 Metatarsal Chondrocyte Proliferation and Differentiation.

4.1 The Influence Of Glucocorticoids And Growth Factors On Metatarsal Growth: Direct Linear Growth And Histomorphometry.

4.1.1 Introduction

Most children who require systemic GCs also suffer from chronic inflammatory disease and, in the clinical scenario it can be difficult to clearly assess the relative contribution of disease and drugs on growth. In these children, maintenance of growth is a complex process that is influenced by a number of different mechanisms that affect the GH/IGF-1 axis by disrupting GH secretion or altering GH/IGF-1 sensitivity (Allen *et al,* 1996).

The dual effector theory of GH/IGF-1 action at the growth plate proposes that GH acts directly on germinal zone precursors of the growth plate to stimulate the differentiation of chondrocytes and then amplify local IGF-I synthesis which, in turn, induces the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (Isaksson *et al,* 1987). Although liver derived IGF-I is the main determinant of serum IGF-I levels, it appears not to be as important for postnatal growth as locally derived IGF-1 (Yakar *et al,* 1999 & 2002).

The complex effects and physiological mechanisms of GC on growth plate chondrocytes are difficult to study solely in live animals where effects cannot be localised to specific cell types. The foetal mouse metatarsal explant culture is a highly physiological model for studying growth as the growth rate of foetal bones in culture is similar to that found *in vivo* whereas bones harvested postnatally from 2-day-old rats arrest in culture after 2-days *in vitro* (Scheven & Hamilton, 1991; Coxam *et al,* 1996). In addition, the metatarsal culture model maintains cell-cell and cell-matrix interactions and the direct assessment of bone growth and histological architecture can be determined. By using the foetal mouse metatarsal assay, the aims of the present studies were to obtain a clearer understanding of

the cellular events underlying GC induced growth retardation and, in addition, determine whether IGF-I and GH can ameliorate the effects of GC on bone growth.

4.1.2 Materials and Methods

a) Foetal metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18 day old embyronic Swiss mice. Bones were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 individually in 24 well plates (Costar, High Wycombe, UK) for up to 10 days. Each well contained 300µl of α-MEM without nucleosides (Invitrogen, Paisley, UK) supplemented with 0.2% BSA Cohn fraction V (Sigma, Dorset, UK), 0.1mmol/L β-glycerophosphate (Sigma), 0.05mg/ml L-ascorbic acid phosphate (Wako, Japan), 0.292mg/ml L-glutamine (Invitrogen), 0.05mg/ml gentamicin (Invitrogen) and 1.25ug/ml fungizone (amphotericin B) (Invitrogen). Dex (Sigma), IGF-I (Bacham, St. Helens, UK) and GH (Bacham), were added at a final concentration of 10^{-6}M, 100ng/ml and 100ng/ml, respectively, to the cultured bones. The control and experimental groups contained 6 metatarsals each and the experiment was repeated at least twice.

b) Morphometric analysis

Images were taken of the metatarsals every second day of culture using a digital camera (COHU, San Diego, USA) attached to an Olympus MO81 microscope. The total length of the bone and width through the centre of the mineralising zone was determined using Image Tool (Image Tool version 3.00, University of Texas Health Life Science Centre in San Antonio). All results are expressed as a percentage change from harvesting length, which was regarded as baseline to demonstrate the rate of growth over time. For the determination of the size (in direction of longitudinal growth) within the growth region of the distinct chondrocyte maturational zones the 4 and 10-day-old metatarsals were fixed in

70% ethanol, dehydrated and embedded in paraffin wax (Haaijman *et al,* 1997). Wax sections (10 μm in thickness) were reacted for ALP activity (Farquharson *et al,* 1992) for the demarcation of the hypertrophic and proliferating zones. Serial sections were stained with von Kossa and haematoxylin & eosin using standard protocols to identify the zone of cartilage mineralisation. Images of the stained metatarsals were captured using Image tool (University of Texas) and the size of the combined (distal and proximal) ALP negative proliferating zone was determined (equation 1). Similarly, the size of the combined ALP positive hypertrophic zone located at either side of the mineralising zone was determined (equation 2). The size of the mineralising zone was determined directly from the von Kossa stained sections. (1). Proliferating zone = total length – (hypertrophic zone + mineralising zone), (2). Hypertrophic zone = (hypertrophic zone + mineralising zone) – mineralising zone.

4.1.3 Statistical analysis

All data are expressed as the mean ± sem and statistical analysis was performed using an analysis of variance (GenStat, Sixth Edition, VSN International Ltd). A p value of <0.05 was considered to be significant.

4.1.4 Results

All foetal mice metatarsals grew in culture and displayed a central core of mineralised cartilage juxtaposed on both sides to a translucent area representing the hypertrophic chondrocytes (Figs. 4.1 & 4.2b,c,d). Digital images were taken every second day and the control metatarsal shown in Fig 4.1 demonstrates an increase in total length and the length of the mineralising zone. The localisation of ALP reactivity within metatarsal sections was restricted to the mineralising and hypertrophic chondrocytes and thus clearly delineated

the boundary between the proliferating and hypertrophic zones (Fig. 4.2e) whereas von Kossa staining was specific to the mineralising zone.

Longitudinal bone growth

All experiments were done on metatarsals from 18-day-old embryos that were cultured for intervals for up to 10 days. Dex-treated bones paralleled control bone growth rate until day 8 when their rate of growth decreased resulting in a total length that was significantly reduced from controls at day 8 ($p<0.05$) and 10 ($p<0.05$), (Fig. 4.3a). IGF-I and combined IGF-I+Dex-treated bones showed a rapid acceleration in growth from day 2 that was significantly higher than the control group ($p<0.05$) and this increased growth rate was maintained throughout the duration of the experiment. At day 10, mean increase from baseline in total length of control, Dex, IGF-1 and IGF1+Dex bones was 50% ± 3, 42% ± 2, ($p<0.05$) 99.3% ± 5, ($p<0.05$) and 87% ± 4 ($p<0.05$), respectively. Compared to the IGF-I treated bones, the length of the metatarsals treated with IGF-I+Dex was also significantly lower at days 8 ($p<0.05$) and 10 ($p<0.05$). The ability of GH to directly influence bone growth in this model system was also studied (Fig. 4.3b). In contrast to the growth promoting effects of IGF-I (Fig. 4.3a), GH was found to have no significant effects on total bone length as compared to control metatarsals.

In control bones there was a significant increase in the length of the mineralising zone by day 6, and by day 10 the mean increase in length from baseline was 122% ± 2, ($p<0.05$) (Fig 4.3c). The mineralising zone length of the IGF-I treated bones changed little throughout the culture period and by day 10 it had only increased from baseline by a mean of 10% ± 2 ($p<0.05$). Also by day 10 the length of the mineralising zone in the Dex-treated metatarsals had increased by a mean of 79% ± 19% ($p<0.05$) from baseline, significantly less than in the control metatarsals ($p<0.05$). The growth rate of the

mineralising zone in the IGF-I+Dex treated metatarsals was also less than control and Dex treated metatarsals with the mineralising zone length significantly decreased at day 6 (11% ± 5, $p<0.05$), day 8 (32% ± 8, $p<0.05$), and day 10 (33% ± 10; $p<0.05$) from the control bones. Overall this data suggests the existence of an inverse relationship between the length of the mineralisation zone and total bone length (Figs 4.3a & c).

The thickness of the control and Dex treated metatarsals did not change with time in culture and were not significantly different from each other at any of the time points examined (Fig. 4.3d). In comparison to the controls, both the IGF-I and IGF-I+Dex treated bones were significantly thicker from day 4 ($p<0.05$) and 6, respectively ($p<0.05$). At day 4, the thickness of the IGF-I treated bones was significantly different from the IGF-I+Dex treated bones. At day 10, the thickness of the IGF-I and IGF-I+Dex treated bones were respectively 51% ± 10 ($p<0.05$) and 35% ± 14 ($p<0.05$) greater than that of their harvesting lengths.

With the exception of the results shown in Fig. 4.3b, the data presented in Figs 4.3a, c & d (and all subsequent results in this chapter) were obtained from metatarsals of embryos from the same mother. The differing growth rates shown in Figs 4.3a & b are likely to be due to variability between the embryos selected for each experiment. The inhibition of growth rate by Dex was observed in both studies.

Assessment of chondrocyte maturational zone sizes

In many of the metatarsal rudiments the boundary between the proliferating and hypertrophic zone of chondrocytes was difficult to delineate whilst in culture, therefore measurements of the size of these individual maturational zones was performed on histological sections of 4 and 10 day-old metatarsals. The lengths of the proliferating, mineralising and hypertrophic zones are shown in (Table 4.1).

Although Dex decreased and IGF-I increased the length of the proliferating zone these changes did not reach statistical significance. However, IGF-I+Dex treatment resulted in a significant increase in the length of the proliferating zone at day 4 ($p<0.05$), which was not sustained by day 10. The length of the mineralising zone was significantly reduced with all treatments at both time points ($p<0.05$) as compared to the controls. At day 4 there was a 10% reduction with all treatments, this decrease became larger by day 10, with Dex, IGF-I and IGF-I+Dex causing a 16, 51 and 42% reduction respectively in the length of the mineralising zone as compared to the control bones ($p<0.05$). Dex caused a non-significant increase in the length of the hypertrophic zone at day 4 and 10. In contrast, IGF-I led to a marked increase in the length of the hypertrophic zone at day 4 (98% increase, $p<0.05$) which became more pronounced by day 10 (346% increase, $p<0.05$) (Table 4.1, Figs 4.4 a & b). The combined effects of IGF-I+Dex were similar to IGF-I exposure alone, resulting in a 74% and 233% increase in length at day 4 and day 10, respectively ($p<0.05$) (Table 4.1). The size of the individual hypertrophic chondrocytes in the 10-day IGF-I treated metatarsals was also much larger than those of the Dex treated metatarsals (Figs 4.4c & d).

4.1.5 Discussion

The growing foetal chondrocytes undergo the three principal stages required for normal foetal growth: proliferation, hypertrophy and mineralisation as shown in this study. The foetal metatarsals have a relatively smaller mineralising zone than the newborn rodent and thus the cartilaginous portions retain a greater capacity for chondrocyte proliferation and hypertrophy; making them an ideal model to study growth plate effects.

The results unequivocally show that Dex and IGF-I have major and opposite effects on longitudinal bone growth with IGF-I clearly reversing the growth inhibitory effects of

Dex. However, the potential for Dex to inhibit bone growth was still present in the IGF-I+Dex cultures where the growth rates did not match those of bones cultured with IGF-I alone. Similar effects with other GC have previously been reported by Picherit *et al,* who demonstrated that hydrocortisone induced growth retardation in foetal rat metatarsals (Picherit *et al,* 2000).

The IGF-I stimulation in linear growth is similar to the results achieved by Scheven and Hamilton (1991), however, these workers reported GH stimulatory effects on metatarsal length, which is in contrast to the data of this present study. Whilst GH is well recognised to stimulate longitudinal bone growth *in vivo* (Isaksson *et al,* 1982, Hunziker *et al,* 1994), its effects *in vitro* are less clear (Lindahl *et al,* 1987; Ohlsson *et al,* 1994). Other studies have strongly suggested that GH effects *in vivo* may be indirect and that IGF-I effects are more pervasive *in vitro* (Vetter *et al,* 1986; Trippel *et al,* 1989).

A morphometric analysis was completed to further characterise the response of metatarsals to both Dex and IGF-I with respect to the size of the individual maturational zones within the growth plate. The reduction in length of the mineralisation zone with Dex was consistent with metatarsals treated with hydrocortisone (Picherit *et al,* 2000). However, the absence of an increase in the length of this zone following IGF-I is at variance to others who have demonstrated an increase in mineralisation zone length with IGF-I in a rat metatarsal model system (Coxam *et al,* 1996). Dex also led to a small, non-significant, increase in the length of the hypertrophic zone which is similar to the findings of Smink and colleagues (2002) who demonstrated an increase in the hypertrophic zone length in mice treated with Dex. They further postulated that this was a likely consequence of an acceleration of the chondrocyte differentiation rate as observed in PTHrP null mice (Kronenberg *et al,* 1997). Alternatively, due to the restriction of cartilage mineralisation, the increased size of the hypertrophic zone may be in part be due to a simple build up of non-mineralised hypertrophic chondrocytes. A similar, more pronounced process, may

explain the more marked reduction in the mineralisation zone observed in the IGF-I treated metatarsals. Additionally another non-significant difference was noted between IGF-I and Dex on the proliferating zone length. It was deemed important to report these trends as subsequent experimentation may confirm or refute these observations, thus reducing the risk of a type 1 or type 2 errors.

In contrast to the effects of Dex, IGF-I rapidly stimulated linear bone growth by increasing the size of the hypertrophic chondrocytes, thus increasing the length of this zone. Within the maturational zones of the growth plate, the major effects of IGF-I were clearly on the length of the hypertrophic chondrocyte zone and also the size of the cells within. This result is in accord with the hypothesis that it is the size of the hypertrophic zone rather than chondrocyte proliferative kinetics that is the single major determinant of bone growth rate (Wilsman *et al,* 1996; Hunziker & Schenk, 1989). Although IGF-I is expressed by chondrocytes situated in all maturational zones of the growth plate, IGF-I mRNA expression is mainly restricted to the hypertrophic zone and the infusion of IGF-I into hypophysectomised rats showed that IGF-I stimulated growth plate chondrocytes at all stages of differentiation including those in the hypertrophic zone (Hunziker et *al,* 1994; Smink *et al,* 2002; Reinecke *et al,* 2000).

The growth retardation in the IGF-I null mouse is associated with an attenuation of chondrocyte hypertrophy and no significant changes in proliferation (Wang *et al,* 1999) and this data further strengthens the hypothesis that the predominant role of IGF-I in growth promotion is in augmenting chondrocyte hypertrophy. This effect of IGF-I can reverse GC induced growth retardation but this apparent ameliorative effect results in an alteration of the relative proportion of proliferative, hypertrophic and mineralised chondrocytes.

In conclusion, this study shows that Dex and IGF-I have opposite effects on linear bone growth. The effects of Dex were time dependent whereas IGF-I effects were immediate.

Dex decreased skeletal mineralisation while IGF-I markedly stimulated chondrocyte hypertrophy in favour of mineralisation and completely reversed Dex induced growth retardation. However, the potential for Dex to inhibit bone growth was still present in the IGF-I+Dex cultures where growth rates did not match those of bones cultured with IGF-I alone. In addition, the IGF-I mediated improvement in growth was at the expense of altering the balance between proliferating and hypertrophic chondrocytes within the metatarsal. GH had no beneficial effect on metatarsal growth at the dose studied.

Figure 4.1. Linear Metatarsal Growth in Culture: The same control bone grown for 10 days in culture is shown. Digital images were taken every two days of every bone and the total length of the metatarsal, mineralising zone (length and width) and thickness were ascertained.

a) Day 0

b) Day 2

d) Day 4

d) Day 6

e) Day 8

f) Day 10

Figure 4.2. Measurements of digital images of foetal mouse metatarsal bones in culture with clearly delineated mineralising zones **(b – d)** were taken using a calibrated ruler **(a)**. These images demonstrate the harvesting day length **(b)** and the increased longitudinal growth at day 4 **(c)**. An IGF-I exposed metatarsal at day 10 is illustrated in **(d)**. Section of an IGF-I treated metatarsal at day 10 reacted for ALP activity showing staining within both the mineralising (MZ) and hypertrophic zone (HZ). The proliferating zone (PZ) is negative for ALP activity **(e)**.

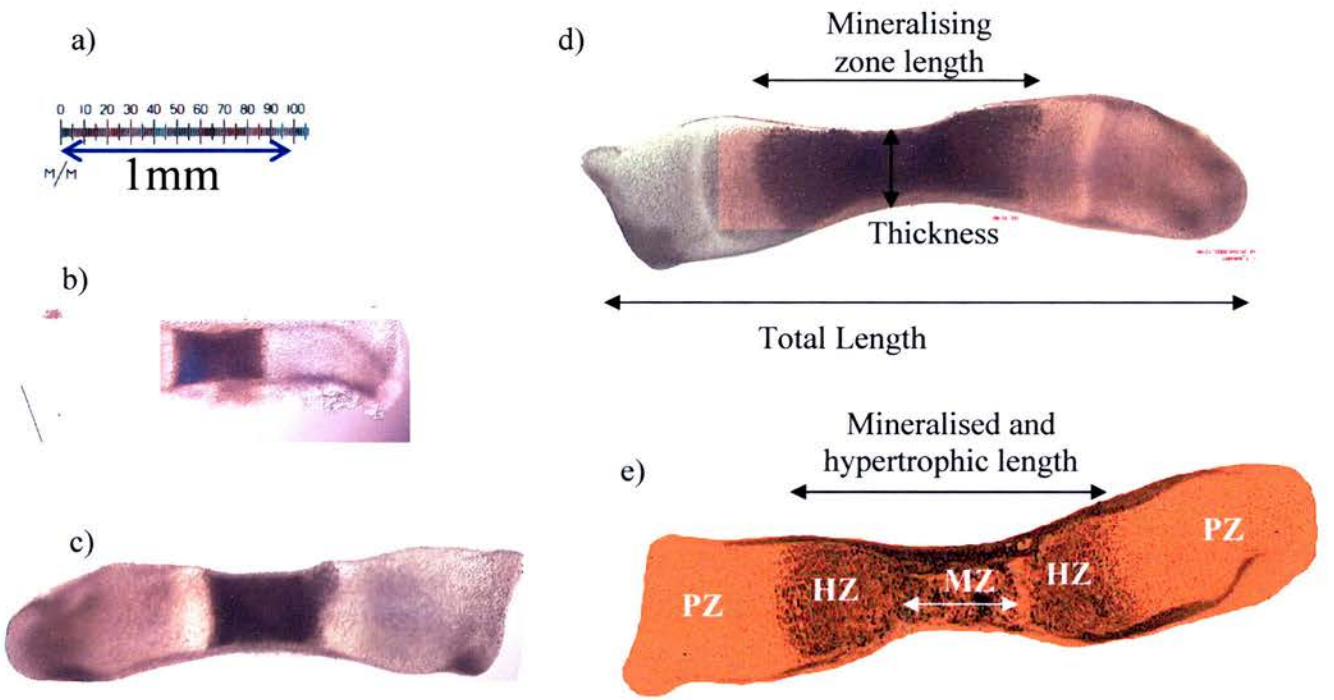


Figure 4.3. Linear Metatarsal Growth after Dex, IGF-I and GH exposure (a) Dex at 10^{-6}M caused a significant decrease in linear growth from 8 days, whereas IGF-I and IGF-I+Dex had significant stimulatory effects from 2 days **(b)** Effects of GH 100ng/ml and Dex 10^{-6}M on total length. GH had no significant effects on total length. Again Dex significantly decreased the total length from day 8 ($p<0.05$). **(c)** Effects of Dex, IGF-I and IGF-I+Dex on the length of the mineralised zone. In the control metatarsals, mineralisation increased from 4 days. All treatments caused a significant reduction in mineralisation from day 6. IGF-I treated bones were the least mineralised, whereas Dex and IGF-I+Dex effects were intermediate. **(d)** Effects of Dex, IGF-I and IGF-I+Dex on metatarsal thickness. Both IGF-I and IGF-I+Dex caused a significant increase in the metatarsal thickness from day 4 and 6 respectively. Results shown in **a, c** and **d** were obtained from the same cultures whereas the data shown in **b** were from a separate experiment. All data is expressed as the mean ± SEM, * = significance compared to control (* $p<0.05$). † = significance of IGF-I compared to IGF-I/Dex († $p<0.05$). Dex 10^{-6}M (Δ); IGF-I 100ng/ml (■); combined IGF-I+Dex (□); GH 100ng/ml (O); Control cultures: (●).

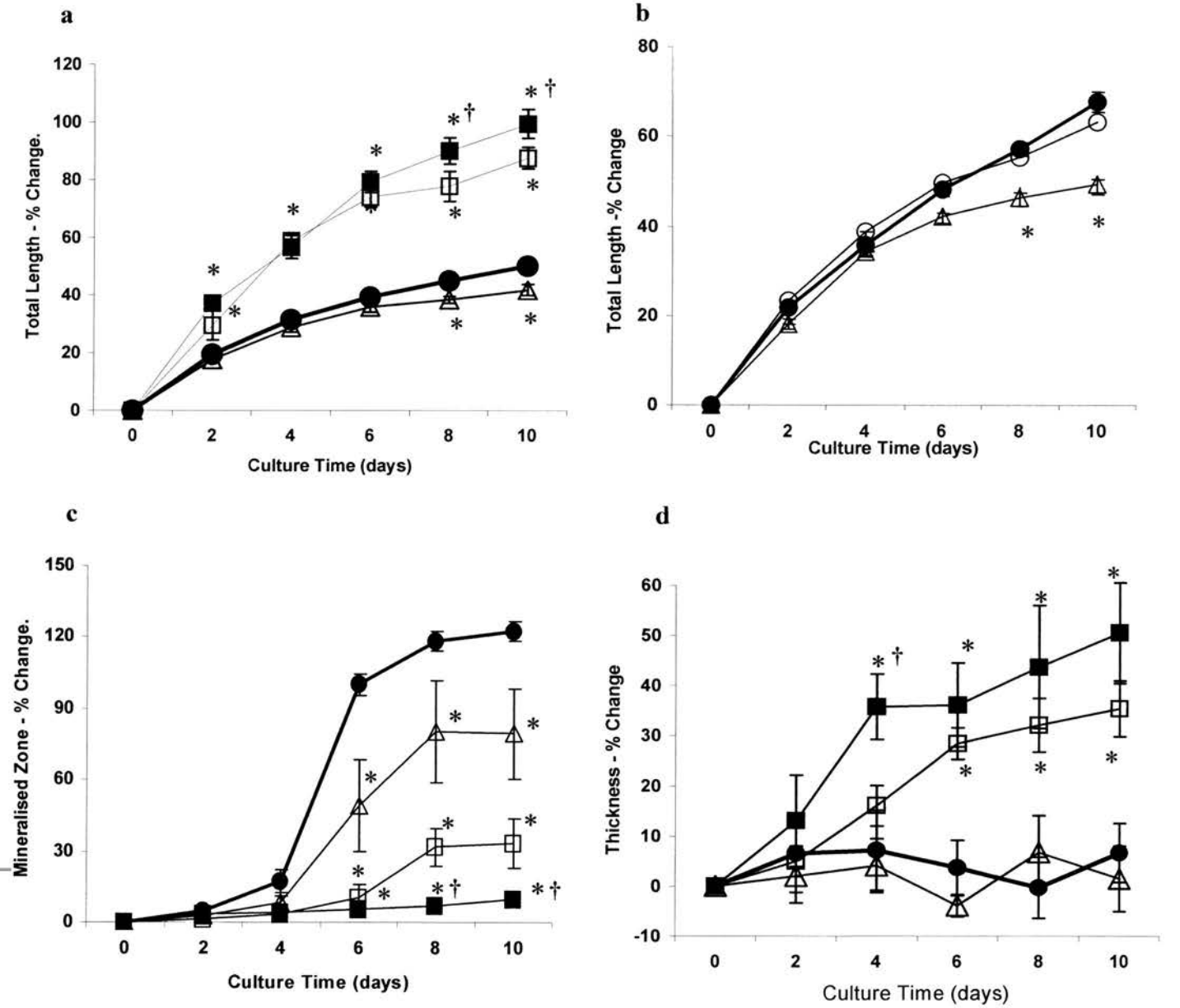


Figure 4.4. Histological assessment of chondrocyte hypertrophy **(a –d)** in metatarsals treated with Dex and IGF-I. Haematoxylin & eosin stained sections of 10-day-old cultures of control **(a & c)** and IGF-I **(b & d)** treated metatarsals. There is an increase in the size of the hypertrophic zone of the IGF-I treated metatarsals **(b)** compared with controls **(a)**. The chondrocytes of the hypertrophic zone of the metatarsals in **a & b** are shown in higher magnification in **c & d**. The chondrocytes juxtaposed to the von Kossa positive mineralised cartilage are larger in the IGF-I treated **(d)** than in the control metatarsals **(c)**. Note the micrographs shown in figs **a & b** are taken at different magnifications to accommodate the increased length of the IGF-I treated metatarsals. P – proliferating chondrocytes; H – hypertrophic chondrocytes; dashed line (---) marks the boundary between the proliferating and hypertrophic zones. Magnifications: **a** = bar 100 μm; **b** = bar 200 μm; **c & d** = 25 μm.

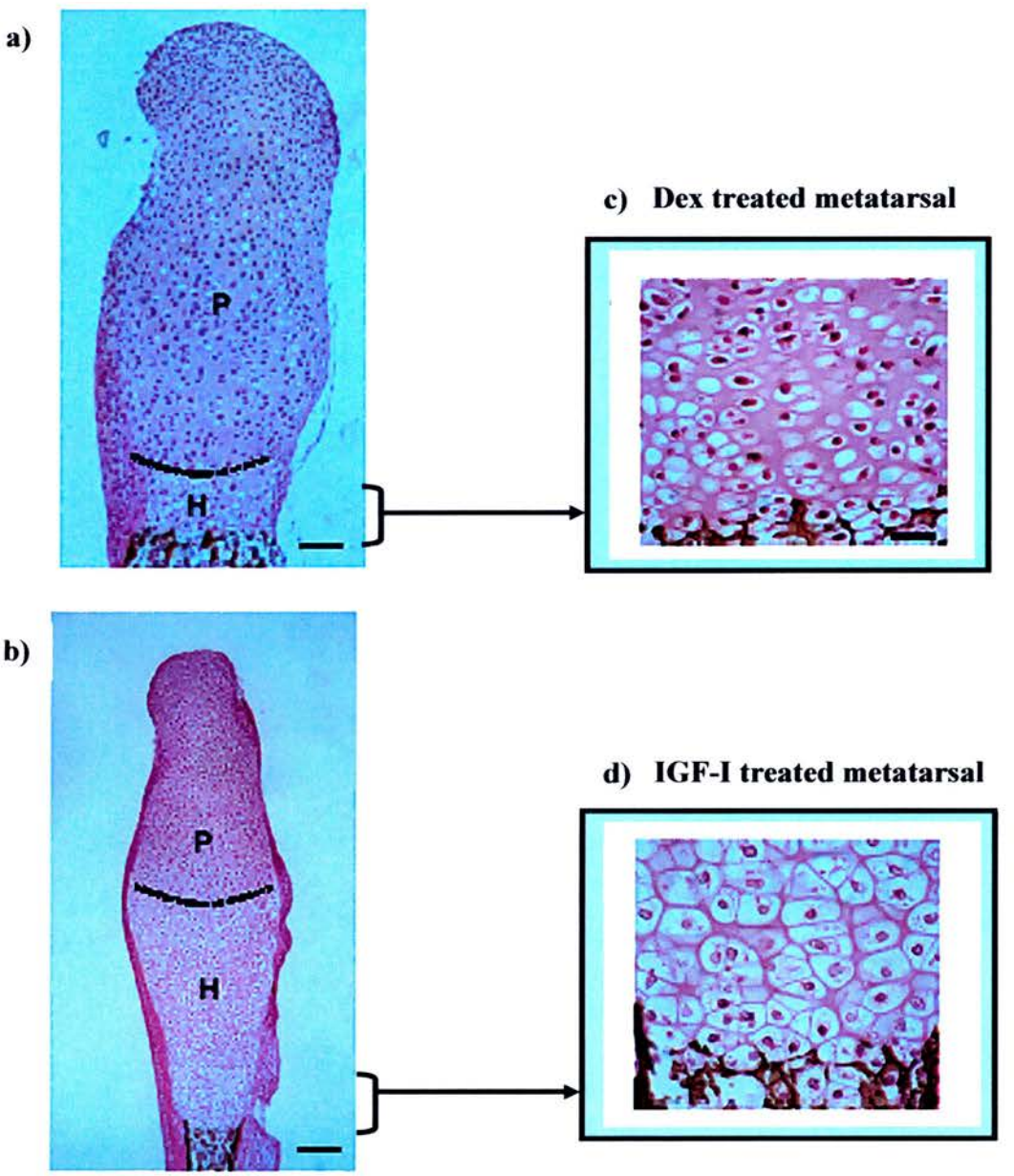


Table 4.1. Lengths of the proliferating, mineralising and hypertrophic zones. Data expressed in micrometers (μm) ± sem, * = significance compared to controls (* $p<0.05$).

Treatment	Proliferating Zone		Mineralising Zone		Hypertrophic Zone	
	Day 4	Day 10	Day 4	Day 10	Day 4	Day 10
Control	140.4 ± 3.0	122.6 ± 7.0	53.7 ± 1.3	102.0 ± 3.4	38.2 ± 1.7	36.7 ± 4.4
Dex	129.0 ± 2.4	120.3 ± 4.2	48.5 ± 1.9 *	84.2 ± 7.4 *	42.6 ± 4.0	40.2 ± 15.1
IGF-I	150.6 ± 5.6	123.8 ± 5.0	48.8 ± 1.5 *	49.3 ± 2.2 *	75.6 ± 1.4 *	163.4 ± 7.6 *
IGF-I+Dex	159.8 ± 8.9 *	137.0 ± 4.7	48.5 ± 1.5 *	57.8 ± 3.5 *	66.6 ± 8.4 *	122.0 ± 3.1 *

4.2. Metatarsal Chondrocyte Proliferation and Differentiation.

4.2.1 Introduction

Having established that Dex retards and IGF-I accelerated linear growth, this experiment set out to ascertain mechanisms by which these two compounds could be having their differential effects on chondrocyte proliferation and hypertrophy.

Undifferentiated progenitors within the reserve stem cell zone differentiate into chondrocytes and progress through to the proliferative phase. However the perichondrium and periosteum are now increasingly known to participate in the regulation of limb growth, serving as potential sources of signalling molecules that are involved in chondrocyte proliferation, maturation and hypertrophy (Colnot *et al,* 2004). The tissues that give rise to the appendicular skeleton namely the cartilage and perichondrium are derived from the same population of mesenchymal cells. During development some mesenchymal cells begin to flatten, elongate and form the perichondrium, whereas cells in the central condensation differentiate into chondrocytes to form the cartilage skeleton. However the nature of the tissue interactions between the cartilage and perichondrium is not well understood, as organ culture work has shown that removal of the perichondrium results in an increase in chondrocyte proliferation and longitudinal growth in chick embryonic bone (Long & Linsenmayer, 1998). Conversely in mice *ex vivo* models the perichondrial removal resulted in growth arrest with little formation of trabecular and periosteal bone (Colnot *et al,* 2004).

Although the previous data indicate that IGF-I is a powerful growth stimulatory and proliferative agent, this metatarsal culture model allowed the direct localisation of the proliferative effects of Dex and IGF-I to the cartilaginous growth plate or the perichondrium. Additionally biochemical corroboration was sought of the observation that

IGF-I has a three-fold increase in the length of the hypertrophic zone, by measuring the alkaline phosphatase activity within the metatarsals.

4.2.2 Materials and Methods

a) Foetal metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18-day old embyronic Swiss mice and Dex and IGF-I were added at a final concentration of 10^{-6}M and 100ng/ml, respectively, to the same cultured bones as described in 4.1.2a.

b) Alkaline Phosphatase (ALP) enzyme activity

At the end of the culture period (day 10), ALP activity within the metatarsals was determined as previously described (Deckers *et al,* 2001). Briefly, each metatarsal was permeabilised in 100 µl of 10 mmol/L glycine (pH 10.5) containing 0.1 mmol/L $MgCl_2$, 0.01 mmol/L $ZnCl_2$ and 0.1% Triton-X-100 by freeze-thawing three times. The extract was assayed for ALP activity by measuring the rate of cleavage of 10 mM p-nitrophenyl phosphate (pNPP). Total ALP activity was expressed as nmoles pNPP hydrolysed/min/metatarsal. Each group contained 3 metatarsals and the experiment was repeated at least twice.

c) Cell proliferation and dry weight determination

[^{3}H]-thymidine uptake.

On day 4 and 10 of culture [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was added (final concentration 10 µCi/ml) to each metatarsal culture for the last 6 hrs of culture. After washing in phosphate buffered saline (PBS) the metatarsals were extracted in trichloroacetic acid (2 x 30 min), acetone (2 x 30 min), ether

(3 x 30 min) and air dried overnight at room temperature. After the determination of dry weight (Sartorious, micro Gottingen, Germany) the tissue was solubilised (NCS-II tissue solubilizer, Amersham) and the DNA incorporated [^{3}H]-thymidine was determined using a scintillation counter (Haaijman *et al,* 1997). The cell proliferation data was expressed as [^{3}H]-thymidine (dpm)/metatarsal. Each group contained 3 metatarsals and the experiment was repeated at least 2 times.

Histological assessment of Bromodeoxyuridine uptake.

Bromodeoxyuridine (BrdU) (Sigma) was added (final concentration 1 mg/ml) to the culture medium of the metatarsals for the last 6 hrs of culture on day 4 and 10 as described previously (Haaijman *et al,* 1997). At the end of the incubation period the tissue was washed in Phosphate Buffered Saline (PBS) and fixed in 70% ethanol, dehydrated and embedded in paraffin wax. Sections, 10 μm in thickness, were cut along the longitudinal axis and chondrocyte nuclei with incorporated BrdU were detected using an indirect immunofluoresence procedure as detailed (Farquharson *et al,* 1993). Briefly, sections were denatured with 1.5 M HCl for 30 min before incubation with an antibody to BrdU (DAKO, Ely, Cambridgeshire, UK) diluted 1:50 in PBS for 1 h. After washing, the sections were incubated for a further 1 h in FITC-labeled goat anti-mouse IgG (Sigma) diluted 1:50 in PBS. The sections were finally mounted in PBS/glycerol (Citifluor, Agar Scientific, Essex, UK). Sections were examined using a Leica BMRB fluorescent microscope and the total number of BrdU positive chondrocytes within both the proximal and distal growth regions was determined. BrdU labelled cells located to the perichondrium were also counted. Three sections from each of 6 bones from each treatment group at both time points were examined to obtain an aggregate value.

4.2.3 Statistical analysis

All data are expressed as the mean ± sem and statistical analysis was performed using an analysis of variance (GenStat, Sixth Edition, VSN International Ltd). A p value of <0.05 was considered to be significant.

4.2.4 Results

Dry weights

At days 4 and day 10 there was no significant difference between the weights of the control and Dex treated metatarsals. They were, however, significantly lighter ($p<0.05$) than the IGF-I and IGF-I+Dex treated bones, which were themselves similar in weight to each other at both time points (Table 4.2).

Metatarsal ALP enzyme activity

The enlargement of the hypertrophic zone seen with IGF-I treatment after 10 days in culture (from previous experiment; section 4.1.4 & Table 4.1) was further studied by determining ALP activity in the metatarsals at the end of the culture period The ALP activity (nmoles/hydrol/min/metatarsal) expressed as mean ± SEM was as follows;

Control	**0.0187 ± 0.009**
Dex	**0.0117 ± 0.009**
IGF-I	**0.038 ± 0.0061 ***
IGF-I+Dex	**0.026 ± 0.0044**

* = significance compared to controls ($p < 0.05$).

In agreement, with the increase in hypertrophic zone length from the previous experiment (Table 4.1; Fig 4.4b), IGF-I treatment resulted in significantly elevated levels of ALP activity within the metatarsals (103% increase, $p<0.05$) compared to the control bones. Combined IGF-I+Dex caused an increase and Dex treatment alone a reduction in ALP activity, these two results were not, however, significantly different from the control values.

Cell Proliferation: [^{3}H]-thymidine incorporation & BrdU staining

The incorporation of [^{3}H]-thymidine into the metatarsals was determined at days 4 and 10, representing two distinct phases of varying growth rates. There was a tailing off in the linear growth curve from day 6 in all bones (Fig.4.3a) and this was reflected in lower [^{3}H]-thymidine incorporation rate in the control metatarsals at day 10 (75131 ± 5864 dpm) compared to the control bones at day 4 (98608 ± 6732 dpm), (Table 4.3). In comparison to control bones, Dex treatment for 4 days resulted in a significant reduction (50%, $p<0.05$) in [^{3}H]-thymidine incorporation, whereas both IGF-I and IGF-I+Dex treatment resulted in significant increases of 43 and 57%, respectively ($p<0.05$). After 10 days there was a significant reduction in [^{3}H]-thymidine incorporation in all treatment groups compared to the control cultures (Table 4.3). However, this reduction, from control bone values, was greater with Dex (80%, $p<0.05$) than that observed with IGF-I (64%, $p<0.05$) or IGF-I+Dex (53%, $p<0.05$).

To further refine the [^{3}H]-thymidine uptake data, the localisation of the proliferating cells (BrdU positive) was determined and quantified within both the growth plate and the perichondrium (Figs 4.5 & 4.6).The total number of proliferating cells in all control metatarsal groups was higher at day 4 than day 10, which is in agreement with the [^{3}H]-thymidine incorporation data and indicative of slower linear growth with time in culture

(Table 4.3 and Fig 4.3a). Compared to day 4 control metatarsals, Dex significantly reduced the number of BrdU positive cells located in the growth plate (42% decrease, $p<0.05$) and perichondrium (76% decrease, $p<0.05$) and therefore also the total number of dividing cells within the whole metatarsal (56% decrease, $p<0.05$), (Figs 4.5a, 4.6a & b). In contrast, IGF-I treatment significantly increased the number of BrdU positive cells in the perichondrium (76% increase, $p<0.05$) but not those within the growth plate (Figs 4.5a & 4.6c). Combined IGF-I+Dex treatment had no significant effect on BrdU incorporation in the perichondrium and growth plate compared to the control metatarsals at day 4 (Fig 4.5a).

Compared to day 10 control metatarsals (Fig. 4.6d), treatment with Dex alone (Fig. 4.6e), or in combination with IGF-I, significantly reduced the number of BrdU positive cells within the perichondrium (Dex: 96% decrease, $p<0.05$; IGF-I+Dex: 71% decrease, $p<0.05$), (Fig. 4.5b). Similarly, treatment with IGF-I alone (Fig. 4.6f) or in combination with Dex resulted in a reduction in BrdU positive cells within the growth plate chondrocytes (IGF-I: 63% decrease, $p<0.05$; IGF-I+Dex: 57%, $P<0.05$) (Fig. 4.5b). No cells out with the perichondrium and growth plates showed any BrdU positive staining. These results indicate that in comparison to chondrocytes within the growth plate, the cells within the perichondrium are more sensitive to stimulation by IGF-I during the period of rapid growth (day 4) and inhibition by Dex at both time points.

4.2.5 Discussion

This study reveals that the Dex-induced reduction in total metatarsal length is likely to be due to a reduction in chondrocyte proliferation as well as a reduction in the length of the mineralising zone seen in the last experiment. This antiproliferative potential of Dex is in agreement with the ATDC5 chondrocyte cell culture data as well as other investigators (Robson *et al,* 1998).

IGF-I rapidly stimulated the chondrocyte proliferation rate during the early phase of bone growth, although this did not persist at 10 days. Scheven & Hamilton (1991) also demonstrated that the increase in cell proliferation in metatarsals is not sustained with IGF-I over time, which could indicate the rapid utilisation of endogenous growth factors needed to support longitudinal growth. Metatarsals treated with IGF-I and Dex displayed greater cell proliferation than IGF-I treatment alone. The synergistic effect of IGF-I and Dex on cell proliferation has not been previously reported. It's clinical significance is unclear but an upregulation of chondrocytes expressing IGF-I following GC exposure has been reported previously and it is possible that IGF-I is an important local growth factor that counteracts the effect of GC at the tissue level (Rooman *et al,* 1999; Borges *et al,* 1999; Smink *et al,* 2002).

In order to understand the cellular mechanisms underlying the opposite effects of Dex and IGF-I on bone length the distribution of BrdU positive cells within metatarsals treated by both Dex and IGF-I alone and in combination was analysed. The number of dividing cells within the perichondrium was greatly reduced by Dex at both 4 and 10 days of culture. In contrast, at 4 days, the number of BrdU positive cells was greater in the perichondrium of IGF-I treated bones. Stimulation of cell proliferation was not observed in the IGF-I treated 10-day-old metatarsals and this may be due to the observed slowing of growth in these rapidly growing bones. In 4-day-old rapidly growing metatarsals, IGF-I completely reversed the inhibitory effects of Dex on cell proliferation within the perichondrium and growth plate. This reversal of the negative effects of Dex by IGF-I co-incubation was also observed, albeit to a lesser extent, in the perichondrial cells of 10-day-old cultures. These results extend the [^{3}H]-thymidine incorporation data and also confirm the ability of IGF-I to reverse the deleterious effects of Dex on cell proliferation. The observation that cell proliferation within the perichondrium was more sensitive to inhibition by Dex and stimulation by IGF-I than chondrocytes within the growth plate has previously not been

recognised. The perichondrium is vital to the endochondral process through its role in mediating the PTHrP-Ihh signalling cascade and it is possible that the marked Dex induced inhibition of proliferation within cells of the perichondrium has a more direct effect on the bone growth process (Kronenberg *et al,* 1997; Long & Linsenmayer 1998; Maeda & Noda, 2003). Although high levels of IGF-I mRNA expression have been shown in the perichondrium, there is no data on the effect of GC on the perichondrial cells (Edmondson *et al,* 1995). The differential sensitivity of cells to Dex treatment within the perichondrium and within the growth plate requires further study.

The IGF-I stimulated increase in the length of the hypertrophic zone was biochemically confirmed by the elevation in ALP. It was of interest to note that the data from the IGF+Dex treated cultures is consistent with the metatarsal length data where the presence of Dex partially reduced the effects of IGF-I. *In vivo* daily IGF-I administration has also been noted to raise the serum ALP, although this is mainly an indicator of osteoblastic activity (Laron *et al,* 1991).

Endochondral bone growth is a dynamic process and studying different time points may more accurately reflect this process. The data shows that Dex decreased and IGF-I increased cell proliferation. This alteration in the proliferation rate by both Dex and IGF-I was most marked within the cells of the perichondrium, with Dex having greater negative effects on the perichondrial chondrocytes at both time points, whereas the marked initial stimulatory effects of IGF-I on these chondrocytes were not sustained over time.

Table 4.2. Dry weights (ug) of the metatarsal bones at day 4 and day 10. Data expressed as mean ± sem, * = significance compared to controls (* $p<0.05$).

Treatment	Dry Weights (ug)	
	DAY 4	DAY 10
Control	34 ± 3	84 ± 2
Dex	33 ± 2	82 ± 5
IGF-I	48 ± 2 *	152 ± 5 *
IGF-I+Dex	59 ± 5 *	147 ± 2 *

Table 4.3. Cell Proliferation: Effect of Dex, IGF-I and IGF-I+Dex on [^{3}H]-thymidine uptake at day 4 and day 10. All data expressed as the mean ± sem, * = significance compared to controls (* $p<0.05$).

Treatment	[^{3}H]-thymidine (dpm)	
	DAY 4	DAY 10
Control	98608 ± 6732	75131 ± 5864
Dex	49521 ± 1124 *	15000 ± 1612 *
IGF-I	140547 ± 9821 *	27631 ± 2594 *
IGF-I+Dex	154941 ± 3695 *	35470 ± 1339 *

Figure 4.5. Effect of Dex, IGF-I and IGF-I+Dex on the number of BrdU positive cells within the growth plate (black bars), the perichondrium (white bars) and the combined number within the growth plate and perichondrium (hatched bars) at **(a)** day 4 and **(b)** day 10. Cell proliferation is higher at day 4 than day 10 with all treatments. At day 4 Dex causes a significant reduction in cell proliferation in the growth plate and perichondrium ($p<0.05$), whereas IGF-I increases the number of proliferating perichondrial cells ($p<0.05$). By day 10, Dex sustains the decrease in cell proliferation, which is significant in the perichondrium ($p<0.05$). Both IGF-I and IGF-I+Dex also cause a decrease in the number of the positive immunofluorescent cells at this time point. All data expressed as the mean ± sem, * = significance compared to controls (* $p<0.05$).

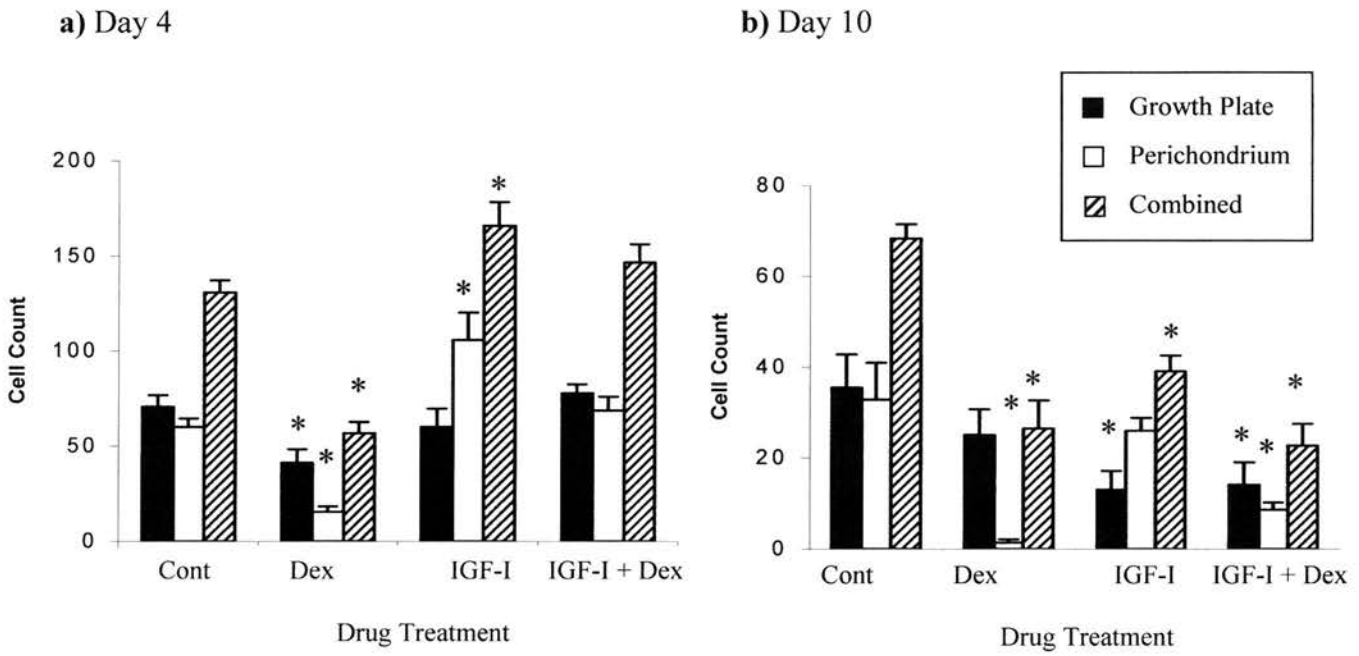
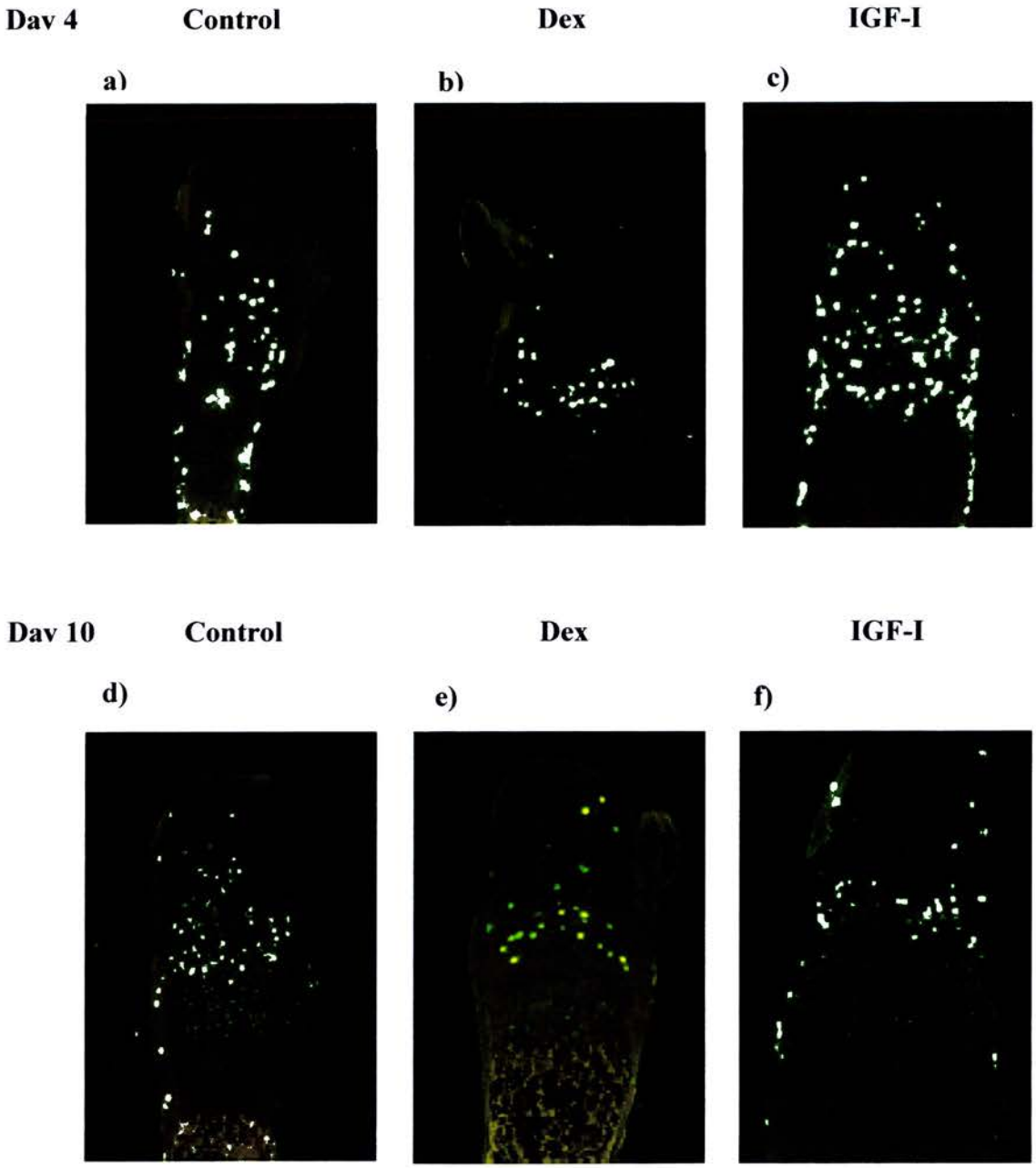


Figure 4.6. Histological assessment of chondrocyte proliferation **(a – f)** in metatarsals treated with Dex and IGF-I. BrdU labelled cells in control **(a & d)**, Dex treated **(b & e)** and IGF-I treated **(c & f)** metatarsals cultured for 4 **(a – c)** and 10 **(d – f)** days. Note the decreased number of proliferating cells in the Dex treated metatarsals and in particular the lack of staining within the perichondrium **(b & e)**. Increased perichondrial staining is observed in the 4-day-old IGF-I treated cultures.
Magnifications = bar 100 μm.



CHAPTER 5

RECOVERY FOLLOWING GLUCOCORTICOID EXPOSURE

5.1 Potential for the ATDC5 Cells to Recover Following Dex Exposure.

5.2 Metatarsal Growth Following Variable Lengths of Dex Exposure.

5.1 Potential for the ATDC5 Cells to Recover Following Dex Exposure

5.1.1 Introduction

Studies in children suggest that growth retardation following a short period of systemic exposure to GC may be followed by a period of CUG and that alternate day therapy may be less adverse for growth (Ahmed *et al,* 1999; Jabs *et al,* 1996). Catch-up growth has also been observed *in vivo* following direct injection of GC into the growth plate of rabbits (Baron *et al,* 1994).

GC induced growth retardation is caused by multiple intertwined factors, including direct effects on the growth plate as demonstrated by Baron et al (1992, 1994) who showed a 77% reduction in growth rate of 5 week old rabbits who had Dex administered directly into the growth plate. Following cessation of Dex, CUG was observed in the affected growth plate only and this ultimately corrected half the growth deficit.

There are no *in vitro* studies looking at the potential for chondrocytes to undergo catch up growth. In this study, the ATDC5 cell line was used to determine the effect of the GCs on the ability of chondrocytes to recover following different intervals of GC exposure to assess the potential for CUG. The ATDC5 chondrocyte cell line undergoes the temporal sequence of events that occur during longitudinal bone growth *in vivo* (Atsumi *et al,* 1990; Shukanami *et al,* 1997). As it had been fully characterised it was considered a useful model to assess if cell recovery following Dex exposure was duration or cell phenotype dependent.

5.1.2 Materials and Methods

a) Chondrocyte cell culture

The fully characterised ATDC5 chondrocyte line was set up and maintained as described previously (3.1.2a).

b) Recovery following GC exposure

For these experiments, a single concentration of Dex at 10^{-6}M was used as it was noted to have the most potent effects in earlier experiments. This dose was added to all cells when confluent (Day 6) and subsequently replaced with differentiation medium without Dex after 1, 3, 7 and 10 days (Recovery plates). Each time point was studied in quadruplicate. All cultures were maintained for a total of 14 days along with a group that was exposed to Dex for the total 14 days duration (no recovery period). Additional culture plates of Dex (10^{-6}M) treated cells and their respective controls (containing 0.01% ethanol) were stopped at the allocated time points (Day 1, 3, 7 and 10) to assess the impact of Dex prior to the period of recovery. Cell number, ALP activity and proteoglycan content were determined as described previously (3.2.2b).

5.1.3 Statistical Analysis

All experiments were performed at least twice. Data was analysed by analysis of variance. All data are expressed as the mean ± sem of four observations within each experiment and statistical analysis was performed using Statview (version 5.0.1). A p value of <0.05 was considered to be significant.

5.1.4 Results: ATDC5 recovery

Recovery following GC exposure

Exposure of the ATDC5 cells to Dex for one or more days resulted in lower cell numbers (protein) on Day 14. These differences however did not reach statistical significance unless the cells were exposed to Dex for all 14 days ($p<0.05$) (Fig 5.1a & b). There was a significant reduction in proteoglycan content after 7, 10 and 14 days of GC exposure (Fig 5.1c). After the recovery period (Fig 5.1d), all Dex exposed cells showed a significant

reduction in proteoglycan content ($p<0.05$). ALP activity was increased after Dex treatment at all days compared to control cultures but this increase was statistically significant only after 7 (65%) and 14 (148%) days of exposure (Fig 5.1e). After the recovery period (Fig 5.1f) ALP remained significantly elevated from day 10 ($p<0.05$).

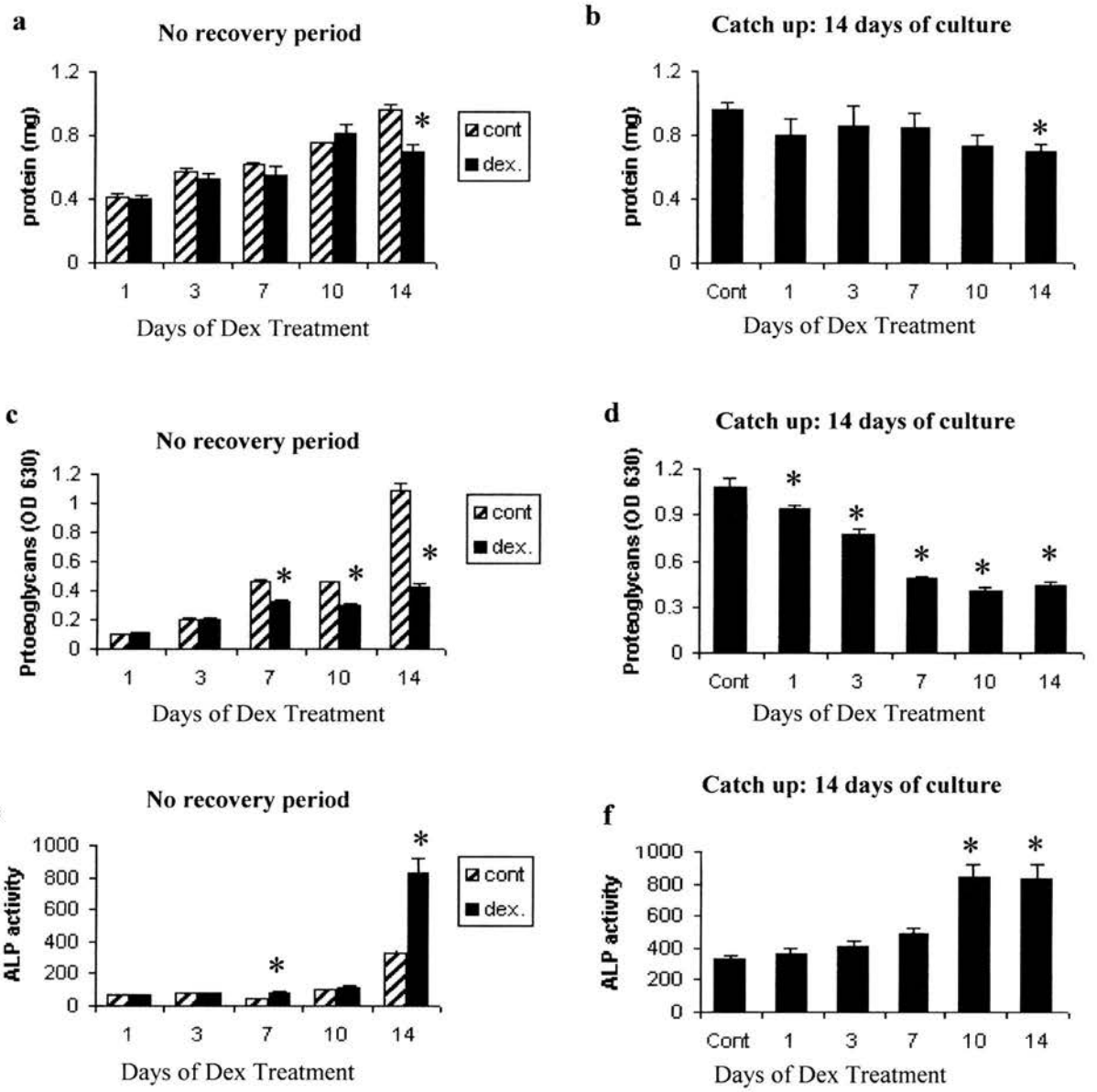
5.1.5 Discussion

In growth failure, amelioration of the growth retarding insult results in a period of supranormal linear growth described as CUG (Prader *et al,* 1963). Following cessation of a unilateral infusion of Dex into rabbit tibial growth plates CUG was observed in the affected growth plate but not in the contralateral tibia (Baron *et al,* 1992). It is postulated that this is due partly to a delay in growth plate senescence by Dex (Gafni *et al,* 2001).

The ATDC5 cell line allowed the study of this recovery phenomenon in greater detail. No differences were observed in proteoglycan content in the culture plates stopped prior to recovery at 24 and 72 hours of treatment. However after the recovery period a reduction in proteoglycans was apparent at all treatment lengths. This would indicate that Dex-induced suppression may take some time to manifest itself even though the stimulus for suppression has been removed. Longer treatments with Dex of 7 and 10 days duration showed unchanged levels of proteoglycans compared to the 14 day treated group and indicates that within the timescale of this experiment the ability to recover after 7 days of Dex treatment was limited. Longer periods of recovery allowed the ALP activity to suppress back towards control levels, although after 10 days there was no further recovery noted as compared to the 14 day Dex treated group. It cannot be ruled out however, that longer periods of recovery could result in suppression of ALP activity back to control levels. *In vivo* it is possible that catch up growth is never complete and merely falls below the statistical detection limit of a study (Silverstein *et al,* 1997).

In conclusion the ability to recover is related to the length of Dex exposure and possibly the chondrocyte phenotype. This study suggests that the potential for recovery of different events of the chondrocyte life cycle may vary.

Figure 5.1. ATDC5 Recovery. Effect of Dex 10^{-6}M on protein **(a & b)**: proteoglycans (PG) **(c & d)** and ALP activity **(e & f)** either after a period of no recovery **(a,c,e)** or where the dex treated cells were allowed to recover and assayed at 14 days **(b,d,f)**. **(a)** Cell numbers increase with time in culture. The only significant reduction is after 14 days Dex treatment ($p<0.05$). **(b)** Shows a reduction in protein at all time points, which is only significant at day14 ($p<0.05$). **(c)** Shows an elevation in proteoglycans with time in the control group, but a significant reduction in proteoglycans ($p<0.05$) from day 7 to day 10 in the Dex treated group as compared to their controls. **(d)** There is a significant reduction in proteoglycan content with 1 day Dex treatment ($p<0.05$) and this is more pronounced with longer periods of treatment **(e)** There is a gradual elevation in ALP activity (nmoles/hydrol/min/mg/protein) at each time point with a significant elevation above the respective control at days 7 and 14. **(f)** After the recovery period ALP activity remains elevated which is significant at days 10 and 14 ($p<0.05$).



5.2 Metatarsal Recovery and Alternate Days Dex Administration

5.2.1 Introduction

Clinically prevention of GC induced growth retardation could be addressed in a number of cases by judicious use of GC therapy. In addition alternate day GC therapy regimens are also useful in reducing the steroid load without having significant adverse disease effects, in conditions such as renal and liver transplant resulting in an improvement in final height (Hasegawa *et al,* 2004; Diem *et al,* 2003; Hochberg, 2002).

As the foetal mouse metatarsal model was thought to be a more physiological model of bone growth, a similar experiment as in the ATDC5 recovery (exp 5.1) was conducted to ascertain discrepancies between recovery following different doses and durations of Dex exposure. In addition to assessing total linear growth following different durations of Dex it was possible to look at alternate day exposure to Dex in these metatarsals.

5.2.2 Methods and Materials

a) Foetal metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18 day old embryonic Swiss mice and cultured at 37°C in individual wells in serum free medium in a humidified atmosphere of 95% air/5% CO_2 as described (4.1.2a).

The foetal mouse metatarsals from one dam were grown in serum free culture. All groups were studied in quadruplicate. One set of control bones was set up along with two sets of metatarsals continuously exposed to Dex 10^{-6}M and Dex 10^{-8}M for a total of 10 days. These were used to compare the effects of the different Dex regimens below.

b) Recovery experiment

On the harvesting day, Dex 10^{-6}M was added in quadruplicate to the wells for a duration of 1, 2, 4 or 6 days. After this allocated time Dex was removed and the metatarsal cultures continued till day 10 to assess recovery.

c) Alternate Day Dex

Alternate day Dex exposure was studied using Dex concentrations of Dex 10^{-6}M and Dex 10^{-8}M. Every day for a total of 10 days the metatarsals were either exposed to Dex or washed once with serum free medium prior to maintaining them in Dex free medium for that corresponding 24-hour period.

d) Morphometric analysis

Images were taken of the metatarsals every second day of culture using a digital camera (COHU, San Diego, USA) and the total length of the bone determined using Image Tool (Image Tool version 3.00, University of Texas Health Life Science Centre in San Antonio) as described (4.1.2b).

5.2.3 Statistical Analysis

Results are expressed as a percentage change in total length from harvesting length. All data are expressed as the mean ± sem and statistical analysis was performed using an analysis of variance (GenStat, Sixth Edition, VSN International Ltd). A p value of <0.05 was considered to be significant.

5.2.4 Results

Recovery Experiment

The metatarsals exposed to 1, 2 and 4 days of Dex 10^{-6}M and then allowed to recover till day 10, were shorter by 3, 5 and 7% than the control group, but these differences were not significant. However the groups given 6 days Dex (followed by a 4 day recovery period) and continuous Dex for 10 days were 27 and 22% shorter respectively as compared to the control metatarsals (Fig 5.2a).

Alternate Day Dex

Both continuous and alternate day Dex treatments resulted in a significant reduction in total length at day 10 ($p<0.05$). By day 10 in the continuously treated Dex 10^{-6}M and Dex 10^{-8}M groups there was a 22% and a 27% reduction in total length respectively when compared to the control metatarsals ($p<0.05$). (Fig 5.2b).

The alternate day Dex groups at 10^{-6}M and 10^{-8}M lengthened by 42% ± 4.4 and 55% ± 4.0 from harvesting length. This represents a 32% and 11% reduction respectively from the control group length at day 10.

Although the Dex 10^{-8}M bones were shorter than the metatarsals exposed to Dex 10^{-6}M, this difference was not significant. Likewise there was no significant difference between continuous and alternate day Dex 10^{-6}M. However alternate day Dex 10^{-8}M treated bones were significantly longer than their continuously treated counterparts ($p<0.05$).

5.2.5 Discussion

After short durations of Dex exposure the metatarsals did not show evidence of growth retardation. However with longer durations of 6 days it is evident that the potential to recover decreases. This may be a reflection of Dex duration or a crucial phenotype after which recovery may not occur. It is also simply possible that there was less recovery time compared to the metatarsals treated for a shorter duration. However if that was the case it

would be anticipated that after a partial recovery period, the metatarsals would be longer than their continuously treated counterparts, which was not the case here.

All metatarsals treated with alternate and continuous Dex were significantly shorter than the control group. Dex at 10^{-6}M did not demonstrate any significant differences between the two groups. However at a lower GC dose, alternate day Dex 10^{-8}M did have significantly longer lengths than the continuously treated cohort.

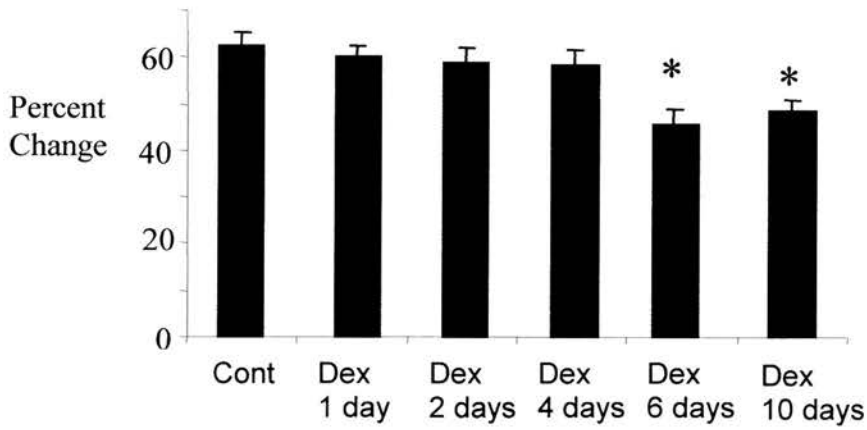
This experiment indicates that the growth-sparing effects of alternate day GC may be dependent on dose as well as pattern GC of administration. This growth sparing effect of alternate day steroids has not been observed by all investigators and it may be influenced not only be the duration of therapy but also the underlying disease process and the sex of the patient (Allen, 1996). In addition, most clinical reports refer to the use of Pred or hydrocortisone whereas these studies employed the use of Dex, which has markedly more potent effects on growth *in vivo* and *in vitro* as demonstrated in earlier experiments.

Figure 5.2 a & b. Metatarsal Growth after variable patterns of Dex Exposure

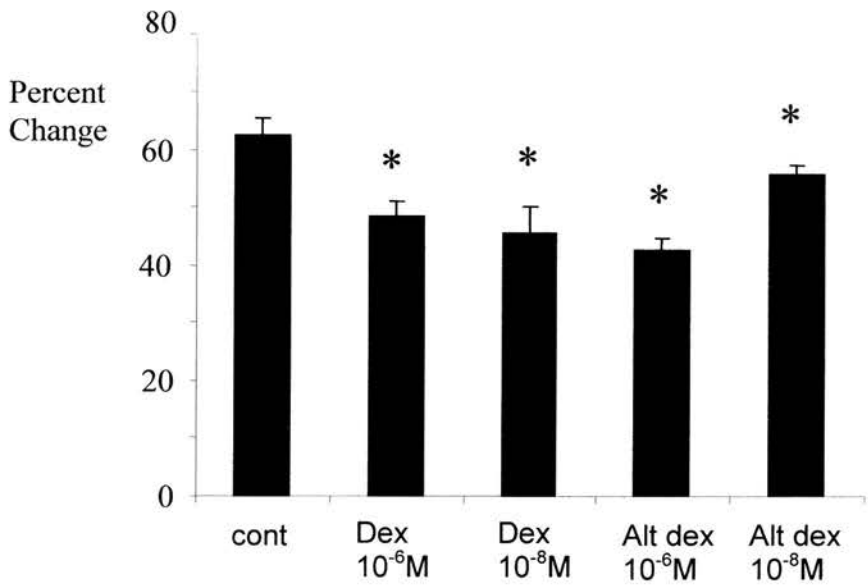
a. Exposure for 1, 2, 4 and 6 days Dex 10^{-6}M followed by period of recovery to day 10. No significant difference in length is noted in the groups allowed to recover after 1, 2 and 4 days Dex treatment. There is a significant reduction in length after 6 days Dex and in the continuously exposed group ($p<0.05$).

b. Effect of continuous and alternate day Dex 10^{-6}M and Dex 10^{-8}M on total length. Both continuous and alternate day Dex cause a significant decrease in length at day 10 ($p<0.05$).

a)



b)



CHAPTER 6

PRENATAL GLUCOCORTICOID EXPOSURE

6.1 Physical Measurements After Prenatal Glucocorticoid Exposure.

6.2 Circulating Levels of IGF-I, IGFBP-2 & Insulin levels after Prenatal Glucocorticoid Exposure.

6.1 Physical Measurements After Prenatal GC Exposure

6.1.1 Introduction

Dex reduced linear growth in foetal mouse metatarsals; further experiments assessed the impact of prenatal GC exposure on the bones and growth of mice exposed to Dex in utero. Thus the following studies attempted to explore the underlying physical and biochemical abnormalities in SGA.

SGA refers to the size of the infant at birth, whereas IUGR suggests diminished growth velocity in the foetus as documented by intrauterine growth measurements and indicates the presence of a pathophysiologic process occurring in utero that inhibits foetal growth. A child who is born SGA has not necessarily suffered from IUGR, and infants who are born after a short period of IUGR are not necessarily SGA (Lee *et al,* 2003). The underlying pathophysiology of IUGR and the failure of CUG in some small-for-gestational-age SGA infants is unclear. Although most SGA newborns catch up during the first 2 years of life, about fifteen percent will display persistent growth failure (Albertsson-Wikland & Karlberg, 1997) and the vast majority of these will remain short in later life, comprising 22% of adults with short stature (Karlberg *et al,* 1997).

Prenatal exposure to GC is known to cause growth restriction in most mammalian species including humans (Mosier *et al* 1982; Reinisch *et al* 1978). Indeed, human IUGR is associated with elevated maternal and foetal levels of endogenous GC (Goland *et al,* 1993), and these levels are more likely to be elevated in those who fail to display catch-up growth (Economides *et al,* 1988; Clark *et al,* 1996; Cianfarani *et al*, 2002).

Administration of synthetic GCs to women at risk for premature delivery is an established, evidenced-based intervention known to accelerate the rate of maturation of various foetal organs, such as the lungs, heart, brain, liver, kidney, and gut. About 10% of pregnant women in North America and Europe are treated with synthetic GCs between weeks 24

and 34 of gestation to promote foetal lung maturation in foetuses at risk of being delivered prematurely (NIH Consensus 1995; Matthews 2000). However, concerns remain about the effect of repeated courses of prenatal Dex and betamethasone on the long-term growth subsequently in childhood (Newnham & Moss, 2001). In rodents, antenatal exposure to GC has been used as a model to study long term effects on a number of physical traits, including hypertension, hyperglycaemia, hyperinsulinaemia, neuroendocrine responses and anxiety-like behaviour (Seckl, 2001). Although there is a substantial amount of literature on the postnatal effects of GC on murine growth (Smink *et al,* 2003), surprisingly little is known of the prenatal effects of GCs on longitudinal growth at the level of the growth plate and its relationship to biochemical markers of GH action.

The current study was performed to investigate the feasibility of using the mouse as a model for studying the effects of prenatal GCs on linear growth.

6.1.2 Methods

a) Animals

Time-mated pregnant C57Bl/6 mice were housed individually with free access to laboratory chow and water. One group (n = 6) received subcutaneous injections of 100μg/kg Dex in 0.9% saline for the last 6 days (E14 – E20) of pregnancy whilst controls (n = 8) were given vehicle injections (0.9% saline). At birth, pups were sexed and weighed, and crown rump length (CRL) was determined using a digital calliper (Mahr GmbH, Esslingen, Germany). The 6 Dex treated dams had 39 pups (20 males, 19 females) and the eight control dams produced 51 pups (32 males, 19 females). A subset of the pups (n = 24; non fasting), randomly chosen, was sacrificed by decapitation and trunk blood obtained. In this subset (6 males and 6 females from three Dex treated mothers; 8 males and 4 females from 3 control dams), tibiae were dissected out and their lengths measured using digital callipers as described above. Additionally, 5 male pups from each group

(Dex or control) were injected intraperitoneally with BrdU (2.5 mg/100g body weight) 1 h before sacrifice. The tibiae from the BrdU injected mice were fixed in 70% ethanol for 24 hours, dehydrated and embedded in paraffin wax. The experimental protocol was approved by the University of Edinburgh Animal Use Committee and the animals were maintained in accordance with university guidelines for the care and use of laboratory animals.

b) Chondrocyte proliferation

Wax sections, 10 μm in thickness, were cut along the longitudinal axis and chondrocyte nuclei with incorporated BrdU were detected using an indirect immunofluoresence procedure as detailed previously (Exp 4.2.2) (Farquharson *et al,* 1993). In brief, sections were denatured with 1.5 M HCl for 30 min before incubation with an antibody to BrdU (DAKO, Ely, Cambridgeshire, UK) diluted 1:50 in phosphate buffered saline (PBS) for 1 h. After washing, the sections were incubated for a further 1 h in FITC-labeled goat anti-mouse IgG (Sigma) diluted 1:50 in PBS. The sections were finally mounted in PBS/glycerol (Citifluor, Agar Scientific, Essex, UK). Sections were examined using a Leica BMRB fluorescent microscope and the total number of BrdU positive chondrocytes within both the proximal and distal growth regions was determined. Two sections from each bone from each treatment group were examined to obtain an aggregate value.

c) Growth plate and maturational zone widths

Wax sections were stained with haematoxylin & eosin, viewed under an Olympus MO81 microscope and images of the growth plate were captured using a COHU digital camera. The width of the growth plate and specific maturational zones were determined using Image Tool as previously described (4.2.2b). Due to the absence of the secondary ossification centre in 1-day-old bones the boundary at the top of the proliferating zone is

not easy to determine. Therefore the growth plate was divided into two broad regions referred to as proliferative and hypertrophic (Fig 6.1). The junction between the proliferating and hypertrophic zone was easily identified by the abrupt change in chondrocyte morphology. The cells within the proliferating zone have an oblate morphology, which is in contrast to the prolate morphology of those within the differentiating hypertrophic zone (Fig. 6.1). The width of the proliferative and hypertrophic zones was presented as a percentage of the whole growth plate width in order to take account of any possible obliqueness of sections. The growth plate width was ascertained from 10 male mice (5 control, 5 Dex). Three sections from each bone from each treatment group were examined to obtain the mean width of the growth plate.

6.1.3 Statistical analysis

Results of body weight, CRL and tibial length are expressed as mean ± sem. Body weight and CRL of the pups exposed to Dex are presented as Standard Deviation Scores (SDS) to examine the gender specific effect of prenatal Dex on growth. SDS for body weight or CRL for each pup was calculated by using the equation (absolute value for that mouse minus mean value for the control cohort of the same sex at same age) divided by one standard deviation from the mean for the control cohort of the same sex at same age. Data were analyzed using SPSS software v10 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2000 SR-2 (Microsoft Corp, Redmond, WA, USA).

6.1.4 Results

Body weight

The mean body weights in male and female pups were 1.377g ± 0.02 in the control group and significantly greater than the pups from the Dex treated dams with a body weight of

1.224g ± 0.02 ($p<0.05$) (Fig 6.2). The difference in body weight was seen in both male (Dex treated, 1.270 ± 0.026g; control, 1.368 ± 0.026g; $p<0.05$) and female pups (Dex treated, 1.175 ± 0.022g; control, 1.392 ± 0.034g; $p<0.05$). However, the effect of Dex on birth weight was more pronounced in female pups, with a mean body weight SDS of -1.46 ± 0.65 compared to -0.66 ± 0.79 for the male mice ($p<0.05$). Five out of 19 (26%) female pups but none of the male pups had a body weight less than –2SDS (Fig 6.3a,b).

Crown Rump Length

The mean CRL in the Dex-treated group was 27.90 ± 0.12mm, compared with 30.00 ± 0.21mm in the control group ($p<0.001$). The difference in CRL was observed in both males (CRLs in Dex-treated group and control group were 28.20 ± 0.20mm and 29.90 ± 0.25mm, respectively; $p<0.05$), and females where CRLs were 27.60 ± 0.12mm and 30.20 ± 0.36mm in the Dex-treated and control groups, respectively ($p<0.05$) (Fig 6.4). The female Dex-treated pups had a mean CRL SDS of -1.59 ± 0.55 compared to -1.19 ± 0.37 in the male mice (not significant). Three out of 19 (16%) female pups but none of the male pups had a CRL less than –2SDS of control mice. Although the effect of Dex was mostly on CRL and body weight, 3 out of 20 (15%) male pups had a CRL of less than –1 SDS but a normal weight (Fig.6.3a,b).

Tibial Length

Tibial lengths were measured in 12 Dex-treated (6 male) and 12 control (8 male) mice. Although the body weight of this subset of Dex-treated (1.29 ± 0.04g) and control (1.34 ± 0.02g) groups was not significantly different (probably reflecting the relatively small sample size), the mean CRL was lower in the former group (27.7 ± 0.2 mm) as compared to 28.8 ± 0.3mm in the control ($p<0.05$). However, the mean tibial lengths in the Dex-

treated and control groups were similar at 4.69 ± 0.08mm and 4.62 ± 0.08mm, respectively. Female mice had significantly shorter tibial lengths than males in both the Dex-treated (4.53 ± 0.12mm versus 4.86 ± 0.08mm; $p<0.05$) and control (4.37 ± 0.09mm versus 4.75 ± 0.08mm; $p<0.05$) groups.

Correlation between Body Weight & CRL and Body Weight & Tibial Length

There was an association between body weight and CRL in both the Dex-treated groups (r,0.5; $p<0.05$) and the control groups (r,0.7: $p<0.05$) (Fig.6.3). Furthermore, in the subgroup of Dex-treated and control mice which had detailed anthropometry, there was an association with body weight and tibial length (r,0.7; $p<0.001$) (Fig.6.5). However, there was no significant relationship between CRL and tibial length in the smaller group of 12 mice, which had tibial length measurements.

Chondrocyte Proliferation and Lengths of Proliferating and Hypertrophic Zones

The length of the individual proliferating and hypertrophic zones were measured in 10 male mice (5 Dex-treated, 5 controls). In this subset, there was no statistically significant difference between the body weights in the Dex-treated group (1.36 ± 0.03g) and the controls (1.38 ± 0.03g). However, the Dex-treated group had significantly shorter CRLs (28.2 ± 0.2mm) compared to the control group (29.3 ± 0.4mm) ($p<0.05$). The total width of the growth plates was 91.9 ± 2.9μm and 86.5 ± 2.3μm in the Dex-treated and control group, respectively (not significant). Mean proliferating zone length expressed as a percentage of the growth plate width was similar at 81.7 ± 1.3% in the Dex-treated group and 81.8 ± 1.1% in the controls. Similarly, the mean length of the hypertrophic zone was 18.3 ± 1.3% and 18.2% ± 1.1 of the growth plate in the Dex-treated and control groups, respectively (not significant). The number of proliferating chondrocytes was reduced in

the pups exposed to prenatal Dex (111.5 ± 5.3) as compared to the control group (140.2 ± 37.1) but this was not significant.

6.1.5 Discussion

The results of the present study not only confirm that prenatal Dex exposure results in growth retardation in the offspring but they also show that it has a greater effect on the female offspring. The extent of reduction in birth weight was similar to that observed by other investigators using other models of IUGR such as bilateral uterine artery ligation, protein restriction, as well as prenatal GC exposure (Harrel & Tannenbaum, 1995; Houdijk *et al*, 2000; Mosier *et al*, 1982).

Sexual dimorphism exists in a number of anthropometric measures at birth and it is possible that these differences are established early during foetal life (Hindmarsh *et al,* 2002). Antenatal GC treatment in sheep produces greater lung maturation in females at risk of preterm delivery (Kovar *et al,* 2001) and in guinea pigs alters the female foetal hypothalamic-pituitary-adrenal (HPA) function (Dean & Matthews, 1999). The effects of prenatal stress or GC exposure in programming the HPA axis have also been shown to be sex-specific, with female rats displaying greater ACTH and corticosterone levels than their male counterparts (McCormick *et al,* 1995). GC exposure in late gestation in the rat permanently programs gender specific differences in adult cardiovascular and metabolic physiology (O'Regan *et al*, 2004). Similarly an increased incidence of SGA in female offspring has been reported in two population-based studies (Hediger *et al* 1998, Thomas *et al,* 2000).

The underlying basis to the sexually dimorphic effect of prenatal GC on growth, with female offspring being more severely affected, is unclear. A gender specific effect on postnatal growth in 3-5 week old rats exposed to prenatal Dex has been reported before by Swolin-Eide *et al*, (2002), where the investigators found an increase in CRL and long

bone length in the male offspring but a decrease in the CRL of the female offspring; the authors did not report any gender-specific differences in size at birth.

Despite a reduction in body weight and crown rump length in the whole cohort of pups, a clear reduction in tibial length was not observed in this study. Furthermore in the subpopulation of mice that had tibial lengths and histomorphometry performed no significant differences in body weight were noted between the control and Dex-treated groups despite a reduction of CRL in the latter. Future studies should include more sensitive and accurate measurements of the rest of the axial skeleton by techniques such as radiogrammetry as it is possible that calliper based measurement of the tibia may not reveal small changes in the length of individual bones that may cumulatively be reflected as reduced CRL. Our finding was similar to that of Mehta *et al* (2002) who did not find any significant reduction in femoral or tibial length in sixteen mice with IUGR secondary to maternal protein restriction. However, they did report an increase in the width of the epiphyseal plate; whereas this study did not reveal any significant increase in the width of the proliferative or hypertrophic zone of the growth plate. Although the number of proliferating chondrocytes was reduced in the prenatal Dex exposed group, this finding was not statistically significant.

Although sexual dimorphisms are well documented, the association with prenatal GC and IUGR has not been previously recognised. This data shows that prenatal GC exposure affects birth weight and length and that this effect is more marked in the female offspring.

Figure 6.1. Photomicrograph of a tibial growth plate, depicting numerous chondrocytes forming the proliferating zone (**PZ**) and the larger, prolate chondrocytes constituting the hypertrophic zone (**HZ**).

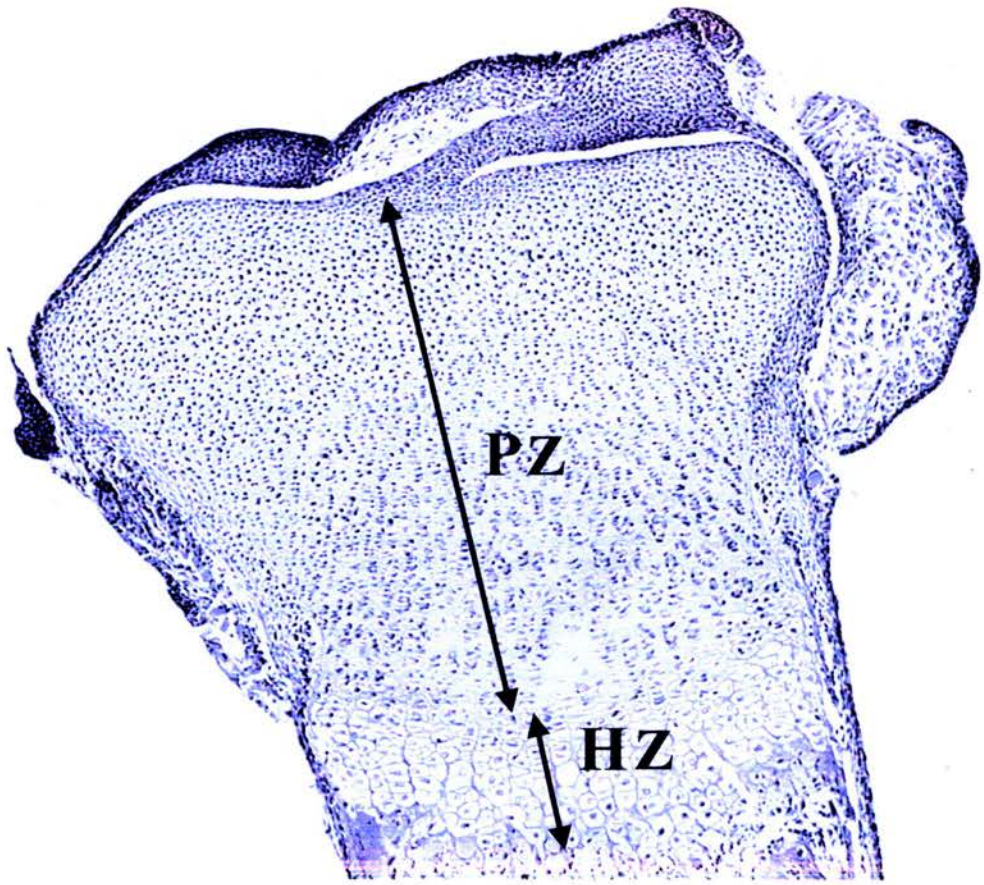


Figure 6.2. Effect of prenatal Dex exposure on **body weight** (g) in the combined, male and female groups. All data expressed as the mean ± sem, * = significance compared to control (* $p<0.05$).

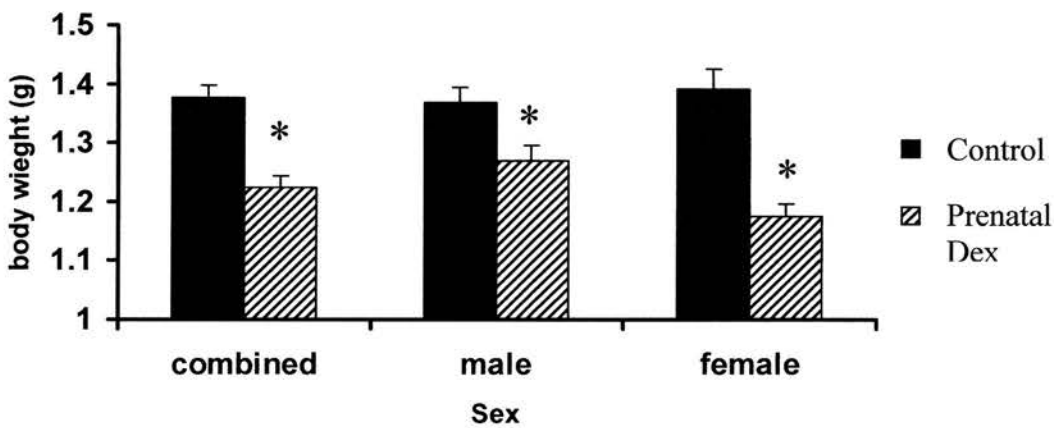
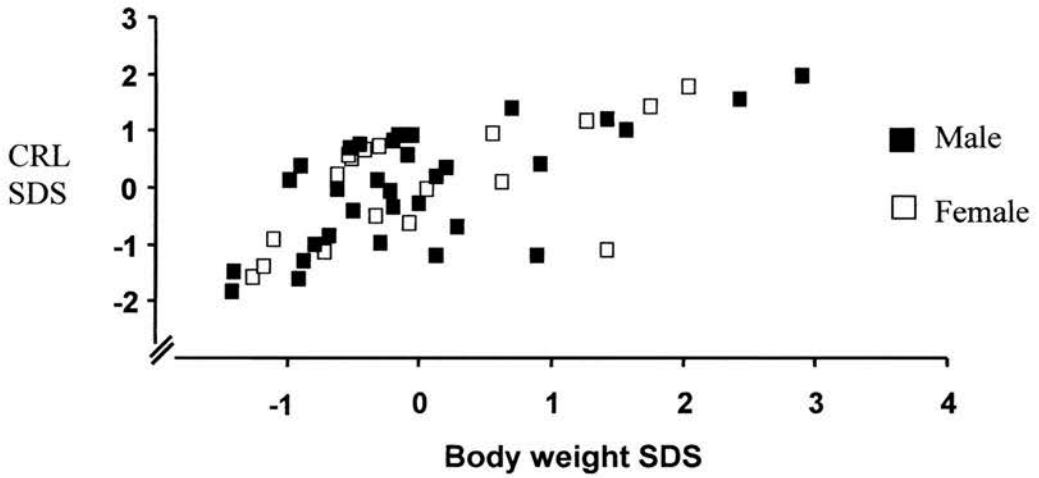


Figure 6.3. a) Relationship between **body weight SDS** and **CRL SDS** in the control group. Correlation score 0.6 ($p<0.05$), 32 males, 19 females. And **b)** Dex exposed group. Correlation score 0.5 ($p<0.05$). 20 males, 19 females. (■) Male, (□) Female.

a) Control group.



b) Prenatal Dex Group

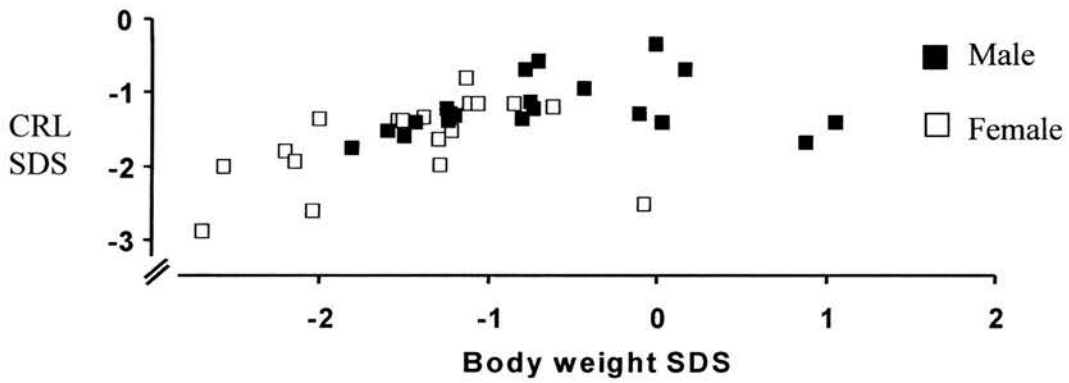


Figure 6.4. Effect of prenatal Dex exposure on **Crown Rump Length** (CRL) in the combined, male and female groups. All data expressed as the mean ± sem, * = significance compared to control (* $p<0.05$).

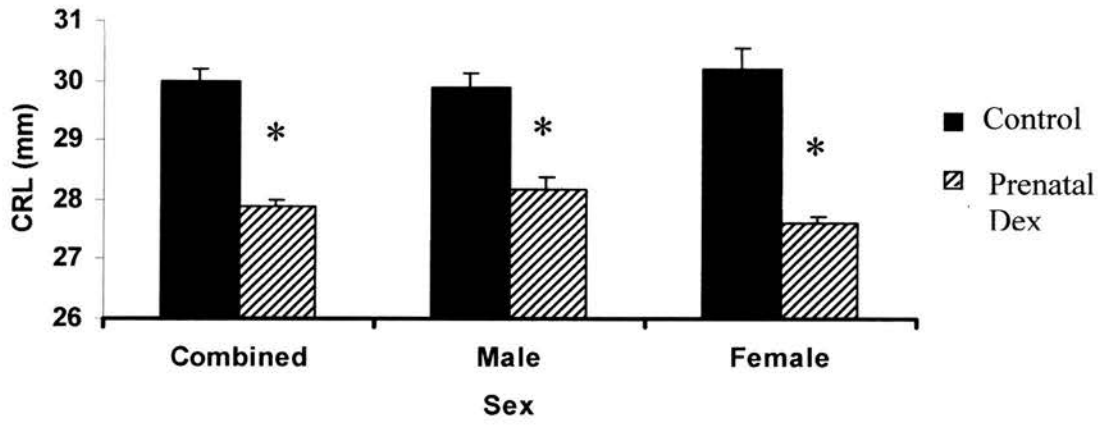
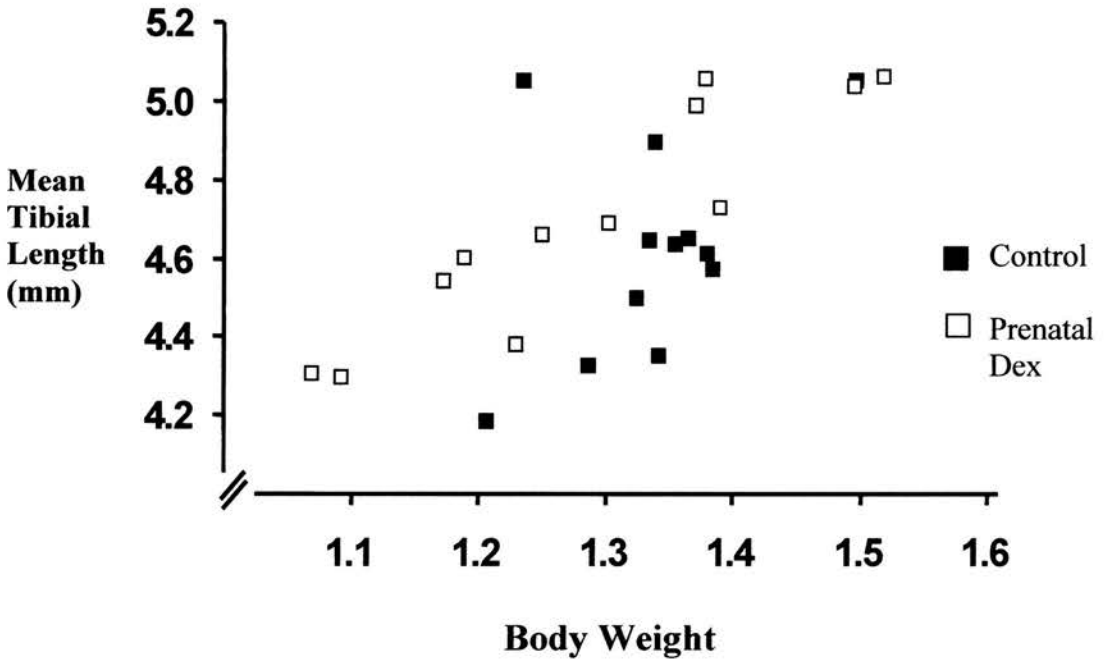


Figure 6.5. Relationship between **body weight (g) and mean tibial length (mm)** in the control and Dex exposed group. Correlation score 0.7 ($p<0.001$). Control (■) , Prenatal Dex (□).



6.2. Circulating Levels of IGF-I, IGFBP-2 & Insulin levels after Prenatal GC Exposure

6.2.1 Introduction

After establishing the effect of prenatal Dex on the physical parameters of growth, this study assesses the impact of prenatal Dex on the levels of circulating growth factors: IGF-I, Insulin and IGFBP-2 in the same offspring.

GH, Insulin, IGF-I, IGF-II and their binding proteins play an important role in influencing prenatal and postnatal growth (LeRoith *et al,* 2001). IGF-II is the dominant IGF during early development and IGF-I becomes more important at a later stages, the growth promoting effects of both factors are mediated through the IGF-I receptor (Gluckman *et al,* 1992, Dechiara *et al,* 1990). Unlike Insulin, which acts predominantly on liver, muscle and adipose tissue, all foetal tissues express IGFs in early gestation. The activity of these peptides is modulated by IGFBPs, which help localise IGFs to particular tissues during differentiation (Gluckman *et al,* 1992; Hill *et al*, 1989).

IGFBP-1,2 and 6 have been reported to act exclusively as growth inhibitors on IGF dependent proliferation of growth plate chondrocytes (Kiepe *et al,* 2002). Amongst these growth inhibitory proteins, IGFBP-2 is the predominant foetal IGFBP (Gluckman *et al,* 1992, Green *et al,* 1994) and may have an inhibitory effect on IGF-I and II activity (Schnieder *et al,* 2000). It is the only binding protein that is expressed in the growth plate of mice (Smink *et al* 2002, 2003) and it is also a crucial factor in avian skeletogenesis, where it has been reported to inhibit chondrogenic differentiation and matrix synthesis (McQueeney & Dealy 2001). Overexpression of IGFBP-2 in mice has been shown to cause a reduction of somatic growth (Hoeflich *et al*, 1999). Increased expression of IGFBP-2 is found after fasting and in other conditions associated with stress (Blum *et al,* 1993, Strasser-Vogel *et al,* 1995). Exogenous GC administration is also associated with

elevated systemic IGFBP-2 levels in children (Crofton *et al,* 2000) and has been reported to be associated with increased tissue expression of IGFBP-2 in some tissues such as human bone marrow stromal cells but not the growth plate (Smink *et al,* 2002 & 2003, Cheng *et al,* 1998).

The aim of this study was to look at the relationship of serum IGF-I, Insulin and IGFBP-2 in the mice offspring who had been exposed to prenatal Dex in the previous experiment (Expt 6.1) and to look for any perturbations of these in the newborn mice.

6.2.2 Methods

a) Animals

A subset of the offspring either exposed to prenatal Dex or vehicle injections from the previous experiment (Expt 6.1) had blood samples collected after their physical characteristics had been ascertained.

b) Serum analysis of IGF-I, IGFBP-2 and Insulin

Serum from 24 pups (control:Dex, 12:12) of the same mother (3 control and 3 Dex treated mothers) were pooled for the assay of Insulin (DRG diagnostics, Germany), IGF-I (Octeia rat/mouse; IDS, Boldon, UK) and IGFBP-2 (OBI-DSL, Upper Heyford, UK) by ELISA. Each sample was assayed in duplicate and followed the manufacturers instructions. Blood samples were centrifuged at 3000g for 15 min at 4°C and the serum was collected and stored at –80°C until assayed for IGF-I, IGFBP-2 and insulin.

6.2.3 Statistical Analysis

Results of serum IGF-I, IGFBP-2 and Insulin were not normally distributed and, therefore, are expressed as median (10^{th}, 90^{th}) centiles. Spearman rank correlations were used to compare any association between variables and Student's paired t-test or the Mann

Whitney U test (for data deviating from the normal distribution) were used to compare differences between groups. Data were analyzed using SPSS software v10 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2000 SR-2 (Microsoft Corp, Redmond, WA, USA).

6.2.4 Results

Serum IGF-I, Insulin and IGFBP-2

Median (10^{th}, 90^{th} centiles) serum IGF-I concentration in the control group was 282ng/ml (281, 291) compared to the Dex-treated groups where it was significantly higher 291ng/ml (282, 297) ($p<0.05$). The median serum IGFBP-2 concentration in the Dex-treated group was also higher than the control values; 3190ng/ml (3001, 3209) and 2982ng/ml (2899, 3282) respectively ($p<0.05$). No significant differences were noted in the serum Insulin concentrations in both groups; control 2.82ng/ml (2.74, 2.85) and Dex 2.82 (2.77, 2.95) (Table 6.1).

6.2.5 Discussion

This study shows that growth retardation following prenatal Dex exposure is associated with raised levels of IGFBP-2 and IGF-I. IGFBP-2 is the most commonly expressed IGFBP in the mouse, the most dominant in the foetal circulation and thereafter the second most abundant IGFBP in the circulation (Rajaram *et al,* 1997) (Smink *et al,* 2002, 2003). Therefore its systemic levels in the newborn mouse were measured to try to understand its relationship to growth.

The experiments show that Dex-induced growth retardation was associated with raised levels of IGFBP-2 and IGF-I. Although the IGFBP-2 knockout mouse does not show any clear growth abnormalities, the transgenic mice that over express IGFBP-2 do show growth retardation (Hoeflich *et al,* 1999). Increased IGFBP-2 expression may contribute

to growth restriction by inhibiting the bioactivity of IGF-I (and IGF-II). Additionally the increased activity of IGFBP-2 has been shown to 'buffer' the effects of IGF-I overexpression in transgenic rabbits (Wolf *et al,* 1997). Direct evidence for an IGF-inhibitory role of IGFBP-2 comes from *in vitro* studies where IGFBP-2 inhibits IGF-dependent growth stimulation of human carcinoma cells but did not affect proliferation of IGF-resistant cells (Hoflich *et al,* 1998). Thus, by inducing IGFBP-2 (and thereby inhibiting IGF-I and/or IGF-II), prenatal Dex may cause a state of IGF resistance.

It is also possible that the raised IGF-I levels that were observed in this study are a reflection of the raised level of bound IGF-I due to the raised IGFBP-2. The finding of raised IGFBP-2 is in concordance with the observations of Price *et al* (1992) who reported increased hepatic expression of IGFBP-2 in rat foetuses exposed to prenatal GCs. Dex has also been reported to increase IGFBP-2 expression in human bone marrow stromal cells (Cheng *et al* 1998). These two studies showed a negligible to a significant fall in IGF-I expression, contrary to our finding of a raised IGF-I level. In addition, Cheng *et al* (1998) reported raised IGF-II expression following Dex exposure and this elevation was in proportion to the rise in IGFBP-2. However, as it has not been observed in other non-skeletal tissues, it is possible that this response may be tissue specific (Beck *et al* 1988; Li *et al* 1993). Interestingly, maternal IGFBP-2 levels also increase in foetal growth restriction in humans (Holmes *et al* 1999). Thus, in this model it is also possible that these raised IGFBP-2 levels in the mother could be reflected in the serum levels of IUGR offspring. Similarly in rabbits, maternal steroid administration causes a marked increase in foetal IGF-I mRNA expression and IGF-I protein levels (Thakur *et al,* 2000). The small serum sample volumes in the day 1 mice offspring restricted the study of IGFBPs to IGFBP-2 and gender specific changes could not be investigated as the samples had to be pooled. The findings would have been further strengthened by the study of the other IGFBPs as well as IGF-II.

Although elevated insulin levels have previously been reported in SGA infants (Veening *et al,* 2002), no difference in serum Insulin levels was noted in this study. However, Cianfarani *et al* (2003) did not demonstrate altered Insulin sensitivity despite a reduction in glucose concentration in SGA children and this effect may be dependent on variables such as age, nutrition and the fasting state of the offspring.

A limitation of this study is the relatively small numbers of mice in each group which then restricted the ability to look for sexual differences in the IGF-I and IGFBP-2 levels in both the control and the Dex exposed group. Future studies should attempt to corroborate whether the small but significant differences in the IGF-I and IGFBP-2 levels remain and indeed if there is a difference between males and females.

In conclusion this study demonstrates elevated IGF-I and IGFBP-2 levels raising the possibility of a state of IGF-I insensitivity. This may be due to GC induced IUGR causing defective IGF-I action which is mediated by an up-regulation of IGFBP-2 and a persistent defect may explain the growth failure that is observed in a proportion of the offspring who remain short.

Table 6.1. Serum concentration (ng/ml) of IGF-I, Insulin and IGFBP-2 in control and prenatal Dex exposed mice. IGF-I and IGFBP-2 levels are significantly increased in the Dex exposed group compared to their respective control. Results expressed as median ± 10^{th}/90^{th} centiles. Significance compared to controls (* $p<0.05$).

	Control			**Prenatal Dex**		
Serum concentration ng/ml	**IGF-I**	**Insulin**	**IGFBP-2**	**IGF-I**	**Insulin**	**IGFBP-2**
Median	**282**	**2.82**	**2982**	**291 ***	**2.82**	**3190 ***
10^{th} centile	281	2.74	2899	282	2.77	3001
90^{th} centile	284	2.85	3282	297	2.95	3209

CHAPTER 7

FINAL DISCUSSION

&

FUTURE DIRECTIONS

7.1 FINAL DISCUSSION

The rate of longitudinal bone growth is determined by a complex interplay of proliferative kinetics, size of the proliferative pool, matrix synthesis and hypertrophic chondrocyte enlargement. The control of these processes is still a matter of debate, however any perturbation of any of these variables may affect normal bone growth. The benefits of GCs often outweigh the risks of potential side effects in the treatment of many diseases in adults and children. However the growing skeleton is susceptible to the additional growth impairing effects of the GC. A greater understanding of these effects may influence the type, duration and dosage schedule that is optimal in controlling disease while minimising the growth impairment. Therefore, the identification of such changes in the present studies may provide a better understanding of the mechanisms underlying Dex induced growth retardation.

GCs regulate many physiological systems and high levels cause growth retardation via their direct effects on the growth plate. In addition to the effect of primary disease and stress on growth retardation, GCs also affect the HPA axis, kidneys, muscle, adrenal and gastrointestinal tract. Many factors interact to cause these negative effects, thus it is essential that the individual mechanisms are carefully dissected out.

The stimulation for this thesis was the relative lack of any *in vivo* data on the potencies of different GC on growth and bone turnover. The clinical findings showed that in children treated for ALL, Dex and Pred affect short-term growth and bone turnover and that Dex may be 18 times more potent than Pred at suppressing short-term linear growth and nine times more potent at suppressing bone turnover. Although the greater potency of Dex had been demonstrated in other organs systems it was the first time that the varying effects on linear growth had been substantiated.

Subsequently these observations were translated into cell culture models and progressed to increasingly physiological models of bone growth such as the metatarsal explants before finally completing the cycle to study the impact of prenatal GC exposure on growth in *in vivo* mouse models.

Cell culture

Data obtained from the cell culture studies also indicate that Dex has more potent effects than Pred. In addition it should be highlighted that GC effects appear to be most apparent during chondrogenesis when the cells are rapidly proliferating – this is an important observation as this is the first study to characterise a chondrocyte cell line so that the GC effects can be studied on a homogeneous population of chondrocytes. This observation could account for the similarities and differences between this data and other investigators, as the major effects of the GC were dependent on the chondrocyte phenotype. Thus although Dex and Pred decreased cell proliferation, cell number, proteoglycan synthesis and increased differentiation these effects were mainly restricted to chondrogenesis with little effects during terminal differentiation. The cell culture data overwhelming supports the issue that the negative GC effects on bone growth are mediated via the their anti-proliferative effects. An additional effect could be the premature progression to the differentiated chondrocyte, as indicated by increased ALP activity. In combination, this decreased proliferation and increased differentiation could account for a net loss of linear growth.

Further experiments revealed that the ATDC5 chondrocyte cell line expressed the GC, IGF-I and GH receptors. This information was utilised to show that the antiproliferative effects of Dex in this cell line could be completely reversed by IGF-I, indeed proliferation was significantly above control levels, with GH having no beneficial effects, possibly due to the fact that the GH receptor was expressed later or due to a suboptimal concentration used. Although Insulin and IGF-I share a high similarity of structure and intracellular

signalling events (Dupont & LeRoith, 2001), it was interesting to note that at similar concentrations IGF-I effects on chondrocyte proliferation were much more profound than insulin.

Metatarsal culture

This thesis has shown that the foetal mouse metatarsal model can closely replicate *in vivo* bone growth and this is the first *in vitro* study to directly demonstrate the pro-hypertrophic effects of IGF-I, and reversibility of Dex induced growth retardation.

The foetal mouse metatarsals provided an ideal model to study the effects of both GC and IGF-I. As these bones have a relatively large percentage of cartilaginous growth plate chondrocytes, maintenance of cellular interactions and a supply of endogenous growth factors it was possible to culture them in medium devoid of serum, thus removing a potential confounding variable. Again on direct linear measurements it was ascertained that Dex and IGF-I have major and opposite effects on longitudinal bone growth with IGF-I clearly reversing the growth inhibitory effects of Dex. The GC effects were dependent on duration of exposure whereas IGF-I showed a rapid acceleration in growth. The mechanism of Dex induced growth retardation in this model appeared to be a decrease in proliferation and mineralisation.

One of the most important results to stem from this thesis is that the increase in linear growth achieved by IGF-I is due to a dramatic increase in the size of the hypertrophic chondrocytes. Although it has been recognised *in vivo* that the growth retardation in the IGF-I null mouse is associated with an attenuation of chondrocyte hypertrophy (Wang *et al,* 1999), it is the first time this observation has been demonstrated in an *in vitro* model.

Longitudinal bone growth is a dynamic process, as the chondrocyte undergoes its life cycle, it is continuously under the influence of a wide and varying range of hormonal influences. Thus it is imperative that to be sure of the GC and growth factor effects, the experimentation should allow for the dynamic nature of bone growth. This was shown in

the cell culture studies undertaken at two distinct time points. In addition the process was repeated in the metatarsal studies. By studying the two time points at days 4 and 10, it can be concluded that proliferation is much more marked in all groups at the earlier time point. Although Dex reduced proliferation at all time points, IGF-I stimulated cell proliferation early, whereas the reverse was true by day 10, indicating a utilisation of growth factors associated with the slower rate of linear growth or a change to the predominant chondrocyte phenotype.

Although IGF-I mRNA has been shown to be concentrated in the murine periosteum and perichondrium (Shinar *et al,* 1993; Wang *et al,* 1995), the observation that cell proliferation within the perichondrium was more sensitive to inhibition by Dex and stimulation by IGF-I than chondrocytes within the growth plate, has previously not been recognised and requires further study, it is possible that the perichondrium could form another area for the convergence of adverse drug effects.

Cell Recovery

The recovery experiments utilising both the cell culture and metatarsal models had essentially similar results; that the detrimental effects of GC are dependent on the duration of the GC exposure – after a certain period the chondrocytes ability to recover from the GC effects is impaired. This was demonstrated in the ATDC5 cell line which showed that cell number and proteoglycans levels gradually decrease with length of exposure, and ALP levels fail to suppress to control levels with increasing GC exposure. It is postulated that alternate day GC may not adversely affect final height potential, but they may still delay puberty and be associated with a delayed growth spurt (Polito *et al,* 1999). This data did not reveal any beneficial effect on metatarsal length in continuous versus alternate day Dex exposure. This could be due to the fact that the high dose of Dex that was used precluded any beneficial effect being noted. Indeed alternate day exposure with Dex 10^{-8}M as compared with Dex 10^{-6}M was associated with less detrimental effects on total

metatarsal length. Additionally it has been noted that animals with shorter gestations such as rodents may provide less clinically applicable information than animals of longer gestations (Jobe, 2003). This would mean that the foetal mouse metatarsal would have had had a higher net steroid load.

Prenatal GC

Prenatal GCs are extensively used in perinatal medicine to increase the maturation of various foetal organs in women at risk of premature delivery. Although concerns remain about the impact of repeated courses of prenatal GC on subsequent childhood growth, little is known of the prenatal effects of GCs on longitudinal growth at the level of the growth plate and its relationship to various biochemical markers of GH action. Although there is a convergence of hormonal, nutritional and environmental effects in the SGA offspring, the IGFs play a crucial role in prenatal and postnatal growth.

To address this, the bone and biochemical profiles in the offspring of mice exposed to prenatal Dex were examined. The mouse is particularly suited to study the role of the IGF-I on prenatal growth as it is known that the foetal growth promoting effects of IGF-I and II are mediated through the IGF-I receptor (Nakae *et al,* 2001). Prenatal GC exposure resulted in a group of SGA mice with reduced weight and length which was greater in the female offspring. This difference was a surprising but not wholly unexpected finding, as sexual dimorphisms exist in a number of physical and biochemical measurements at birth. This sexual dimorphism can exist in birth size as well as other parameters such as the urinary excretion of GH and IGF-I in children (Fall *et al,* 2000). Although sexual dimorphisms are well documented, the association with prenatal GCs and IUGR has not been previously recognised. It is increasingly recognised that IGF-I action is regulated by a number of binding proteins, which may also have IGF-I independent effects. This group of SGA mice had raised levels of IGF-I and IGFBP-2; the latter is the main binding protein that inhibits IGF-I action. The small sample size did not allow for gender

differences to be extracted. Together this data indicates the possibility of a state of IGF-I insensitivity mediated by an up-regulation of IGFBP-2 action. As IGF-2 is the main prenatal growth factor it is also probable that its regulation is also disrupted.

An issue that arose during this thesis that has not been resolved is the relative contribution of GH and IGF-I to linear growth. In these experiments IGF-I was consistently shown to have growth stimulatory effects, over-riding all the Dex effects. However GH had no effects in the two chondrocyte models. This may have been related to the stage of differentiation of the chondrocyte or the GH concentration used. As GH is required for postnatal growth it is also possible that the foetal metatarsals are unresponsive to the GH effects at the gestation used. It seems likely that local IGF-I production may be the more important regulator of linear growth (Yakar *et al,* 1999; Ueki *et al,* 2000). Although infusion of GH into a limb causes an increase in *in vivo* bone length (Isaksson *et al,* 1982), it is postulated that these effects are nonetheless mediated via an elevation in local IGF-I (Hunziker *et al,* 1994). Similarly GHR knock out mice show reduced bone growth caused by premature reduction in chondrocyte proliferation and cortical bone growth at 2 weeks of age, which is reversed by raising serum IGF-I levels (Sims *et al,* 2000). In the IGF-I null mice the hypertrophic zone is shortened but the reserve zone is expanded (Wang *et al,* 1999). The latter is considered to be due to the increased GH levels, thus supporting the view that GH expands the pool of chondrocyte progenitors but this contradicts the hypothesis that IGF-I is responsible for clonal expansion of the proliferating cells. Further studies have indicated that as well as local IGF-I production there may be a minimal IGF-I threshold concentration that is necessary for normal growth (Yakar *et al,* 2002).

In conclusion, this thesis took the clinical observation showing that Dex and Pred have different potencies on short term linear growth and bone turnover. This was further investigated in increasingly physiological models of bone growth demonstrating that *in vitro*, Dex and IGF-I have opposite effects on direct linear growth and markers of bone

growth. These effects are dependent on the chondrocyte phenotype, in addition the effects of Dex are time dependent whereas IGF-I effects are immediate. Dex decreases proliferation, cell number, matrix production and skeletal mineralisation while IGF-I markedly stimulates chondrocyte hypertrophy in favour of mineralisation and proliferation and completely reverses the Dex induced growth retardation. However, IGF-1 administration alters the balance of the different chondrocyte phenotypes and it is unclear whether this has any long-lasting implications. Recovery after GC exposure is related to the duration of Dex exposure and alternate-day Dex administration did not have any growth sparing effects and GH had no beneficial effects at the dose studied. Finally the thesis completed the cycle back to *in vivo* data demonstrating that prenatal GC administration leads to SGA offspring, which is worse in females, and these SGA offspring may have a state of IGF-I resistance.

7.2 FUTURE DIRECTIONS

This thesis has opened up some intriguing avenues for further research which in the future may have clinical significance. In my opinion tackling and understanding the underlying principles of GC induced bone growth should proceed using a combination of models of longitudinal growth. It was inevitable that as the work progressed further avenues for research would be identified.

Observational studies

IGF-II is a key regulator of prenatal bone growth and the circulating levels increase with gestation. There is little data about the effects of IGF-II in these models of bone growth and its relative and/or synergistic effects to IGF-I would provide useful additional information regarding the regulation of longitudinal bone growth.

Once a ligand binds to the GCR, it undergoes a conformation change and the receptor either causes transcriptional activation or repression (or both events) via different downstream pathways – the latter is central to GC mediated anti-inflammatory and anti-proliferative effects. Selective GCR modulators (SGRM) are in development which have the same anti-inflammatory activity but without the side effects. The synthetic GC; Deflazacort (an oxazoline derivative of Pred) was believed to have less impact on growth and GC induced osteoporosis but fell out of favour as it was subsequently shown to be little different from standard GC once the anti-inflammatory activity was optimised (Markham A, 1995). Newer compounds such as AL-438 may have a similar anti-inflammatory profile to standard GC but with lesser effects on glucose metabolism and bone turnover (Rosen & Miner 2005). The models described in this thesis could be used to corroborate the bone and growth effects of these newer generation of SGRMs.

Pathophysiology

Dex partially reversed some of the IGF-I effects in both culture systems. The cellular actions of IGF-I are mediated by a receptor tyrosine kinase (IGF-IR). Binding of IGF-I to its receptor utilises a family of soluble receptors, known as IRSs which results in the activation of two distinct signalling pathways, phosphatidylinositol 3-kinase and p42/p44 mitogen-activated protein kinase, leading to proliferative and antiapoptotic effects. These pathways can be inhibited by LY-294002 and PD-98059 respectively. Therefore the models could be used to ascertain if the deleterious effects of Dex on chondrocyte proliferation and linear bone growth are mediated by an inhibition of IGF-I signalling.

Clinical and in vivo studies

It is unclear which subset of SGA infants do not undergo catch up growth and if a relationship exists between birthweight and length and future growth potential. Further studies could ascertain what the spectrum and size reduction is in mice offspring following GC exposure, that is. A) reduced weight, reduced length, B) reduced weight, normal length, C) normal weight, reduced length, D) normal weight, normal length. And what the relationship of the first three phenotypes (A – C) is to the presence or absence of CUG after repeat measurements in the growing offspring. Differences related to dose and duration of GC exposure could be further explored.

Furthermore the models described respond to various factors present in the serum, including cytokine and growth factors and it is possible that the effects of inflammatory disease, growth inhibitory or stimulatory agents can be directly measured using a combination of these models and thus provide information on linear growth regarding the disease process and subsequent response to therapy.

CHAPTER 8

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BIBLIOGRAPHY

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PUBLICATIONS

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Short-term effects on linear growth and bone turnover in children randomized to receive prednisolone or dexamethasone

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Summary

AIM To compare the relative potency of prednisolone (Pred) and dexamethasone (Dex) on short-term growth and bone turnover.

METHOD Prospective study over 16 weeks of children randomized to receive Pred (40 mg/m^2) or Dex (6·5 mg/m^2) for the first 5 weeks as part of the MRC-ALL97/99 induction chemotherapy for acute lymphoblastic leukaemia (ALL).

MEASUREMENTS Lower leg length velocity (LLLV) and weight, serum IGF-I, serum bone alkaline phosphatase (bALP) levels and creatinine-adjusted, urinary excretion of deoxypyridinoline cross-links (DPD).

SUBJECTS Nineteen children (eight boys, 11 girls) with a median age of 5·9 years (range 2·6–13) and with a diagnosis of ALL.

RESULTS At week 2 of therapy, median LLLV in the Dex group was −1·5 mm/week (range 0·7 to −2·1) and significantly lower than the LLLV in the Pred group which was −0·1 mm/week (range 0·20 to −0·28; $P < 0·05$). In the Dex group, LLLV remained lower at week 8 (med LLLV, −0·3 mm/week, range 0 to −1·3) compared to LLLV in the Pred group at 0·3 mm/week (range 0·2–1·0; $P < 0·05$). Body weight showed an increase after week 2 and reached a peak in both groups of children at week 6. The change in weight from baseline was greater in the Dex group than the Pred group reaching a maximum change by week 5 of 17·5% (range 5–25) and 8·7% (range −3 to 18), respectively ($P < 0·05$). At presentation, median IGF-I level for the whole group was 83·5 μg/l (range 31·8–293). IGF-I levels fell markedly during Dex therapy and continued to remain lower than baseline. At weeks 4, 6 and 8, median change in IGF-I from baseline was lower in the Dex group than the Pred group. From week 1 to week 3, median change in bALP was 72% (range −8 to 304) in the Pred group, whereas in the Dex group change in bALP was −1% (range 23 to −28; $P < 0·005$). By week 3, median bALP was higher in the Pred group at 65 U/l (range 36–187) than in the Dex group at 39 U/l (range 26–60; $P < 0·05$) but by week 6 median bALP in the Pred group had fallen to a similar level to the Dex group. At presentation, median DPD was 22 nmol/l (range 17–38) and 20 nmol/l (range 12–26) in the Pred and Dex groups, respectively (ns), reaching a nadir between weeks 3 and 6. The median percentage change in DPD in the Pred and Dex group from week 1 to week 3 was −34% (range −7 to 14) and −53% (range −6 to −69), respectively (ns). By week 8, DPD excretion had started to rise more dramatically in the Pred group such that the median DPD was 35 nmol/l (range 10–53) in the Pred group and 22 (range 9–30) in the Dex group ($P < 0·05$). On average, between weeks 2 and 8, LLLV was three times lower, percentage gain in weight was three times higher, bALP was 1·3 times lower and DPD was 1·5 times lower in the Dex group than the Pred group.

CONCLUSION Pred and Dex both affect short-term growth and bone turnover. The mechanism of the effect on bone formation may be different between the two drugs. Dex may be about 18 times more potent than Pred at suppressing short-term linear growth and stimulating weight gain, and about nine times more potent at suppressing bone turnover. Glucocorticoids have a variable effect on different parameters of growth and bone turnover and the intensity may depend on the steroid used.

Glucocorticoid therapy (GC) is commonly used as anti-inflammatory therapy and as part of immunosuppressive regimens in childhood. It is estimated that 5–10% of children may require some form of GC at some time in childhood (Warner, 1995).

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The functional effects of steroids on target tissues is difficult to predict and their use is hampered in some individuals more than others because of side-effects such as growth retardation, osteoporosis, hypertension, altered body composition and blood glucose homeostasis. Impairment of childhood growth with long-term GC was described almost 50 years ago by Blodgett *et al.* (1956). Alterations in growth and bone turnover, as assessed by knemometry and markers of GH secretion and bone turnover, can also occur during relatively short periods of GC therapy (Crofton *et al.*, 1998; Ahmed *et al.*, 1999). The onset and severity of these GC-induced effects may be dependent on the duration of therapy and the nature of the steroid compound, and the comparative biological potency of GC such as prednisolone (Pred) and dexamethasone (Dex) may be tissue-specific (Orth & Kovacs, 1998). For instance, in children and young adults with congenital adrenal hyperplasia, Dex may be about 25 and 80 times more adrenal suppressive than Pred and hydrocortisone, respectively (Hansen & Loriaux, 1976; Hughes & Read, 1982). Dex is also reported to be four times more potent at suppressing the hypothalamo–pituitary–adrenal axis than Pred but it may be 16 times more lymphocytotoxic than Pred (Kaspers *et al.*, 1996). Dex also displays better CNS penetration (Balis *et al.*, 1987), and this feature and has led to a randomized trial to look at its efficacy in the treatment of ALL (Gaynon & Carrel, 1999). The relative potency of different GC on growth and bone turnover is unclear as there are no *in vivo* studies in children. The current study was performed on children entering a national trial of acute lymphoblastic leukaemia (ALL) therapy in which they were randomized to receive Pred or Dex as part of induction of remission and continuing treatment. By accounting for possible confounding factors such as other concurrent chemotherapy and the effects of the disease, the randomization process provided a suitable opportunity to compare the short-term effects of Pred and Dex on growth and bone turnover by the same methods as employed in earlier studies (Crofton *et al.*, 1998; Ahmed *et al.*, 1999).

Patients and methods

Patients

All children presenting to a paediatric oncology centre with a diagnosis of ALL were eligible for the study. Out of a total of 22 eligible children, one child was excluded because of CNS disease at presentation and two children and their families declined participation in the study. Nineteen children (eight boys, 11 girls) with a median age of 5·9 years (range 2·6–13 years) were recruited.

Design

The children were entered into the national UK trial of ALL – MRCALL97/99 – and randomized to receive Pred (40 mg/m^2, daily) or Dex (6·5 mg/m^2, daily) as induction chemotherapy for 5 weeks (Table 1). The comparative doses of the GC were chosen based on previous lymphocytotoxic data. GC were also administered as 5-day blocks in weeks 9 and 13. Brief details of the chemotherapy regimens are outlined in Fig. 1. Seventeen out of 19 children received either Group A or B chemotherapy, which was determined by their age and white cell count at presentation. The remaining two children received a more intensive regimen of chemotherapy as they failed to remit over the first 4 weeks of induction therapy. Data from the first 4 weeks were analysed up to 5 weeks in these children. The study was approved by the local research ethics committee and informed consent was given by all parents and their children, where appropriate.

Table 1 Details of Patients who were randomised to receive Prednisolone (Pred) or Dexamethasone (Dex) as steroid therapy.

Sex	Pred	Dex
N	12	7
Age (yrs)	5·6	6·3
(range)	(2·6, 12·3)	(4·3, 13)
Prepubertal	11/12	6/7
Grp A, B, C	7, 4, 1	4, 2, 1

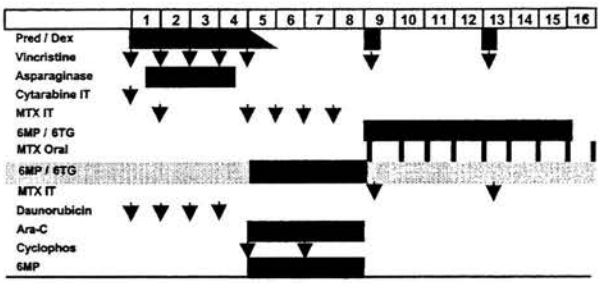

Fig. 1 A flow diagram of chemotherapy schedule over the first 16 weeks of MRC-ALL97/99 in children receiving regimen A or B. Children entering the trial were randomized to receive dexamethasone or prednisolone. Drugs in the stippled area were only administered to Group A, drugs in the shaded area were only administered to Group B and drugs in the open area were administered to both groups. Drug doses and route of administration: prednisolone (Pred), 40 mg/m^2/day oral; dexamethasone (Dex), 6·5 mg/m^2/day oral; vincristine, 1·5 mg/m^2 i.v.; L-Asparaginase, 6000 U/m^2; cytarabine intrathecal (i.t.) age < 2 years, 30 mg; 2 years, 50 mg; > 3 years, 70 mg; methotrexate (MTX) i.t., age < 2 years, 8 mg; 2 years, 10 mg; > 3 years, 12 mg; methotrexate (MTX) oral, 20 mg/m^2 weekly; mercaptopurine (6MP), 75 mg/m^2/day oral; 6-Thioguanine (6TG), 40 mg/m^2/day oral; Daunorubicin, 25 mg/m^2 i.v.; cytarabine (Ara-C), 75 mg/m^2/day i.v.; cyclophosphamide (Cyclophos), 1000 mg/m^2 i.v.

Samples and anthropometric measurements

Collection of blood samples coincided with vascular access for clinical management and were performed every 1–2 weeks at

approximately the same time in late morning (Fig. 1). Lower leg length (LLL) was measured weekly by a portable knemometer using the random zero method (Ahmed *et al.*, 1995). Briefly, the knemometer consists of two main parts, a rigid metal frame holding a footplate, measuring ruler and a moveable chair that moves backwards and forwards on the frame. The LLL is measured while the subject sits on the seat and the right leg is placed on the footplate and the measuring ruler with its platform rests on the top of the knee. The measurer moves the chair back and forth until the maximum length is recorded. The precision of the measurement was assessed by calculating the technical error (TE), i.e. 1SD from the mean of a set of triplicate measurements. The overall mean TE (± 1SD) was 0·15 mm (0·13). Knemometry was performed in 13 (six Pred : seven Dex) out of the 19 children. The remainder of the children had a median age of 3·1 years and were too young to cooperate with the measurements. Body weight with undergarments was measured by an electronic scale.

IGF-I

IGF-I concentrations were measured using a two-site immunoenzymometric (IEMA) assay incorporating a sample pretreatment to inactivate binding proteins (Immunodiagnostic Systems Ltd, Tyne and Wear, UK). The intra-assay and interassay coefficients of variaton were < 5% and < 8%, respectively, over the sample concentration range. The detection of the assay was 10 ng/ml.

Bone markers

All samples were analysed in duplicate and samples from each patient were analysed in a single run to minimize analytical variation. Bone alkaline phosphatase (bALP) was measured in plasma by enzyme-linked immunosorbent assay (ELISA; Alkphase-B, Metra Biosystems Inc, Mountain View, CA, USA). The sensitivity of the assay was 0·7 U/l and within-run and between-run coefficients of variation were < 5% and < 8%, respectively. Deoxypyridinoline cross-links (DPD) were measured in urine by ELISA (Pyrilink-D, Metra Biosystems Inc). Assay sensitivity was 1·1 nmol/l and within-run and between-run coefficients of variation were < 6% and < 11%, respectively. The results were expressed in relation to creatinine measured on the same urine sample.

Statistical analyses

For the knemometry data, lower leg length velocity (LLLV) was calculated for each time point by subtracting the LLL at that time point from that measured at the previous time point, and dividing by the time interval (in weeks) between the two measurements. LLLV was expressed as mm/week. Change in LLL was also expressed as a percentage of the previous LLL. Body weight was expressed as a percentage change in body weight from the pretreatment body weight. Serum IGF-I, bALP and urinary DPD were expressed as absolute values as well as percentage change in IGF-I, bALP and DPD (%IGF-I, %bALP, %DPD). The data were expressed as medians and ranges and analysed using non-parametric tests. Comparison between groups was performed using the Mann–Whitney *U*-testm and Spearman rank correlations were used to compare any association between variables at each time point. Data were analysed using SPSS software v9·0.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 97 SR-2 (Microsoft Corp, Redmond, WA, USA).

Results

Lower leg length velocity

At week 2 of therapy, median LLLV in the Dex group was significantly lower than the LLLV in the Pred group ($P < 0·05$). During GC therapy, LLLV rose temporarily before falling by week 6 when GC therapy ended. In the Dex group, LLLV remained lower at week 8 (med LLLV, −0·3 mm/week, range 0 to −1·3) compared to LLLV in the Pred group at 0·3 mm/week (range 0·2–1·0; $P < 0·05$). By weeks 12 and 16 of the chemotherapy protocol, LLLV was similar in both groups (Fig. 2a). Mean LLLV between weeks 2 and 8 in the Dex and Pred groups were −0·27 mm/week and 0·18 mm/week, respectively. Compared to previous studies of healthy children where the mean LLLV was 0·39 mm/week (1SD, 0·12) (Ahmed *et al.*, 1995), the LLLV in the Dex and Pred groups were 5·7 SD and 1·8 SD below the mean.

Body weight

During GC therapy, body weight showed an increase after week 2 and reached a peak in both groups of children at week 6 (Fig. 2b). Although the increase in weight from baseline was generally greater in the Dex group, the difference did not reach statistical significance until week 6 when the median change in weight from baseline in the Dex group was twice that of the Pred groups at 17·5% (range 5–25) and 8·7% (range −3 to 18), respectively ($P < 0·05$). Children in the Dex group continued to remain at a higher level of weight gain from baseline until week 16 when their weight gain became similar to that of the Pred group (Fig. 2b). The extra weight gain observed in the Dex group in this study was a transient phenomenon.

IGF-I

At presentation, median IGF-I level for the whole group was 83·5 μg/l (range 31·8–293); median IGF-I level in the Pred and Dex groups were 69·3 μg/l (range 33·8–175) and 166 μg/l (range 39–293), respectively ($P = 0·12$). During the study period,

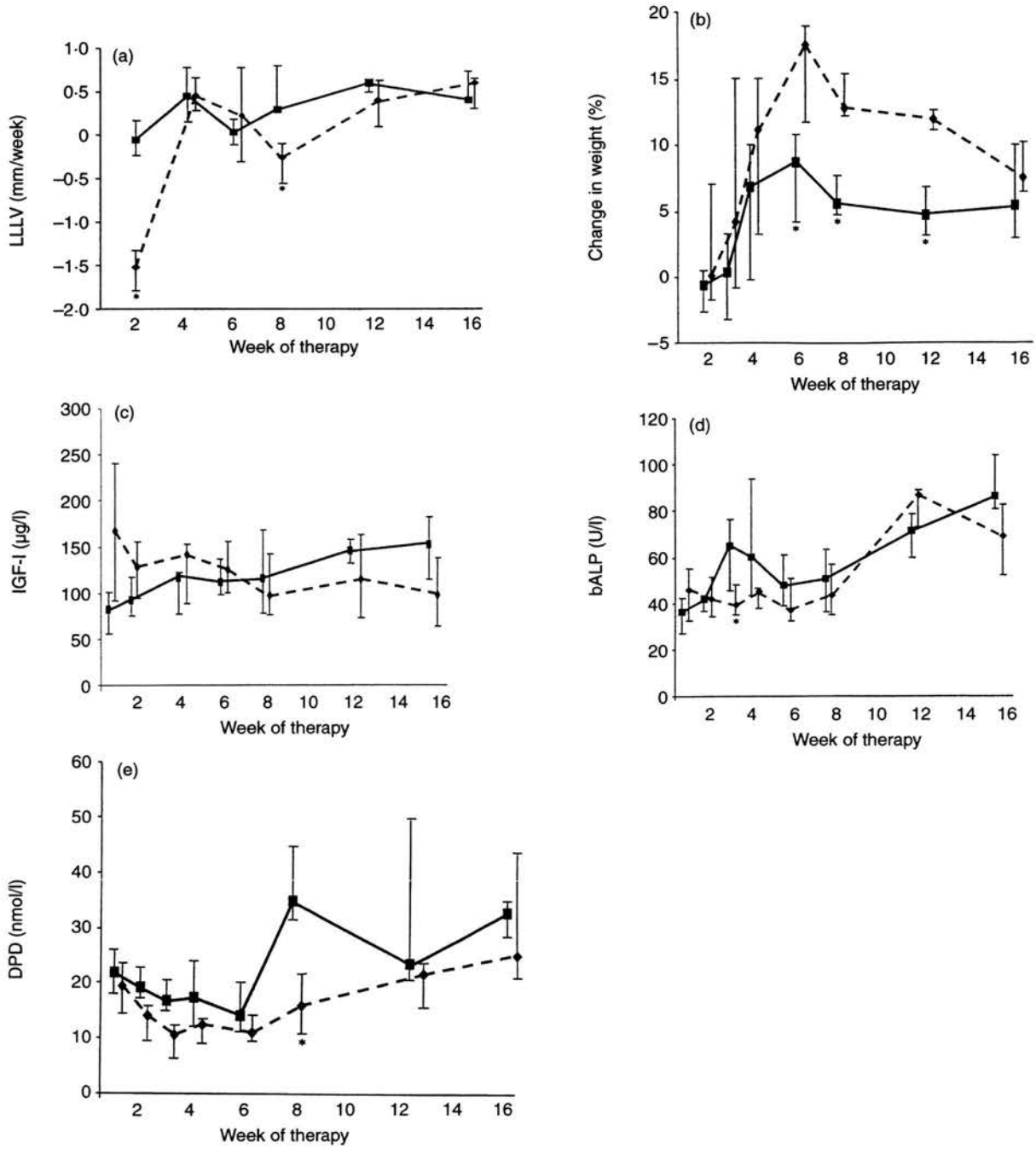

Fig. 2 The effect of prednisolone (■) and dexamethasone (◆) on (a) lower leg length velocity (LLLV, mm/week), (b) percentage change in body weight from baseline, (c) serum IGF-I concentration (IGF-I, µg/l), (d) serum bone ALP concentration (bALP, U/l), and (e) Urinary DPD excretion corrected for creatinine excretion (DPD, nmol/L/creat, nmol/l) over the first 16 weeks of ALL97. Glucocorticoid therapy was administered as induction of remission therapy for 4 weeks at full dose and for an additional week as a tapering dose. Glucocorticoids were also administered as 5-day blocks in weeks 9 and 13. The results are presented as median and 25th and 75th centile values and measurements at any one time point in the two groups are clustered in pairs. $*P < 0{\cdot}05$.

median IGF-I levels remained between 100 and 150 μg/l in the whole group and the absolute values were similar in the Pred and Dex groups. However, in the Dex group, IGF-I levels fell much more markedly during the period of steroid therapy and continued to remain lower than baseline (Fig. 2c) At weeks 4, 6 and 8, median change in IGF-I from baseline was lower in the Dex group than the Pred group at −16% (range −9 to −45) *vs.* 19% (range −50 to 195), −3% (range −58 to 43) *vs.* 56% (range −43 to 146) and −42% (range −74 to 3) *vs.* 44% (range −73 to 415), respectively ($P < 0{\cdot}05$).

Bone alkaline phosphatase

At presentation, median bALP concentration was low but similar in the Pred and Dex groups at 37 U/l (range 17–159) and 46 U/l (range 23–69). From week 1 to week 3, change in bALP, as median %bALP, was 72% (range −8 to 304) in the Pred group, whereas in the Dex group %bALP was −1% (range −28 to 23; $P < 0{\cdot}005$). By week 3 of therapy, median bALP concentration was higher in the Pred group ($P < 0{\cdot}05$) and, by the end of therapy at week 6, median bALP concentration had fallen to a similar level in both groups (Fig. 2d). By weeks 12 and 16, pooled bALP concentrations had risen to a median value of 80 U/l (range 36–123) compared to a median pooled value of 44 U/l (range 17–187) between weeks 1 and 8 ($P < 0{\cdot}005$). At weeks 2, 3, 4, 6 and 8, bALP levels were 1, 1·7, 1·4, 1·3 and 1·2 times lower in the Dex group than the Pred group, respectively. Therefore, on average, bALP was 1·3 times lower in the Dex group than the Pred group.

Deoxypyridinoline

At presentation, median DPD excretion was similar in the Pred and Dex groups at 22 nmol/l (range 17–38) and 20 nmol/l (range 12–26). DPD excretion fell in both groups reaching a nadir between weeks 3 and 6 (Fig. 2c). The %DPD in the Pred and Dex group from week 1 to week 3 was −34% (range −7 to 14) and −53% (range −6 to −69), respectively (ns). By week 8, DPD excretion had started to rise more dramatically in the Pred group, and subsequently DPD excretion continued to rise but there was wide variation during this recovery period (Fig. 2e). At weeks 2, 3, 4, 6 and 8, DPD excretion was 1·4, 1·6, 1·4, 1·2 and 2·2 times lower in the Dex group than the Pred group, respectively. Therefore, on average, DPD was 1·5 times lower in the Dex group than the Pred group.

Discussion

Pred and Dex are two GCs that are commonly used in immunosuppressive therapy. As Dex has a longer half-life, higher lymphocytoxicity and penetrates better into the CSF, it may be better suited for treatment in ALL (Veerman *et al.*, 1990; Kaspers *et al.*, 1996; Gaynon & Carrel, 1999). The improved CNS penetration may explain the recent finding of an increased risk of neurocognitive late effects in those children who received Dex rather than Pred during ALL treatment (Waber *et al.*, 2000). Besides ALL, there may be other conditions where Dex may be a more suitable drug than Pred but a lack of data on relative efficacy as well as adverse effects of the two drugs has hindered an objective choice. By evaluating a number of short-term physical and biochemical changes, this study has attempted to quantify the effects of Pred and Dex in children.

The changes documented in short-term growth and bone turnover in this study were generally similar to our previous study of children undergoing treatment for ALL using Pred during induction of remission (Crofton *et al.*, 1998; Ahmed *et al.*, 1999). By the end of the induction period there was a reduction in short-term growth and suppression of markers of bone turnover.

The current study shows that, at the dose used, Dex had a more profound suppressive effect on bone turnover and short-term growth than Pred. Short-term growth as assessed by knemometry, and bone formation as assessed by bALP levels, fell lower in the Dex group. Similar changes were also observed in urinary DPD excretion, a marker of bone resorption (Calvo *et al.*, 1996). Not only did they fall further, bone formation and resorption remained suppressed for longer in the Dex group. In addition, the rise in bALP seen over the first 2 weeks of Pred was not observed in the group who received Dex. This paradoxical rise in bALP has been attributed to premature maturation of osteoblasts and our data suggest that this effect may be specific to Pred (Stein *et al.*, 1990; Canalis, 1996). The rise and subsequent fall in bALP emphasizes the importance of sequential measurements of bALP during monitoring of GC effects on bone formation. These results showing an increased potency of Dex are in keeping with the recent observation of an increased cumulative incidence of fractures in those children who received Dex compared to Pred during ALL therapy (Strauss *et al.*, 2001).

GC-induced changes in growth may be due to a combination of factors such as a disruption in the GH–IGF-I axis and direct effects on the growth plate (Robson, 1999; Mushtaq *et al.*, 2001). High-dose GC therapy alters pulsatility of GH secretion through an elevation of somatostatin tone and may alter GH binding protein activity (Gabrielsson *et al.*, 1995; Tonshoff *et al.*, 1996). Our previous studies have suggested that a state of GH resistance may exist in children undergoing ALL treatment (Crofton *et al.*, 1998) and the current study shows that Dex is more potent at depressing IGF-I levels than Pred. Absolute IGF-I levels are age-dependent and may be easier to assess following standardization for age as standard deviation scores. However, this standardization was not employed in this study as the age range of children in the two randomized groups was similar and the aim of the study was to assess the change in IGF-I levels over a short period of a few weeks.

GC promotes food consumption both directly and through stimulation of NPY and inhibition of CRH release (Tempel & Leibovitz, 1994; Tataranni *et al.*, 1996). Short-term changes in energy intake secondary to GC administration during maintenance treatment in children with ALL have recently been reported by Reilly *et al.* (2001). This group did not show any significant differences in energy intake or weight gain between Dex and Pred and this may have been due to the short, 5-day period of steroid administration. Our study shows that the changes in weight gain were more marked in those children who were randomized to receive Dex for a longer period of 4 weeks.

Previous studies of short-term growth by knemometry have shown that changes in body weight may independently influence LLL due to the action of gravity on the soft tissues of the lower leg (Hermanussen *et al.*, 1988; Ahmed *et al.*, 1996). Previous studies in a pregnant adult subject showed that the increase in body weight was associated with a reduction in LLL until the subject developed dependent oedema, at which point LLL started to increase (Ahmed *et al.*, 1996). Although changes in general body weight may explain the negative growth or actual shrinkage that was observed in some children, weight changes cannot solely account for the observed LLLV differences between the two groups as some of the major differences in LLLV between the Pred and Dex groups preceded the major differences in body weight in the current study. Some of the early shrinkage may be explained by recent ultrasound studies that show that systemic steroid therapy may have an early water-depleting effect on connective tissues and may lead to a reduction in the subcutis thickness (Schou *et al.*, 2001). This water-depleting affect may similarly effect the growth plate, which is already known to be directly susceptible to the effect of corticosteroids by a variety of mechanisms (Mushtaq & Ahmed, 2002).

The dose of Pred in milligrams was approximately six times that used of Dex and our studies show that LLLV was about three times lower in the Dex group over the period of treatment and the subsequent few weeks. We therefore estimate that, between weeks 1 and 8 of the 5-week period of GC therapy, Dex was 18 times more potent at suppressing short-term growth. Similar calculations estimate that Dex was 19 times more potent at raising body weight and about 8–9 times more potent at suppressing bone turnover as assessed by serum bALP concentration and urinary DPD excretion. After 8 weeks all the above parameters, except change in body weight, were similar in the two groups.

In summary, this study has attempted to quantify the relative effect of the two corticosteroids, Pred and Dex, on short-term growth and bone turnover. Compared to Pred, Dex may be almost 20 times more potent at suppressing short-term growth and at raising body weight and almost 10 times more potent at suppressing bone turnover. GCs have a variable effect on different parameters of growth and bone turnover and the intensity may depend on the steroid used.

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Endocrinology

The impact of corticosteroids on growth and bone health

T Mushtaq, S F Ahmed

An examination of current evidence

Glucocorticoids (GC) are important regulators of diverse physiological systems and are often used in the treatment of a number of chronic inflammatory, autoimmune, and neoplastic diseases. It is estimated that 10% of children may require some form of GC at some point in their childhood.[1] Impairment of childhood growth with an approximate cortisone dose of 1.5 mg/kg/day was first described over 40 years ago; osteopenia in children receiving a prednisolone dose of less than 0.16 mg/kg/day has also been reported.[2,3] The maintenance of growth and bone health is a complex process that can be influenced not only by drugs, but also by the nutritional status of the patient and the underlying disease process. The purpose of this review is to examine the current evidence for linking GC to adverse growth and bone health in childhood disorders that commonly require GC therapy.

PATHOPHYSIOLOGY

Loss of bone and deterioration in short term growth are dependent on the type and dose of GC and occur most prominently over the first six months of treatment.[4-6] Although it is generally believed that GC affect trabecular bone more than cortical bone, a recent study of fractures in children following steroid exposure as part of acute lymphoblastic leukaemia (ALL) treatment showed a high incidence of cortical bone involvement, suggesting that the disease process may interact with GC usage in influencing site of bone loss.[7,8]

GC have a suppressive effect on osteoblastogenesis in the bone marrow and promote the apoptosis of osteoblasts and osteocytes, thus leading to decreased bone formation.[9] Accumulation of apoptotic osteocytes may also explain the so called "osteonecrosis", also known as aseptic or avascular necrosis. There is some evidence to suggest that GC may also increase bone resorption by extending the lifespan of pre-existing osteoclasts.[10] GC may also promote calcium loss through the kidneys and gut, and this negative calcium balance can itself lead to increased bone remodelling and osteoclastic activity due to secondary hyperparathyroidism.[11]

High dose GC therapy can attenuate physiological growth hormone (GH) secretion via an increase in somatostatin tone, and the GH response to GH stimulation tests may be reversibly impaired in some cases of steroid exposure.[12,13] However, GC induced growth failure may also be due to direct effects on the growth plate. Infusion of GC into the growth plate leads to a temporary reduction in the growth rate of that leg and may disrupt the growth plate vasculature.[14,15] GC exposed chondrocytes show reduced proliferation rates and a reversible, prolonged resting period. In vitro studies suggest that local somatotrophic action of GH and IGF-1 may be affected by a number of different mechanisms, including alterations in the activity of the GH binding protein, down regulation of GH receptor expression and binding capacity, and a reduction in local IGF-1 production and activity.[16-19]

GC may also impair the attainment of peak bone mass and delay growth through alterations in gonadal function at the level of the pituitary and through direct effects on the gonads. Studies in adults show that GC therapy may be associated with testosterone deficiency as well as reversible gonadotrophin deficiency.[20,21] Levels of other sex steroids such as androstenedione and oestrogen may also be depressed due to adrenal inactivity following chronic GC therapy.[22] In addition, there is in vitro evidence suggesting that GC impair FSH action, thus reducing oestrogen secretion.[23]

Figure 1 summarises the mechanisms of GC induced bone loss and growth retardation.

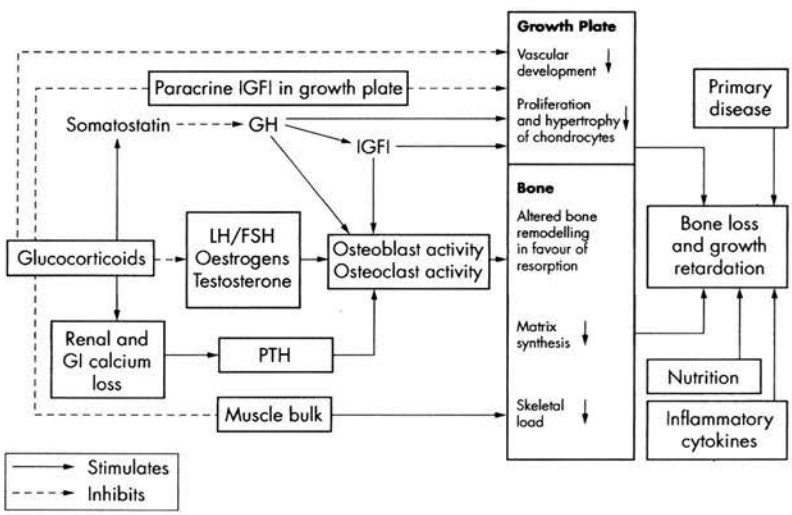

Figure 1 Mechanisms of GC induced bone loss and growth retardation.

ASTHMA, ECZEMA, AND HAY FEVER

The increasing incidence and prevalence of childhood atopy and the more widespread use of inhaled steroid therapy for asthma prophylaxis probably accounts for the largest group of children who are chronically exposed to steroids. Oral GC therapy in asthma is associated with a delay in growth and puberty, and there is some evidence to suggest that final height may also be compromised.[24] Systemic exposure to inhaled steroids may be higher with metered dose inhalers and dry powder devices where 80% of the drug is deposited in the oropharynx. Although earlier studies did not show a relation between inhaled steroids and growth, there is now good evidence in children with relatively mild asthma that inhaled steroids can temporarily slow growth and alter bone and collagen turnover. The magnitude of this effect may be influenced by the dose delivery system as well as the systemic bioavailability of the inhaled steroid used.[25] This effect may be most pronounced over the first few weeks of treatment.[26] Long term studies are difficult due to a number of

Abbreviations: ALL, acute lymphoblastic leukaemia; DEXA, dual energy x ray absorptiometry; GC, glucocorticoid; GH, growth hormone; JIA, juvenile idiopathic arthritis

confounding factors including the plethora of drugs, delivery systems, compliance, and disease severity, but there is no clear evidence that final height is compromised following inhaled GC therapy in children with asthma. Studies of bone mineral density in children with asthma have not shown any significant abnormality but have only concentrated on those children who are on relatively low doses of inhaled steroids.[27]

Some intranasal GC such as budesonide have a very high level of systemic absorption when applied directly to the nasal mucosa; short and intermediate term studies of children on intranasal steroids such as budesonide and beclomethasone also show a deterioration in growth velocity.[28] Like asthma, there is some suggestion that the effects of these intranasal steroids may be dose dependent and that the newer forms of intranasal steroids such as mometasone furoate may not have these adverse growth effects.[29] Assessment of short term growth during topical steroid treatment for eczema has also been studied, but the results have been inconclusive.[30]

INFLAMMATORY BOWEL DISEASE

Longitudinal studies show that the growth velocities of children in the year preceding diagnosis are reduced; growth retardation frequently complicates the clinical course in children.[31] There is an increased recognition of osteoporosis in adult patients with chronic inflammatory bowel disease. In children with inflammatory bowel disease, retardation of growth and skeletal maturation are widely reported and may be related to disease activity as well as to its treatment.[32] Vertebral fractures have also been described in children with Crohn's disease with a short or absent history of steroid usage.[33 34] A cross sectional study of bone mineralisation using dual energy *x* ray absorptiometry (DEXA) showed evidence of osteopenia even when corrected for sex, height, weight, and puberty.[35] In this study, the bone status was related to steroid usage but had no relation to disease activity. In a longitudinal study of 55 children, uncorrected total body bone mineral density standard deviation score correlated negatively to cumulative steroid dosage and positively to body mass index.[36] A reduction in bone mineral density of the lumbar spine, femoral neck, and radius may be more prominent in children with Crohn's disease and those children who are of a pubertal or post-pubertal age. The recent introduction of budesonide enemas for treatment of distal colitis has also been reported to be associated with suppression of markers of bone formation.[37]

RENAL DISEASE

Impaired linear growth is one of the major complications of childhood onset chronic renal insufficiency and its treatment. Final height may be less than the third centile in 50% of children who enter end stage renal failure in childhood. It is unclear whether such children who are on appropriate vitamin D supplements have a poorer bone mineralisation status. Children with a history of renal insufficiency who receive GC may grow more slowly, have a poorer bone mineralisation status, and may not respond satisfactorily to vitamin D replacement compared to those who do not receive GC.[38] The prolonged use of GC is also associated with growth failure and reduced bone mineral density in other childhood chronic renal disease, such as nephrotic syndrome.[39] Post-transplantation, the cumulative GC dosage may be inversely related to the change in relative height, but this finding is not universal.[40] Interindividual differences in the handling of GC as assessed by area under the curve estimation rather than dose have shown a stronger association with adverse growth in post-transplantation patients. In nephrotic syndrome, it is not clear whether intermittent GC therapy over a number of years has an adverse effect on growth correlates with final height.[41] Following transplantation, a given cumulative dose of GC has a lower inhibitory effect on growth velocity, without compromising graft function, when given on alternate days.[42] Although alternate day GC may not adversely affect final height potential, it may still delay puberty and be associated with a delayed growth spurt.[43] Substitution of GC with other immunosuppressants improves growth but leads to a higher rate of rejection. The effect of these newer agents on growth and bone health while reducing the need of GC are unclear. Alternative forms of GC, such as deflazacort, an oxazoline derivative of prednisolone, appear to have fewer effects on growth and corticosteroid induced osteoporosis, but are not used in common practice.[44]

ARTHRITIS

GC are widely used for treating chronic connective tissue diseases in children; as with other inflammatory conditions, there is considerable overlap between the inflammatory process and steroid induced effects on bone health. A failure to develop adequate bone mineralisation is virtually universal in children with juvenile idiopathic arthritis (JIA) and is characterised by a failure of bone formation, with a subsequent failure to undergo the normal increase in bone mass during puberty.[45] The reduction in bone mineral density and bone mineral gain may be adversely affected if the child was on steroids.[46] Other studies have not shown any statistical significance of the cumulative dose of corticosteroids on growth, although they did note a reduction in the growth velocity during the first year of treatment which was more apparent in the polyarticular group.[47] It does appear that good control of disease activity in systemic onset JIA can be achieved by high dose alternate day prednisolone with minimal side effects.[48] The use of high doses of oral steroids in autoimmune conditions is associated with a prompt decrease of bone formation and collagen synthesis, without any significant changes in bone resorption; however, these negative effects seem to revert after lowering GC dosage.[49]

ACUTE LYMPHOBLASTIC LEUKAEMIA

GC have been a mainstay of the therapy for childhood ALL. Dexamethasone is now replacing prednisolone as the drug of choice because of its greater lymphocytotoxicity and higher CNS penetration. Recent studies have shown that bone mineralisation status as assessed by bone mineral density, corrected as well as uncorrected for body size, may be adversely affected immediately after completion of treatment.[50 51] The fracture incidence during leukaemia treatment has been reported to be as high as 39%; this has been recently confirmed by Strauss *et al* who have shown a five year cumulative incidence of 28% for fractures and 7% for osteonecrosis, with a median follow up of over seven years.[8 50 52] The median time from diagnosis to first fracture was 15 months and most occurred within two years of diagnosis. Older age, pubertal age, the male sex, and dexamethasone have been shown to be independent risk factors for fractures and reduced bone mineralisation.[8] Previous studies by our group have shown alterations in bone turnover and short term growth of children during leukaemia treatment; these changes were most marked during periods of intensive chemotherapy and high dose systemic glucocorticoid administration.[4 5 53] More recently, we have also reported that the biological potency of dexamethasone in suppressing growth may be 18 times higher than that of prednisolone.[6]

MANAGEMENT

A number of childhood conditions that require chronic GC therapy may themselves predispose the child to abnormalities of growth and bone health. For instance, poor linear growth and osteoporosis may be presenting features in a child with inflammatory bowel disease. The interaction with other factors such as inflammatory cytokine production, diminished physical activity, alterations in nutritional status, and the use of other

immunomodulatory agents that may also have bone adverse effects greatly increase the risks posed to the growth and bone health of the child on chronic GC therapy.

The clinical effects on bone health can be divided into those occurring in the short term (fractures and avascular necrosis) and those that may occur over the longer term—that is, increased predisposition for osteoporosis and skeletal deformity. Current studies and clinical observations suggest that children who require long term systemic GC therapy (for more than three months) have a higher incidence of fractures during therapy. For adults, the Royal College of Physicians has issued guidelines on indications for assessing and managing bone health.[54] In the absence of any clear guidelines for children, it would seem prudent to monitor susceptible children carefully with regular review of bone symptomatology, GC dosage, nutrition (including calcium and vitamin D status), anthropometry (including sitting height), pubertal status, and assessment of bone mineralisation status. Bone mineral status can be assessed by a number of methods and DEXA is by far the most popular method. Unlike adults where a single assessment of bone mineral density by DEXA can predict likelihood of fracture in age related osteoporosis, this relation is not so clear in children with GC induced osteoporosis. Children at risk of GC induced osteoporosis and those displaying growth failure should, therefore have serial bone mineral density assessment to assess a change in status; results need to be carefully interpreted in relation to their sex, age, height, and weight, as well as their disease and its treatment. Current studies of long term follow up of children treated with chemotherapy only regimens for ALL do not show disturbances in final height or bone mineral status, and there are no data to support or refute the claim that prolonged GC therapy in childhood may lead to early osteoporosis in adulthood.[55–57]

Skeletal disproportion has been reported as a possible long term effect of ALL chemotherapy; it is not clear whether this phenomenon of skeletal disproportion is observed in other groups of children requiring chronic GC therapy.[58] In the absence of any convincing evidence for or against long term osteopenia, it would, again, seem prudent to consider assessing growth and bone mineral status in all patients with a past history of prolonged GC exposure when they reach the end of their second decade and should have acquired peak bone mass. Failure to acquire peak bone mass should prompt longer term monitoring.

Prevention of GC induced growth retardation and adverse bone health could be addressed in a number of cases by judicious use of GC therapy coupled with improved nutrition and promotion of weight bearing activities. In addition, alternate day GC regimens and consideration of GC sparing drugs at an earlier stage than before may be possible preventive measures but need further evaluation. For reasons mentioned earlier, calcium and vitamin D supplementation is generally recommended in patients on GC therapy, although there is little objective clinical evidence to suggest that this practice prevents GC induced osteoporosis in adults or children. As hypogonadism may contribute both to poor growth and impaired bone mineral accretion, addressing hypogonadism in pubertal children on GC therapy should be an important consideration. Recent studies show that recombinant GH treatment may be of benefit in halting the growth retardation and bone loss observed in children on chronic GC therapy.[59 60]

In children, the role of the antiresorptive group of drugs, bisphosphonates has been mostly studied in the field of osteogenesis imperfecta where their use is associated with a reduction in the frequency of fractures, improved bone mass, and mobility.[61] Bisphosphonate therapy is now used regularly in adults for prevention and treatment of glucocorticoid induced osteoporosis and needs to be carefully evaluated in the paediatric setting.[62] Acute vertebral fractures can be a debilitating condition that may be associated with a prolonged period of immobility during which the patient may become increasingly susceptible to further fractures. An early resumption of aerobic as well as weight bearing activity, with good analgesic control will require the support of a child oriented physiotherapy and pain relief service.

GC are an effective and necessary form of therapy for a large number of children. In some children, their use may be associated with adverse effects; effective management of GC induced growth retardation and bone health will require improved awareness and better access for monitoring and managing these children, as well as an improved understanding of the contributory factors.

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Glucocorticoid effects on chondrogenesis, differentiation and apoptosis in the murine ATDC5 chondrocyte cell line

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Abstract

Glucocorticoids (GC) are used extensively in children and may cause growth retardation, which is in part due to the direct effects of GC on the growth plate. We characterised the ATDC5 chondrocyte cell line, which mimics the *in vivo* process of longitudinal bone growth, to examine the effects of dexamethasone (Dex) and prednisolone (Pred) during two key time points in the chondrocyte life cycle - chondrogenesis and terminal differentiation. Additionally, we studied the potential for recovery following Dex exposure. During chondrogenesis, Dex and Pred exposure at 10^{-8} M, 10^{-7} M and 10^{-6} M resulted in a significant mean reduction in cell number (28% vs 20%), cell proliferation (27% vs 24%) and proteoglycan synthesis (47% vs 43%) and increased alkaline phosphatase (ALP) activity (106% vs 62%), whereas the incidence of apoptosis was unaltered. Minimal effects were noted during terminal differentiation with both GC although all concentrations of Dex lowered apoptotic cell number. To assess catch-up growth the cells were incubated for a total of 14 days which included 1, 3, 7, 10 or 14 days exposure to 10^{-6} M Dex, prior to the recovery period. Recovery of proteoglycan synthesis was irreversibly impaired following just one day exposure to Dex. Although cell number showed a similar pattern, significant impairment was only achieved following 14 days exposure. Irreversible changes in ALP activity were only noticed following 10 days exposure to Dex.

In conclusion, GC have maximal effects during chondrogenesis; Dex is more potent than Pred and cells exposed to Dex recover but this may be restricted due to differential effects of GC on specific chondrocyte phenotypes.

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Introduction

Glucocorticoids (GC) are commonly used as anti-inflammatory therapy and in immunosuppressive regimens and it is estimated that 5–10% of children may require some form of GC therapy at some time in childhood (Warner 1995). The functional effects of steroids on target tissues are difficult to predict and their use is hampered in some individuals more than others because of side-effects such as growth retardation and osteoporosis. Impairment of childhood growth with long-term GC was described almost 50 years ago but more recent studies have shown that altered growth and bone turnover also occur during relatively short periods of GC therapy and that these effects may vary depending on the type of corticosteroid used (Blodget *et al.* 1956, Crofton *et al.* 1998, Ahmed *et al.* 1999, 2002).

At the level of the growth plate, local and systemic factors regulate longitudinal bone growth which involves the differentiation of committed stem cells into proliferating chondrocytes; after a finite number of cell divisions these cells terminally differentiate into the hypertrophic phenotype that deposit a matrix which is mineralised and eventually replaced by bone (Green *et al.* 1985, Isaksson *et al.* 1991). GC-induced growth failure may be due to a combination of factors such as a disruption of the growth hormone–insulin-like growth factor-I (GH–IGF-I) axis, a defect in sex steroid action, a disturbance in calcium and phosphate homeostasis as well as direct effects on the growth plate (Crilly *et al.* 1978, Unterman & Phillips 1985, Baron *et al.* 1992, Jux *et al.* 1998).

Studies in children suggest that growth retardation following a short period of systemic exposure to GC may be followed by a period of catch-up growth and that alternate day therapy may be less adverse for growth (Jabs *et al.* 1996, Ahmed *et al.* 1999). Catch-up growth has also been observed following direct injection of GC into the growth plate of rabbits (Baron *et al.* 1994).

The direct effects of GC on chondrocytes are not clearly understood and previous studies that have examined the effect of GC on primary growth plate chondrocytes have

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Table 1 Primer pairs used for specific gene analysis

Gene	Primer sequence	Cycles	Product size (bp)
18S	Unknown, purchased commercially from Ambion	15	488
Collagen type II	TTAGAAAGGGGAGCACAGTCC TACACTGCCATGAAGCATGG	35	323
Collagen type X	CAGAGGAAGCCAGGAAAGC GGTGTCCAGGACTTCCATAGC	32	330

Ambion, Huntingdon, Cambs, UK.

been unable to examine the effect of GC on the different stages of the chondrocyte life cycle due to the heterogeneous mixture of maturational phenotypes (Robson *et al.* 1998, Koedam *et al.* 2000). Recently, the murine ATDC5 chondrocyte cell line has been shown to undergo the temporal sequence of events that occur during longitudinal bone growth *in vivo* and thereby provide a good model to study the molecular mechanisms underlying regulation of endochondral bone formation (Atsumi *et al.* 1990, Shukunami *et al.* 1997). In this study, this cell line was used to explore the effects of two commonly used glucocorticoids, dexamethasone (Dex) and prednisolone (Pred), on cell number, proliferation, differentiation and apoptosis at key maturational time points (chondrogenesis and terminal differentiation), within the chondrocyte life cycle. The effect of the GC on the ability of chondrocytes to recover following GC exposure was also studied to assess the potential for catch-up growth.

Materials and Methods

Chondrocyte cell culture

The ATDC5 chondrocyte line was obtained from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al.* (1990). Cells were cultured at a density of 12 000 cells per cm^2 in multi-well plates (Costar, High Wycombe, Bucks, UK) in a maintenance medium of DMEM/Ham's F12 (Invitrogen, Paisley, Strathclyde, UK) supplemented with 5% FCS (Invitrogen), 10 μg/ml human transferrin and 3×10^{-8} M sodium selenite (Sigma, Poole, Dorset, UK) until confluent (day 6). Thereafter, differentiation was induced by the addition of insulin (10 μg/ml; Sigma) and ascorbic acid (20 μg/ml) to the maintenance medium (differentiation medium). Incubation was at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and the medium was changed every second day.

Gene expression

For the determination of chondrocyte phenotype, cells were grown for up to 20 days as above and RNA was extracted, reverse transcribed and analysed for collagen type II and collagen type X expression at days 6, 8, 10, 13, 15, 17 and 20 by semi-quantitative RT-PCR.

RNA extraction

Total RNA was extracted from chondrocytes by repeated aspiration through a 25-gauge syringe needle in 1·5 ml Ultraspec (Biotecx, Houston, TX, USA). After extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack resin (Biotecx) following the manufacturer's protocol. After washing with 75% ethanol, the RNA was eluted in 100 μl ribonuclease-free water (Houston *et al.* 1999). In each case the 260/280 ratio was 1·9–2·0, confirming the purity of the RNA. All preparations were diluted to a concentration of 50 ng/μl and stored at −70 °C.

Semiquantative RT-PCR

Gene expression was analysed by semiquantitative RT-PCR (Farquharson *et al.* 1999, Houston *et al.* 1999, Jefferies *et al.* 2000). Aliquots of 500 ng RNA (or an equivalent volume of water as a control) were reverse transcribed in 20-μl reactions with 200 ng random hexamers and 200 U Superscript II reverse transcriptase using the Superscript preamplification protocol (Invitrogen). PCR was performed in 20-μl reactions containing cDNA equivalent to 10 ng RNA and 200 nM gene-specific primers in 11·1 × PCR buffer (Jefferies *et al.* 1998) (Table 1). The cycling profile was 1 min at 92 °C (first cycle, 2 min), 1 min at 55 °C, and 1 min at 70 °C. The number of cycles performed was carefully titrated to ensure that the reactions were in the exponential phase. Reaction products were analysed on 1·5% agarose gels in the presence of ethidium bromide (250 μg/l), and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories, Inc., Hemel Hempstead, Herts, UK).

Chondrocyte number, proliferation, differentiation and matrix production

Dex and Pred (Sigma) were added to the cells at a final concentration of 10^{-8} M, 10^{-7} M and 10^{-6} M, in 0·01% ethanol and compared with control cultures which

contained 0·01% ethanol only. Collagen type II and collagen type X expression was first noted at 10 and 15 days respectively. The GC were added from day 6 or day 11 for the 4 days leading up to the expression of these two chondrocyte phenotypic markers.

Cell layers were rinsed with phosphate buffered saline (PBS) and lysed with 0·9% NaCl and 0·2% Triton X-100 and centrifuged at 12 000 *g* for 15 min at 4 °C. The supernatant was assayed for protein content and alkaline phosphatase (ALP) activity as a measure of cell number and chondrocyte differentiation respectively. The protein content of the supernatant was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories) based on the Bradford dye binding procedure, and gamma globulin was used as standard (Farquharson *et al.* 1995). Enzyme activity was determined by measuring the cleavage of 10 mM p-nitrophenyl phosphate (pNPP) at 410 nm. Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein (Farquharson *et al.* 1999). The rate of chondrocyte proliferation was assessed by incubating the chondrocytes with 0·2 μCi/ml [^{3}H]thymidine (37 MBq/ml; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) for the last 18 h of the culture period and the amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates measured (Farquharson *et al.* 1999). Proteoglycan synthesis was evaluated by staining with Alcian Blue as previously described (Shukunami *et al.* 1997). In brief, cells were washed twice with PBS, fixed in 95% methanol for 20 min and stained with 1% Alcian Blue 8 GX (Sigma) in 0·1 M HCl overnight and rinsed with distilled water. Alcian Blue-stained cultures were extracted with 1 ml 6 M guanidine-HCl for 6 h at room temperature and the optical density (O.D.) was measured at 630 nm using a Jenway 6105 spectrophotometer.

Apoptosis

Apoptosis of the cells was measured by two complementary methods: (1) APOPercentage Apoptosis assay, (Biocolor Ltd, Belfast, N Ireland), which quantifies dye uptake by apoptotic cells only after the translocation of phosphatidylserine to the outer surface of the cell membrane (Fadok *et al.* 1992) and (2) Nucleosome ELISA kit (Oncogene Research Products, Nottingham, Notts, UK), which allows the quantification of apoptotic cells *in vitro* by DNA affinity-mediated capture of free nucleosomes followed by their anti-histone-facilitated detection. Both kits were used according to the manufacturers' instructions. Dex and Pred at concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M were added to the cell cultures on day 6 or day 13 for a period of 24 h. As a positive control, cells were incubated as above with 5% ethanol.

Recovery following GC exposure

For these experiments, a single concentration of Dex at 10^{-6} M was used as it was noted to have the most potent effects in the above experiments. This dose was added to all cells at confluency (day 6) and subsequently replaced with differentiation medium without Dex after 1, 3, 7 and 10 days. All cultures were maintained for a total of 14 days along with a group that was exposed to Dex for the whole 14 days duration (no recovery period). Additional culture plates of Dex (10^{-6} M)-treated cells and their respective controls (containing 0·01% ethanol) were stopped at the allocated time points (days 1, 3, 7 and 10) to assess the impact of Dex prior to the period of recovery. Cell number, ALP activity and proteoglycan content were determined as described above.

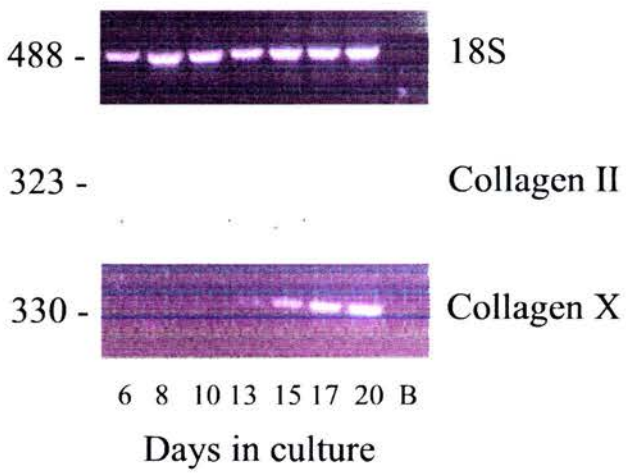

Figure 1 Semiquantative RT-PCR analysis of the expression of chondrocyte marker genes. Collagen type II is expressed from day 10 and collagen type X from day 15. B, blank.

Statistical analysis

All experiments were performed at least twice. Data were analysed by one way analysis of variance. All data are expressed as the mean ± S.E.M. of four observations within each experiment and statistical analysis was performed using Statview (SAS Institute Inc., Cary, NC, USA; version 5.0.1). $P<0·05$ was considered to be significant.

Results

Temporal expression of chondrocyte phenotype specific markers

Using gene-specific primers, collagen type II expression by the ATDC5 cells was first noted after 10 days in culture indicating that the differentiation of mesenchymal cells to the chondrocyte phenotype (chondrogenesis) had occurred. Similarly, collagen type X expression was noted from day 15 onwards indicating that terminal differentiation of the chondrocytes occurred from day 10 to day 15 (Fig. 1). This information was used to study the effects of

Table 2 Effect of Dex and Pred on cellular protein, proteoglycans and alkaline phosphatase activity during chondrogenesis and terminal differentiation. All data are expressed as means ± S.E.M.

		10^{-8} M		10^{-7} M		10^{-6} M	
	Control	Dex	Pred	Dex	Pred	Dex	Pred
Chondrogenesis							
Cellular protein (mg)	0·66 ± 0·01	0·54 ± 0·05*	0·59 ± 0·04	0·44 ± 0·01*	0·52 ± 0·01*	0·45 ± 0·03*	0·46 ± 0·02*
Proteoglycan (O.D.)	0·26 ± 0·02	0·15 ± 0·01*	0·17 ± 0·005*	0·13 ± 0·003*†	0·15 ± 0·01*	0·13 ± 0·003*	0·12 ± 0·002*
ALP (nmoles pNPP hydrol/min/mg protein)	331 ± 8	660 ± 32*†	440 ± 15*	723 ± 27*†	585 ± 10*	716 ± 36*†	586 ± 19*
Terminal Differentiation							
Cellular protein (mg)	0·92 ± 0·01	0·91 ± 0·02	0·85 ± 0·05	0·84 ± 0·03	0·79 ± 0·03*	0·92 ± 0·02†	0·82 ± 0·04*
Proteoglycan (O.D.)	0·82 ± 0·07	0·89 ± 0·05	0·82 ± 0·03	0·7 ± 0·02	0·77 ± 0·04	0·73 ± 0·05	0·72 ± 0·04
ALP (nmoles pNPP hydrol/min/mg protein)	262 ± 11	265 ± 17	244 ± 34	292 ± 21	270 ± 9	245 ± 10	253 ± 23

*P<0·05 compared with control cultures; †P<0·05 Dex cultures compared with equivalent dose of Pred.

Dex and Pred during the periods leading up to the expression of these maturation markers.

Effects of GC on cell number and proliferation

In comparison with control cultures the addition of Dex and Pred to cells during the chondrogenic period (days 6–10) caused a significant reduction in cell number as indicated by cell protein data (Table 2). The reduction in cell number from control values for the Dex concentrations tested were: 10^{-8} M, 18·2%; 10^{-7} M, 33·3%; and 10^{-6} M, 31·8% (P<0·05). The apparent plateau noted at 10^{-7} M for Dex was not seen with Pred, where a dose-dependent reduction was observed over the three concentrations tested: 10^{-8} M, 10·6% (not significant); 10^{-7} M, 21·2% (P<0·05); and 10^{-6} M, 30·3% (P<0·05). The mean reduction in cell number over the three concentrations was 28% with Dex and 20% with Pred.

The effect of Dex and Pred on [^{3}H]thymidine uptake during the chondrogenesis period is shown in Fig. 2. Both GC caused a significant concentration-dependent decrease in cell proliferation from control values – Dex: 10^{-8} M, 11·7%; 10^{-7} M, 33·8%; and 10^{-6} M, 36·6%; Pred: 10^{-8} M, 9·6%; 10^{-7} M, 24·7%; and 10^{-6} M, 37% (P<0·05). As was noted for cell number, the apparent plateau noted at 10^{-7} M for Dex was not seen with Pred, where a dose-dependent decrease was observed over the three concentrations tested. The mean reduction over the three concentrations for Dex and Pred was 27% and 24% respectively. Dex at 10^{-7} M was significantly more antiproliferative than Pred at 10^{-7} M (P<0·05).

During the terminal differentiation phase (days 10–15) Dex did not significantly alter cell numbers when compared with control values (Table 2), whereas Pred caused a significant reduction (P<0·05) at both 10^{-7} M (14·1%) and 10^{-6} M (10·9%). The cell proliferation rate in control cultures was sixfold less during the terminal differentiation phase than during the chondrogenic stage and the addition of GC led to a significant suppression of proliferation with Dex at 10^{-8} M (40·9%), 10^{-7} M (24·1%) and 10^{-6} M (40·3%) whereas a reduction in proliferation by Pred was noted at 10^{-8} M (26·3%), with a rise in proliferation at 10^{-6} M (21·6%, P<0·05) (Fig. 2).

Effects of GC on proteoglycan production

In comparison with control cultures during the chondrogenesis period, there was a concentration-dependent reduction in proteoglycan synthesis ranging from 42 to 50% with Dex and 35 to 54% with Pred (Table 2). An apparent plateau was noted at 10^{-7} M for Dex which was not seen with Pred, where a dose-dependent reduction was observed over the three concentrations tested.

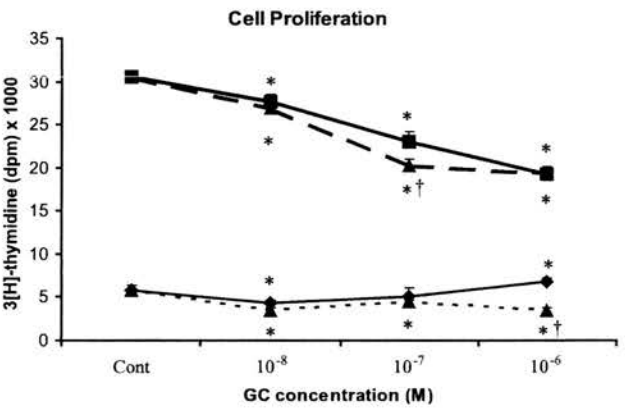

Figure 2 Effect of Dex and Pred on cell proliferation as assessed by [^{3}H]thymidine uptake during chondrogenesis and terminal differentiation phases. Effect of Dex on chondrogenesis (▲ and long dashed lines); effect of Dex on terminal differentiation (▲ and short dashed lines); effect of Pred on chondrogenesis (■ and solid line); effect of Pred on terminal differentiation (◆ and solid line). All data are expressed as means ± S.E.M. *P<0·05 compared with control (Cont); †P<0·05 significance level between Dex and equivalent dose of Pred.

Comparing Dex and Pred at equivalent concentrations, Dex at 10^{-7} M caused a significantly greater fall in proteoglycans than Pred at 10^{-7} M ($P<0{\cdot}05$) (Table 2). Over the three concentrations, Dex caused a mean reduction in proteoglycan synthesis of 47% compared with 43% with Pred. No significant differences were noted during terminal differentiation.

Effect of GC on chondrocyte differentiation

The effect of GC on terminal chondrocyte differentiation as assessed by ALP activity is shown in Table 2. During chondrogenesis, enzyme activity in comparison with control values was significantly increased with both Dex: 10^{-8} M, 83%; 10^{-7} M, 118%; and 10^{-6} M, 116% and Pred: 10^{-8} M, 39%; 10^{-7} M, 77%; and 10^{-6} M, 77% ($P<0{\cdot}05$). The mean elevations in ALP with all concentrations of Dex and Pred were 106% and 62% respectively and at equimolar concentrations of GC, Dex caused significantly larger increases in ALP than Pred. No significant differences in ALP activity were noted during the terminal differentiation phase.

Effects on apoptosis

Using the APOPercentage Apoptosis assay the number of apoptotic cells was higher in the terminally differentiating chondrocytes in comparison with cultures in the chondrogenesis phase. No evidence was detected for an effect of Dex and Pred on apoptosis during the chondrogenesis phase (Fig. 3a), however during terminal differentiation all Dex concentrations, and Pred at 10^{-6} M caused a significant decrease in apoptotic cell numbers ($P<0{\cdot}05$) (Fig. 3b). Ethanol acted as a positive control and caused an elevation in apoptosis at both developmental phases ($P<0{\cdot}05$). These data were confirmed with the use of the nucleosome ELISA kit (results not shown).

Recovery following GC exposure

Exposure of the ATDC5 cells to Dex for one or more days resulted in lower cell numbers on day 14. These differences, however, did not reach statistical significance unless the cells were exposed to Dex for all 14 days ($P<0{\cdot}05$) (Fig. 4a and b). There was a significant reduction in proteoglycan content after 7, 10 and 14 days of GC exposure (Fig. 4c). After the recovery period (Fig. 4d), all Dex-exposed cells showed a significant reduction in proteoglycan content ($P<0{\cdot}05$). ALP activity was increased after Dex treatment at days 7, 10 and 14 compared with control cultures but this increase was statistically significant only after 7 (65%) and 14 (148%) days of exposure (Fig. 4e). After the recovery period (Fig. 4f) ALP remained significantly elevated from day 10 ($P<0{\cdot}05$).

Discussion

GC are known to exert effects on many physiological systems and can retard growth in children (Loeb 1976). While they may do this by altering GH secretion or GH sensitivity (Luo & Murphy 1989, Lima *et al.* 1993, Devesa *et al.* 1995), it is very likely that they may also exert direct effects on growth plate chondrocytes (Baron *et al.* 1992). *In vivo* studies in rats and *in vitro* studies using primary cultured rat epiphyseal chondrocytes show a down-regulation of GH receptor mRNA expression after GC treatment as well as an inhibition of IGF-I production and secretion into the culture medium (Gabriellsson *et al.* 1995, Jux *et al.* 1998). However, Heinrichs *et al.* (1994) reported an increase in GH receptor gene expression levels after treatment of rabbits with Dex. A reduction in rat growth cartilage width after GC treatment has also been observed and these authors have suggested that this is a likely consequence of the lower chondrocyte proliferation rate and increased hypertrophic chondrocytes apoptosis (Silvestrini *et al.* 2000).

Studies using rat chondrocyte cultures showed that Dex and Pred reduced both cell proliferation and colony formation and also that Dex was more potent than Pred at equimolar concentrations (Dearden *et al.* 1986, Robson *et al.* 1998). This culture data is in accord with *in vivo* observations where Dex appears to be more potent than Pred at causing impairment of normal bone growth (Strauss *et al.* 2001, Ahmed *et al.* 2002).

Our present experiments used the ATDC5 chondrocyte cell line, which has less phenotypic diversity than cultures containing a heterogeneous population of primary chondrocytes (Robson *et al.* 1998, Koedam *et al.* 2000). Furthermore, it allows the study of two critical events during cartilage formation: the early differentiation of committed mesenchymal cells into chondrocytes (chondrogenesis) and the terminal differentiation of proliferating to hypertrophic chondrocytes (Cancedda *et al.* 1995). Cell numbers were reduced by both Dex and Pred during the chondrogenesis period, but little effect of either GC was noted during the terminal differentiation period. Cell numbers may be reduced by GC by mechanisms such as loss of proliferative activity, increased apoptosis and cytostasis. Our present data strongly support the proposal that loss of proliferative activity is, at least in part, responsible for the decrease in chondrocyte numbers by GC treatment. We found no evidence of increased apoptosis, which is in accordance with Mehls *et al.* (2001). In the growth plate it is well recognised that apoptotic chondrocytes are most prevalent in the terminally differentiated zone (Ohyama *et al.* 1997) and this is also reflected in the ATDC5 cell line as shown in the present study. Interestingly, GC reduced apoptosis in the terminally differentiated cells whilst having no effect on the chondrogenesis phenotype, suggesting that GC control of chondrocyte apoptosis is phenotype dependent. This observation

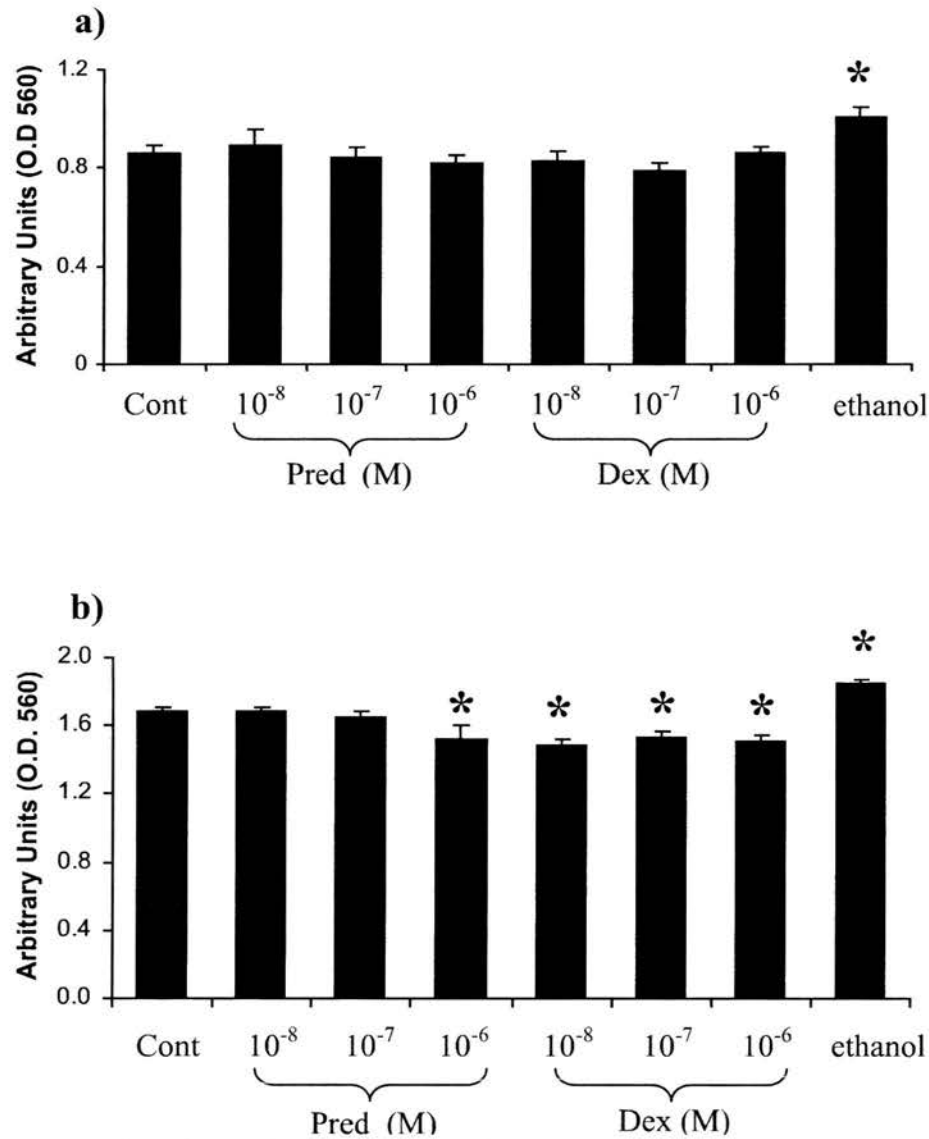

Figure 3 Effect of Dex and Pred treatment for 24 h on the incidence of apoptosis during (a) chondrogenesis and (b) terminal differentiation. All data are expressed as means ± S.E.M. *$P<0.05$ compared with control (Cont). No effects of the GC were observed during the chondrogenesis phase whereas all concentrations of Dex, and Pred at 10^{-6} M, caused a significant reduction in apoptosis during terminal differentiation. Ethanol (5%) caused a significant elevation in apoptosis at both developmental time points.

requires further study. Cell proliferation rates and cell numbers were more greatly affected by Dex and Pred during the chondrogenesis period when the chondrocytes were rapidly proliferating. These results extend the data from cultures containing chondrocytes of various maturational phenotypes (Robson *et al.* 1998) and are also in agreement with studies on other bone cell types which indicate that Dex was more potent than Pred in reducing osteoblast cell number and DNA synthesis (Kasperk *et al.* 1995, Davies *et al.* 2002). Davies and colleagues (2002) also reported that osteoblast precursor cells (HCC1) were more chemosensitive to Dex than fully differentiated osteoblasts and together with our present data suggest that in bone cells, GC exert their maximum effect at the cell precursor stage. Over the three concentrations, Dex was also more potent than Pred as it caused a 44% greater increase in ALP activity and greater reductions in proteoglycan synthesis, cell number and cell proliferation. Annefeld (1992) also showed that Dex treatment in rats results in inhibition of both chondrocyte proliferation and

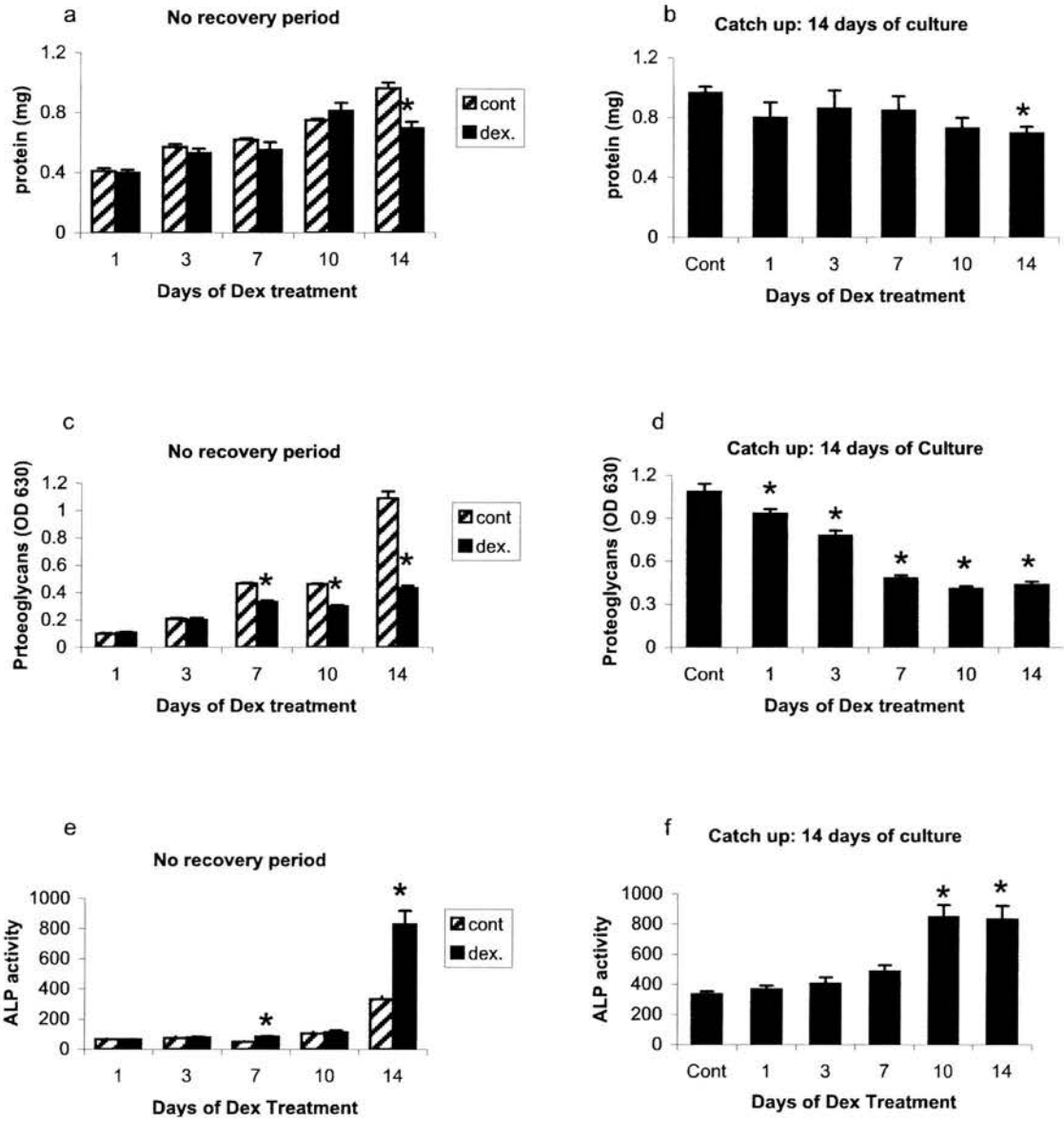

Figure 4 Effect of 10^{-6} M Dex on protein (a and b), proteoglycans (c and d) and ALP activity (e and f) either after a period of no recovery (a, c, e) or where the Dex-treated cells were allowed to recover and were assayed at 14 days (b, d, f). (a) The cell number increased with time in culture. The only significant reduction was after 14 days Dex treatment ($P<0{\cdot}05$). (b) Protein was reduced at all time points, but this was only significant at day 14 ($P<0{\cdot}05$). (c) Proteoglycan levels were elevated with time in the control group, but there was a significant reduction in proteoglycans ($P<0{\cdot}05$) from day 7 to day 14 in the Dex-treated group as compared with their controls. (d) A significant reduction in proteoglycan content occurred after 1 day Dex treatment ($P<0{\cdot}05$) and this was more pronounced with longer periods of treatment. (e) There was a gradual elevation in ALP activity (nmoles pNPP hydrolysed/min/mg protein) at each time point with a significant elevation above the respective control at days 7 and 14. (f) After the recovery period, ALP activity remained elevated and reached significance at days 10 and 14 ($P<0{\cdot}05$). Cont, control.

cartilage matrix production. The pro-differentiating effects of Dex are in agreement with studies using costo-chondral cultures, in which Dex promoted ALP activity (Schwartz *et al.* 1995). However, the results are at variance with other studies where Dex lowered enzyme activity in prehypertrophic chondrocytes but had minimal effects on hypertrophic or mineralising chondrocytes (Robson *et al.* 2001). Although these results are in contrast to those presented here, they do substantiate our own observations and those of others that the response of chondrocytes to

Dex is dependent on their stage of differentiation (Yasuda *et al.* 1995).

In growth failure, amelioration of the growth retarding insult results in a period of supranormal linear growth described as catch-up growth (Prader *et al.* 1963). Two underlying mechanisms for this phenomenon have been suggested – a neuroendocrine model which adjusts the growth rate to an age appropriate set point, and a local mechanism intrinsic to the growth plate (Prader *et al.* 1963, Baron *et al.* 1994). Baron *et al.* (1994) demonstrated a 77% reduction in the growth rate of 5-week-old rabbit limbs that had Dex infused into the proximal tibial growth plate. Following cessation of Dex, catch-up growth was observed in the affected growth plate and not in the contralateral tibia. It is postulated that this is due partly to a delay in growth plate senescence by Dex (Gafni *et al.* 2001).

The ATDC5 cell line allowed us to study this recovery phenomenon in greater detail. No differences were observed in proteoglycan content in the culture plates stopped prior to recovery at 24 and 72 h of treatment. However, after the recovery period a reduction in proteoglycans was apparent at all treatment lengths. This would indicate that Dex-induced suppression may take some time to manifest itself even though the stimulus for suppression has been removed. Longer treatments with Dex of 7 and 10 days duration showed unchanged levels of proteoglycans compared with the 14-day treated group and indicates that within the timescale of this experiment the ability to recover after 7 days of Dex treatment was irreversible. Longer periods of recovery allowed the ALP activity to be suppressed towards control levels, although after 10 days there was no further recovery noted as compared with the 14-day Dex-treated group. It cannot be ruled out, however, that longer periods of recovery could result in suppression of ALP activity back to control levels. It is possible that catch-up growth is never complete and merely falls below the statistical detection limit of a study (Silverstein *et al.* 1997).

In conclusion, Dex and Pred reduce cell number, cell proliferation and proteoglycan content whilst stimulating chondrocyte differentiation. The GC have maximal effects during chondrogenesis with minimal effects during terminal differentiation. The ability to recover is related to the length of Dex exposure and possibly the chondrocyte phenotype. Our studies suggest that the potential for recovery of different events of the chondrocyte life cycle may vary. Our findings in the ATDC5 cell line will allow a more focussed approach towards studying the mechanisms underlying GC-induced growth retardation as well as investigating the potential benefit of growth-promoting therapy.

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Insulin-Like Growth Factor-I Augments Chondrocyte Hypertrophy and Reverses Glucocorticoid-Mediated Growth Retardation in Fetal Mice Metatarsal Cultures

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The study aims were to improve our understanding of the mechanisms of glucocorticoid-induced growth retardation at the growth plate and determine whether IGF-I could ameliorate the effects. Fetal mouse metatarsals were cultured for up to 10 d with dexamethasone (Dex; 10^{-6} M) and/or IGF-I and GH (both at 100 ng/ml). Both continuous and alternate-day Dex treatment inhibited bone growth to a similar degree, whereas IGF-I alone or together with Dex caused an increase in bone growth. GH had no effects. These observations may be explained at the cellular level; cell proliferation within the growing bone was decreased by Dex and increased by IGF-I and these effects were more marked in the cells of the perichondrium than those in the growth plate. However, the most prominent observation was noted in the hypertrophic zone where all treatments containing IGF-I significantly increased (3-fold) the length of this zone, whereas Dex alone had no significant effect. In conclusion, Dex impaired longitudinal growth by inhibiting chondrocyte proliferation, whereas IGF-I stimulated chondrocyte hypertrophy and reversed the growth-inhibitory Dex effects. However, the IGF-I-mediated improvement in growth was at the expense of altering the balance between proliferating and hypertrophic chondrocytes within the metatarsal. (*Endocrinology* 145: 2478–2486, 2004)

CLINICAL STUDIES BY our own group, as well as others, have shown that growth and skeletal development are impaired during treatment with prednisolone and dexamethasone (Dex) (1, 2). Most children who require systemic glucocorticoids (GCs) also suffer from chronic inflammatory disease, and in the clinical scenario, it can be difficult to clearly assess the relative contribution of disease and drugs on growth. In these children, maintenance of growth is a complex process that is influenced by a number of different mechanisms that influence the GH/IGF-1 axis by disrupting GH secretion or altering GH/IGF-1 sensitivity (3, 4). Although catch-up growth often follows cessation of GC therapy, children with systemic chronic inflammatory diseases who are on long-term GCs may have reduced final height (5, 6). Concomitant high-dose recombinant GH therapy may prevent a further deterioration in height velocity, but there is no evidence that it can normalize height in this group of children (7–9). Alternate-day GC treatment may have a lesser impact on childhood growth velocity than continuous GC treatment, but permanent growth impairment has also been noted in children receiving this form of therapy (10, 11). Optimization of growth-promoting therapy requires an improved understanding of the biological effects of GCs and GH/IGF-1 at the level of the growth plate.

The dual-effector theory of GH/IGF-1 action at the growth plate proposes that GH acts directly on germinal zone precursors of the growth plate to stimulate the differentiation of chondrocytes and then amplify local IGF-I synthesis, which, in turn, induces the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (12). Although liver-derived IGF-I is the main determinant of serum IGF-I levels, it is not as important for postnatal growth as locally derived IGF-1 (13, 14).

In the ATDC5 chondrogenic cell line, our group's recent *in vitro* studies show that GC effects may be dependent on the stage of chondrocyte maturation with maximal effects during chondrogenesis and minimal effects during terminal differentiation (15). It also seems that, although the progenitor cells may become quiescent when exposed to GC, their capacity to undergo chondrogenesis is maintained and the program is reactivated when the GC is removed (16). These data are consistent with the *in vivo* model of catch-up growth that is observed after cessation of GC administration directly into the growth plate (17).

The complex effects and physiological mechanisms of GC on growth plate chondrocytes are difficult to study solely in live animals where effects cannot be localized to specific cell types. The fetal mouse metatarsal explant culture is a highly physiological model for studying growth as the growth rate of fetal bones in culture is similar to that found *in vivo*, whereas bones harvested postnatally from 2-d-old rats arrest in culture after 2 d *in vitro* (18, 19). In addition, the metatarsal culture model maintains cell-cell and cell-matrix interactions, and the direct assessment of bone growth and histological architecture can be determined. By using the fetal mouse metatarsal assay, the aims of the present study were to obtain a clearer understanding of the cellular events underlying GC-induced growth retardation and, in addition, determine whether IGF-I can ameliorate the effects of GC on bone growth. This model has also allowed a comparison of

Abbreviations: ALP, Alkaline phosphatase; BrdU, bromodeoxyuridine; Dex, dexamethasone; GC, glucocorticoid.

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the effect of continuous *vs.* alternate-day GC exposure. Our studies reveal that the fetal mouse metatarsal model can replicate *in vivo* bone growth, and these experiments represent the first *in vitro* study to demonstrate the prohypertrophic effects of IGF-I and reversibility of Dex-induced growth retardation.

Materials and Methods

Fetal metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18-d-old embryonic Swiss mice. Bones were cultured at 37 C in a humidified atmosphere of 95% air/5% CO_2 individually in 24-well plates (Costar, High Wycombe, UK) for up to 10 d. Each well contained 300 μl of α-MEM without nucleosides (Invitrogen, Paisley, UK) supplemented with 0.2% BSA Cohn fraction V (Sigma, Dorset, UK), 0.1 mmol/liter β-glycerophosphate (Sigma), 0.05 mg/ml L-ascorbic acid phosphate (Wako, Fukuoka, Japan), 0.292 mg/ml L-glutamine (Invitrogen), 0.05 mg/ml gentamicin (Invitrogen), and 1.25 μg/ml fungizone (amphotericin B) (Invitrogen). Dex (Sigma), IGF-I (Bacham, St. Helens, UK), and GH (Bacham) were added at a final concentration of 10^{-6} M, 100 ng/ml, and 100 ng/ml, respectively, to the cultured bones. In addition, the effects of continuous Dex 10^{-6} M *vs.* alternate-day Dex 10^{-6} M exposure on total metatarsal length was also studied. The control and experimental groups contained six metatarsals each, and the experiment was repeated at least two times.

Morphometric analysis

Images were taken of the metatarsals every second day of culture using a digital camera (COHU, San Diego, CA) attached to an Olympus MO81 microscope. The total length of the bone and width through the center of the mineralizing zone was determined using Image Tool (Image Tool version 3.00, University of Texas Health Life Science Centre, San Antonio, TX). All results are expressed as a percentage change from harvesting length, which was regarded as baseline to demonstrate the rate of growth over time. For the determination of the size (in the direction of longitudinal growth) within the growth region of the distinct chondrocyte maturational zones, the 4- and 10-d-old metatarsals were fixed in 70% ethanol, dehydrated, and embedded in paraffin wax (20). Wax sections (10 μm in thickness) were reacted for alkaline phosphatase (ALP) activity (21) for the demarcation of the hypertrophic and proliferating zones. Serial sections were stained with von Kossa and hematoxylin and eosin using standard protocols to identify the zone of cartilage mineralization. Images of the stained metatarsals were captured using Image Tool (University of Texas), and the size of the combined (distal and proximal) ALP-negative proliferating zone was determined: proliferating zone = total length – (hypertrophic zone + mineralizing zone. Similarly, the size of the combined ALP-positive hypertrophic zone located at either side of the mineralizing zone was determined: hypertrophic zone = (hypertrophic zone + mineralizing zone) – mineralizing zone. The size of the mineralizing zone was determined directly from the von Kossa-stained sections.

ALP enzyme activity

At the end of the culture period (d 10), ALP activity within the metatarsals was determined as previously described (22). Briefly, each metatarsal was permeabilized in 100 μl of 10 mmol/liter glycine (pH 10.5) containing 0.1 mmol/liter $MgCl_2$, 0.01 mmol/liter $ZnCl_2$, and 0.1% Triton X-100 by freeze-thawing three times. The extract was assayed for ALP activity by measuring the rate of cleavage of 10 mM *p*-nitrophenyl phosphate. Total ALP activity was expressed as nanomoles *p*-nitrophenyl phosphate hydrolyzed per minute per metatarsal. Each group contained three metatarsals, and the experiment was repeated at least twice.

Cell proliferation and dry weight determination

[^{3}H]Thymidine uptake. On d 4 and 10 of culture [^{3}H]thymidine (Amersham Biosciences, Little Chalfont, UK) was added (final concentration, 10 μCi/ml) to each metatarsal culture for the last 6 h of culture. After washing in PBS, the metatarsals were extracted in trichloroacetic acid (twice for 30 min), acetone (twice for 30 min), and ether (three times for 30 min) and air dried overnight at room temperature. After the determination of dry weight (Sartorious Micro, Gottingen, Germany) the tissue was solubilized (NCS-II tissue solubilizer, Amersham) and the DNA incorporating [^{3}H]thymidine was determined using a scintillation counter (20). The cell proliferation data were expressed as [^{3}H]thymidine (dpm) per metatarsal. Each group contained three metatarsals, and the experiment was repeated at least two times.

Histological assessment of bromodeoxyuridine (BrdU) uptake. BrdU (Sigma) was added (final concentration, 1 mg/ml) to the culture medium of the metatarsals for the last 6 h of culture on d 4 and 10 as described previously (20). At the end of the incubation period, the tissue was washed in PBS and fixed in 70% ethanol, dehydrated, and embedded in paraffin wax. Sections, 10 μm in thickness, were cut along the longitudinal axis, and chondrocyte nuclei with incorporated BrdU were detected using an indirect immunofluorescence procedure as detailed previously (23). Briefly, sections were denatured with 1.5 M HCl for 30 min before incubation with an antibody to BrdU (Dako, Ely, UK) diluted 1:50 in PBS for 1 h. After washing, the sections were incubated for an additional 1 h in fluorescein isothiocyanate-labeled goat antimouse IgG (Sigma) diluted 1:50 in PBS. The sections were finally mounted in PBS/glycerol (Citifluor, Agar Scientific, Essex, UK). Sections were examined using a Leica BMRB fluorescent microscope, and the total number of BrdU-positive chondrocytes within both the proximal and distal growth regions was determined. BrdU-labeled cells located to the perichondrium were also counted. Three sections from each of six bones from each treatment group at both time points were examined to obtain an aggregate value.

Statistics

All data are expressed as the mean ± SEM, and statistical analysis was performed using an ANOVA (GenStat, sixth edition, VSN International Ltd., Hemel Hempstead, UK). $P < 0.05$ was considered to be significant.

Results

All fetal mice metatarsals grew in culture and displayed a central core of mineralized cartilage juxtaposed on both sides to a translucent area representing the hypertrophic chondrocytes (Fig. 1, B–D). The localization of ALP reactivity within metatarsal sections was restricted to the mineralizing and hypertrophic chondrocytes and thus clearly delineated the boundary between the proliferating and hypertrophic zones (Fig. 1E) whereas von Kossa staining was specific to the mineralizing zone (not shown).

Longitudinal bone growth

All experiments were done on metatarsals from 18-d-old embryos that were cultured for intervals for up to 10 d. Dex-treated bones paralleled control bone growth rate until d 8 when their rate of growth decreased resulting in a total length that was significantly reduced from controls at d 8 ($P < 0.05$) and 10 ($P < 0.05$), (Fig. 2A). IGF-I and combined IGF-I plus Dex (IGF-I+Dex)-treated bones showed a rapid acceleration in growth from d 2 that was significantly higher than the control group ($P < 0.05$), and this increased growth rate was maintained throughout the duration of the experiment. At d 10, mean increase from baseline in total length of control, Dex, IGF-1, and IGF1+Dex bones was 50 ± 3%, 42 ± 2% ($P < 0.05$), 99.3 ± 5%, ($P < 0.05$), and 87 ± 4% ($P < 0.05$), respectively. Compared with the IGF-I-treated bones, the length of the bones treated with IGF-I+Dex was also significantly lower at d 8 ($P < 0.05$) and 10 ($P < 0.05$). The

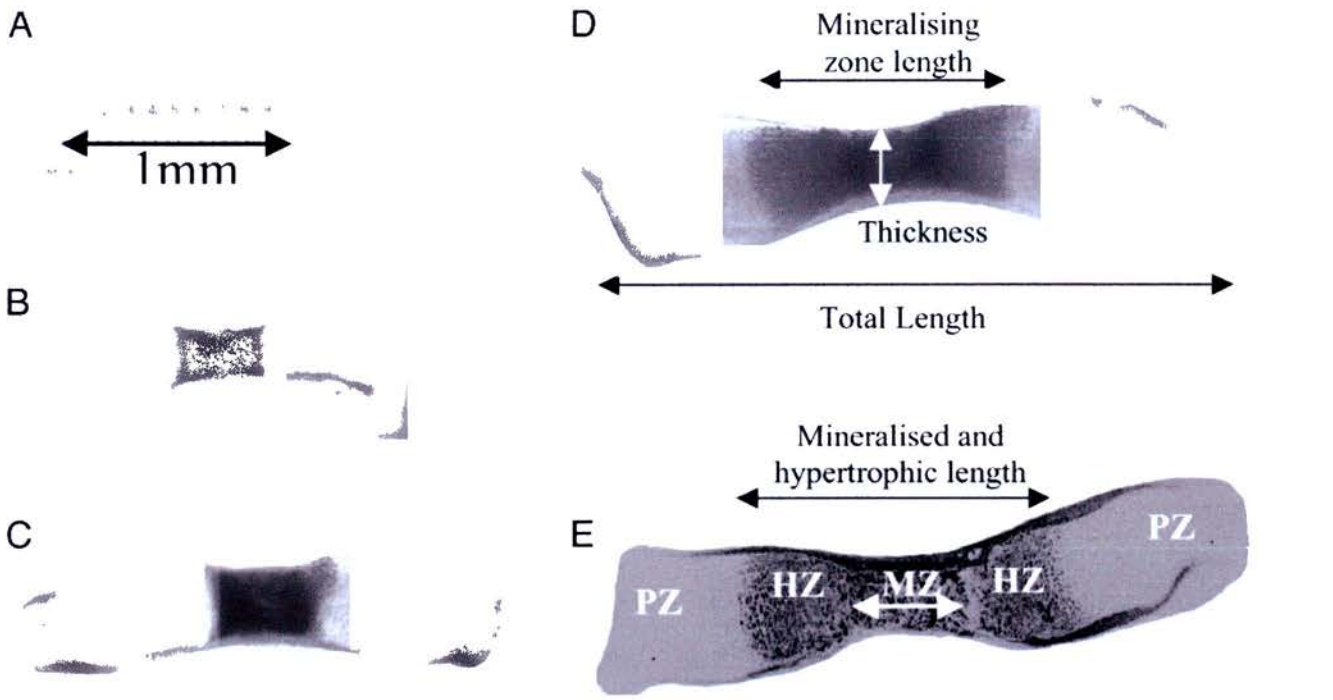

FIG. 1. Measurements of digital images of fetal mouse metatarsal bones in culture with clearly delineated mineralizing zones (B–D) were taken using a calibrated ruler (A). These images demonstrate the harvesting day length (B) and the increased longitudinal growth at d 4 (C). An IGF-I-exposed metatarsal at d 10 is illustrated in D. Section of an IGF-I-treated metatarsal at d 10 reacted for ALP activity showing staining within both the mineralizing and hypertrophic zone. The proliferating zone is negative (E). The location of the proliferating (PZ), mineralizing (MZ), and hypertrophic (HZ) zones are also illustrated in E.

ability of GH to directly influence bone growth in this model system was also studied (Fig. 2B). In contrast to the growth-promoting effects of IGF-I (Fig. 2A), GH was found to have no significant effects on total bone length compared with control metatarsals. We also determined whether alternate-day Dex had a lesser deleterious impact on growth rate than continuous Dex treatment (Fig. 2B). Both modes of Dex treatments resulted in a significant reduction in total length from d 8 ($P < 0.05$). By d 10 in the continuously treated Dex group there was a 27 ± 2% ($P < 0.05$) reduction in total length *vs.* a 21 ± 3% decrease in the alternate-day Dex group ($P < 0.05$) when compared with the control metatarsals. No significant differences were detected between the continuous and alternate-day Dex lengths.

In control bones, there was a significant increase in the length of the mineralizing zone by d 6, and by d 10 the mean increase in length from baseline was 122 ± 2% ($P < 0.05$) (Fig. 2C). The mineralizing zone length of the IGF-I-treated bones changed little throughout the culture period and by d 10 it had increased from baseline by only a mean of 10 ± 2% ($P < 0.05$). Also by d 10 the length of the mineralizing zone in the Dex-treated metatarsals had increased by a mean of 79 ± 19% ($P < 0.05$) from baseline, significantly less than in the control metatarsals ($P < 0.05$). The growth rate of the mineralizing zone in the IGF-I+Dex-treated metatarsals was also less than control, and Dex-treated metatarsals with the mineralizing zone length significantly decreased at d 6 (11 ± 5%; $P < 0.05$), d 8 (32 ± 8%; $P < 0.05$), and d 10 (33 ± 10%; $P < 0.05$) from the control bones. Overall, these data suggest the existence of an inverse relationship between the length of the mineralization zone and total bone length (Fig. 2, A and C).

The thickness of the control and Dex-treated metatarsals did not change with time in culture and were not significantly different from each other at any of the time points examined (Fig. 2D). In comparison with controls, both the IGF-I and IGF-I+Dex-treated bones were significantly thicker from d 4 ($P < 0.05$) and 6, respectively ($P < 0.05$). At d 4, the thickness of the IGF-I-treated bones was significantly different from the IGF-I+Dex-treated bones. At d 10, the thickness of the IGF-I and IGF-I+Dex-treated bones were, respectively, 51 ± 10% ($P < 0.05$) and 35 ± 14% ($P < 0.05$) greater than that of their harvesting lengths.

With the exception of the results shown in Fig. 2B, the data presented in Fig. 2, A, C, and D (and all subsequent results), were obtained from metatarsals of embryos from the same mother. The differing growth rates shown in Fig. 2, A and B, are likely to be due to variability between the embryos selected for each experiment. The inhibition of growth rate by Dex was observed in both studies.

Assessment of chondrocyte maturational zone sizes

In many of the metatarsal rudiments, the boundary between the proliferating and hypertrophic zone of chondrocytes was difficult to delineate while in culture; therefore, measurements of the size of these individual maturational zones was performed on histological sections of 4- and 10-

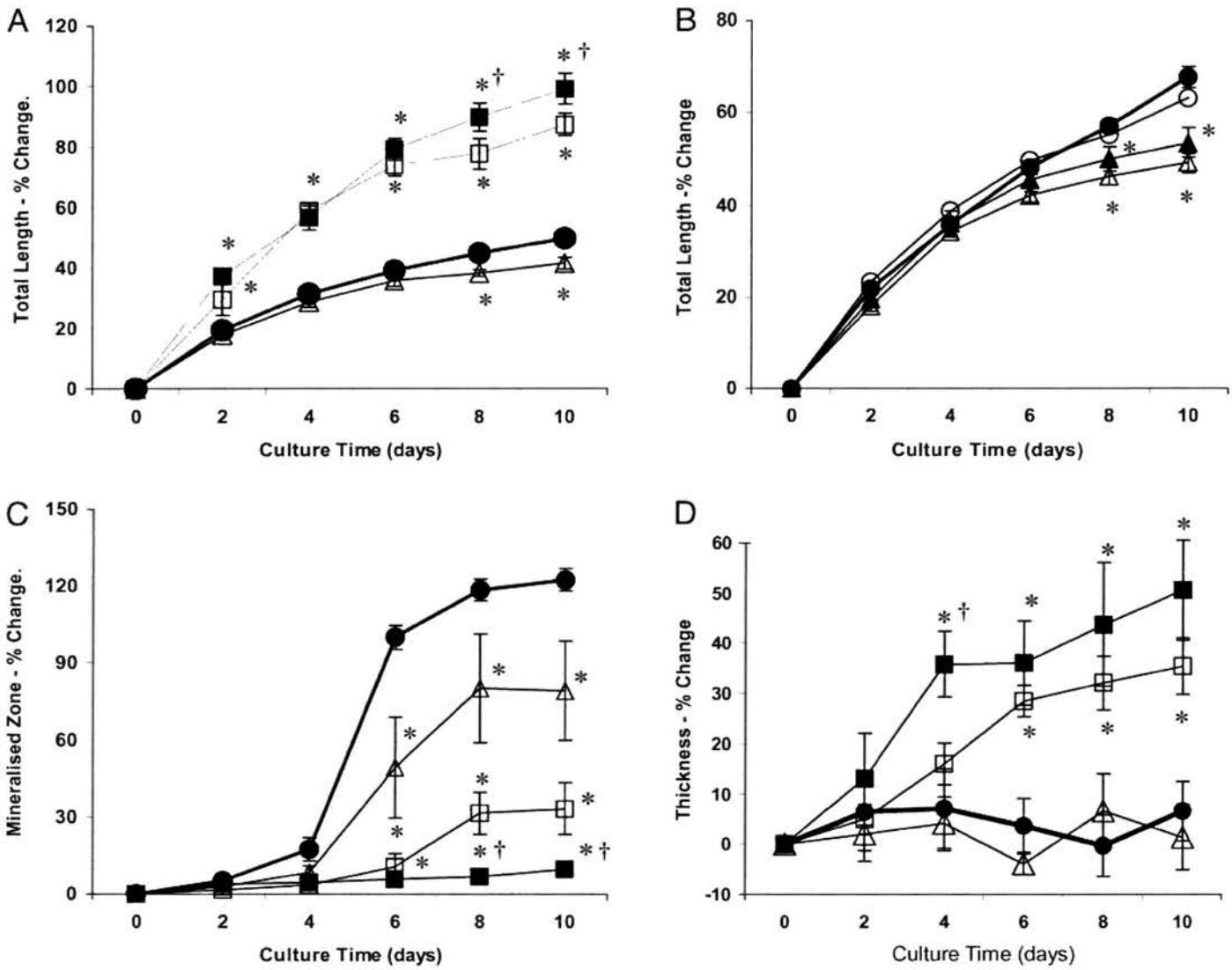

FIG. 2. A, Continuous Dex at 10^{-6} M caused a significant decrease in linear growth from 8 d, whereas IGF-I and IGF-I+Dex had significant stimulatory effects from 2 d. B, Effects of GH (100 ng/ml) and continuous and alternate-day Dex on total length. GH had no significant effects on total length. However, both continuous and alternate-day Dex caused a significant decrease in length from d 8 ($P < 0.05$). C, Effects of Dex, IGF-I, and IGF-I+Dex on the length of the mineralized zone. In the control metatarsals, mineralization increased from 4 d. All treatments caused a significant reduction in mineralization from d 6. IGF-I-treated bones were the least mineralized, whereas Dex and IGF-I+Dex effects were intermediate. D, Effects of Dex, IGF-I, and IGF-I+Dex on metatarsal thickness. Both IGF-I and IGF-I+Dex caused a significant increase in the metatarsal thickness from d 4 and 6, respectively. Results shown in A, B, and D were obtained from the same cultures, whereas the data shown in B were from a separate experiment. All data are expressed as the mean ± SEM; *, $P < 0.05$ compared with control; †, significance of IGF-I compared with IGF-I/Dex ($P < 0.05$); △, continuous Dex; ▲, alternate-day Dex; ■, IGF-I (100 ng/ml); □, combined IGF-I+Dex; ○, GH (100 ng/ml); ●, control cultures.

d-old metatarsals. The lengths of the proliferating, mineralizing, and hypertrophic zones are shown in Table 1.

Although Dex decreased and IGF-I increased the length of the proliferating zone, these changes did not reach statistical significance. However, IGF-I+Dex treatment resulted in a significant increase in the length of the proliferating zone at d 4 ($P < 0.05$), which was not sustained by d 10. The length of the mineralizing zone was significantly reduced with all treatments at both time points ($P < 0.05$) compared with the controls. At d 4, there was a 10% reduction with all treatments; this decrease became larger by d 10, with Dex, IGF-I, and IGF-I+Dex causing a 16, 51, and 42% reduction, respectively, in the length of the mineralizing zone compared with the control bones ($P < 0.05$). Dex caused a nonsignificant increase in the length of the hypertrophic zone at d 4 and 10. In contrast, IGF-I led to a marked increase in the length of the hypertrophic zone at d 4 (98% increase; $P < 0.05$), which became more pronounced by d 10 (346% increase; $P < 0.05$) (Table 1; Fig. 3, A and B). The combined effects of IGF-I+Dex were similar to IGF-I exposure alone, resulting in a 74% and 233% increase in length at d 4 and d 10, respectively ($P < 0.05$) (Table 1). The size of the individual hypertrophic chondrocytes in the 10-d IGF-I-treated metatarsals was also much larger than those of the control metatarsals (Fig. 3, C and D).

TABLE 1. Lengths of the proliferating, mineralizing and hypertrophic zones

Treatment	Proliferating zone		Mineralizing zone		Hypertrophic zone	
	d 4	d 10	d 4	d 10	d 4	d 10
Control	140 4 + 3.0	122.6 ± 7.0	53.7 ± 1.3	102.0 ± 3.4	38.2 ± 1.7	36.7 ± 4.4
Dex	129.0 ± 2.4	120.3 ± 4.2	48.5 ± 1.9[a]	84.2 ± 7.4[a]	42.6 ± 4.0	40.2 ± 15.1
IGF-I	150.6 ± 5.6	123.8 ± 5.0	48.8 ± 1.5[a]	49.3 ± 2.2[a]	75.6 ± 1.4[a]	163.4 ± 7.6[a]
IGF-I + Dex	159.8 ± 8.9*	137.0 ± 4.7	48.5 ± 1.5[a]	57.8 ± 3.5[a]	66.6 ± 8.4[a]	122.0 ± 3.1[a]

Data are expressed in micrometers ± SEM.
[a] $P < 0.05$ compared with controls.

Metatarsal ALP enzyme activity

The enlargement of the hypertrophic zone with IGF-I treatment after 10 d in culture was further studied by determining ALP activity in the metatarsals at the end of the culture period The ALP activity (nmoles hydrolyzed per minute per metatarsal) expressed as mean ± SEM was as follows: control, 0.0187 ± 0.009; Dex, 0.0117 ± 0.009; IGF-I, 0.038 ± 0.0061 ($P <$ 0.05); and IGF-I+Dex, 0.026 ± 0.0044. In agreement with the increase in hypertrophic zone length (Table 1; Fig. 3B), IGF-I treatment resulted in significantly elevated levels of ALP activity within the metatarsals (103% increase; $P < 0.05$) compared with the control bones. Combined IGF-I+Dex caused an increase and Dex treatment alone a reduction in ALP activity, although these results were not significantly different from the control values. It was of interest to note that the data from the IGF+Dex-treated cultures are consistent with the metatarsal length data (Fig. 2A) where the presence of Dex partially reduced the effects of IGF-I.

Cell proliferation: [^{3}H]thymidine incorporation and BrdU staining

The incorporation of [^{3}H]thymidine into the metatarsals was determined at d 4 and 10, representing two distinct phases of varying growth rates. There was a tailing off in the linear growth curve from d 6 in all bones (Fig. 2A), and this was reflected in a lower [^{3}H]thymidine incorporation rate in the control metatarsals at d 10 (75131 ± 5864 dpm) compared with the control bones at d 4 (98608 ± 6732 dpm) (Table 2). In comparison with control bones, Dex treatment for 4 d resulted in a significant reduction (50%; $P < 0.05$) in [^{3}H]thymidine incorporation, whereas both IGF-I and IGF-I+Dex treatment resulted in significant increases of 43 and 57%, respectively ($P < 0.05$). After 10 d, there was a significant reduction in [^{3}H]thymidine incorporation in all treatment groups compared with the control cultures (Table 2). However, this reduction, from control bone values, was greater with Dex (80%; $P < 0.05$) than that observed with IGF-I (64%; $P < 0.05$) or IGF-I+Dex (53%; $P < 0.05$).

To further refine the [^{3}H]thymidine uptake data we determined the localization of the proliferating cells (BrdU positive) and quantified their number in both the growth plate and the perichondrium (Figs. 3 and 4). The total number of proliferating cells in all control metatarsal groups was higher at d 4 than d 10, which is in agreement with the [^{3}H]thymidine incorporation data and indicative of slower linear growth with time in culture (Table 2 and Fig. 2A). Compared with d 4 control metatarsals, Dex significantly reduced the number of BrdU-positive cells located in the growth plate (42% decrease; $P < 0.05$) and perichondrium (76% decrease; $P < 0.05$) and therefore also the total number of dividing cells within the whole metatarsal (56% decrease; $P < 0.05$), (Figs. 3, E and F, and 4A). In contrast, IGF-I treatment significantly increased the number of BrdU-positive cells in the perichondrium (76% increase; $P < 0.05$) but not those within the growth plate (Figs. 3G and 4A). Combined IGF-I+Dex treatment had no significant effect on BrdU incorporation in the perichondrium and growth plate compared with the control metatarsals at d 4.

Compared with d 10 control metatarsals (Fig. 3H), treatment with Dex alone (Fig. 3I) or in combination with IGF-I, significantly reduced the number of BrdU-positive cells within the perichondrium (Dex, 96% decrease, $P < 0.05$; IGF-I+Dex, 71% decrease, $P < 0.05$) (Fig. 4B). Similarly, treatment with IGF-I alone (Fig. 3J) or in combination with Dex resulted in a reduction in BrdU-positive cells within the growth plate chondrocytes (IGF-I, 63% decrease, $P < 0.05$; IGF-I+Dex, 57%, $P < 0.05$) (Fig. 4B). No cells outside of the perichondrium and growth plates showed any BrdU-positive staining. These results indicate that in comparison with chondrocytes within the growth plate, the cells within the perichondrium are more sensitive to stimulation by IGF-I during rapid growth (d 4) and inhibition by Dex at both time points.

Dry weights

At d 4 and 10, there was no significant difference between the weights of the control and Dex-treated metatarsals They were, however, significantly lighter ($P < 0.05$) than the IGF-I and IGF-I+Dex-treated bones, which were themselves similar in weight to each other at both time points (Table 3).

Discussion

The rate of longitudinal bone growth is determined by a complex interplay of proliferative kinetics, size of the proliferative pool, matrix synthesis, and hypertrophic chondrocyte enlargement (24, 25). The control of these processes is still a matter of debate, and the individual contribution of each variable to bone growth differs with growth rate and is not uniform for all bones (26). Any perturbation of these synchronized variables may underlie the growth-modulatory effects of both Dex and IGF-I. Therefore, the identification of such changes in this present study will provide a better understanding of the mechanisms underlying Dex-induced growth retardation.

Our results unequivocally show that Dex and IGF-I have major and opposite effects on longitudinal bone growth with IGF-I clearly reversing the growth-inhibitory effects of Dex. However, the potential for Dex to inhibit bone growth was

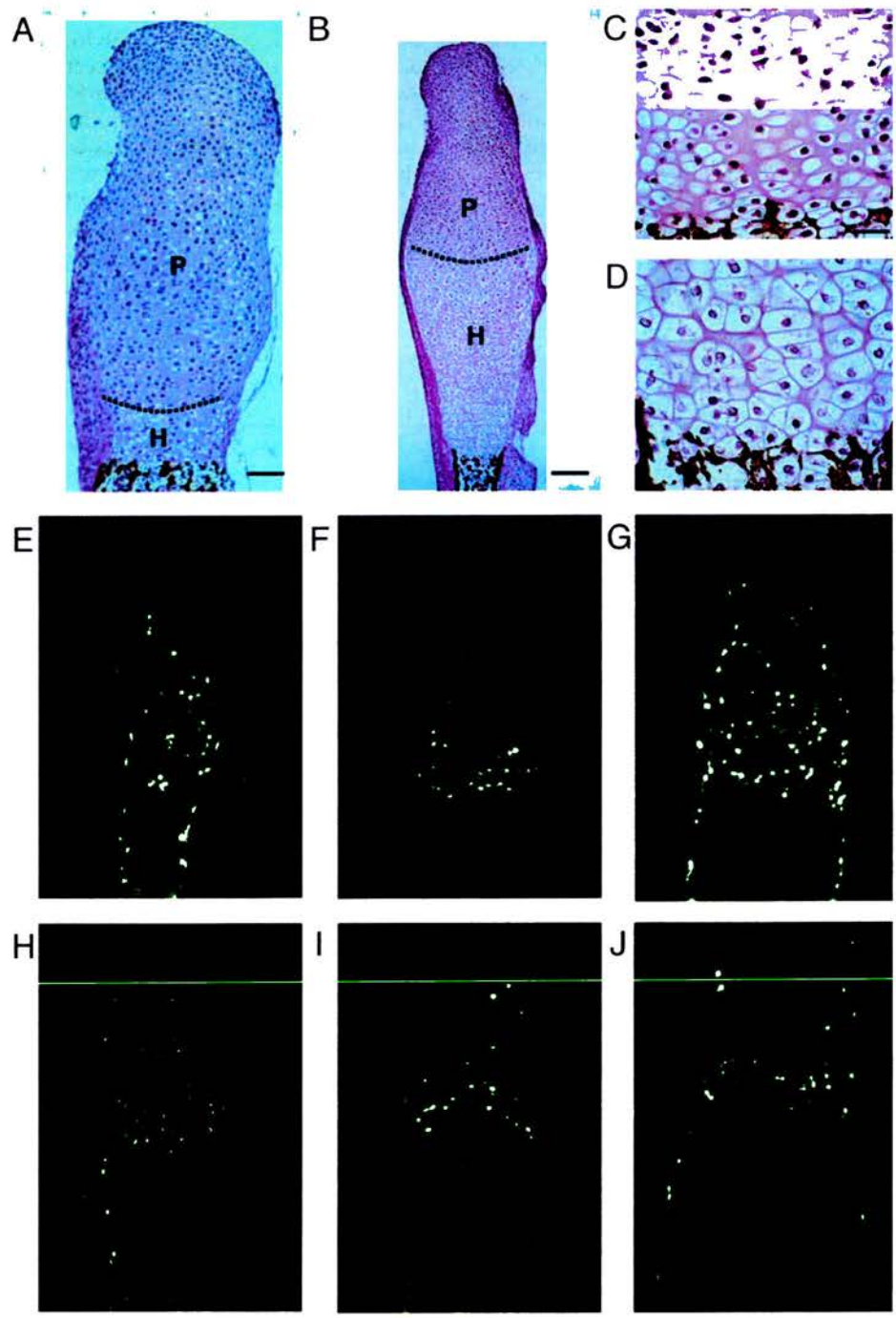

FIG. 3. Histological assessment of chondrocyte hypertrophy (A–D) and proliferation (E–J) in metatarsals treated with Dex and IGF-I. A–D, Hematoxylin- and eosin-stained sections of 10-d-old cultures of control (A and C) and IGF-I-treated (B and D) metatarsals. There is an increase in the size of the hypertrophic zone of the IGF-I-treated metatarsals (B) compared with controls (A). The chondrocytes of the hypertrophic zone of the metatarsals in A and B are shown in higher magnification in C and D. The chondrocytes juxtaposed to the von Kossa-positive mineralized cartilage are larger in the IGF-I-treated (D) than in the control metatarsals (C). Note the micrographs shown in A and B are taken at different magnifications to accommodate the increased length of the IGF-I-treated metatarsals. P, Proliferating chondrocytes; H, hypertrophic chondrocytes. The *dashed line* marks the boundary between the proliferating and hypertrophic zones. E–J, BrdU-labeled cells in control (E and H), Dex-treated (F and I), and IGF-I-treated (G and J) metatarsals cultured for 4 d (E–G) and 10 d (H–J). Note the decreased number of proliferating cells in the Dex-treated metatarsals and in particular the lack of staining within the perichondrium (F and I). Increased perichondrial staining is observed in the 4-d-old IGF-I-treated cultures. *Bars*, 100 μm (A and E–J), 200 μm (B), and 25 μm (C and D).

still present in the IGF-I+Dex cultures where the growth rates did not match those of bones cultured with IGF-I alone. The Dex-induced reduction in total metatarsal length from d 8 was due to reduced chondrocyte proliferation and a reduction in the growth of the mineralizing zone. Similar effects with other GCs have previously been reported by Picherit *et al.* (27) who demonstrated that hydrocortisone induced growth retardation in fetal rat metatarsals. Interestingly, our present data did not reveal a significant growth-sparing effect of alternate-day glucocorticoids. The growth-sparing effect of alternate-day steroids is not a universal observation, and it may be influenced not only by the duration of therapy but also by the underlying disease process and the sex of the patient (4). In addition, most clinical reports refer to the use of prednisolone or hydrocortisone, whereas our studies employed the use of Dex, which has markedly more potent effects on growth *in vivo* and *in vitro* (2, 15).

In contrast to the effects of Dex, IGF-I rapidly stimulated

linear bone growth by increasing both the size of the hypertrophic zone and the chondrocyte proliferation rate during the early phase of bone growth. Stimulation of bone growth by IGF-I has also been reported by Scheven and Hamilton (18), but they further demonstrated that the stimulation of cell proliferation in cultured rat metatarsals by IGF-I was not sustained with IGF-I over time. This may indicate the rapid use of endogenous growth factors needed to support longitudinal growth. However, these workers (18) reported GH stimulatory effects on metatarsal length, which is in contrast to the data of this present study. Although GH is well recognized to stimulate longitudinal bone growth *in vivo* (28, 29) its effects *in vitro* are less clear (30, 31). Other studies have strongly suggested that GH effects *in vivo* may be indirect and that IGF-I effects are more pervasive *in vitro* (32, 33).

To understand the cellular mechanisms underlying the opposite effects of Dex and IGF-I on bone length we analyzed the distribution of BrdU-positive cells within metatarsals treated by both Dex and IGF-I alone and in combination. The number of dividing cells within the perichondrium was greatly reduced by Dex at both 4 and 10 d of culture. In contrast, at 4 d, the number of BrdU-positive cells was greater in the perichondrium of IGF-I-treated bones. Stimulation of cell proliferation was not observed in the IGF-I-treated 10-d-old metatarsals, and this may be due to the observed slowing of growth in these rapidly growing bones. In 4-d-old rapidly growing metatarsals, IGF-I completely reversed the inhibitory effects of Dex on cell proliferation within the perichondrium and growth plate. This reversal of the negative effects of Dex by IGF-I coincubation was also observed, albeit to a lesser extent, in the perichondrial cells of 10-d-old cultures. These results extend the [^{3}H]thymidine incorporation data and also confirm the ability of IGF-I to reverse the deleterious effects of Dex on cell proliferation. Our observation that cell proliferation within the perichondrium was more sensitive to inhibition by Dex and stimulation by IGF-I than chondrocytes within the growth plate has previously not been recognized. The perichondrium is vital to the endochondral process through its role in mediating the parathyroid hormone-related peptide-indian hedgehog (PTHrP-Ihh) signaling cascade, and it is possible that the marked Dex-induced inhibition of proliferation within cells of the perichondrium has a more direct effect on the bone growth process (34–36). The differential sensitivity of cells to Dex treatment within the perichondrium and within the growth plate requires additional study.

A morphometric analysis was completed to further characterize the response of metatarsals to both Dex and IGF-I with respect to the size of the individual maturational zones

TABLE 2. Cell proliferation: effect of Dex, IGF-I, and IGF-I + Dex on [^{3}H]thymidine uptake at d 4 and 10

Treatment	[^{3}H]thymidine (dpm)	
	d 4	d 10
Control	98608 ± 6732	75131 ± 5864
Dex	49521 ± 1124[a]	15000 ± 1612[a]
IGF-I	140547 ± 9821[a]	27631 ± 2594[a]
IGF-I + Dex	154941 ± 3695[a]	35470 ± 1339[a]

Data are expressed as mean ± SEM.
[a] $P < 0.05$ compared with controls.

TABLE 3. Dry weights (microgram) of all bones at d 4 and 10

Treatment	Dry weights (μg)	
	d 4	d 10
Control	34 ± 3	84 ± 2
Dex	33 ± 2	82 ± 5
IGF-I	48 ± 2[a]	152 ± 5[a]
IGF-I + Dex	59 ± 5[a]	147 ± 2[a]

Data are expressed as mean ± SEM.
[a] $P < 0.05$ compared with controls.

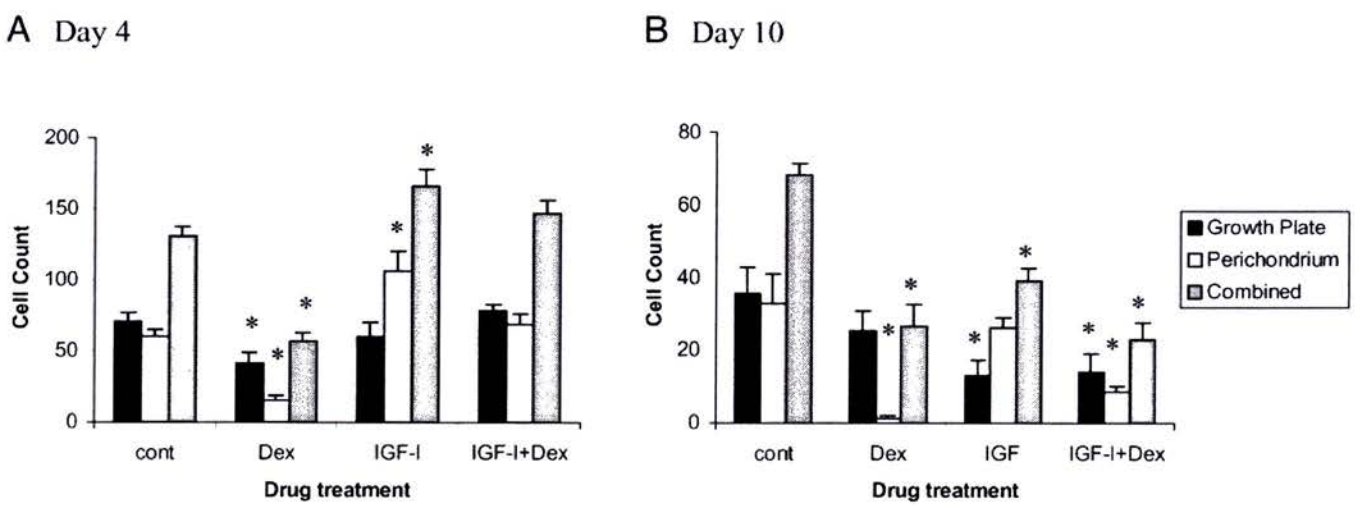

FIG. 4. Effect of Dex, IGF-I, and IGF-I+Dex on the number of BrdU-positive cells within the growth plate (*black bars*), the perichondrium (*white bars*), and the combined number within the growth plate and perichondrium (*hatched bars*) at d 4 (A) and d 10 (B). Cell proliferation is higher at d 4 than d 10 with all treatments. At d 4, Dex causes a significant reduction in cell proliferation in the growth plate and perichondrium ($P < 0.05$), whereas IGF-I increases the number of proliferating perichondrial cells ($P < 0.05$). By d 10, Dex sustains the decrease in cell proliferation, which is significant in the perichondrium ($P < 0.05$). Both IGF-I and IGF-I+Dex also cause a decrease in the number of the positive immunofluorescent cells at this time point. All data are expressed as the mean ± SEM; *, $P < 0.05$ compared with controls.

within the growth plate. The reduction in length of the mineralization zone with Dex was consistent with metatarsals treated with hydrocortisone (27). However, the absence of an increase in the length of this zone after IGF-I is at variance to others who have demonstrated an increase in mineralization zone length with IGF-I in a rat metatarsal model system (19). Dex also led to a small, nonsignificant increase in the length of the hypertrophic zone, which is similar to the findings of Smink and colleagues (37) who demonstrated an increase in the hypertrophic zone length in mice treated with Dex. They further postulated that this was a likely consequence of an acceleration of the chondrocyte differentiation rate as observed in PTHrP null mice (34, 37). Alternatively, due to the restriction of cartilage mineralization, the increased size of the hypertrophic zone may be in part due to a simple buildup of nonmineralized hypertrophic chondrocytes. A similar, more pronounced process may explain the more marked reduction in the mineralization zone observed in the IGF-I-treated metatarsals.

Within the maturational zones of the growth plate, the major effects of IGF-I were clearly on the length of the hypertrophic chondrocyte zone and also the size of the cells within. This result is in accord with the hypothesis that it is the size of the hypertrophic zone rather than chondrocyte proliferative kinetics that is the single major determinant of bone growth rate (26, 38). Although IGF-I is expressed by chondrocytes situated in all maturational zones of the growth plate, IGF-I mRNA expression is mainly restricted to the hypertrophic zone, and the infusion of IGF-I into hypophysectomized rats showed that IGF-I stimulated growth plate chondrocytes at all stages of differentiation including those in the hypertrophic zone (29, 37, 39). The growth retardation in the IGF-I null mouse is associated with an attenuation of chondrocyte hypertrophy and no significant changes in proliferation (40), and our data further strengthen the hypothesis that the predominant role of IGF-I in growth promotion is in augmenting chondrocyte hypertrophy rather than proliferation. This effect of IGF-I can reverse GC-induced growth retardation, but this apparent ameliorative effect results in an alteration of the relative proportion of proliferative, hypertrophic, and mineralized chondrocytes.

The opposite effects of IGF-I and Dex on cell proliferation and bone growth and the ability of IGF-I to reverse the growth-inhibitory effects of Dex have not been previously reported. Its clinical significance is unclear, but an upregulation of chondrocytes expressing IGF-I after GC exposure has been reported previously, and it is possible that IGF-I is an important local growth factor that counteracts the effect of GCs at the tissue level (37, 41, 42). Besides GH and IGF-I, GC exposure may also alter the GH and IGF binding proteins that modulate tissue exposure to these growth factors, and this requires additional study, especially now that a complex of IGF-I and IGFBP3 is available for treatment of GH insensitivity (37, 43–45).

In conclusion, we have shown that Dex and IGF-I have opposite effects on linear bone growth. The effects of Dex were time dependent, whereas IGF-I effects were immediate. During the phase of rapid growth, Dex decreased and IGF-I increased cell proliferation. The alteration in proliferation rate by both Dex and IGF-I were most marked within the cells of the perichondrium. Dex decreased skeletal mineralization, whereas IGF-I markedly stimulated chondrocyte hypertrophy in favor of mineralization and completely reversed Dex-induced growth retardation. However, the potential for Dex to inhibit bone growth was still present in the IGF-I+Dex cultures where growth rates did not match those of bones cultured with IGF-I alone. In addition, the IGF-I-mediated improvement in growth was at the expense of altering the balance between proliferating and hypertrophic chondrocytes within the metatarsal. Alternate-day Dex administration did not have a growth-sparing effect, and GH had no beneficial effect on metatarsal growth at the dose studied. The fetal mouse metatarsal model can replicate *in vivo* bone growth, and this is the first *in vitro* study to demonstrate the prohypertrophic effects of IGF-I and reversibility of Dex-induced growth retardation.

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