

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

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Abstract

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

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

Analysis of the tibial growth plate showed that the total growth plate width significantly decreased to 84.5% of control values, caused by a significant decrease in the

proliferative zone. The number of proliferating cell nuclear antigen (PCNA)-positive chondrocytes in the proliferative zone decreased significantly (to 40%) and TUNEL staining showed a significant 1.6-fold increase in apoptotic hypertrophic chondrocytes.

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

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Introduction

The process of endochondral ossification, which occurs at the growth plates of the long bones, results in longitudinal growth. During endochondral ossification, chondrocytes within the growth plate proliferate, differentiate, mature and eventually die by apoptosis and are replaced by bone (Stevens & Williams 1999). This process has to be tightly controlled in order to maintain normal growth.

The insulin-like growth factor (IGF) system has been proposed to be the major determinant of postnatal longitudinal growth (Baker *et al.* 1993, LeRoith *et al.* 2001, Lupu *et al.* 2001, Yakar *et al.* 2002). The IGF system consists of IGF-I, IGF-II, six IGF-binding proteins (IGFBP-1 to -6), which modulate the IGF bioavailability, and the IGF receptors (Jones & Clemmons 1995). The IGFs are produced by multiple tissues and can act both in an endocrine and autocrine/paracrine fashion (Jones & Clemmons 1995). The IGFBPs act mainly as autocrine/paracrine factors at or close to their sites of synthesis (Jones & Clemmons 1995).

Whether IGF-I and IGF-II are both expressed by growth plate chondrocytes was an issue of debate for several years (Nilsson *et al.* 1990, Shinar *et al.* 1993, Wang *et al.* 1995, Reinecke *et al.* 2000). Recently, we and others showed expression of both IGF-I and IGF-II in the growth plate (Olney & Mougey 1999, de los Rios & Hill 1999, Smink *et al.* 2002a). However, due to differences in species and ages of the models used, discrepant results for localization of IGF-I (Reinecke *et al.* 2000, Lupu *et al.* 2001, Smink *et al.* 2002a) and IGF-II (Nilsson *et al.* 1990, Shinar *et al.* 1993, Wang *et al.* 1995, Smink *et al.* 2002a) were reported. Nevertheless, IGF-I and IGF-II are suggested to each have a unique and complementary role in augmenting longitudinal bone growth (LeRoith *et al.* 2001, Smink *et al.* 2002a).

Glucocorticoids (GCs), which are widely used as anti-inflammatory and immuno-suppressive drugs, result in growth inhibition as a side-effect in both children (Allen 1996) and experimental animal models (Price *et al.* 1992, Leili & Scanes 1998, Rooman *et al.* 1999, Stevens & Williams 1999). GCs act locally to inhibit growth,

suggesting a mechanism which is intrinsic to the growth plate (Baron *et al.* 1992, Silvestrini *et al.* 2000). GC-induced growth retardation is suggested to be due to impaired action of the components of the IGF system (Price *et al.* 1992, Jux *et al.* 1998, Klaus *et al.* 2000, Smink *et al.* 2002a). GCs are known to regulate the expression of the components of the IGF system in the circulation *in vivo* (Price *et al.* 1992, Miell *et al.* 1993, Rooman *et al.* 1999, Ward *et al.* 1999) and in chondrocytes *in vitro* (Jux *et al.* 1998, Koedam *et al.* 2000, Smink *et al.* 2002b). While the known *in vivo* data often describe other effects than the effects on the growth plate, *in vitro* data obtained from chondrocytes usually show very short-term effects of GCs for several hours and these results do not reflect growth retardation *in vivo*. In view of the local action of GCs in causing growth retardation, it is important to study the regulation of the IGF system by GCs in the growth plate itself.

At first, to resolve the discrepancy in IGF localization in the growth plate, we investigated the distribution of the IGFs in growth plates of 4-week-old (prepubertal) mice, and compared them with data obtained in our previous study on 7-week-old (postpubertal) mice (Smink *et al.* 2002a). To study the possible involvement of the IGF system in GC-induced growth retardation, we have previously studied the effects of dexamethasone (DXM) treatment for 4 weeks on the expression of the IGFs and IGF-BPs in the growth plates of postpubertal mice and showed an increased expression of IGF-I in the growth plate, whereas IGF-II and IGF-BP-2 mRNA were not affected (Smink *et al.* 2002a). During the first week of this treatment, however, growth velocity decreased markedly, while during the further duration of treatment, DXM had little effect on growth (Rooman *et al.* 1999). Therefore, to study the effects of GCs on general growth and the IGF system in the growth plate, when the most marked effect on growth is observed, we have treated 3-week-old mice for 7 days with 20 µg/day DXM and analysed the short-term *in vivo* effects on the growth plate.

Materials and Methods

Materials

Restriction enzymes and modifying enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The DeadEnd colorimetric Apoptosis Detection system (TUNEL assay) was obtained from Promega (Leiden, The Netherlands). Monoclonal antibody specific for proliferating cell nuclear antigen (PCNA) (PC-10) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The biotinylated secondary antibody and the Vectastain ABC kit were purchased from Vector Laboratories (Burlingame, CA, USA) and the 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Sigma

Chemical Co. (St Louis, MO, USA). PVDF membranes (Immobilon-P) were from Millipore Corp. (Bedford, MA, USA). Polyvinyl alcohol was obtained from Aldrich (Milwaukee, WI, USA). Euparal and DPX mounting medium were purchased from Klinipath (Duiven, The Netherlands).

Mice and tissue preparation

Three-week-old female FVB mice were divided into 2 groups of 5 mice each. The mice were selected to ensure equal means and standard deviations for total body length and weight in each group at the start of the experiment. The animals were injected subcutaneously with 0.1 ml vehicle (phosphate buffered saline (PBS), pH 7.4), or with 20 µg dexamethasone (DXM), once a day for 7 days. The mice were killed by decapitation after ether anaesthesia, 2 h after the last injection. The protocol received approval of the committee for Animal Experiments of the University Medical Centre, Utrecht, The Netherlands.

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

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

Western ligand blot analysis

Sera of the five mice in each group were pooled and loaded on a 12% gel for non-reducing SDS-PAGE. IGF-BPs were visualized by electrotransfer to PVDF membranes followed by incubation with [¹²⁵I]IGF-II (Van Buul-Offers *et al.* 1994). Molecular weights were calculated using BioRad (Hercules, CA, USA) broad range markers as standard. Bands were quantified by densitometry using the GS-363 Molecular Imager and the Molecular Analyst software program, version 1.5 (BioRad).

Determination of serum IGF-I

Sera of the five mice in each group were pooled and serum IGF-I was measured by heterologous radioimmunoassay after Sep-Pak C18 chromatography (Waters Corp., Milford, MA, USA), as described previously (Van Buul-Offers *et al.* 1994).

Morphometry of the growth plate

Growth plate sections were stained with haematoxylin and eosin and pictures of the sections were taken with a Zeiss Axiomat HRC camera equipped with the Axio-Vision software version 3.0 (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width. Corresponding sections of the tibia were used to ensure correct comparison between the different groups. Total width of the growth plate (distance between the epiphysis and the chondro-osseous junction) was determined from the entire transverse area excluding the periphery of the growth plate. Of the images, measurements at 100 µm intervals were performed (about 20 measurements per growth plate) and averaged. The measuring lines were subsequently shortened to the first appearance of regular chondrocyte columns (the boundary between the resting and the proliferative zone) and to the first enlargement of the flattened cells (the boundary between the proliferative and the hypertrophic zones). From the lengths of these lines the widths of the three different zones of the growth plate were calculated. Five animals per treatment group were analysed (2 sections per animal).

Probes

Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP as substrate (Melton *et al.* 1984). cDNAs encoding human IGF-I and IGF-II (Jansen *et al.* 1983, 1985) and cDNAs encompassing mouse IGFBP-1, -2, -3, -4, -5 and -6 cDNAs (kindly provided by Prof. Dr S L S Drop and Dr J W van Neck, Dept. of Paediatrics, Erasmus University, Rotterdam, The Netherlands) (Schuller *et al.* 1993), were used as templates for the synthesis of antisense and sense digoxigenin-labelled cRNA probes. All probes used were specific for the mRNAs analysed. Probes were checked for possible cross-hybridization using *in situ* hybridization on different types of mouse tissues (spleen, thymus) and complete mice embryos (Schuller *et al.* 1993, Smink *et al.* 1999) for the IGFBP probes, and brain for the IGF probes (C Reijnders, personal communication). The various probes displayed distinct expression patterns in these tissues. Northern blot analysis of different tissues using the same probes yielded bands of the expected sizes, verifying the correct identity of the probes.

In situ hybridization

Corresponding sections of the tibia of the different groups were used for the same probes to ensure reliable comparison between the groups. The *in situ* hybridization using digoxigenin-labelled cRNA probes, was performed as described previously (Smink *et al.* 2002a). As a control for

specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals.

Five different animals per treatment group were used per analysed mRNA. Analysis of the five different animals of both groups was performed in the same *in situ* hybridization, for each analysed mRNA. Each analysed glass slide contained 6 sections, 3 of a PBS- and 3 of a DXM-treated mouse. For quantitative evaluation of the number of chondrocytes expressing mRNA, sections were coded and the number of positive cells in a fixed window was determined using the Image-Pro Plus software program (version 4.5) from Media Cybernetics, L.P. (Silver Spring, MD, USA). The total number of all positive cells (including weakly stained cells) in all zones of the growth plate (excluding the periphery of the growth plates) was counted and expressed relative to the total number of cells in the area that was counted.

Immunohistochemistry (PCNA)

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

Determination of apoptosis

Apoptotic cell death was determined by the TUNEL reaction, using the DeadEnd colorimetric Apoptosis Detection system, which was performed according to the procedures of the manufacturer with some minor modifications. Sections were fixed with 4% (w/v) paraformaldehyde/PBS for 15 min and treated with 20 µg/ml proteinase K for 10 min at room temperature. Subsequently, sections were postfixed with 4% (w/v) paraformaldehyde/PBS for 5 min. The TdT reaction was performed at 37 °C for 60 min. For negative controls, the TdT enzyme was replaced by water, which did not show any signal. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide/PBS for 10 min at room temperature. Biotinylated nucleotides were detected by streptavidin-horseradish peroxidase conjugate. Diaminobenzidine (DAB) staining was performed for 30 min at room temperature in the dark, resulting in an insoluble coloured substrate at the site of

Table 1 Effect of dexamethasone (DXM) treatment on body and organ weight and body length. Values are means \pm S.E.M. ($n=5$)

	PBS	DXM	<i>P</i> value*
Total body weight (g)	16.7 \pm 0.3	13.6 \pm 0.6	<0.01
Total body length (cm)	15.9 \pm 0.1	15.3 \pm 0.2	<0.05
Total weight gain (g/wk)	6.1 \pm 0.1	3.0 \pm 0.3	<0.001
Total length gain (cm/wk)	1.9 \pm 0.1	1.3 \pm 0.1	<0.001
Tibia weight (mg)	70.3 \pm 1.0	65.8 \pm 2.5	0.13
Tibia length (mm)	14.8 \pm 0.2	14.4 \pm 0.2	0.20
Liver (mg)	852.3 \pm 19.0	678.9 \pm 49.7	<0.05
Kidney (mg)	197.5 \pm 6.1	187.3 \pm 4.6	0.22
Thymus (mg)	100.2 \pm 5.9	21.5 \pm 1.9	<0.001
Spleen (mg)	82.0 \pm 3.0	35.8 \pm 2.7	<0.001

*DXM-treated compared with PBS-treated control animals (Student's *t*-test).

DNA fragmentation. Subsequently, sections were counterstained with 0.1% light green, dehydrated and mounted with DPX.

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

Statistical analysis

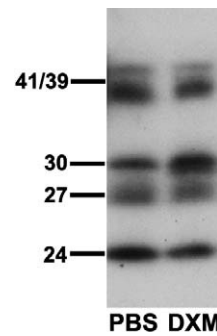
Data are expressed as the mean \pm S.E.M. The effects of the DXM treatment were statistically tested using Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

Results

Dexamethasone-induced growth retardation

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

The IGF-I serum levels decreased from 511.8 ng/ml in PBS-treated control animals to 390.4 ng/ml in DXM-treated animals. Western ligand blot analysis of normal mice sera showed the expected presence of 4 IGFBPs (Fig. 1) (Rooman *et al.* 1999), a doublet of 41/39 kDa (most probably IGFBP-3), a 30-kDa band (probably IGFBP-2), a 27-kDa band (probably IGFBP-5) and a 24-kDa band (probably IGFBP-4). DXM treatment did not affect the IGFBP protein levels (Fig. 1).

**Figure 1** IGFBPs present in pooled sera of control (PBS) and dexamethasone (DXM)-treated mice, as indicated. IGFBPs were detected using Western ligand blot analysis using [¹²⁵I]IGF-II as described in Materials and Methods. Numbers on the left are molecular weights (in kDa) of the detected IGFBPs.**Table 2** Effect of dexamethasone (DXM) treatment on total growth plate width and width of the 3 zones (μ m) of the growth plates of 4-week-old mice. Values are means \pm S.E.M. ($n=5$)

Treatment	Total growth plate	Resting zone	Proliferative zone	Hypertrophic zone
PBS	322.6 \pm 15.5	32.4 \pm 0.4	133.4 \pm 5.6	152.3 \pm 9.3
DXM	272.6 \pm 10.9*	32.5 \pm 1.2	98.4 \pm 6.8*	140.5 \pm 5.4

**P*<0.05 compared with PBS-treated control animals (Student's *t*-test).

Growth plate morphology

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

Immunostaining for PCNA, a marker for proliferating cells, showed a significant decrease in the number of proliferating chondrocytes from 24.8% \pm 2.2% in control animals (Fig. 2A) to 9.8% \pm 1.5% in DXM-treated animals (Fig. 2B) (*P*<0.001) (expressed as a percentage of the total number of cells in the growth plate). Staining for PCNA was predominant in the proliferative zone; however, some staining in the prehypertrophic and hypertrophic zones was also seen.

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

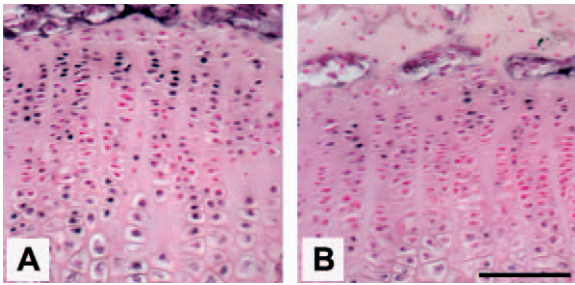


Figure 2 Immunohistochemical analysis of PCNA in representative sections of tibial growth plates of control (A) and DXM-treated mice (B). The protein signal is shown as a dark precipitate. Scale bar represents 100 µm.

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

To analyse the expression of the IGFs and the IGFBPs in the growth plates of 4-week-old normal mice, we performed non-radioactive *in situ* hybridization on tibial epiphyseal growth plates. As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals (Fig. 3C, F and I). IGF-I mRNA was predominantly expressed in the proliferative zone and in the early hypertrophic zone (or prehypertrophic zone) of the growth plate in 4-week-old normal control mice. Weak staining was observed in some of the resting and hypertrophic chondrocytes.

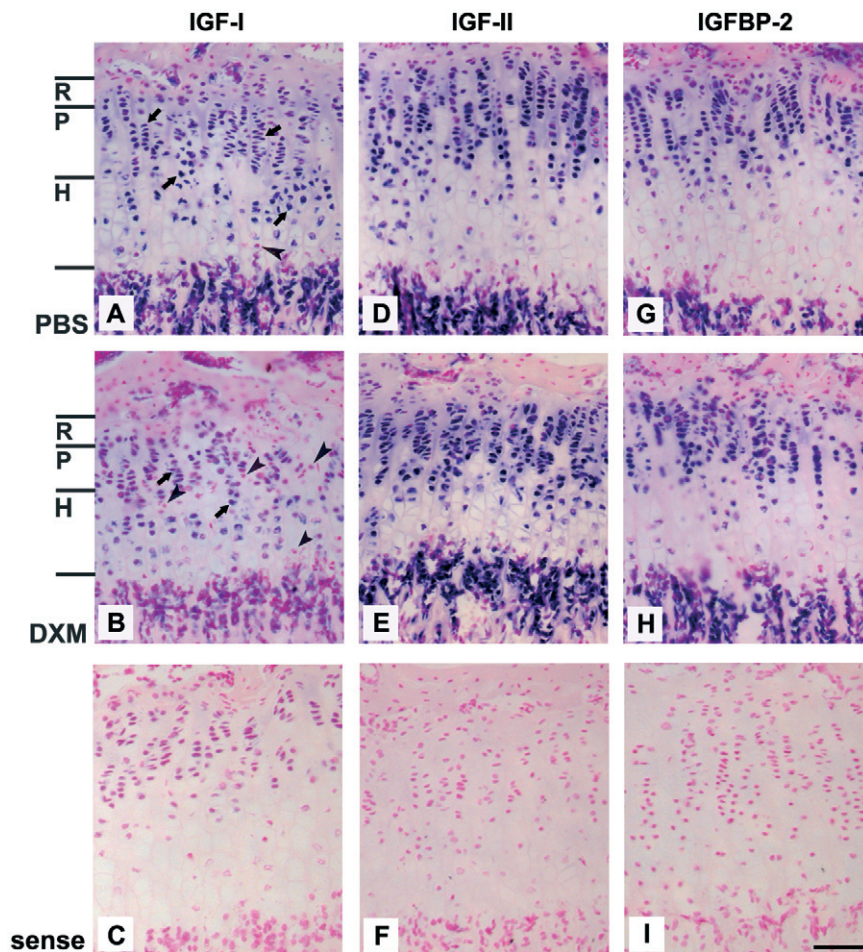


Figure 3 Expression of the IGF system in the postnatal growth plate. Expression patterns of IGF-I mRNA (A, B), IGF-II mRNA (D, E) and IGFBP-2 mRNA (G, H) in representative sections of the growth plates of 4-week-old mice. (A, D, G) PBS-treated mice (PBS), (B, E, H) DXM-treated mice (DXM), as analysed by non-radioactive *in situ* hybridization. Sections were counterstained with nuclear fast red. The mRNA signal is shown as a blue (dark) precipitate. Sections of the PBS-treated mice hybridized with the appropriate sense probes of IGF-I (C), IGF-II (F) and IGFBP-2 (I) are also shown. R, resting zone; P, proliferative zone; H, hypertrophic zone. Scale bar represents 100 µm. Arrows indicate IGF-I mRNA, arrowheads indicate chondrocytes expressing no IGF-I mRNA.

In control animals, $56.5\% \pm 4.7\%$ of the chondrocytes in the growth plate expressed IGF-I (Fig. 3A), which was significantly decreased in all zones of the growth plate, to $35.4\% \pm 4.0\%$, in the DXM-treated animals (Fig. 3B) ($P < 0.01$). The expression pattern (presence in the various zones) of IGF-I was not affected by the DXM treatment.

IGF-II mRNA showed a similar expression pattern as IGF-I and was also predominantly expressed in the proliferative and prehypertrophic zones. Some staining was observed in the resting and hypertrophic zones. IGF-II mRNA was detected in $53.0\% \pm 2.6\%$ of the chondrocytes in control animals (Fig. 3D). The number of IGF-II-expressing chondrocytes was not affected by the DXM treatment ($49.0\% \pm 7.6\%$) (Fig. 3E).

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

Discussion

In our study, we showed that short-term GC treatment of prepubertal mice (i) induced growth retardation at body and organ level, (ii) significantly decreased tibial growth plate width, caused by a decrease in the width of the proliferative zone, (iii) decreased the number of proliferating chondrocytes and increased the number of apoptotic chondrocytes and (iv) decreased the number of IGF-I-expressing chondrocytes in the growth plate. This study also allowed us to compare the localization of components of the IGF axis in prepubertal mice (4-week-old) with our earlier study (Smink *et al.* 2002a) in postpubertal mice (7-week-old). Likewise, short-term GC treatment (1 week) could be compared with longer GC treatment (4 weeks) as performed in our earlier study (Smink *et al.* 2002a).

In the growth plates of prepubertal mice, we detected expression of IGF-I, IGF-II and IGFBP-2, while the other IGFBPs were not detected. This confirms our previous study, where we detected the same three components of the IGF system in the growth plates of postpubertal mice (Smink *et al.* 2002a). IGF-I, IGF-II and IGFBP-2 mRNAs were predominantly detected in the proliferative and prehypertrophic zones. In contrast, in our previous report on postpubertal mice, IGF-I and IGF-II were predominantly present in the hypertrophic zone and not in the proliferative zone (Smink *et al.* 2002a). The expression

of IGFBP-2 also shifts from being predominantly present in the proliferative and prehypertrophic zones in prepubertal mice to hypertrophic chondrocytes in postpubertal mice (Smink *et al.* 2002a). In previous reports on prepubertal animals, predominant expression of IGF-I (Lupu *et al.* 2001) and IGF-II (Wang *et al.* 1995) was also shown in the proliferative and prehypertrophic zones of the growth plate, whereas in rats of 9 weeks of age, IGF-I was predominantly present in the hypertrophic zone (Reinecke *et al.* 2000). We are the first to describe expression of IGFBP-2 in the postnatal growth plate; previously only low levels of IGFBP-6 were shown in murine growth plates, using radioactive *in situ* hybridization (Wang *et al.* 1995). The age-dependent expression pattern of the IGF system in the postnatal growth plate might indicate a shift in the function of the IGF system during chondrogenesis and postnatal development. Early in postnatal development, the local expression of the IGF system in the growth plate is probably important for the clonal expansion of chondrocyte columns in the proliferative zone, as postulated for IGF-I in the 'dual effector theory' (Ohlsson *et al.* 1998), as well as for the differentiation of proliferative chondrocytes into hypertrophic chondrocytes (Wang *et al.* 1999, LeRoith *et al.* 2001, Lupu *et al.* 2001, Siebler *et al.* 2001, Smink *et al.* 2002a). IGF-I has indeed been shown to stimulate growth plate chondrocytes at all stages of differentiation (Hunziker *et al.* 1994), which might also be true for the other components of the IGF system. It could well be that later on in postnatal development, after puberty, the main function of the IGF system is to augment chondrocyte hypertrophy (LeRoith *et al.* 2001, Smink *et al.* 2002a) and, to a much lesser extent, to stimulate the proliferation of chondrocytes. This latter function might become less important as the growth rate at postpubertal age is much lower.

The GC treatment of the prepubertal mice for 1 week inhibited total body length, weight and organ weights to a similar extent as observed after 4 weeks of treatment (Rooman *et al.* 1999). This suggests that most of the inhibiting effects of GCs on growth occur during the early period of the treatment. Tibial length (as a measure of longitudinal bone growth) was decreased after 1 week of GC treatment, although this decrease only became statistically significant after 4 weeks of treatment (Smink *et al.* 2002a). Clearly, the changes observed in the growth plate in response to GC treatment (see below) take some time before they result in a significant retardation of long bone growth. Other differential effects on organ growth are observed, since the thymus and spleen are more severely affected than the liver and the kidneys by the GC treatment, as also shown after the long-term GC treatment (Rooman *et al.* 1999).

GC-induced growth retardation is suggested to involve a mechanism intrinsic to the growth plate (Baron *et al.* 1992, Silvestrini *et al.* 2000). Therefore, we studied the effects of the short-term GC treatment on the growth

plate. The short-term GC treatment significantly decreased the total tibial growth plate width, confirming previous studies in rats (Silvestrini *et al.* 2000) and mice (Altman *et al.* 1992). The decrease in total growth plate width was caused by a significant decrease in the width of the proliferative zone. Proliferating cell nuclear antigen staining decreased, indicating a decreased proliferation which is likely to contribute to the decrease in the width of the proliferative zone. Several *in vitro* studies confirm the susceptibility of proliferative chondrocytes to GCs, in which GCs caused a decrease in proliferation (Robson *et al.* 1998, Klaus *et al.* 2000, Smink *et al.* 2002b). The GC treatment also affected the hypertrophic zone, increasing the number of apoptotic chondrocytes 1.6-fold. This finding confirms previous reports which showed a similar increase in apoptotic chondrocytes in GC-treated rats (Silvestrini *et al.* 2000, Sanchez & He 2002). Both the decreased proliferation and increased apoptosis in the growth plate probably contribute to the observed GC-induced growth retardation.

The growth retarding effects of GCs are suggested to involve impaired action of the IGF system (Price *et al.* 1992, Jux *et al.* 1998, Klaus *et al.* 2000). The short-term GC treatment decreased IGF-I serum levels, which confirms previous data in chickens (Leili & Scanes 1998). However, in humans (Miell *et al.* 1993, Ward *et al.* 1999) and during long-term GC treatment of mice (Rooman *et al.* 1999), GC treatment increased IGF-I serum levels. These discrepancies are probably due to species, age and/or treatment differences. The IGF-II serum levels are very low in mice and are not affected by GC treatment (Rooman *et al.* 1999). IGFBP serum levels were not affected by the DXM treatment, confirming previous reports (Leili & Scanes 1998, Rooman *et al.* 1999). Serum levels of the IGF system, however, are reported to provide little insight into the involvement of the IGF system in GC-induced growth retardation (Ward *et al.* 1999), indicating the importance of studying the local effects of GCs on the growth plate and the IGF system.

The short-term GC treatment decreased IGF-I expression in the growth plate whereas the number of IGF-II and IGFBP-2 expressing chondrocytes was not affected. The decrease in IGF-I-expressing chondrocytes is probably related to the decrease in proliferation, as indicated by the decreased number of PCNA-positive cells. In IGF-I knock-out mice, proliferation in the growth plate is decreased (Lupu *et al.* 2001) and also chondrocyte hypertrophy is inhibited (Wang *et al.* 1999, Lupu *et al.* 2001). This further strengthens our suggestion that a decrease in IGF-I expression in the growth plate, caused by the GC treatment, results in a decreased proliferation of chondrocytes. The differentiation of chondrocytes to the hypertrophic phenotype might also be inhibited, as IGF-I expression in the prehypertrophic zone is also decreased. The decreased proliferation, and possibly the decreased differentiation, would then result in a retardation of

the endochondral ossification, causing the retardation of longitudinal bone growth.

The decrease in IGF-I due to the short-term GC treatment is in contrast with the increase of IGF-I in the growth plate after long-term GC treatment (Smink *et al.* 2002a). This difference could be linked to a decrease in severity of the effects of GCs on growth and on the growth plate over time. However, it cannot be excluded that differences in the age of the treated mice at the termination of the experiment may also play a role. We showed in this study that the growth retarding effect of GCs after short-term treatment is almost equal to the growth retardation after long-term treatment (Rooman *et al.* 1999). This suggests that the different organs as well as the growth plate no longer seem to respond to the GCs. Indeed, it has been shown that the growth plate becomes less sensitive to GCs after a period of time, as the number of glucocorticoid receptors is decreased after a 3-week GC treatment in rats (Silvestrini *et al.* 2000). Thus the observed increase in IGF-I levels in the growth plate and serum after long-term treatment might be part of a compensatory mechanism for the induced growth retardation (Smink *et al.* 2002a), although the difference in age of the mice could also be involved in this observed difference. After both the short-term and the long-term GC treatment (Smink *et al.* 2002a), IGF-II and IGFBP-2 mRNA levels in the growth plate were unaffected. This suggests that, in contrast to IGF-I, IGF-II and IGFBP-2 are not involved in GC-induced growth retardation. In conclusion, we suggest that the action of IGF-I is impaired by GC treatment, as IGF-I levels are decreased after short-term GC treatment, and that this contributes to the GC-induced growth retardation.

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