IGF and IGF-binding protein expression in the growth plate of normal, dexamethasone-treated and human IGF-II transgenic mice

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Abstract

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

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

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

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

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

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Introduction

Long-term high-dose glucocorticoid (GC) treatment as anti-inflammatory and immunosuppressive therapy is associated with marked skeletal growth retardation in children (Allen 1996, Ward *et al.* 1999). In experimental animal models, high doses of GCs also have a growth-suppressive effect on longitudinal bone growth (Price *et al.* 1992, Leili & Scanes 1998, Rooman *et al.* 1999, Silvestrini *et al.* 2000). GCs have been shown to act locally to inhibit longitudinal growth, suggesting a local mechanism within the growth plate (Baron *et al.* 1992, Silvestrini *et al.* 2000).

Locally produced insulin-like growth factor (IGF) axis components, including both IGF-I and -II, their binding proteins (IGFBP)-1 to -6 and their receptors, play a key role in longitudinal bone growth (Ohlsson *et al.* 1998, Olney & Mougey 1999). Disruption of the IGF signaling results in growth retardation (Baker *et al.* 1993). Besides being produced by growth plate chondrocytes, exogenously added IGFs have a stimulatory effect on longitudinal bone growth (Hunziker 1994, Van Buul-Offers *et al.* 1994). IGF-I and IGF-II both have a unique and complementary role in augmenting longitudinal bone growth (LeRoith *et al.* 2001). However, there still exists some doubt as to whether both IGF-I and IGF-II are produced in the growth plate. Several *in vitro* data have shown expression of both IGFs (Bhaumick 1993, Olney & Mougey 1999), while several *in vivo* studies have shown conflicting results (Nilsson *et al.* 1990, Shinar *et al.* 1993,

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Wang *et al.* 1995, de los Rios & Hill 1999, Reinecke *et al.* 2000).

It has been suggested that the growth-inhibiting side-effect of GCs is partially mediated by impaired action of the IGF axis components in *in vitro* studies (Jux *et al.* 1998, Klaus *et al.* 2000, Koedam *et al.* 2000, Smink *et al.* 2002). GCs and IGF axis components have opposite effects on growth, as described above. In addition, GCs regulate the expression of IGFs (Jux *et al.* 1998) and IGFBPs (Koedam *et al.* 2000) in chondrocytes *in vitro*. Few data are available, however, concerning the regulation by GCs *in vivo* (Price *et al.* 1992) and it has been reported that serum levels of IGF axis components provide little insight into the mechanisms of GC-induced growth retardation and the possible involvement of the IGF axis components therein (Ward *et al.* 1999). Therefore, in order to elucidate the possible involvement of IGF axis components in GC-induced growth retardation, it is important to study locally produced IGFs and IGFBPs and their regulation by GCs in the growth plate.

We have previously treated 3-week-old female mice for 4 weeks with 20 μg dexamethasone (DXM)/day or saline (phosphate-buffered saline; PBS) (Rooman *et al.* 1999). These mice showed general growth retardation and a significant reduction in tibial length and weight. We have now studied the effects of this GC treatment on growth plate morphology. We have also studied the possible involvement of locally expressed IGFs and IGFBPs in this GC-induced growth retardation, by analyzing their expression in the growth plates of these mice using non-radioactive *in situ* hybridization. This study also served to analyze whether only IGF-II or both IGF-I and IGF-II are expressed by growth plate chondrocytes.

We previously suggested a possible protective effect of IGF-II against GC-induced growth retardation (Rooman *et al.* 1999). It was shown that DXM did not affect tibial length and weight to the same degree in human (h) IGF-II transgenic mice as in normal mice (Rooman *et al.* 1999). This suggested that hIGF-II overexpression might be able to partially counteract the growth-inhibitory effect of DXM at the level of the growth plate. In order to study the possible role of the hIGF-II transgene in GC-induced growth retardation, we also analyzed the morphology of the growth plates of these mice and the expression patterns of the IGFs and IGFBPs, which were compared with normal PBS- and DXM-treated animals.

Materials and Methods

Materials

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

Mice and tissue preparation

hIGF-II transgenic mice were generated by the introduction of a human IGF-II gene, under the control of the H-2K^b promoter and containing the SV40 small t intron and early polyadenylation signal, into FVB mice as described in detail previously (Van Buul-Offers *et al.* 1995). Throughout the study, the transgenic line designated 5′-74 (TgII) and normal FVB/N mice were used for our experiments. The animals were kept under standardized laboratory conditions.

Three-week-old female FVB and TgII mice were divided into two groups of five mice each. The animals were injected subcutaneously with 0·1 ml vehicle (PBS; pH 7·4) or 20 μg DXM once a day, 5 days a week for 4 weeks (experiment 2 in Rooman *et al.* 1999). The mice were killed by decapitation after ether anesthesia 2 h after the last injection, at the age of 7 weeks. At this age the animals are maturing sexually and are still growing (van Buul & Van den Brande 1978). The protocol received the approval of the Committee for Animal Experiments of the University Medical Center Utrecht, The Netherlands.

The tibiae were dissected and fixed in buffered 3·8% formalin for 24 h. Tibiae were subsequently decalcified for 24 h in 0·45 M phosphate-buffered EDTA, pH 8·0, washed in PBS, dehydrated through a series of ethanol and embedded in paraffin in a standardized manner to ensure proper orientation.

Morphometry of the growth plate

Growth plate sections were stained with hematoxylin and eosin and photographs of the sections were taken with a Zeiss Axiomat HRC camera equipped with AxioVision software version 3·0 (Zeiss, München-Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width. Corresponding sections of the tibia of the different groups were used to ensure correct comparison between the different groups. Total width of the growth plate (distance between the epiphysis and the chondro-osseous junction) was determined from one image (magnification × 50) per growth plate section, covering the entire transverse area excluding the periphery of the growth plate. Five animals per treatment group were analyzed (two sections per animal). From the images, measurements at 100 μm intervals were performed (about 18 measurements per growth plate) and averaged. The measuring lines were subsequently shortened to the first appearance of regular chondrocyte columns (the boundary between the resting and the proliferative zone) and to the first enlargement of the flattened cells (the boundary between the proliferative

and the hypertrophic zones). From the lengths of these lines the widths of the three different zones of the growth plate were calculated.

Probes

Standard RNA synthesis reactions using T7- or T3-RNA polymerase were carried out using digoxigenin-UTP as substrate (Melton *et al.* 1984). cDNAs encoding hIGF-I and IGF-II (Jansen *et al.* 1983, 1985), cDNA encompassing the 1 kb BamHI–BamHI SV40 fragment (Van Buul-Offers *et al.* 1995) and mouse IGFBP-1, -2, -3, -4, -5 and -6 cDNAs (kindly provided by S L S Drop and J W van Neck, Department of Pediatrics, Rotterdam, The Netherlands) (Schuller *et al.* 1993) were used as templates for the synthesis of antisense and sense digoxigenin-labeled cRNA probes. All probes used were specific for the mRNAs analyzed. The hIGF-II probe detects both endogenous mouse IGF-II as well as the hIGF-II transgene mRNA (Smink *et al.* 1999). The SV40 fragment detects specifically the hIGF-II transgene, making it possible to discriminate between the endogenous and transgene IGF-II mRNAs. Probes were checked for possible cross-hybridization using *in situ* hybridization on different types of mouse tissues (spleen, thymus and complete mice embryos) (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

Paraffin tissue sections (10 μm) were cut in a standardized way and mounted on 2% amino-propyl-triethoxy silane/3% glutaraldehyde-coated glass slides. Corresponding sections of the tibia of the different groups were used for the same probes to ensure reliable comparison between the groups. Sections were dewaxed, hydrated, rinsed in PBS and treated with proteinase K (0·21 units/ml) for 30 min at 37 °C, treated with 0·2 M HCl for 10 min at room temperature and subjected to an acetylation treatment (Wilkinson 1992). Sections were rinsed in 2 × SSC and kept in this solution until the start of the hybridization.

Hybridization was performed in a solution containing 50% formamide, 2 × SSC, 1 × Denhardt's solution, 1 μg/μl yeast RNA and 10% dextran sulphate and the digoxigenin-labeled cRNA probe at a concentration of 1000–1500 pg/μl. Sections were hybridized overnight at 51 °C, except for IGF-II where 58 °C was used. After hybridization, sections were washed with 50% formamide in 2 × SSC at the hybridization temperature for 30 min and treated with RNase A (1 unit/ml) for 30 min at 37 °C. Subsequently, sections were rinsed in 2 × SSC, treated with 10% lamb serum for 30 min and incubated with sheep anti-digoxigenin Fab-fragments coupled to alkaline phosphatase (1:1250) overnight at 4 °C.

Chromogenesis was performed with 0·38 mg/ml NBT and 0·19 mg/ml BCIP in the presence of 6% (w/v) polyvinylalcohol (De Block & Debrouwer 1993) at room temperature in the dark, resulting in a blue precipitate. Sections were counterstained with nuclear fast red, dehydrated through a series of ethanol and mounted with Euparal.

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

Statistical analysis

Data are expressed as means ± S.E.M. The effects of the DXM treatment and the hIGF-II transgene were statistically tested by one-way ANOVA with Bonferroni–Dunn post hoc tests using InStat version 3·00 (GraphPad Software, Inc., San Diego, CA, USA). The effects of the DXM treatment on body and tibial length of both mice strains (FVB and hIGF-II transgenic) were calculated using Student's *t*-test. A P value of less than 0·05 was considered statistically significant.

Results

Growth plate morphology

Normal FVB mice were treated for 4 weeks with 20 μg/day DXM, which resulted in a significant inhibition of body length and weight (Rooman *et al.* 1999). Tibial length was also significantly inhibited, to a similar extent as total body length as summarized in Table 1. DXM treatment of hIGF-II transgenic mice resulted in a similar inhibition of body length as in FVB mice. However, tibial length was not significantly affected in the hIGF-II transgenic mice (Table 1).

DXM treatment of FVB mice had no effect on the total width of the proximal tibial growth plate. However, the width of the proliferative zone was significantly decreased (by 11%), whereas the width of the hypertrophic zone was significantly increased (by 17%). The resting zone was not affected by the DXM treatment (Table 2). hIGF-II

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

	Body				**Tibia**			
	Length (cm)	S.E.M.	*P* value[1]	Inhibition by DXM (%)	Length (mm)	S.E.M.	*P* value[1]	Inhibition by DXM (%)
Group								
FVB PBS	18·06	0·26			17·11	0·23		
FVB DXM	17·24	0·06	<0·02	4·5	16·49	0·10	<0·05	3·6
TgII PBS	17·86	0·07			16·58	0·13		
TgII DXM	17·00	0·09	<0·01	4·8	16·27	0·18	NS	1·9

[1]Compared with PBS control within the same strain.

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

	Total (μm)	S.E.M.	**R** (μm)	S.E.M.	**P** (μm)	S.E.M.	**H** (μm)	S.E.M.
Group								
FVB PBS	168·2	4·9	21·9	1·1	85·6	3·7	59·7	1·8
FVB DXM	170·9	1·8	24·4	0·8	76·6[1]	1·1	69·9[1]	1·5
TgII PBS	188·0[1]	2·6	25·6	1·8	93·2	1·5	68·9	1·8
TgII DXM	177·0	4·6	26·4	1·1	80·2[2]	1·6	70·3	3·9

Total, total growth plate width; R, resting zone; P, proliferative zone; H, hypertrophic zone. [1]$P<0·05$ compared with the normal, PBS group; [2]$P<0·05$ compared with the TgII, PBS group.

transgenic mice showed a significantly larger width of the growth plate compared with untreated FVB mice (12% increase). This increase was caused by a non-significant increase in the width of all three zones of the growth plate. Treatment with DXM caused a significant decrease in the width of the proliferative zone (14%), a similar decrease as that seen in FVB mice. In contrast, in hIGF-II transgenic mice, DXM did not affect the width of the hypertrophic zone (Table 2). As in FVB mice, the resting zone was not affected by the DXM treatment.

Expression of IGF axis components in the postnatal growth plate

To analyze the endogenous expression of the IGFs and their binding proteins, IGFBP-1 to -6, in the postnatal epiphyseal growth plate of mice, non-radioactive *in situ* hybridization was performed on tibial epiphyseal growth plates of 7-week-old PBS-treated normal mice. At the age of 7 weeks, the mice are maturing sexually and are still growing (van Buul & Van den Brande 1978). As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals in any of the treatment groups (Figs 1E, 2E and 3E).

In the growth plates of these mice, IGF-I, IGF-II and IGFBP-2 transcripts were present, each showing a similar expression pattern. However, no expression of IGFBP-1, -3, -4, -5 and -6 could be detected (data not shown). Using the same probes, we have previously demonstrated the expression of IGFBP-2, -3, -4, -5 and -6 in lymphoid tissues (Smink *et al*. 1999).

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

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

Of the IGFBPs, only IGFBP-2 could be detected in the postnatal growth plate (Fig. 3A). IGFBP-2 transcripts

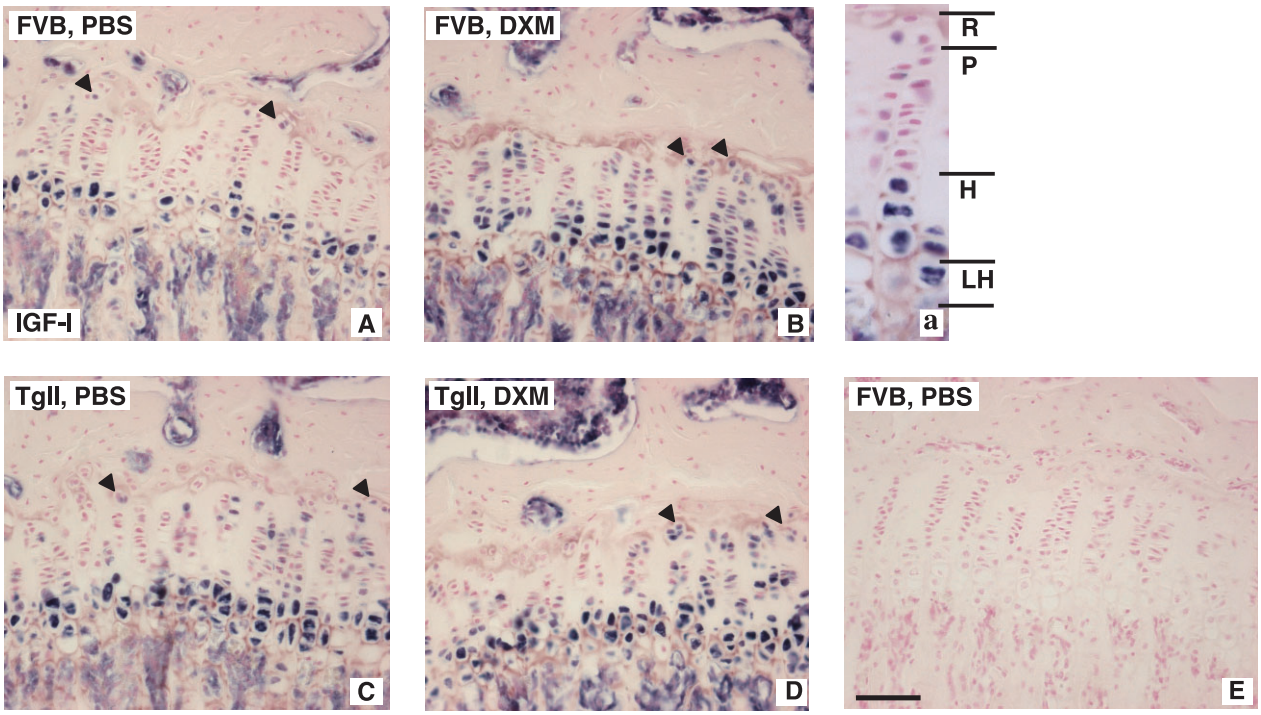

Figure 1 IGF-I expression in the postnatal growth plate. Expression patterns of IGF-I mRNA in representative sections of the growth plates of 7-week-old mice. (A) PBS-treated FVB mice, (B) DXM-treated FVB mice, (C) PBS-treated hIGF-II transgenic mice, (D) DXM-treated hIGF-II transgenic mice and (E) section of the PBS-treated FVB mice hybridized with an IGF-I sense probe, as analyzed by non-radioactive *in situ* hybridization, under brightfield illumination. Magnification × 200. Sections were counterstained with nuclear fast red. The mRNA signal is shown as a blue precipitate under brightfield illumination. (a) Detail of signal in PBS-treated FVB mice, magnification × 400. R, resting zone; P, proliferative zone; H, hypertrophic zone; LH, late hypertrophic zone. Arrowheads indicate IGF-I mRNA in the resting zone. Scale bar, 100 μm.

were mainly detected in the hypertrophic zone and fewer cells in the late hypertrophic zone expressed IGFBP-2 mRNA. As for IGF-I and IGF-II, IGFBP-2 mRNA was also detected in the proliferative cells located at the periphery of the growth plate, but not in the proliferative cells in the center of the tibia. No IGFBP-2 mRNA was detected in the resting zone. IGFBP-2 was also expressed in osteoblasts lining the trabeculae.

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

DXM treatment resulted in a significant 2·4-fold (± 0·3, $P<0·001$) increase in the number of IGF-I-expressing chondrocytes in the epiphysial tibial growth plate (Fig. 1B). In the center of the growth plate, more rows of cells were positive for IGF-I, including proliferative chondrocytes. In contrast, in PBS-treated FVB mice, no proliferative chondrocytes were positive for IGF-I. IGF-I expression in the resting zone was not altered because of the DXM treatment. Although the width of the hypertrophic zone increased because of the DXM treatment, this could only partially account for the increase in IGF-I-expressing cells as, besides hypertrophic chondrocytes, proliferative chondrocytes also expressed IGF-I. The expression patterns and levels of IGF-II (Fig. 2B) and IGFBP-2 (Fig. 3B) were similar to those found in the growth plates of control mice.

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

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

To analyze the influence of hIGF-II overexpression on the expression of IGF axis components in the growth plate, sections of the growth plates of PBS-treated hIGF-II transgenic mice were also analyzed. The hIGF-II transgene itself was detected using a transgene specific probe (SV40 probe). The transgene was not detected in the epiphyseal growth plate, although transgene mRNA was detected in osteoblasts lining the trabeculae (Fig. 4C). The number of IGF-I-expressing cells was significantly increased 1·9-fold (± 0·3, $P<0·05$) in hIGF-II transgenic mice (Fig. 1C). This included positive cells in the proliferative zone. In contrast, the number of cells expressing

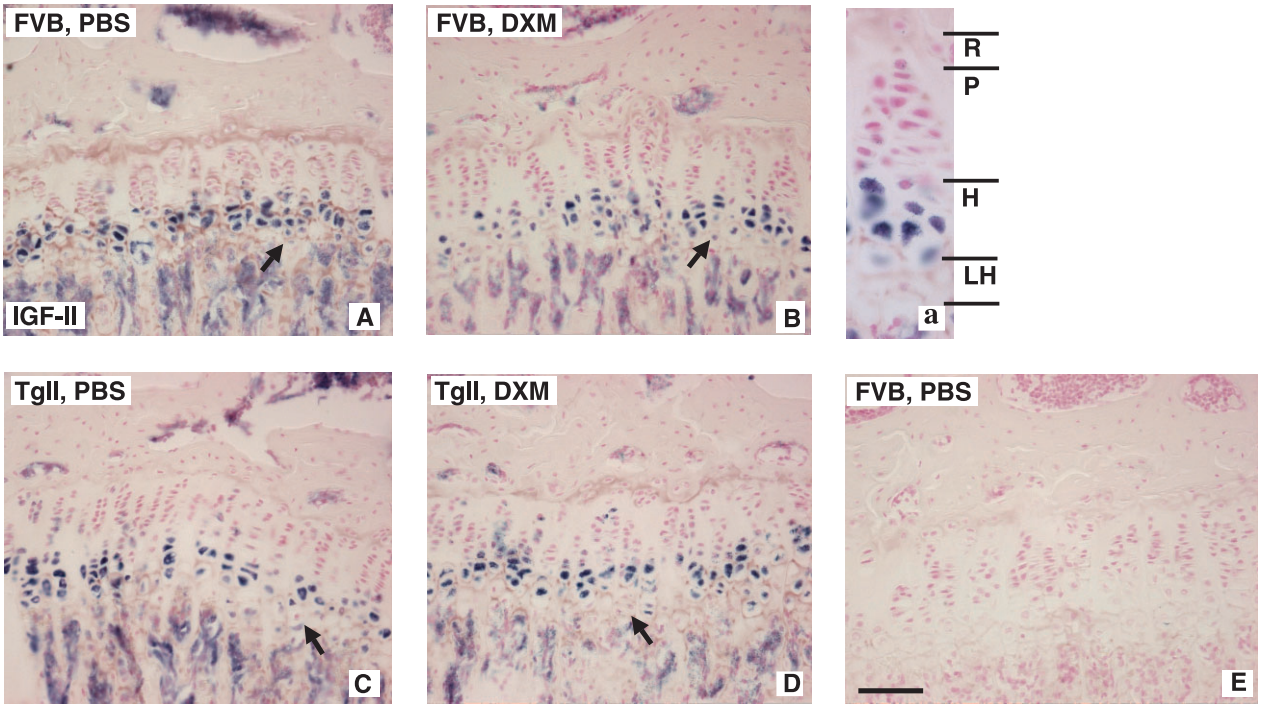

Figure 2 IGF-II expression in the postnatal growth plate. Expression patterns of IGF-II mRNA in representative sections of the growth plates of 7-week-old mice. (A) PBS-treated FVB mice, (B) DXM-treated FVB mice, (C) PBS-treated hIGF-II transgenic mice, (D) DXM-treated hIGF-II transgenic mice and (E) section of the PBS-treated FVB mice hybridized with an IGF-II sense probe. Sections were treated as described in Fig. 1. Magnification × 200. (a) Detail of signal in PBS-treated FVB mice, magnification × 400. Arrows indicate the lower level of expression in the late hypertrophic (LH) zone, compared with the hypertrophic (H) zone. Scale bar, 100 μm.

endogenous IGF-II (Fig. 2C) and IGFBP-2 (Fig. 3C) in the growth plate were not changed in hIGF-II transgenic mice as compared with normal mice. No expression of IGFBP-1, -3, -4, -5 and -6 was detected in the postnatal growth plates of hIGF-II transgenic mice (data not shown).

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

When DXM-treated hIGF-II transgenic mice were compared with PBS-treated normal mice, the number of IGF-I-expressing chondrocytes in the epiphyseal tibial growth plate increased 2·6-fold (± 0·3), to a level similar to that observed in DXM-treated normal mice, including proliferative chondrocytes (Fig. 1D). This increase was significant when compared with control normal mice ($P<0·001$), but not significant when compared with PBS-treated hIGF-II transgenic mice. Endogenous IGF-II and IGFBP-2 gene expression were not affected by the DXM treatment (Figs 2D and 3D respectively). IGFBP-1, -3, -4, -5 and -6 transcripts were not detected in hIGF-II transgenic mice treated with DXM (data not shown). We observed no change in transgene expression levels caused by the DXM treatment (Fig. 4D).

Discussion

There are strong indications that GC-induced growth retardation involves impaired action of the IGF axis components (Price *et al.* 1992, Jux *et al.* 1998, Klaus *et al.* 2000, Koedam *et al.* 2000, Smink *et al.* 2002). In the present study, we analyzed the *in vivo* effects of GCs on the expression of the IGF axis components at the local level of the epiphyseal growth plate, where longitudinal growth is regulated and adverse effects of GCs on growth are expected to be targeted (Siebler *et al.* 2001). Besides studying the effects of GCs on the expression of the IGF axis components, the possible protective effect of IGFs against GCs was also studied using hIGF-II transgenic mice.

The analyses performed in this study on the endogenous expression of the IGF axis components in the mouse postnatal growth plate (postnatal day (P) 49), showed expression of IGF-I, IGF-II and IGFBP-2. Until now, there have been conflicting reports whether *both* the IGFs are expressed in the postnatal epiphyseal growth plate. Expression of IGF-I was shown in the rat epiphyseal growth plate (P10, P28, P35) (Nilsson *et al.* 1990, Reinecke *et al.* 2000) and costochondral growth plate (Lin & Oberbauer 1999). In contrast, Wang *et al.* (1995)

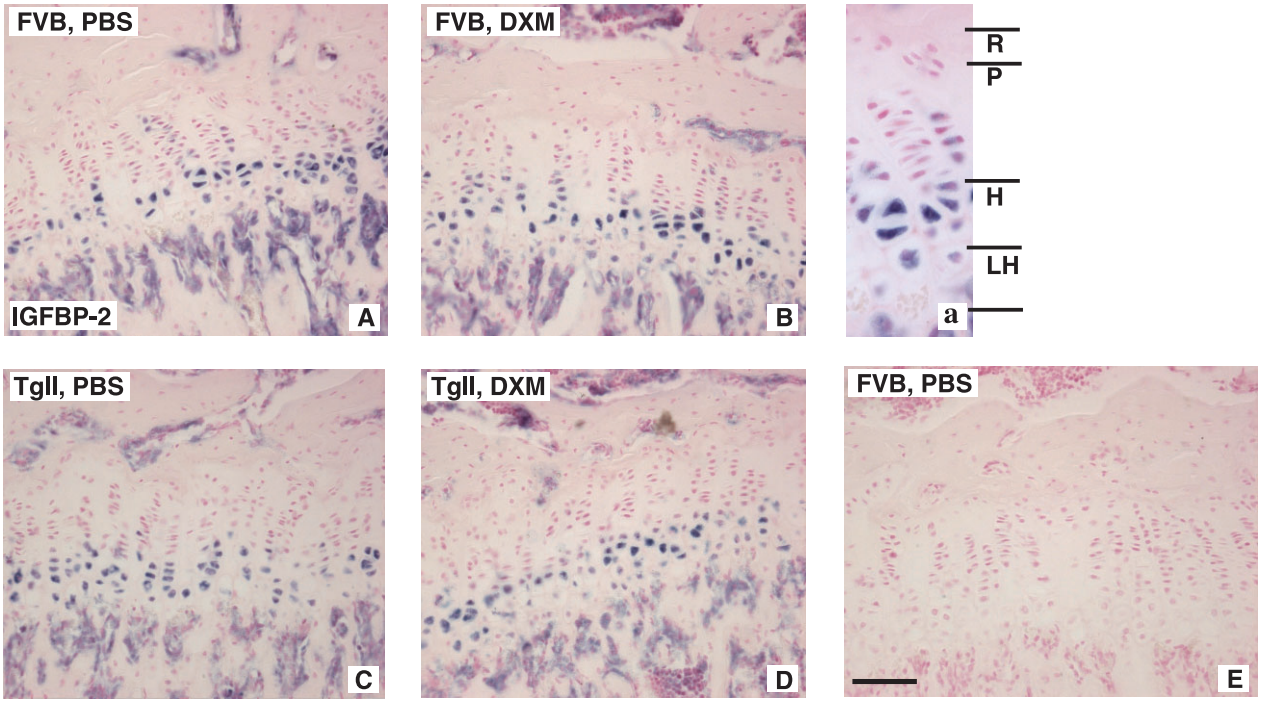

Figure 3 IGFBP-2 expression in the postnatal growth plate. Expression patterns of IGFBP-2 mRNA in representative sections of the growth plates of 7-week-old mice. (A) PBS-treated FVB mice, (B) DXM-treated FVB mice, (C) PBS-treated hIGF-II transgenic mice, (D) DXM-treated hIGF-II transgenic mice and (E) section of the PBS-treated FVB mice hybridized with an IGFBP-2 sense probe. Sections were treated as described in Fig. 1. Magnification × 200. (a) Detail of signal in PBS-treated FVB mice, magnification × 400. Scale bar, 100 μm.

detected only IGF-II in murine growth plates (P25) and Shinar *et al.* (1993) reported similar results in rat growth plates (P10–P35). Although species differences and age variables will influence the observed expression signals, the conflicting data are more likely caused by the detection method used. In previous studies (Nilsson *et al.* 1990, Shinar *et al.* 1993, Wang *et al.* 1995), in which radioactive *in situ* hybridization was used, less clear expression of IGF-I and IGF-II was found. This was confirmed by Reinecke *et al.* (2000), who also showed a better visible signal of IGF-I expression using non-radioactive *in situ* hybridization than that shown previously by the radioactive method (Nilsson *et al.* 1990). It is unlikely that the detection of both IGF-I and IGF-II is due to cross-hybridization between the probes used (described in Materials and Methods). In addition, there are differences in the expression patterns and responses to DXM treatment and the presence of the hIGF-II transgene, as discussed below.

IGF-I was mainly detected in cells of the hypertrophic zone and fewer positive cells were detected in the late hypertrophic zone. Only a few cells expressing IGF-I were detected in the resting and proliferative zones. This distribution confirms previous data (Reinecke *et al.* 2000). Local IGF-I synthesis of proliferative chondrocytes was previously thought to stimulate the clonal expansion of chondrocyte columns in the proliferative zone in an autocrine/paracrine manner, as described by the 'dual effector theory' (Ohlsson *et al.* 1998). It should be borne in mind, however, that the mitogenic properties of the IGFs have only been demonstrated in cultured cells, and not in *in vivo* models. In our study, only a small number of cells in the proliferative zone expressed IGF-I, whereas IGF-I expression was mainly detected in the hypertrophic zone, suggesting that IGF-I could be involved in the differentiation of proliferative to hypertrophic chondrocytes. In IGF-I knock-out mice, hypertrophic chondrocytes are reduced by 30% in linear dimension, accounting for most of the observed decrease in longitudinal growth (Wang *et al.* 1999). This further strengthens the notion that IGF-I plays an important role in augmenting chondrocyte hypertrophy (LeRoith *et al.* 2001, Lupu *et al.* 2001, Siebler *et al.* 2001). Furthermore, infusion of IGF-I in hypophysectomized rats showed that IGF-I stimulates growth plate chondrocytes at all stages of differentiation, including hypertrophic chondrocytes (Hunziker *et al.* 1994), rather than acting specifically upon proliferative chondrocytes as postulated in the 'dual effector theory'. Previously, there was uncertainty as to the source of the IGF-I that would promote the chondrocyte hypertrophy (LeRoith *et al.*

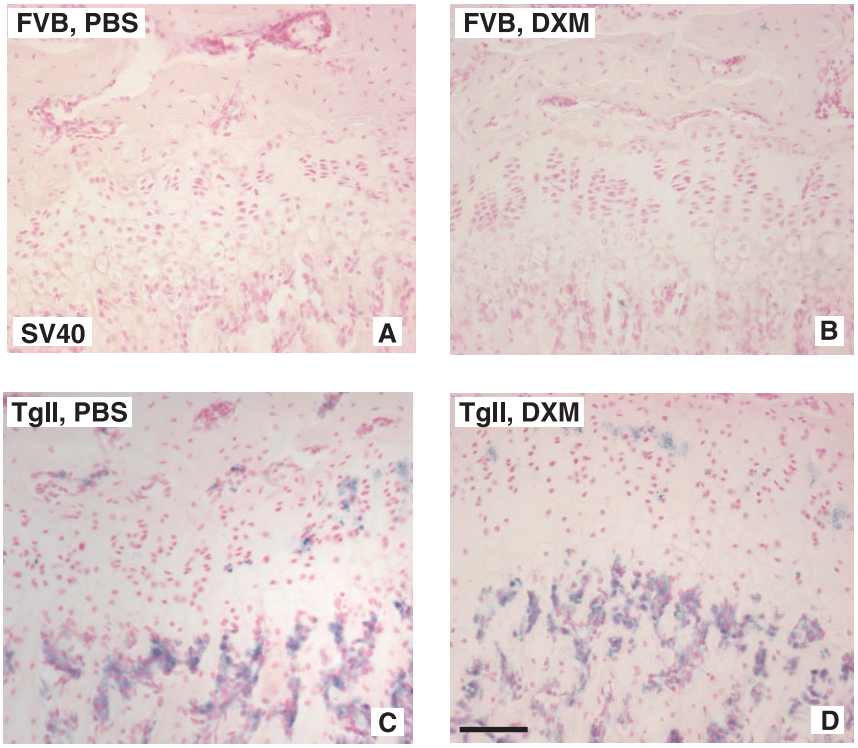

Figure 4 Transgene expression in the postnatal growth plate. Expression patterns of transgene mRNA in representative sections of the growth plates of 7-week-old mice. (A) PBS-treated FVB mice, (B) DXM-treated normal mice, (C) PBS-treated hIGF-II transgenic mice and (D) DXM-treated hIGF-II transgenic mice. Sections were treated as described in Fig. 1. Magnification × 200. Blue staining observed in the extracellular matrix of the growth plate is non-specific. Scale bar, 100 μm.

2001). Our study provides strong evidence that the source of the IGF-I is the growth plate itself. However, besides having a role in differentiation of chondrocytes, IGF-I may still play a role in the clonal expansion of proliferative chondrocytes, in a paracrine manner. Our data, however, suggest that the 'dual effector theory' should be extended with a role for IGF-I in chondrocyte differentiation. The type I IGF receptor, which mediates the intracellular effects of both IGF-I and IGF-II (Baserga *et al.* 1997), is mainly expressed in the proliferative and hypertrophic zones of the growth plate (Wang *et al.* 1995, Siebler *et al.* 2001). However, in the hypertrophic zone, distinctly higher levels of type I IGF receptor mRNA levels are present compared with the proliferative zone (Wang *et al.* 1995), supporting the suggestion that in addition to stimulating proliferation, IGFs play a role in differentiation of chondrocytes.

The distribution of IGF-II transcripts confirms previous data, which showed predominant expression in the hypertrophic zone and no (in our study, very low) expression in the terminally differentiated chondrocytes at the osteochondral junction (Wang *et al.* 1995). IGF-II has been suggested to play a role in the proliferation of chondrocytes, and also in their differentiation (Hill 1992). The presence of both IGFs, their distinct expression patterns and the difference in response to GC treatment (see below) strengthens the suggestion that they have a unique and complementary role in regulating bone growth in a paracrine manner (LeRoith *et al.* 2001).

Concerning the IGFBPs, we are the first to detect expression of IGFBP-2 in the postnatal growth plates of sexually maturing mice (P49). The detection of IGFBP-2 is specific and is not due to cross-hybridization with other IGFBPs (described in Materials and Methods). This contrasts with the fetal situation where expression of IGFBP-2 to -6 in the growth plate *in vivo* has been reported previously in sheep (de los Rios & Hill 1999) and cows (Olney & Mougey 1999). However, expression was at such a low level that detection was only possible using RT-PCR (Olney & Mougey 1999, de los Rios & Hill 1999). In fetal murine growth plates (Wang *et al.* 1995), radioactive *in situ* hybridization only showed expression of IGFBP-5 and -6 in the growth plate, which rapidly declined with age. IGFBP-5 was no longer detectable after embryonic day 18 and only low levels of IGFBP-6 were detectable up to at least P25 (Wang *et al.* 1995).

IGFBP-1 has never been detected in the growth plate, neither pre- nor postnatally (Luo & Murphy 1989, Wang *et al.* 1995, Olney & Mougey 1999). In our study, IGFBP-2 is mainly expressed in the hypertrophic zone in co-localization with IGF-I and IGF-II. Various studies have suggested that IGFBP-2 plays a role in growth and development (Streck *et al.* 1992, Hoeflich *et al.* 1999). In addition, a role for IGFBP-2 in the growth plate is suggested by its presence in cultured chondrocytes (Borromeo *et al.* 1996, Smink *et al.* 2002). The specific localization of IGFBP-2 in the pre- and hypertrophic zone of the growth plate and the fact that IGFBP-2 is the only member of the IGFBPs present at this stage of development suggests a modulating role for this IGFBP in chondrocyte differentiation and maturation, in collaboration with the IGFs.

Treatment of normal mice with DXM induced general growth retardation and a decrease in tibial length (experiment 2 in Rooman *et al.* 1999). We now report that total growth plate width was not affected, while the proliferative zone decreased and the hypertrophic zone increased. In contrast, in hIGF-II transgenic mice, DXM decreased only total body length, whereas tibial length was not affected. The DXM-induced reduction in the proliferative zone of the growth plate remained, whereas the hypertrophic zone was no longer affected in the hIGF-II transgenic mice. Thus, there appears to be a correlation between tibial growth retardation and an increase in the hypertrophic zone of the growth plate. This increase of the hypertrophic zone could be caused by an accelerated differentiation of proliferative chondrocytes. Premature maturation of chondrocytes has previously been shown to lead to growth retardation. In parathyroid hormone-related protein (PTHrP) receptor knockouts for example, an accelerated chondrocyte differentiation led to premature ossification (Lanske *et al.* 1996, Jobert *et al.* 1998). However, the effect of GCs on the proliferative zone could also be important in GC-induced growth retardation, as shown in *in vitro* studies (Jux *et al.* 1998, Robson *et al.* 1998, Smink *et al.* 2002).

Treatment of normal mice with DXM also increased the number of chondrocytes expressing IGF-I. The increase of IGF-I-expressing cells can only partially be explained by the increase of the hypertrophic zone as, besides hypertrophic chondrocytes, proliferative chondrocytes also express IGF-I after DXM treatment. In addition, we also reported elevated serum levels of IGF-I (Rooman *et al.* 1999). We have shown no effects on IGF-II and IGFBP-2 mRNA levels in the growth plate, and no effects on serum IGF-II and IGFBP-2 levels have been detected (Rooman *et al.* 1999). The increase in IGF-I due to GC treatment is surprising, since IGFs are thought to promote growth. It could be that the increase of IGF-I in the growth plate might counteract the adverse effects of GCs on the growth plate in an attempt to diminish growth retardation. The observed increase, however, is not sufficient to completely abolish the observed growth deficit. IGF-I has indeed been shown previously to be able to counteract the anti-proliferative effects of GCs, both *in vitro* as well as *in vivo* (Klaus *et al.* 2000). However, this is the first study to describe an upregulation of IGF-I in response to GC treatment, possibly as a compensatory mechanism. Another possibility could be that the increase of IGF-I in the growth plate might result in an increased differentiation of chondrocytes, resulting in thickening of the hypertrophic zone, accelerated ossification and growth retardation. Probably other factors are involved as well, such as, for example, the Indian hedgehog–PTHrP negative feedback loop (van der Eerden *et al.* 1999), core binding factor α-1 (Cbfa1), transforming growth factor-β, fibroblast growth factors and others (Hering 1999, Stevens & Williams 1999). The effects of GC treatment on the type I IGF receptor levels in the growth plate *in vivo* are not known. However, *in vitro* data from chondrocytes showed that DXM had no effect on type I IGF receptor levels (Jux *et al.* 1998). However, a possible involvement of the type I IGF receptor in GC-induced growth retardation cannot be excluded.

It is noteworthy that the hIGF-II transgenic mice also have increased IGF-I mRNA levels in the growth plate as compared with normal mice, whereas IGF-II and IGFBP-2 levels are not affected. However, this increase in IGF-I has no effect on tibial growth of the hIGF-II transgenic mice (Rooman *et al.* 1999). Transgene-specific hybridization showed that the hIGF-II transgene is not expressed in the growth plate, but only in osteoblasts lining the trabeculae. The transgene in osteoblasts could be responsible for the increase in IGF-I levels in the growth plate in a paracrine manner as has been shown by us for IGFBP expression in lymphoid tissues (Smink *et al.* 1999). In addition, the transgene is also thought to be able to regulate circulating IGF-I as in the hIGF-II transgenic mice serum IGF-I levels are elevated (Rooman *et al.* 1999). The hIGF-II transgene in the osteoblasts does not result in an increase of IGFBP-2 in the growth plate, and no effect on IGFBP-2 serum levels has been shown (Rooman *et al.* 1999). In contrast, in the lymphoid tissues, the presence of the hIGF-II transgene results in an increase of IGFBP-2 (Smink *et al.* 1999). This difference might be due to the localization of the hIGF-II transgene and IGFBP-2, which are more co-localized in the lymphoid tissues than in the growth plate. This difference in localization apparently does not hamper the effect of the transgene on IGF-I expression. These hIGF-II transgenic mice do show a similar growth retardation to that of the normal mice when treated with DXM. However, while this treatment resulted in a significant reduction in tibial length and weight of normal mice, no significant effects on the tibia and the hypertrophic zone of the growth plate of hIGF-II transgenic mice were observed. The high levels of IGF-I already present in the growth plate of the untreated hIGF-II transgenic mice might be responsible for the

observed partial protection against the GCs with regard to growth retardation of the tibia. This is supported by data that showed that increased IGF-I levels, in the presence of GCs, could counterbalance the growth-inhibiting effect of the GCs (Klaus *et al.* 2000).

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