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

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Abstract

High (pharmacological) doses of glucocorticoids inhibit the proliferation of growth plate chondrocytes, which leads to one of the side-effects of these steroids, namely suppression of longitudinal growth. Growth inhibition by glucocorticoids is thought to be mediated in part by impaired action of components of the IGF axis, which are important for chondrocyte regulation and hence for longitudinal growth. The aim of the present study was to determine whether glucocorticoid-induced growth retardation involves changes in IGF axis components. Chondrocytes were isolated from epiphyseal growth plates of neonatal piglets and treated with pharmacological doses of dexamethasone (DXM) for 24 h to study glucocorticoid-induced growth retardation. Under IGF-I-supplemented (10 nM) culture conditions, IGF-binding proteins (IGFBPs)-2, -4 and -5 were secreted by the growth plate chondrocytes and IGFBP-2 protein and mRNA levels were decreased by the DXM treatment, whereas IGFBP-4 and -5 were not affected. Proliferation of the chondrocytes, as measured by [3H]thymidine incorporation, was 3-5-fold higher in serum-supplemented medium in contrast to IGF-I-supplemented (10 nM) medium. In the presence of serum, DNA synthesis was significantly inhibited by 50–63% when treated with 100 nM DXM, which was prevented by the glucocorticoid-receptor antagonist Org34116. mRNA levels of IGF axis components were determined using Northern blot analysis. IGFBP-2 to -6 were expressed in the chondrocytes, IGFBP-1 was absent and both IGF-I and IGF-II, and the type I and type II IGF receptors were expressed. Treatment with DXM (100 nM) resulted in a 2-fold increase in mRNA levels of both IGFBP-5 and the type I IGF receptor, whereas IGFBP-2 mRNA levels decreased by 55%, in concert with the decrease in protein level observed under IGF-I-supplemented culture conditions. The changes in mRNA levels due to the DXM treatment were prevented by the glucocorticoid receptor antagonist.

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

Introduction

Longitudinal bone growth results from the proliferation, differentiation, maturation and eventually apoptosis of chondrocytes within the growth plates of the long bones (Stevens & Williams 1999). These processes must be correctly coordinated in order to maintain normal growth.

Glucocorticoids (GCs), although effective drugs in the treatment of several diseases, such as asthma, respiratory distress syndrome and rheumatoid arthritis, may induce growth retardation in children as a side-effect (Allen 1996). Studies in experimental animal models have also shown that high levels of GCs have a growth suppressive effect on longitudinal bone growth (Price et al. 1992, Leili & Scanes 1998, Rooman et al. 1999, Stevens & Williams 1999). Several in vivo studies showed that GCs act locally to restrain growth, suggesting a local mechanism which is intrinsic to the growth plate (Baron et al. 1992, Silvestrini et al. 2000).

The inhibitory actions of GCs on longitudinal growth are suggested to be due to impaired action of the components of the insulin-like growth factor (IGF) axis (Price et al. 1992, Jux et al. 1998, Klaus et al. 1998). IGF-I and IGF-II are important mediators of longitudinal growth, as shown by in vitro and in vivo experiments (Van Buul-O ffers et al. 1988, D’Ercole 1996, Ohlsson et al. 1998). The IGFs are potent mitogenic and differentiation-promoting growth factors (Cohick & Clemmons 1993). They are produced by multiple tissues, and can act in both an endocrine and autocrine/paracrine fashion. IGFs are...
bound to members of high-affinity IGF-binding proteins (IGFBPs), which modulate the IGF availability and bioactivity (Clemmons 1997, Rajaram et al. 1997). They act mainly as autocrine and/or paracrine factors at or close to their sites of synthesis and have different affinities for the IGFs (Jones & Clemmons 1995). Growth plate chondrocytes produce both IGFs and IGFBPs, which are presumed to be important for chondrocyte regulation and hence for longitudinal growth (Price et al. 1992, Bhaumick 1993, Sunic et al. 1995, Olney & Mougey 1999). Both IGFs and several IGFBPs are regulated by GCs (Dell et al. 1999), both in vitro, e.g. in fibroblasts (Conover et al. 1995) and in osteoblasts (Okazaki et al. 1994), as well as in vivo, i.e. determined in serum (Price et al. 1992, Miell et al. 1993, Rooman et al. 1999, Ward et al. 1999) and in liver and lung tissue (Price et al. 1992). However, scarce data are available with respect to the regulation of IGFBPs by GCs on growth plate chondrocytes. We have reported that in rabbit costal chondrocytes, IGFBP-5 expression is down-regulated and IGFBP-3 expression is induced by dexamethasone (DXM) (Koedam et al. 2000). In rat growth plate chondrocytes, GCs impair growth hormone (GH)-induced stimulation of local secretion and paracrine action of IGF-I, which would contribute to GC-induced growth retardation (Jux et al. 1998). However, in patients treated with GCs, serum IGF-I levels are usually not altered, while its bioactivity is (Cautri & Copinschi 1986), indicating that the IGFBPs might be the IGF axis components that are involved in GC-induced growth retardation (Price et al. 1992). Since locally produced IGFs are suggested to have an autocrine/paracrine action on growth and GCs act locally to inhibit longitudinal growth, serum levels of IGF axis components provide little insight into the mechanisms by which GCs induce growth retardation (Ward et al. 1999).

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

Materials and Methods

Materials

Restriction enzymes, modifying enzymes, Tripure isolation reagent and Agarose Gel DNA extraction kit were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Nylon membranes (Hybond-N’), RediPrime Random Primer labeling mixture, [α-32P]dCTP (10 mCi/ml), [3H]methyl-thymidine (1 mCi/ml), the Hyperfilm ECL film and the horseradish peroxidase-conjugated secondary antibody were obtained from Amersham Pharmacia Biotech. Collagenase (C-9891) and protease (P-6911) enzymes were purchased from Sigma Chemical Co. Dulbecco’s modified Eagle’s medium (DMEM), t-glutamine, penicillin, streptomycin, fetal calf serum (FCS) and SuperScript II Reverse Transcriptase were obtained from Life Technologies. The TOPO TA Cloning kit was obtained from Invitrogen.

Cell strainers were purchased from Becton Dickinson. Centricron-10 ultrafiltration membranes and PVDF (Immobilon-P) were from Millipore Corp. BioMax MR X-ray films were obtained from Kodak. Supersignal chemiluminescent substrate was purchased from Pierce. Org34116 (a GC receptor (GR) antagonist) was provided by Organon (Oss, The Netherlands). Dexamethasone disodium phosphate (DXM) from Merck Sharp & Dohme was from our hospital pharmacy.

Recombinant human IGF-I was kindly provided by Eli Lilly & Co. The human IGFBP-2 antibody (rabbit antibody directed against a peptide sequence from bovine IGFBP-2), the human IGFBP-5 antibody (rabbit antibody directed against a peptide sequence from human IGFBP-5) and the recombinant human IGFBP-2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Recombinant human IGFBP-5 was obtained from GroPep (Adelaide, Australia). Human IGF-I to -6 cDNAs were kindly provided by Dr S Shimasaki (La Jolla, CA, USA) (Han et al. 1996). GAPDH cDNA was a gift from Dr H van Teefelen (Utrecht, The Netherlands) (Dufourny et al. 1997). Rat type I (Werner et al. 1989) and mouse type II IGF receptor cDNAs (Ludwig et al. 1992) were a gift from Dr J W van Neck (Rotterdam, The Netherlands). Human IGF-I (Jansen et al. 1983) and -II (Jansen et al. 1985) cDNAs were obtained from Dr M Jansen (Utrecht, The Netherlands).

Cell culture

Epiphyseal growth plate chondrocytes were isolated from the distal and proximal tibial growth plate of neonatal piglets by splitting the growth plate transversally and dissecting the growth plate free under sterile conditions. The growth plates were minced with a scalpel and digested with 1 mg/ml protease for 30 min at 37°C with constant shaking in DMEM containing 4.5 g/l glucose, 4 mM t-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, followed by digestion with 0.5 mg/ml collagenase for 6 h at 37°C in the same medium supplemented with 10% FCS. Cells were filtered through a sterile 70 µm nylon cell strainer, centrifuged, counted and seeded in 75 cm2 tissue culture flasks. The cells were grown as a monolayer culture at 37°C in a 5% CO2 and humid atmosphere.

The chondrocyte phenotype of the cells was verified by Alcian blue staining for sulfated proteoglycans (Leonard et al. 1991), expression of type II collagen (Cancedda et al. 1995) and cartilage-derived retinoic acid-sensitive protein
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(1996) by Northern blot analysis (Koedam et al. 2002).

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

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

\[ {\text{H}} \text{thymidine incorporation} \]

Cells were grown on 24-well plates and incubated with increasing concentrations of DXM for 24 h, with or without the GR antagonist, as described above and each experiment was conducted in triplicate, both in serum-free conditions supplemented with 10 nM IGF-I/0·005% BSA and in 10% FCS conditions. The rate of chondrocyte proliferation was assessed by incubating the cells in 0·5 µCi/ml of \[ {\text{H}} \text{thymidine] for the final 4 h of incubation. Cells were subsequently washed with PBS to remove unincorporated isotope and the DNA and protein were precipitated with three incubations of 1 ml 10% (w/v) ice-cold trichloroacetic acid for 15 min at 4 °C and solubilized by incubation at room temperature with 0·4 M NaOH for 1 h. Isotope incorporation was measured by liquid scintillation counting.

\[ \text{Western blotting} \]

Cells were first preincubated with serum-free medium for 2 h, after which medium was changed for medium supplemented with 10 nM IGF-I and 0·005% BSA. Subsequently, cells were treated with increasing concentrations of DXM. Chondrocyte media were collected after 48 h (to ensure enough accumulation of protein to be measured) and centrifuged for 10 min at 3000 g (4 °C) and stored at −80 °C. The chondrocyte-conditioned medium (1 ml) was concentrated 10-fold on Centricon-10 ultrafiltration membranes and loaded on a 12% gel for non-reducing SDS-PAGE. IGFBPs were visualized by electrotransfer to PVDF membranes followed by incubation with \[ {\text{I}} \text{IGF-II (ligand blotting) (Van Buul-Offer et al. 1994). Molecular masses were calculated using BioRad (Hercules, CA, USA) broad range markers as standard. Bands were quantified by densitometry using a GS-363 Molecular Imager and Molecular Analyst software program, version 1·5 (BioRad).

For immunoblotting, 10 ml conditioned medium from 75 cm² tissue culture flasks was concentrated 10-fold on Centricon-10 ultrafiltration membranes. IGFBP-2 was detected using a bovine IGFBP-2 antibody at 1/2500 dilution, followed by a horseradish peroxidase-conjugated secondary antibody at 1/7500 dilution. IGFBP-5 was detected using a human IGFBP-5 antibody at 1/2000 dilution, followed by a horseradish peroxidase-conjugated secondary antibody at 1/7500 dilution. The bands were visualized using the SuperSignal chemiluminescent substrate and Hyperfilm ECL film.

\[ \text{Northern blot analysis} \]

\[ \text{RNA extraction} \]

Cells (3 x 10⁶) were cultured in 75 cm² tissue culture flasks and treated with DXM for 24 h. Cells were directly lysed with TriPure solution reagent and total RNA was extracted according to the procedures of the manufacturer, based on the single-step acid guanidinium–thiocyanate method (Chomczynski & Sacchi 1987).

\[ \text{Probes} \]

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

\[ \text{Northern blot hybridization} \]

Twenty micrograms of total RNA were separated by electrophoresis in a 1% (w/v) agarose/2·2 M formaldehyde gel in 1 x 3-(morpholino)propanesulfonic acid buffer, transferred to a 0·2 µm nylon membrane and crosslinked to the membrane by UV radiation. The membranes were prehybridized for 2 h at 60 °C in a solution containing 0·1% SDS, 3 x SSC, 5 x Denhardt’s solution, 10% dextran sulfate and 50 µg/ml denaturated salmon sperm DNA. Hybridization was performed at 60 °C overnight in the same solution, containing the \[ {\text{P}} \text{dCTP-labeled probe. Following hybridization, the membranes were washed to a stringency of 0·2 x SSC, 0·1% (w/v) SDS at 60 °C. The hybridization signals were analyzed by densitometry using the GS-363 Molecular Imager and the Molecular Analyst software program (BioRad) and the relative abundance of the different mRNAs was subsequently normalized to the GAPDH
values. The signals were also visualized by autoradiography on BioMax MR X-ray films.

**GR RT-PCR**

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

**Statistical analysis**

Data are expressed as means ± S.E.M. Statistical differences between two groups were determined by Student’s t-test. Statistical differences between multiple treatments were determined by one-way ANOVA using InStat version 3·00 (GraphPad Software, Inc., San Diego, CA, USA). The Dunnett post-hoc test was used to determine significance between the control and the treatment groups. Significance of the difference between the various treatments with or without the GR antagonist in the [3H]thymidine incorporation were determined using the Bonferroni post-hoc test. A P value of less than 0·05 was considered statistically significant. Growth plate cultures of at least three different animals were used per analyzed mRNA (both for the Northern Blot analysis and for the semi-quantitative RT-PCR) and for the [3H]thymidine incorporation.

**Results**

**Characterization of secreted IGFBPs and regulation by DXM**

The production of IGFBPs by the primary porcine growth plate chondrocytes was determined using Western ligand blot analysis. Under serum-free incubations, two IGFBPs of 34 and 24 kDa were detected (Fig. 1A). When the medium was supplemented with 10 nM IGF-I the expression of a 31/32 kDa doublet was induced and the expression of the 24 kDa protein was increased 2·9-fold (Fig. 1A). At 100 nM DXM, the intensity of the 34 kDa IGFBP, in both the absence and presence of DXM (100 nM). Total RNA was isolated and subjected to Northern blot analysis (as described in Materials and Methods). Subsequently, hybridization with a GAPDH probe was performed, which served as an internal control.
decreased to respectively 35 and 50% of control levels. The 24 kDa band and the 31/32 kDa doublet were not influenced by DXM treatment in either of the two incubations (Fig. 1A).

Immunoblotting identified the 34 kDa band as IGFBP-2 (Fig. 1B) and the 31/32 kDa doublet as IGFBP-5 (Fig. 1B). The band of 24 kDa probably represents IGFBP-4 (Koedam et al. 2000). Therefore, the DXM-sensitive protein was identified as IGFBP-2 and the major IGF-I-inducible IGFBP produced by growth plate chondrocytes was identified as IGFBP-5. IGFBP-4 was increased by IGF-I, but not affected by the DXM treatment.

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

**DXM-induced growth retardation**

To determine the effect of DXM on chondrocyte proliferation, [³H]thymidine incorporation into DNA was measured. The [³H]thymidine incorporation decreased significantly to 66 ± 3% of the control values when the chondrocytes were cultured in IGF-I-supplemented medium and subsequently treated with 100 nM DXM (Fig. 2). However, when growth plate chondrocytes were cultured in the presence of 10% FCS, basal proliferation (without any further treatment) increased 3·5-fold when compared with chondrocytes cultured in IGF-I-supplemented medium. Subsequent treatment with 100 nM DXM resulted in a 2·0 ± 0·1-fold reduction in proliferation (Fig. 2). Culturing in 10% FCS, therefore, resulted in higher cell proliferation and a larger decrease in proliferation when treated with DXM.

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

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

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

**mRNA levels of IGF axis components and regulation by DXM**

Northern blot analysis of primary porcine growth plate chondrocytes cultured in 10% FCS showed expression of the same IGFBP species as detected at the protein level (Fig. 1). The major transcripts detected (Fig. 5A) were: IGFBP-2 (1.8 kb), IGFBP-4 (2.0 kb) and IGFBP-5 (7.0 kb). IGFBP-6 mRNA was detected at low levels (2.0 kb). No IGFBP-1 and only very weak IGFBP-3
expression (5·0 kb, in one out of five conducted experiments) was found (not shown).

We determined the effects of pharmacological doses of DXM on the expression of IGF axis components in growth plate chondrocytes cultured in 10% FCS using Northern blot analysis. The IGFBP-2 mRNA levels decreased to 45 ± 9% of the control values, when treated with 100 nM DXM (Fig. 5A). The 7·0 kb transcript of IGFBP-5 was significantly increased by 2·0 ± 0·2-fold at 100 nM DXM (Fig. 5A). The mRNA levels of IGFBP-4 and -6 were not affected by the DXM treatment (Fig. 5A). The levels of IGFBP-3 were too low to be quantified.

Besides the IGFBPs, we also studied the presence and effects of DXM treatment on the remaining IGF axis components. The growth plate chondrocytes of neonatal piglets expressed very low levels of a 5·0 kb IGF-I transcript (data not shown). Northern blots hybridized with a 32P-labeled human IGF-II cDNA probe revealed four transcripts of IGF-II of 4·6, 2·6, 1·8 and 1·5 kb respectively (Fig. 5B). Low levels of a transcript of 11 kb of the type I IGF receptor were detected (Fig. 5B), and low levels of a transcript of 9·0 kb of the type II IGF receptor (mannose-6-phosphate receptor) (Fig. 5B).

DXM treatment did not affect the expression levels of the four detected transcripts of IGF-II (Fig. 5B). Also, the mRNA levels of the type II IGF receptor were not affected (Fig. 5B). The levels of IGF-1 were too low to be quantified. In contrast, type I IGF receptor expression levels increased when treated with 100 nM DXM (approximately 2- to 3-fold) (Fig. 5B); due to the low expression level of the untreated chondrocytes, no precise quantification of the increase was possible.

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

Discussion

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

First, we characterized the production and regulation of the IGFBPs by the primary porcine growth plate chondrocytes as the IGFBPs could be the IGF axis components that are involved in GC-induced growth retardation (Unterman & Phillips 1985, Price et al. 1992). We have previously reported that IGFBP-5 is the major IGF-I-inducible IGFBP produced by rabbit costal chondrocytes (Koedam et al. 2000). The present result in porcine epiphyseal growth plate chondrocytes confirmed this finding. In contrast to the costal chondrocytes, the growth plate chondrocytes expressed IGFBP-2, and showed upregulation of IGFBP-4 by IGF-I. Further differences are noted with respect to the regulation by DXM. While DXM inhibited the expression of IGFBP-5 in the costal chondrocytes (Koedam et al. 2000), this binding protein was unaffected by DXM at the protein level. In addition, we reported an induction of IGFBP-3 in the costal chondrocytes, while IGFBP-3 remained undetected in the growth plate chondrocytes in the presence of DXM. As costal chondrocytes of the neonatal piglets showed similar
results to the porcine tibial growth plate chondrocytes (data not shown), we suggest that species and/or age differences can account for these differences and not the difference in the origin of the tissue of the chondrocytes. The decrease in IGFBP-2 protein levels was paralleled by a decrease of IGFBP-2 mRNA levels, suggesting a regulation at the transcriptional level. GCs have indeed been shown to affect IGF axis components primarily at the level of gene expression (Price et al. 1992, Okazaki et al. 1994, Dell et al. 1999, Koedam et al. 2000).

GC treatment of our chondrocytes resulted in inhibition of proliferation without an increase in cell death, in
accordance with other in vitro studies, e.g. in osteoblasts (Okazaki et al. 1994) and in tibial growth plate chondrocytes (Jux et al. 1998, Robson et al. 1998). It has been previously reported that the decreased proliferation of chondrocytes treated with GCs is due to an increase in cell doubling times with a reduction in the number of S phase cells; in addition, no signs of increasing cell death or increase in morphological signs of apoptosis were shown (Jux et al. 1998, Robson et al. 1998).

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

As a consequence of the presence of serum in the experimental conditions, measurements of IGFBP protein production by the porcine tibial growth plate chondrocytes were unreliable. Further study of the effects of DXM on the IGF axis components (including also both IGFs and IGF receptors) on the growth plate chondrocytes was therefore only performed at the mRNA level. Northern blot analysis showed that not all components of the IGF axis were expressed in neonatal porcine growth plate chondrocytes. IGFBP-1 was not detected, as described previously (de los Rios & Hill 1999), and only very low levels of IGFBP-3 and low levels of IGFBP-6 were shown. IGFBP-2, -4 and -5 were the predominant IGFBPs expressed by the growth plate chondrocytes, which was in accordance with the IGFBPs detected at the protein level in IGF-I-supplemented serum-free culture conditions. This is in accordance with data from others, who also showed expression of IGFBP-2 to -6 in growth plate chondrocytes (Olney & Mougey 1999, de los Rios & Hill 1999). In accordance with others, we found IGF-II to be the most prominent IGF expressed in the growth plate (Shinar et al. 1993). Only very low levels of IGF-I were detected. Expression of the type I IGF receptor was also previously observed in rat growth plate chondrocytes (Klaus et al. 2000). This is the first study to describe expression of the type II IGF receptor in growth plate chondrocytes.

Pharmacological doses of DXM did not influence the expression of IGFBP-3, -4 and -6, of both IGFs and of the type II IGF receptor. This contrasts with the observed effects of GCs on other cell types as downregulation by GCs of the type II IGF receptor (Rydziel & Canalis 1995) and IGFBP-4 (Okazaki et al. 1994) in osteoblasts. IGFBP-6 was shown both to be stimulated in osteoblasts (Gabbitas & Canalis 1996) and to be not affected by GCs in osteoblasts (Okazaki et al. 1994) and in fibroblasts (Conover et al. 1995). IGFBP-3 expression was stimulated by GCs in costal chondrocytes (Koedam et al. 2000). Obviously, the regulation of expression of IGF axis components is cell type specific, and also depends on developmental stage and species used (Dell et al. 1999).

DXM treatment did influence the expression levels of the type I IGF receptor, IGFBP-5 and IGFBP-2. We showed that pharmacological doses of DXM increased the type I IGF receptor mRNA levels in the growth plate chondrocytes. This increase of type I IGF receptor mRNA by DXM has previously been shown in vivo (Price et al. 1992) and might be a response to counteract the effect of the GC-induced growth retardation. However, this response is insufficient to compensate for the growth inhibition observed.

DXM treatment increased IGFBP-5 mRNA levels in the presence of 10% FCS, which has not been described previously. In contrast, reduction of IGFBP-5 levels was reported in costal chondrocytes (Koedam et al. 2000), osteoblasts (Okazaki et al. 1994, Gabbitas et al. 1996) and in fibroblasts (Conover et al. 1995), a discrepancy which is probably due to a difference in cell type and species used as discussed above (Dell et al. 1999). In the rabbit costal chondrocytes we described before (Koedam et al. 2000), a decrease in IGFBP-5 was shown due to DXM treatment, whereas an increase in IGFBP-3 was observed. In contrast, our porcine growth plate chondrocytes expressed almost no IGFBP-3, but expressed IGFBP-5 at a high level. It has been suggested that IGFBP-3 and IGFBP-5 could have similar functions (Booth et al. 1995, Schedlich et al. 1998, Twigg & Baxter 1998). An increase in IGFBP-5 due to DXM treatment in piglet growth plate chondrocytes could therefore resemble the increase in IGFBP-3 due to DXM treatment in rabbit costal chondrocytes (Koedam et al. 2000). Furthermore, IGFBP-5 inhibits IGF-II-dependent DNA synthesis in growth plate chondrocytes (de los Rios & Hill 1999). An increase in IGFBP-5 thus can inhibit proliferation of growth plate chondrocytes by inhibiting IGF-II activity, which is the predominant IGF in our growth plate chondrocytes. IGFBP-5 could therefore account in part for the GC-induced growth retardation observed in our growth plate chondrocytes.

We observed a decrease in IGFBP-2 due to DXM treatment, both in IGF-I-supplemented experimental conditions as well as in the presence of 10% FCS. This observation is in accordance with data on osteoblasts (Chen et al. 1991) and in vivo experiments (Orłowski et al. 1990). Treatment of chondrocytes with GH increased IGFBP-2 levels (Borromeo et al. 1996). Together with our data, this implies that IGFBP-2 levels are correlated with growth in chondrocytes. IGFBP-2 can inhibit, but also stimulate, IGF activity and it has a preference for binding IGF-II (Jones & Clemmons 1995), the predominant IGF in the growth plate. IGFBP-2 was also suggested to facilitate targeting of the IGFs, in particular IGF-II pro-hormone, to skeletal tissues, resulting in stimulation of proliferation (Khosla et al. 1998). A decrease in IGFBP-2
could result in a decrease in IGF targeted to the chondrocytes, resulting in a decreased proliferation. Downregulation of IGFBP-2 expression in chondrocytes might therefore contribute to the inhibition of growth. This strengthens our conclusion that IGFBP-2, the only IGFBP which is affected by DXM in different culture conditions, could play a role in the regulation of growth in the growth plate, the mechanisms of which needs to be further elucidated.

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

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