Chapter 5

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

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

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

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ABSTRACT

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

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

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

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

INTRODUCTION

Endochondral bone formation and longitudinal bone growth are the result of proliferation, differentiation, maturation and eventually apoptosis of chondrocytes within the growth plate. Apoptosis of terminal hypertrophic chondrocytes (i.e., adjacent to the ossification front) is associated with extracellular matrix degradation and vascular invasion of the growth plate. It results in a cartilaginous scaffold on which new bone will be formed by invading osteoblasts. To allow osteoblasts to invade the growth plate, vascularization (i.e., angiogenesis) at the chondro-osseous junction between metaphyseal bone and the growth plate is required. It has been suggested that a mutual control exists between apoptosis and angiogenesis. Endothelial cells express growth factors that promote the differentiation of chondrocytes to a hypertrophic phenotype. Conversely, the growth plate secretes factors which promote angiogenesis. One of these factors, vascular endothelial growth factor (VEGF), is a key regulator of angiogenesis. VEGF inactivation in mice results in suppression of blood vessel invasion, impaired bone formation and expansion of the hypertrophic zone of the growth plate, demonstrating that VEGF is essential for attraction of capillaries to the growth plate and appears necessary for growth plate function. Matrix metalloproteinases (MMPs) are among
the other factors, which are important for angiogenesis and bone formation. They degrade the cartilage extracellular matrix and release angiogenic factors such as VEGF from the growth plate matrix. In MMP-9 knock-out mice, abnormal vascularization of the growth plate, delayed apoptosis of hypertrophic chondrocytes and expansion of the hypertrophic zone are observed. The growth plates of these mice show similar abnormalities as the growth plates of mice in which VEGF is inactivated. These results suggest a link between cartilage matrix degradation, apoptosis, angiogenesis, and VEGF and MMP-9 expression.

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

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

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

**MATERIALS AND METHODS**

**Animal studies**

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

**Tissue preparation**

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

Sections of 10 µm were cut on an ultramicrotome and mounted on 2% amino-propyl-triethoxy silane/3% glutaraldehyde coated glass slides. Sections were deparaffinized and hydrated prior to histochemical analyses (see below).
Morphometry
Growth plate sections were stained with haematoxylin and eosin and pictures of the growth plate sections were taken with a Zeiss Axiomat HRC camera equipped with the Axiovision software, version 3.0 (Zeiss, München-Hallbergmoos, Germany). The interactive measurement module was used for measurements of the growth plate width.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In the PRDL-treated piglets, VEGF mRNA and protein in the hypertrophic zone were not detectable. In the osteoblasts aligning the trabeculae, the levels of VEGF mRNA and protein did not appear to be affected by the PRDL treatment (Fig. 2D, F). In control animals, MMP-9 protein was detected in cells aligning the bone trabeculae and at the transverse septae of the cartilage-bone junction (Fig. 2G). PRDL treatment did not alter this expression pattern (Fig. 2H).
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Figure 1 Haematoxylin and eosin staining of representative sections of proximal tibial growth plates of 6-week-old prepubertal control (A, D) and PRDL-treated piglets (B, E). (C) result of measurements of total growth plate width of control and PRDL-treated piglets and of the three different zones of the growth plate. The data represent the mean ± SEM. *P < 0.05, **P < 0.02, PRDL relative to control. Bars represent 500 µm in (A, B), and 200 µm in (D, E). T, denotes the total growth plate; R, resting zone; P, proliferative zone; H, hypertrophic zone.

(F, G) Apoptosis, as shown by TUNEL-staining, in representative sections of tibial growth plates. (F) control piglets; and (G) PRDL-treated piglets; TUNEL staining is shown as a dark precipitate. Arrows indicate examples of TUNEL-positive cells. The scale bar represents 50 µm.
DISCUSSION

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

GC treatment results in decreased growth in children\textsuperscript{10,11}, several experimental animal models\textsuperscript{12-14} and also in piglets\textsuperscript{15,16}, which are a good model for growing children\textsuperscript{20}. GC-induced growth retardation occurs at the local level of the growth plate\textsuperscript{4,17} and as the pig growth plate more closely resembles the human growth plate\textsuperscript{21,22}, it is important to study the effects of GCs on the pig growth plate. We found a 27% reduction in the width of the proliferative zone in the piglet tibial growth plate due to GC treatment. In rats, GC treatment...
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suppressed the proliferation rate without changing the width of this zone and in mice we found a 10% decrease in the proliferative zone, concomitant with dexamethasone-induced growth inhibition. Also in rabbits, dexamethasone decreased bone growth together with the width of the proliferative zone. In addition to these in vivo data, also in vitro experiments using cultured rodent and porcine chondrocytes have shown that GCs inhibit their proliferation.

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

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

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

Many genes have been described to be involved in angiogenesis, with VEGF being one of the main modulators. In our control piglets, VEGF mRNA and protein are predominantly expressed in the hypertrophic zone, which is in accordance with other studies. In the PRDL-treated animals, VEGF mRNA and protein were no longer detectable in the hypertrophic zone. In prepubertal rats, GC treatment also resulted in a down-regulation of VEGF in the growth plate, although this decrease was not as severe as shown in our piglets. We have previously shown a down-regulation of VEGF expression by GCs in piglet chondrocytes in vitro. Together, our findings suggest that GCs decrease VEGF expression in the growth plates of growing piglets, which could be the cause of the disturbed angiogenesis, resulting in the observed defects in capillary architecture and metaphyseal bone. Besides the involvement of VEGF, possibly other angiogenic and/or anti-angiogenic factors could be involved in the disturbed angiogenesis in our PRDL-treated piglets. We examined MMP-9, which is known to make VEGF bioavailable and is important for angiogenesis to
occur \(^6,8\), but found no effect of the GC treatment on the MMP-9 expression pattern. Still other factors could be postulated as intermediates in GC action. For instance, TGF-\(\beta\) inhibits angiogenesis \(^40\) and stimulates apoptosis \(^41\). IGF-I, on the other hand, protects against GC-induced apoptosis \(^42\), stimulates production of VEGF in growth plate chondrocytes \(^19\) and is important for angiogenesis \(^43\). We have previously suggested that IGF-I in the growth plate could contribute to or counteract the adverse effects of GCs on growth \(^26\).

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

REFERENCES
