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

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Summary

Objective: Glucocorticoid treatment of children often leads to growth retardation, and the precise target(s) in the growth plate responsible for this effect are unknown. Angiogenesis is an important part of the endochondral ossification process, and VEGF expressed in the growth plate is essential for proper angiogenesis to occur. Since glucocorticoid treatment down-regulates VEGF expression in cultured chondrocytes, we hypothesized that in vivo glucocorticoid treatment could result in VEGF down-regulation in the growth plate and disturbed angiogenesis, thus contributing to the growth retardation.

Design: We treated 6-week-old prepubertal piglets (10 kg) for 5 days with prednisolone (50 mg/day). Tibial growth plate sections were studied for apoptosis and the expression of VEGF protein and mRNA and MMP-9 protein. Capillaries in the metaphysis were visualized by CD31 immunostaining. Growth plate morphology (width of various zones) was determined by interactive measurements on hematoxylin/eosin stained sections and apoptotic cells were detected by TUNEL assay.

Results: In the prednisolone-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 prednisolone-treated animals, 40.5% of the hypertrophic chondrocytes was apoptotic (P<0.02), with apoptotic chondrocytes also appearing higher in the hypertrophic zone.

We observed fewer capillaries and loss of their parallel organization in the metaphysis in the prednisolone-treated animals. The capillaries were shorter and chaotic in appearance. In contrast to controls, in prednisolone-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. No changes were observed in the expression pattern of MMP-9, a matrix metalloproteinase, which is also important for angiogenesis and bone formation.

Conclusions: These results indicate that short-term glucocorticoid 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 glucocorticoid-induced growth retardation.

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Key words: Growth plate, Angiogenesis, Glucocorticoid, VEGF, Porcine.

Abbreviations: MMP, matrix metalloproteinase, TUNEL, TdT-mediated dUTP nick-end labeling, VEGF, vascular endothelial growth factor.

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. The growth plate secretes factors, which promote angiogenesis, including vascular endothelial growth factor (VEGF). VEGF inactivation in mice results in suppression of blood vessel invasion, impaired bone formation and expansion of the hypertrophic zone of the growth plate.
growth plate, demonstrating that VEGF is essential for attraction of capillaries to the growth plate and for growth plate function.\(^6\) 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.\(^5,6\) 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.\(^10\) The growth plates of these mice show similar abnormalities as the growth plates of mice in which VEGF is inactivated.\(^6\) These results suggest a link between cartilage matrix degradation, apoptosis, angiogenesis, and VEGF and MMP-9 expression.

Agents that disturb longitudinal growth could potentially act through interference with apoptosis and angiogenesis. Glucocorticoids are effective drugs in anti-inflammatory and immuno-suppressive therapy, but are well known to result in growth retardation in children\(^11,12\) and in experimental animal models.\(^13–17\) Glucocorticoids act locally to inhibit longitudinal bone growth, suggesting a mechanism intrinsic to the growth plate.\(^4,18\) Treatment of rats with glucocorticoids resulted in an increase of apoptosis of hypertrophic chondrocytes\(^4,19\), indicating that glucocorticoids can interfere with apoptosis in the growth plate. We have previously shown that glucocorticoid treatment of cultured porcine growth plate chondrocytes resulted in a down-regulation of VEGF expression.\(^20\) Given the pivotal role of growth plate-derived VEGF in vascularization, it is conceivable that, in addition to increasing apoptosis, glucocorticoids could target vascularization of the growth plate. Although a 30% decrease in VEGF mRNA expression in the growth plate was reported as a result of glucocorticoid treatment in rats\(^19\), the effects on the actual vascular bed in the metaphysis are unknown.

The growing piglet is a good model for growing children.\(^21\) 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.\(^22,23\) In this model, we aimed to study the effects of a short-term (5 days) glucocorticoid treatment on a combination of factors, which could influence growth. These include architecture of the growth plate, apoptosis, VEGF expression and angiogenesis.

**Methods**

**ANIMAL STUDIES**

Twelve 6-week-old (prepubertal) female cross-bred (Landrace×Yorkshire) piglets were used 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, NL). One group of six piglets received 5 mg/kg bodyweight prednisolone daily (orally), for a period of 5 days. The average weight gains of the groups did not differ significantly from each other (0.89±0.35 kg for the control group and 0.75±0.45 kg for the prednisolone group). 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 (APES)/3% glutaraldehyde coated glass slides. Sections were dewaxed and hydrated prior to histochemical analyses (see below).

**MORPHOMETRY**

Growth plate sections were stained with hematoxylin and eosin and pictures of the growth plate sections were taken with the Zeiss Axiomat HRC camera equipped with the Axio Vision software, version 3.0. 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 four images (magnification 50 times) 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 Dr M.F. Gebbink and Dr E.E. Voest (University Medical Center Utrecht, the Netherlands)\(^25\) 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.\(^28\)

The *in situ* hybridization was performed as described previously.\(^27\) As a control for specific hybridization, sections were hybridized with the appropriate sense probes, which showed no signals.

**IMMUNOHISTOCHEMISTRY**

Endogenous peroxidase in sections was blocked by a 20 min incubation in 0.3% H\(_2\)O\(_2\) in methanol. Antigen retrieval was performed by heating at 90°C for 3 min in 10 mM Na-citrate, pH 6.0. Sections were blocked with 10% serum (Vector Laboratories, Inc., Burlingame, CA) 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) and goat anti-MMP-9 (R&D Systems, Inc., Minneapolis, MN). 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) as substrate was used. Sections were counterstained with nuclear fast red, dehydrated and mounted with DPX (Klinipath, Duiven, NL).

DETERMINATION OF APOPTOSIS

Apoptotic death was determined by the TdT-mediated dUTP nick-end labeling (TUNEL) reaction (Promega, Leiden, NL), 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 mean±S.E.M. Statistical differences between the control and the prednisolone-treated group were determined by the unpaired t-test, using InStat version 3.00 (GraphPad Software, Inc., San Diego, CA). A P-value of <0.05 was considered statistically significant.

Results

GROWTH PLATE MORPHOLOGY

A 5-day prednisolone 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. 1(A and B)], caused by a significant decrease of the proliferative zone to 73±9% of control values (P<0.05) [Fig. 1(C)]. The hypertrophic zone showed a small, non-significant decrease, whereas the resting zone was not affected by the prednisolone treatment. The prednisolone 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. 1(A and B)].

Trabecular bone length in the primary spongiosa of the prednisolone-treated piglets was clearly diminished when compared to the untreated controls [Fig. 1(D and 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. 1(F)]. Prednisolone 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. 1(G)]. In addition to the terminal hypertrophic zone, the higher layers of the hypertrophic zone also contained apoptotic chondrocytes in the prednisolone-treated piglets [Fig. 1(G)].

ANGIOGENESIS

Immunohistochemistry for CD31/PECAM-1, a transmembrane glycoprotein in endothelial cells, was used to detect capillaries. In the control piglets, metaphyseal blood vessels ran parallel to the chondrocyte columns in the growth plate and ended at the osteochondral junction [Fig. 2(A)]. Prednisolone treatment resulted in fewer, disorganized and short blood vessels, although they still penetrated the hypertrophic zone of the growth plate [Fig. 2(B)]. We studied whether this disturbed angiogenesis could be due to changes in expression of VEGF and/or MMP-9 proteins. 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. 2(C)]. Immunohistochemistry showed a similar expression pattern for the VEGF protein [Fig. 2(E)]. In the prednisolone-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 prednisolone treatment [Fig. 2(D and F)]. In control animals, MMP-9 protein was detected in cells (most likely osteoclasts and osteoclast-like cells)10) aligning the bone trabeculae and at the transverse septa of the cartilage–bone junction [Fig. 2(G)]. Prednisolone treatment did not alter this expression pattern [Fig. 2(H)].

Discussion

In our study, piglets treated with glucocorticoids 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. These changes likely contribute to the growth retardation frequently observed in children receiving glucocorticoid treatment11,12. Although rodents and chickens can be used as animal models for glucocorticoid-induced growth retardation13–16, piglets are a better model for growing children17 and their growth plates more closely resemble the human growth plate22,23. Some of the present findings have been reported before by us1,2,27,28 and others4,19,29, but never before have the effects of glucocorticoids on the morphology, apoptosis and angiogenesis of the piglet growth plate been studied in an integrated approach.

The width of the proliferative zone in the piglet tibial growth plate was reduced by 27% due to the glucocorticoid treatment. We found a similar reduction of the proliferative zone after a 1-week treatment of mice with dexamethasone28, and a 10% decrease in the proliferative zone, concomitant with dexamethasone-induced growth inhibition, after 4 weeks of treatment15,27. In rats, glucocorticoid treatment suppressed the proliferation rate without changing the width of this zone19,29, while in rabbits, dexamethasone decreased bone growth together with the width of the proliferative zone30. In addition to these in vivo data, also in vitro experiments using cultured rodent and porcine...
chondrocytes have shown that glucocorticoids inhibit their proliferation\textsuperscript{31–34}. In addition to the proliferative zone, the hypertrophic zone was also affected by the glucocorticoid treatment: there was a sevenfold increase in apoptosis in the prednisolone-treated piglets, with apoptotic chondrocytes also appearing higher in the hypertrophic zone. In rats, glucocorticoid treatment resulted in a twofold increase in apoptosis of hypertrophic chondrocytes, but it remained limited to the terminal hypertrophic chondrocytes\textsuperscript{4,19}. 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 glucocorticoid treatment. Interestingly, cell death of terminal chondrocytes has been found to inversely correlate with the growth rate of the bone\textsuperscript{35}. Glucocorticoids, 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\textsuperscript{36}

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Fig. 1. Hematoxylin and eosin staining of representative sections of proximal tibial growth plates of 6-week-old prepubertal control (A, D) and prednisolone (PRDL)-treated piglets (B, E). (C) Result of measurements of total growth plate width of control and prednisolone-treated piglets and of the three different zones of the growth plate. The data represent the mean±S.E.M. *$P<0.05$, **$P<0.02$, 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) prednisolone-treated piglets; TUNEL staining is shown as a dark precipitate. Arrows indicate examples of TUNEL-positive cells. The scale bar represents 50 µm.
Fig. 2. Angiogenesis in representative sections of tibial growth plates of control (A, C, E, G) and prednisolone (PRDL)-treated piglets (B, D, F, H). (A, B) CD31 immunohistochemistry for detection of blood capillaries; (C, D) VEGF mRNA expression, as analyzed by *in situ* hybridization; (E, F) VEGF protein expression, as analyzed by immunohistochemistry; (G, H) MMP-9 protein expression, as analyzed by immunohistochemistry. The mRNA signal is shown as a blue precipitate and the protein signal as a dark precipitate. Scale bars represent 100 µm.
compensates for the enhanced apoptosis, leaving the number of chondrocytes in the hypertrophic zone almost unchanged. Glucocorticoid-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, prednisolone 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 glucocorticoid 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 prednisolone treatment on growth plate cartilage, the structure of primary trabecular and trabecular bone formation of the primary spongiosa were also severely disturbed, already after this short-term glucocorticoid treatment. Glucocorticoids have been shown to affect bone formation in the secondary spongiosa by decreasing bone formation and increasing bone resorption while increasing apoptosis of bone cells. Our study demonstrates that the primary spongiosa is affected as well.

The severe defect in formation and organization of the metaphyseal blood vessels in the tibia is perhaps the most striking finding of this study. Although changes in the growth plate apoptosis and VEGF gene expression (see below) probably are the key factors behind this defect, also the loss of trabecular architecture and direct effects of glucocorticoids on endothelial cells could play a role in the disturbed angiogenesis. In any event, normal blood vessel invasion of the growth plate is required for endochondral ossification and longitudinal bone growth, as demonstrated by the delay in apoptosis and expansion of the hypertrophic zone, which followed the impaired vascular invasion resulting from inactivation of VEGF. A reduced bone regional blood flow was reported in pigs, which had been treated with high-dose methylprednisolone for 2 weeks. Since especially the epiphysis of the femur head was affected, this could be an important pathogenetic factor in glucocorticoid-induced osteonecrosis in this region.

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 prednisolone-treated animals, VEGF mRNA and protein were no longer detectable in the hypertrophic zone. In prepubertal rats, glucocorticoid 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 glucocorticoids in piglet chondrocytes in vivo and in vitro. The importance of growth plate-derived signals in the induction of capillary ingrowth was elegantly described by Abad et al., who excised, inverted and reimplemented rabbit growth plates. The epiphyseal bone, which became adjacent to the hypertrophic zone of the growth plate, became the site of blood vessel invasion. Although in that study the involvement of VEGF was not directly addressed, our results, together with the sequestration of VEGF leading to impaired vascular invasion, strongly suggest that down-regulation of VEGF by glucocorticoids is directly responsible for the disturbed angiogenesis, resulting in the observed defects in capillary architecture. In this context, also the role of MMP-9 is important, because it degrades the matrix and makes VEGF bioavailable. We found no effect of the glucocorticoid treatment on the MMP-9 expression pattern. In a variety of experimental models, glucocorticoids did inhibit cytokine-induced expression of MMP-9 by interfering with expression and/or binding of transcription factors AP-1, NFκB and Ets. The actions of glucocorticoids on gene expression could depend on the type of cell and/or its location, as exemplified by the strong inhibition of VEGF expression in the growth plate, without an apparent inhibition in the metaphysis. Although MMP-9 expression has been reported in the hypertrophic zone of the growth plate, we did not detect it there. Rather, the pattern of expression is in accordance with its reported expression by osteoclasts, chondroclasts and endothelial cells.

It would be of interest, however, to characterize the expression of MMP-9 and its regulation by glucocorticoids in cultured growth plate chondrocytes. In addition, other MMPs, in particular MMP-13, may play a role in endochondral ossification and would be worth investigating.

Interestingly, the glucocorticoid treatment did not result in the rachitic type of enlargement of the growth plate as was observed after disrupting VEGF signaling or MMP-9 expression. In both these experimental models, as well as in vitamin D receptor ablated mice, the expansion of the hypertrophic zone appears to be caused by a delayed or decreased apoptosis. The glucocorticoid treatment, on the other hand, resulted in increased apoptosis, which could explain the loss of cartilage even in the absence of proper endochondral ossification. Although it is difficult to quantitatively compare the defects in vascularization among the various animal models, it is possible that in the glucocorticoid-treated piglets there is still sufficient vascular invasion for the delivery of MMP-9-expressing osteoclasts and subsequent induction of apoptosis. Indeed, MMP-9 has been suggested to cause chondrocyte apoptosis, in addition to its functioning as an angiogenic signal.

Still other factors could be postulated as intermediates in glucocorticoid action. For instance, TGF-β inhibits angiogenesis and stimulates apoptosis. IGF-I, on the other hand, protects against glucocorticoid-induced apoptosis, stimulates production of VEGF in growth plate chondrocytes and is important for angiogenesis. We have previously suggested that IGF-I in the growth plate could contribute to or counteract the adverse effects of glucocorticoids on growth. It is of interest that vitamin D upregulates VEGF expression both in cultured chondrocytes and in the hypertrophic zone of the growth plate in vivo. Together with an upregulation of MMP-9 at the chondro-osseous junction, this results in enhanced vascular invasion of the growth plate. Considering these findings, and the use of vitamin D to treat glucocorticoid-induced osteoporosis, it will be of interest to study whether vitamin D can prevent the changes which we have now described in the growth plate.

References


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