

Growth retardation induced by dexamethasone is associated with increased apoptosis of the growth plate chondrocytes

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Abstract

Glucocorticoids cause significant growth retardation in mammals and humans and decreased proliferation of chondrocytes has been considered as the main local mechanism. Death by apoptosis is an important regulator of homeostasis in multicellular organisms. Here we chose to study the role of apoptosis in growth retardation caused by glucocorticoid treatment. We treated 7-week-old male rats with dexamethasone (5 mg/kg/day) for 7 days. Apoptosis was studied in tibiae growth plates by the TUNEL method. Immunoreactivity for parathyroid hormone-related peptide (PTHrP), caspase-3, and the anti-apoptotic proteins Bcl-2 and Bcl-x was also studied. Apoptosis was mainly localized in terminal hypertrophic chondrocytes (THCs) in both control and dexamethasone-treated animals. Dexamethasone caused an increase in apoptosis which was fourfold in THCs (2.45 ± 0.12 vs

0.62 ± 0.09 apoptotic cells/mm growth plate, $P < 0.001$), and 18-fold in proliferative chondrocytes (0.18 ± 0.04 vs 0.01 ± 0.007 apoptotic cells/mm growth plate, $P < 0.001$). Increased apoptosis after dexamethasone treatment was accompanied by increased immunoreactivity for caspase-3 and decreased immunoreactivity for the anti-apoptotic proteins Bcl-2 and Bcl-x, which further supports our apoptosis results. Dexamethasone also decreased the immunoreactivity for PTHrP, suggesting a role in the mechanism by which glucocorticoids induce apoptosis in the growth plate. We conclude that apoptosis is one mechanism involved in growth retardation induced by glucocorticoids. Premature loss of resting/proliferative chondrocytes by apoptosis could contribute to incomplete catch-up seen after prolonged glucocorticoid treatment.

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Introduction

Impaired longitudinal growth is a well-established side effect of glucocorticoid treatment in humans and animals (Altman *et al.* 1992, Allen 1996). The decreased growth is accompanied by morphological changes in the growth plate. It becomes thinner (Altman *et al.* 1992, Anfeld 1992), which has been attributed to decreased proliferation of the chondrocytes (Kember & Walker 1971, Anfeld 1992). Glucocorticoids also alter the synthetic capability of chondrocytes with consequent alterations in the cartilage matrix (Anfeld 1992) and enhanced formation of electron-dense, dark-staining degenerate-appearing chondrocytes (Dearden *et al.* 1986).

Multiple mechanisms, both endocrine and paracrine, have been implicated as the cause of growth retardation caused by glucocorticoids. Alterations of the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis at multiple levels (Allen 1996, Mehls *et al.* 2001) have been described. In addition to systemic effects, glucocorticoids also have local effects on the growth plate, the structure responsible for longitudinal bone growth (Baron *et al.* 1994). Decreased proliferation of epiphyseal chondrocytes

and alterations in the local production of GH/IGF-I have been reported as possible mechanisms (Kember & Walker 1971, Anfeld 1992, Mehls *et al.* 2001).

Apoptosis acts as an important regulator of cell and tissue homeostasis by eliminating cells through a genetically programmed cell death. It has been suggested that apoptosis plays a role in growth plate homeostasis during development (Chrysis *et al.* 2002) and that apoptosis is the fate of the terminal hypertrophic chondrocytes (Franum & Wilsman 1987, Gibson *et al.* 1995). Furthermore, strong evidence that apoptosis is a regulator of growth plate homeostasis comes from *in vivo* experiments. As an example, mice with targeted disruptions of both alleles for the anti-apoptotic protein Bcl-2 have short limbs and accelerated ossification of their growth plates (Amling *et al.* 1997). In addition, two chondrodysplastic conditions (parathyroid hormone-related peptide (PTHrP) knock-out mice and activating mutations of the fibroblast growth factor-3 (FGFR-3)) are associated with increased apoptosis of the growth plate chondrocytes (Amizuka *et al.* 1994, Legeai-Mallet *et al.* 1998).

In a multicellular organism or tissue, growth is accomplished when cell proliferation exceeds cell death.

Therefore, one could assume that cell death or apoptosis plays a role in growth retardation after glucocorticoid treatment. Indeed, based on the TUNEL method, increased apoptosis of the terminal hypertrophic chondrocytes (THCs) has been reported in growth plates of rats after corticosterone administration (Silvestrini *et al.* 2000). However, conflicting results have been reported concerning the magnitude and location of apoptosis in the growth plate, based solely on the TUNEL method (Bronckers *et al.* 1996, Aizawa *et al.* 1997, Gibson 1998). In addition, the underlying mechanisms and the role of apoptosis in the growth plate after glucocorticoid treatment have not been elucidated. Therefore, we studied apoptosis in the growth plate after glucocorticoid treatment following an integrated approach. We studied not only apoptosis by the TUNEL method but also proteins important in the apoptotic pathway such as caspase-3 (one of the executors of apoptosis), and the anti-apoptotic proteins, Bcl-2 and Bcl-x. Furthermore, because Bcl-2 lies downstream after PTHrP signaling we hypothesized that apoptosis induced by glucocorticoids is associated with decreased expression of PTHrP.

Materials and Methods

Animals and sample preparation

After approval by the local ethical committee, 7-week-old male Sprague Dawley rats (B&K Universal, Sollentuna, Sweden) were housed in our animal care facility under a 12-h light, 12-h darkness schedule; food pellets were available *ad libitum*. After an adaptation period, 5 animals received dexamethasone (5 mg/kg body weight, s.c.) for 7 days, whereas the control group of 5 animals received vehicle only. Tibiae were removed and the upper part was fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 18 h at 4 °C. After fixation, specimens were decalcified in 5% EDTA and were then paraffin embedded. Sections, 5 µm thick, were cut longitudinally and mounted on histological glass slides (Superfrost +/+; Menzel-Gloeser, Braunschweig, Germany). On each slide, two sections were mounted, one from each group, so that all samples were treated under the same conditions during TUNEL and immunohistochemistry. Slides were dried at 37 °C overnight.

Determination of growth rate

For the determination of growth rate, the fluorescent marker calcein was administered i.p. as a single injection (10 mg/kg) at the start of a separate experiment which included 6 animals divided into two groups of 3 animals each. One group received vehicle during the 7 days of the experiment, whereas the other group received dexamethasone. Proximal tibiae were prepared as described

above, non-decalcified tissues were embedded in methyl-methacrylate blocks and 5 µm thick sections were cut longitudinally. The distance between the proximal borders of the calcein and the chondro-osseous junction of the growth plate was measured; this corresponds to the growth accomplished during 7 days.

Detection of apoptosis

Apoptotic cells in the growth plates were identified by terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL), according to the manufacturer's instructions (TdT-FragEL kit; Oncogene Research Products, Boston, MA, USA) with slight modifications (Chrysis *et al.* 2002): deparaffinized and rehydrated sections were treated with proteinase K (20 mcg/ml) at 37 °C for 10 min instead of 20 min. Treatment of sections with DNase labeled all cells, whereas sections treated with distilled water instead of TdT were negative. At least 5 sections from each animal, 25 µm apart (to avoid counting the same cells twice) were used to determine the number of apoptotic cells. The number of apoptotic chondrocytes was counted in the growth plate. Only cells in closed lacunae were counted and were expressed as their number per mm length of the chondro-osseous junction. The length of the chondro-osseous junction from each section was measured by the Image-Pro image analysis system (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry

The rabbit caspase-3 polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the Bcl-2 rabbit polyclonal antibody was from Upstate Biotechnology (1:600; Upstate Biotechnology, Lake Placid, NY, USA), and the rabbit polyclonal Bcl-x antibody was from Transduction Laboratories (1:500; Becton Dickinson AB, Stockholm, Sweden). For the immunodetection of PTHrP a rabbit polyclonal antibody raised against amino acids 34–53 of human PTHrP was used (1:80; Oncogene Science, Cambridge, MA, USA). Control experiments were carried out with omission of the primary antibodies, incubation with non-specific rabbit IgG and competition of the primary antibody with corresponding peptide when available (PTHrP).

Sections were dewaxed in xylene and rehydrated in graded alcohol baths. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) at 95–98 °C for 20 min (for caspase-3 only) or with neuraminidase digestion for PTHrP (0.025 U/ml neuraminidase for 50 min at room temperature). Immunohistochemistry was carried out as previously described (Chrysis *et al.* 2002). Biotinylated goat anti-rabbit IgG (1:1000) was purchased from Jackson ImmunoResearch Lab. (West Grove, PA, USA),

avidin-conjugated peroxidase was purchased from Vector Laboratories (Burlingame, CA, USA), and a DAB-kit which generates a brown color for the detection of peroxidase activity was from Vector Laboratories. Digital microphotographs were captured by a Nikon Eclipse 800 microscope (Nikon, Bergström Instruments, Stockholm, Sweden) equipped with a Hamamatsu Orca IIIc CCD camera (Hamamatsu Inc, Stockholm, Sweden).

Immunopositive chondrocytes were detected by automatic thresholding using an image analysis system (Image-Pro; Media Cybernetics). For each antibody, at least 300 cells were analyzed in two separate sections of each specimen. Immunopositive cells are expressed as a percentage of the total number of cells analyzed in the proliferative and hypertrophic zones respectively. For each antibody, all slides were processed in parallel in order for comparisons to be valid.

Statistical analysis

Results are expressed as means \pm s.e. Differences between groups were tested by Student's *t*-test. $P < 0.05$ was considered significant.

Results

Body weight and growth rate

Before treatment was initiated, body weight was similar in the control (199.6 ± 4.6 g) and in the dexamethasone-treated (197.0 ± 2.5 g) group of animals. When killed after 7 days of treatment, body weight was increased by 32% in the control group (263.1 ± 5.0 g), whereas the dexamethasone group had lost 23% of the initial body weight (152.3 ± 2.9 g, $P < 0.001$ vs control). Growth rate over the 7-day period of treatment was significantly higher in controls compared with dexamethasone-treated animals (207 ± 14 $\mu\text{m}/\text{day}$ and 68 ± 5 $\mu\text{m}/\text{day}$ respectively, $P < 0.001$).

Apoptosis

Seven days of treatment with dexamethasone increased the number of apoptotic chondrocytes in all layers of the growth plate (Fig. 1). In both control and dexamethasone-treated animals most of the apoptotic chondrocytes were THCs. Compared with controls, dexamethasone caused a fourfold increase in apoptosis in THCs (2.45 ± 0.12 vs 0.62 ± 0.09 apoptotic cells/mm growth plate, $P < 0.001$). Apoptotic proliferative chondrocytes were rare in sections from control animals and only occasional apoptotic cells were observed in a few sections. In contrast, sections from dexamethasone-treated animals regularly had some apoptotic proliferative chondrocytes, mainly early proliferative

(Fig. 1), resulting in an 18-fold increase (0.18 ± 0.04 vs 0.01 ± 0.007 apoptotic cells/mm growth plate, $P < 0.001$) compared with controls.

Caspase-3

In control sections, caspase-3 immunoreactivity was localized in proliferative and early hypertrophic chondrocytes with no staining in mature and THCs. Dexamethasone treatment caused a significant increase in caspase-3 immunoreactivity in all layers of the growth plate and, more significantly, mature chondrocytes and THCs were also immunopositive (Fig. 1, Table 1).

Bcl-2 and Bcl-x

Immunoreactivity for the anti-apoptotic protein, Bcl-2, was localized throughout the growth plate in sections from control animals, although it was less abundant in mature and was almost absent in late/THCs (Fig. 1). Dexamethasone treatment caused a dramatic decrease in Bcl-2 immunoreactivity in all layers of the growth plate (Fig. 1, Table 1).

Immunoreactivity for Bcl-x, another anti-apoptotic protein, was localized mainly in late proliferative and hypertrophic chondrocytes, especially at the interface between the proliferative and hypertrophic zones (Fig. 1). Dexamethasone caused a significant decrease in Bcl-x immunoreactivity throughout the whole growth plate (Fig. 1, Table 1).

PTHrP

In control animals, PTHrP immunoreactivity was present in all layers of the growth plate but was more prominent in the hypertrophic and resting zones (Fig. 1). Dexamethasone treatment caused a significant decrease in PTHrP immunoreactivity in all layers (Fig. 1, Table 1). Similar results were obtained with a second PTHrP antibody (mouse monoclonal antibody raised against amino acids 38–64 of human PTHrP; Oncogene Science).

Discussion

We report that dexamethasone treatment increases apoptosis in growth plate chondrocytes. The vast majority of the apoptotic cells were THCs and to a lesser extent early proliferative chondrocytes. In addition, increased apoptosis was associated with increased expression of caspase-3, and decreased expression of the anti-apoptotic proteins Bcl-2 and Bcl-x, which further support the activation of apoptotic signals in the growth plate after dexamethasone treatment.

Growth retardation after glucocorticoid treatment is characterized by decreased proliferation of the growth

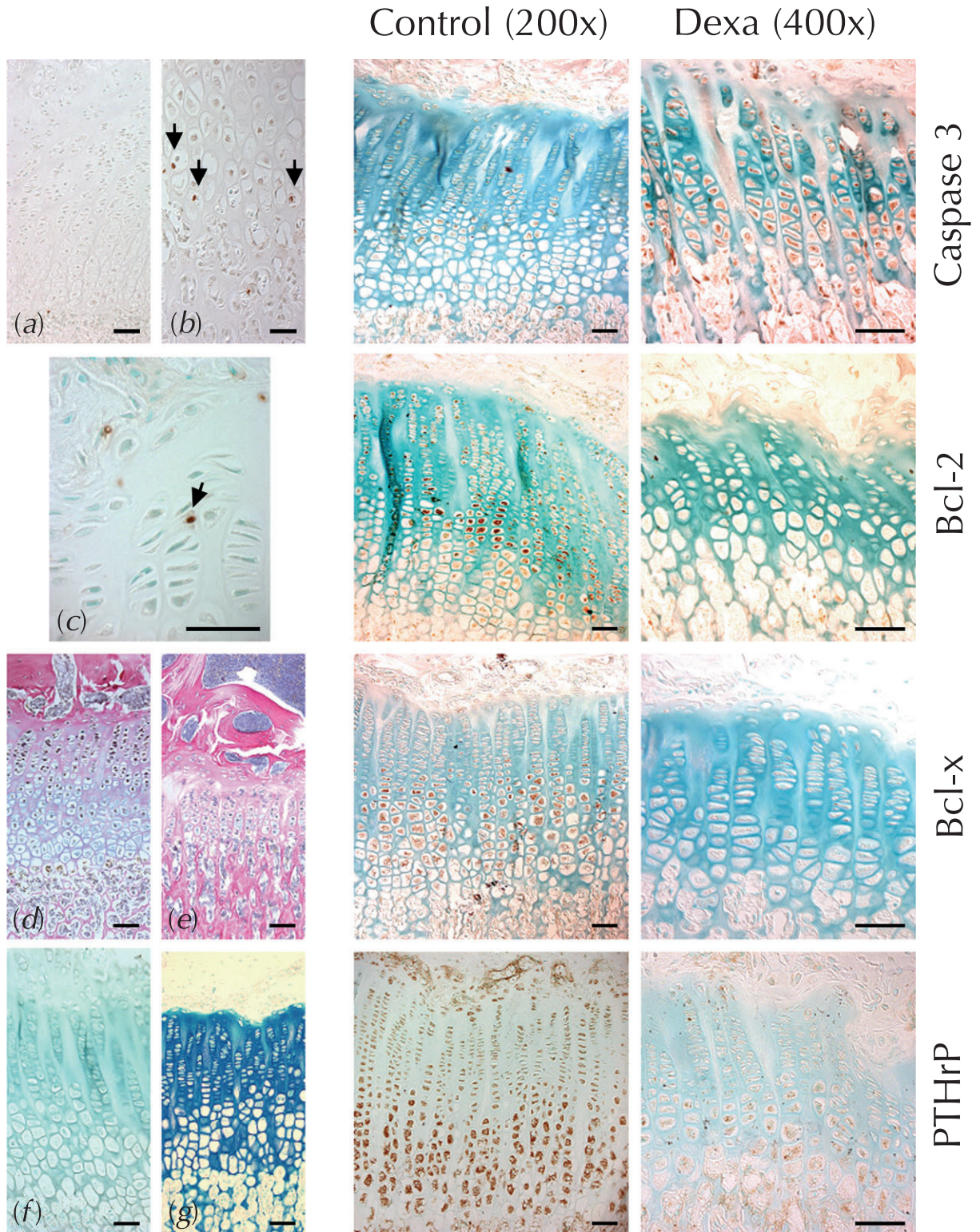


Table 1 Percentage (mean \pm S.D.) of immunoreactive chondrocytes in the different zones in control and dexamethasone (Dexa)-treated rats

	Proliferative		Hypertropic	
	Control	Dexa	Control	Dexa
Caspase-3	45 \pm 8	77 \pm 11*	18 \pm 2	94 \pm 9*
Bcl-2	78 \pm 6	2.0 \pm 0.1*	39 \pm 5	5.0 \pm 1.0*
Bcl-x	35 \pm 2	1.0 \pm 0.1*	81 \pm 13	3.0 \pm 0.1*
PTHrP	73 \pm 9	32 \pm 4*	94 \pm 12	58 \pm 7*

* $P < 0.001$ vs control.

plate chondrocytes, which has been considered to be the main local mechanism by which glucocorticoids decrease growth rate and thickness of the growth plate (Kember & Walker 1971, Anfeldt 1992). As growth is the excess of cell proliferation compared with cell death, glucocorticoids could also slow growth by inducing apoptosis of growth plate chondrocytes. Indeed, we found that the number of apoptotic THCs was significantly increased in dexamethasone-treated compared with control animals. Therefore, the combination of decreased proliferation and increased cell death could, together, explain glucocorticoid-induced growth retardation. Our data are further supported by a previously published study in older rats where increased apoptosis was observed after long treatment (3 weeks) with corticosterone (Silvestrini *et al.* 2000). Based on several observations we believe that the observed increased rate of apoptosis in the THCs reflects a real increase rather than an artifact. First, the immunoreactivity for caspase-3 was increased concomitantly with apoptosis and more specifically in the hypertropic and THCs where apoptosis was mainly observed. Secondly, both anti-apoptotic proteins, Bcl-2 and Bcl-x, were dramatically decreased in dexamethasone-treated rats. Finally, apoptosis was increased by dexamethasone not only in THCs but also in early proliferative chondrocytes although less frequently. The antibody for caspase-3 recognizes both proactive and active caspase-3 and therefore the immunoreactivity does not indicate which form is present. The several-fold increase in caspase-3 in the hypertropic chondrocytes where most apoptosis was observed, in

association with the remarkable decrease in the anti-apoptotic proteins Bcl-2 and Bcl-x, could suggest an increase in the activation of the apoptotic pathway and therefore activation of caspases rather than an increase in the proactive caspase alone.

Decreased growth rate is associated with a longer lifespan of the growth plate chondrocytes (Huzniker *et al.* 1987). Therefore, one could argue that the observed increase in apoptosis after dexamethasone treatment is an artifact as the cells remain longer as THCs and thereby are more likely to be detected as apoptotic. We believe that this hypothesis is not valid because the final stage of apoptosis, as detected by TUNEL, is irreversible and there are no indications from the literature that this stage can be elongated. Moreover, our integrated data from caspase-3, Bcl-2, and Bcl-x clearly show that the growth plate is more susceptible to apoptosis after dexamethasone treatment.

In vivo experiments have shown the importance of apoptosis for normal growth. Bcl-2 knockout mice (Amling *et al.* 1997) have short limbs with skeletal deformities. These experiments indicate that despite the existence of several members of the Bcl family of proteins in the growth plate, Bcl-2 has a pivotal role in growth plate apoptosis. In addition, elegant *in vivo* and *in vitro* experiments have shown that Bcl-2 lies downstream of the PTHrP signaling pathway (Amling *et al.* 1997). PTHrP stimulates the expression of Bcl-2 protein in the growth plate chondrocytes leading to delayed maturation. On the other hand, mice homozygous for PTHrP gene ablation (Amizuka *et al.* 1994, 1996), similar to Bcl-2 knock-out mice, have skeletal deformities with decreased proliferation and accelerated maturation of chondrocytes, and a large population of immature chondrocytes were apoptotic. Furthermore, the survival role of PTHrP has been confirmed not only *in vivo* but also *in vitro* (Henderson *et al.* 1995). Based on these data, we hypothesized that the decreased proliferation of chondrocytes after dexamethasone treatment, as well as the decreased immunoreactivity for Bcl-2 and consequently increased apoptosis could be mediated through diminished expression of PTHrP in the growth plate. Indeed, with two different antibodies we observed that the immunoreactivity for PTHrP was decreased after dexamethasone treatment. Our data

Figure 1 Representative microphotographs of TUNEL assays from growth plates from control (a) and dexamethasone-treated (b, c) rats. Apoptosis was observed mainly in terminal hypertropic chondrocytes (THCs) (a, b) and dexamethasone caused a fourfold increase in apoptosis of THCs (b). Arrows indicate apoptotic chondrocytes in closed lacunae. Apoptotic proliferative chondrocytes were rare and an 18-fold increase was observed after dexamethasone (c). Bars represent 100 μ m. Representative microphotographs of caspase-3, Bcl-2, Bcl-x and PTHrP immunohistochemistry from tibial growth plates in control and dexamethasone-treated rats are shown. Microphotographs from control sections have a magnification of 200 \times , whereas those from the dexamethasone-treated group have a magnification of 400 \times because the height of the growth plate is significantly smaller than that of controls. Immunoreactivity for caspase-3 was increased in the dexamethasone-treated group, whereas for Bcl-2, Bcl-x and PTHrP it was decreased. Microphotographs of growth plates stained with Alcian blue/van Gieson show the differences in the growth plate height in control (d) and dexamethasone (e) groups at 200 \times magnification. The specificity of primary antibodies was assessed by replacement of primary antibodies with normal rabbit serum (f) and with preabsorption of the PTHrP antibody with blocking peptide (g).

suggest an underlying mechanism for apoptosis induced by glucocorticoids which involves the PTHrP/Bcl-2 pathway. This is further supported by many *in vitro* studies in different cell lines, where dexamethasone downregulates the expression of parathyroid hormone (PTH)/PTHrP (Glatz *et al.* 1994, Southby *et al.* 1996).

Discontinuation of systemic glucocorticoid treatment in humans and mammals or treatment of Cushing's syndrome in children leads to catch-up growth which is often incomplete and does not fully compensate for the growth deficit induced by glucocorticoid excess (Baron *et al.* 1994, Magiakou *et al.* 1994). We observed an increased number of apoptotic resting/early proliferative chondrocytes after dexamethasone treatment. Apoptotic resting/early proliferative chondrocytes were rare in occasional sections from control animals but after dexamethasone treatment an 18-fold increase was observed. It has been suggested that the observed catch-up growth after dexamethasone treatment is due to delayed senescence of the growth plate as the chondrocytes have undergone fewer replications due to the inhibitory effect of dexamethasone on cell proliferation (Gafni *et al.* 2001). In every dynamic model of growth there are two parameters for optimal growth: cell proliferation and cell death. Therefore, we propose that cell death should be considered in the model of growth plate senescence. Premature loss of resting or early proliferative cells could diminish the growth potential in spite of increased numbers of remaining replications of the chondrocytes. This premature loss of resting/early proliferative chondrocytes could account for the incomplete catch-up growth after glucocorticoid excess and for a final height which is often shorter than the predicted height.

In summary, we show that growth retardation after dexamethasone treatment is associated with increased apoptosis in the growth plate, which occurs through a down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-x and an increase in caspase-3. Our data support a model of glucocorticoid-induced apoptosis where the inhibition of the PTHrP expression causes a reduction of the anti-apoptotic protein Bcl-2 which triggers death signals in the growth plate chondrocytes. Finally, we propose that incomplete catch-up growth after prolonged exposure to supraphysiological concentrations of glucocorticoids could be secondary to premature loss of resting/proliferative chondrocytes by apoptosis.

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