Catch-up growth after dexamethasone withdrawal occurs in cultured postnatal rat metatarsal bones

Andrei S Chagin, Elham Karimian, Katja Sundström, Emma Eriksson and Lars Sävendahl

Pediatric Endocrinology Laboratory, Department of Women's and Children's Health, Karolinska Institutet, Stockholm SE-17176, Sweden

(Correspondence should be addressed to A S Chagin who is now at Endocrine Unit, Thier 1101, Massachusetts General Hospital, 50 Blossom Street, Boston, Massachusetts 02114, USA; Email: achagin@partners.org)

Abstract

Children exposed to systemic glucocorticoids often exhibit growth retardation and after the cessation of therapy catch-up growth occurs in many, but not all patients. The developmental regulation and underlying cellular mechanisms of catch-up growth are not fully understood. To clarify this issue, we established an *in vitro* model of catch-up growth. Here we present a protocol for the long-term culture (up to 160 days) of fetal (E20) as well as postnatal (P8) rat metatarsal bones which allowed us to characterize *ex vivo* the phenomenon of catch-up growth without any influence by systemic factors. The relevance of the model was confirmed by the demonstration that the growth of fetal and postnatal bones were stimulated by IGF1 (100 ng/ml) and inhibited by dexamethasone

(Dexa; 1 μ M). We found that the capacity to undergo catch-up growth was restricted to postnatal bones. Catch-up growth occurred after postnatal bones had been exposed to Dexa for 7 or 12 days but not after a more prolonged exposure (19 days). Incomplete catch-up growth resulted in compromised bone length when assessed at the end of the 4-month period of culture. While exposure to Dexa was associated with decreased chondrocyte proliferation and differentiation, catch-up growth was only associated with increased cell proliferation. We conclude that the phenomenon of catch-up growth after Dexa treatment is intrinsic to the growth plate and primarily mediated by an upregulation of chondrocyte proliferation.

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Introduction

Longitudinal bone growth occurs at the growth plate by a process called endochondral ossification where a continuously formed cartilage template is substituted by newly formed bone tissue (Kronenberg 2003, Chagin & Savendahl 2007). Dexamethasone (Dexa) is a well-known inhibitor of longitudinal bone growth. It exerts growth-inhibiting effects both systemically through downregulation of the GH/insulin-like growth factor 1 (IGF1) axis, and directly through inhibition of chondrocyte proliferation, mineralization, and increased apoptosis (van der Eerden *et al.* 2003, Mushtaq *et al.* 2004, Nilsson *et al.* 2005). The direct effects of Dexa are believed to be partly caused by downregulation of the GH receptor and/or the IGF1 receptor at the growth plate level (van der Eerden *et al.* 2003, Nilsson *et al.* 2005).

Catch-up growth after glucocorticoid exposure is a less understood phenomenon where growth velocity rises above the corresponding control after cessation of treatment (Boersma & Wit 1997). Catch-up growth was first proposed to occur via a neuroendocrine mechanism where an organism recognizes the degree of mismatch between its target size and its actual size, and consequently adjusts growth rate according to the degree of mismatch (Tanner 1963). Later this hypothesis was challenged by experiments where partial

catch-up growth of one leg was observed after local infusion of Dexa into the rabbit growth plate (Baron *et al.* 1992, 1994a). This observation gave rise to the senescence hypothesis which proposes that glucocorticoids delay senescence of chondrocytes, where senescence is a function of cell division and delayed chondrocyte proliferation preserves the proliferative capacity. Therefore, after cessation of treatment, chondrocytes proliferate like younger cells demonstrating increased growth velocity compared with controls (Baron *et al.* 1994a). However, the extent to which local and systemic mechanisms contribute to the phenomenon of catch-up growth is still unclear.

In the present work, we characterized an *in vitro* model that mimics the clinically observed catch-up growth after cessation of glucocorticoid exposure. For this purpose, we optimized a well-known model of fetal rat metatarsal bone culture (Scheven & Hamilton 1991, Mancilla *et al.* 1998, De Luca *et al.* 2001, Martensson *et al.* 2004) and characterized the growth pattern of postnatal metatarsal bones, which have not previously been successfully grown *ex vivo* (Scheven & Hamilton 1991, Coxam *et al.* 1996). Our results demonstrate that the catch-up growth is specific to the postnatal growth plate and is attributed to local mechanisms. Moreover, catch-up growth is clearly associated with increased chondrocyte proliferation.

Materials and Methods

Materials

IGF1, Dexamethasone, BSA, and β -glycerophosphate were all purchased from Sigma-Aldrich, Inc. PBS, DMEM/F12, MEM, fetal bovine serum, and gentamycin were all purchased from Invitrogen, Inc. Ascorbic acid was purchased from ICN Corp. (Aurora, OH, USA).

Bone culture

The hindpaws of Sprague–Dawley rat fetuses at day 20 of gestation (E20) or from Sprague–Dawley rats at day 8 of age were collected in DMEM/F12 supplemented with 20 μ g/ml gentamycin on ice. The three middle metatarsal bones were microdissected and then cultured in 24-well plates in either MEM or DMEM/F12 medium changed every 2–3 days. Both culture media were supplemented with 1 mM β -glycerophosphate, 50 μ g/ml ascorbic acid, 0.2% BSA, and 20 μ g/ml gentamycin. These experiments were pre-approved by the Local Ethics Committee at Karolinska Institutet, Stockholm, Sweden.

Length measurements

Digital images of cultured bones were captured at days 0, 2, 5, 7, 12, 19, and 26 and thereafter once a week using a stereo microscope (Nikon SMZ-U) connected to a digital camera (Hamamatsu C4742–95) and a computer. Images were analyzed as previously described (Martensson *et al.* 2004) using the Image-ProPlus image analysis software (Media Cybernetic, Glen Head, NY, USA) by a person blinded to the nature of the treatment groups. Bone lengths were expressed in millimeters. For a given time point, growth velocity was calculated by dividing the increase in bone length since the previous measurement with the time interval between the two measurements.

BrdU incorporation

For BrdU labeling, 2.5 h (or 24 h) prior to fixation, rat metatarsal bones were washed once in PBS and the DMEM/F12 medium was changed to MEM medium supplemented with BrdU labeling reagent (1:200 dilution of labeling reagent, Cell Proliferation Kit RPN20, Amersham Biosciences). BrdU detection and quantification were performed as previously described (Chagin *et al.* 2007).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling

Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) *in situ* using the TdT-FragEL DNA

fragmentation kit according to the manufacturer's instructions (Oncogene Research, Boston, MA, USA). The following modifications were previously introduced: sections were treated with 5 μ g/ml proteinase K for 10 min (Martensson *et al.* 2004), DNA incorporation of biotin-labeling nucleotides was detected by streptavidin conjugated to Alexa Fluor 546 (Invitrogen Inc.), and slides were embedded in DAPI-containing mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). The level of apoptosis was calculated as the percentage Alexa-546 positive cells of DAPI positive cells (total cell number) employing the Image-ProPlus image analysis software (Media Cybernetic) as previously described (Chagin *et al.* 2007). Distilled water was used instead of TdT as a negative control for cell labeling.

Quantitative histology

Four micrometer sections of the metatarsal bones were counterstained with Alcian blue/van Gieson or hematoxylin/eosin. Distal growth plates were exclusively used for histological analysis. The proliferative zone was defined as the layer with columnar appearance between rounded chondrocytes in the resting and hypertrophic zones. The heights of both proliferative and hypertrophic zones were calculated as an average of 10 measurements per bone. Hypertrophic chondrocytes were defined by a height of more than 7 μ m (Tivesten *et al.* 2004). The size of hypertrophic chondrocytes was averaged by measuring 25 hypertrophic chondrocytes per distal growth plate in the hypertrophic zone between the third and seventh cell layers (counted from the metaphyseal side; Chagin *et al.* 2009). Measurements were performed using the Image-ProPlus image analysis software (Media Cybernetic). All histological measurements were made by a single person unaware about the origin of the images.

Immunohistochemistry

Rat metatarsal bones were fixed in 4% formaldehyde, paraffin embedded and 4 μ m-thick sections were obtained. Immunohistochemistry was performed as described elsewhere (Nilsson *et al.* 2002). The primary rabbit polyclonal anti-IGF1 (H-70) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at a dilution of 1:100 (Chagin *et al.* 2006, Shao *et al.* 2007). Secondary polyclonal goat anti-rabbit biotinylated antibody was purchased from DakoCytomation A/S (Glostrup, Denmark) and applied in a 1:400 dilution. For detection of collagen type X, antigen retrieval was performed in citrate buffer at 95 °C for 15 min followed by peroxidase quench in 3% H₂O₂ for 10 min. Sections were subsequently treated with 5 mg/ml hyaluronidase (Sigma-Aldrich, Inc.) for 30 min at 37 °C. Anti-collagen type X antibody (Quartett GmbH, Berlin, Germany) was used at a dilution of 1:30 overnight.

Figure 1 Fetal rat metatarsal bones grow better when cultured in DMEM/F12 than in MEM medium. Chondrocyte proliferation (A; BrdU incorporation) and increase in bone length (B) were determined in bones cultured for 2 days in MEM or DMEM/F12. Atrophy of the middle part (arrows) was observed in bones cultured for 14 days in MEM (C) but not in DMEM/F12 (D). Scale bars 100 μm. Data represent means $\pm$ S.E.M. *** $P < 0.001$ versus MEM.

Statistical analysis

Statistical differences were calculated by one-way ANOVA followed by the Holm–Sidak test if more than two groups were compared. The unpaired Student's *t*-test was used to calculate statistical differences between two groups (Fig. 1). For determination of growth pattern three to four bones were used per treatment group for every experiment and every experiment was repeated at least three times. For determination of proliferation, apoptosis or cell morphometry four to five bones were analyzed for every treatment or time-point.

Results

Establishment of long-term cultures of fetal and postnatal rat metatarsal bones

Based on our previous studies on short-term cultures of fetal rat metatarsal bones, we believe that bones cultured in DMEM/F12 medium (Chagin *et al.* 2006, 2007, Heino *et al.* 2008) grow better than those cultured in MEM (Mancilla *et al.* 1998, Martensson *et al.* 2004, Zaman *et al.* 2007) or alpha MEM (Chagin *et al.* 2009) media. However, a direct formal comparison has never been made before. To facilitate the establishment of long-term metatarsal bone cultures, we first compared the growth of fetal metatarsal bones when cultured in either MEM or DMEM/F12 medium. Already after 2 days in culture, bone growth and chondrocyte proliferation rate were significantly higher in those bones cultured in DMEM/F12 medium ($P < 0.001$ versus MEM, Fig. 1A and B). Furthermore, a central depletion of chondrocytes was

observed in a majority of bones cultured for more than 2 weeks in MEM medium (Fig. 1C), a phenomenon which was never observed when cultured in DMEM/F12 medium (Fig. 1D). When cultured in DMEM/F12 medium, fetal (E20) rat metatarsal bones continued to grow throughout the 3-month observation period (Fig. 2A and B) although the growth velocity gradually declined over time (Fig. 2C). The significant improvement of fetal bone growth linked to the introduction of the more enriched DMEM/F12 medium enabled us to expand our *in vitro* studies, also including cultures of postnatal metatarsal bones dissected between 5-day and 8-day (P8) old rats as well as from 4-day-old mice (Fig. 2 and data not shown for bones dissected from 5-day old rats and 4-day-old mice). When comparing E20 and P8 bones, we found that the growth velocities were comparable throughout the 3-month period of culture with the exception of a more rapid initial decline in the growth of P8 bones (Fig. 2C) which was accompanied by a parallel decline in chondrocyte proliferation rate (Fig. 2D). All columnar chondrocytes turn into hypertrophic cells somewhere after 70–90 days in culture and, therefore, essentially no BrdU incorporation (24 h labeling) was observed after this time point (Fig. 2D and data not shown). Bone growth was primarily attributed to growth of the cartilage template while the length of the diaphysis did not significantly change and no secondary ossification center was formed (Supplementary Figure 1, see section on supplementary data given at the end of this article). Thus, there was no bone tissue formation

Figure 2 Growth of fetal and postnatal rat metatarsal bones cultured for 90 days. The bones were dissected from fetuses at day 20 of gestation (E20) or from postnatal rats at day 8 (P8) of age and cultured *ex vivo* in DMEM/F12 medium. Total bone length (A), length increase (B), and growth velocity (C) were calculated after 2, 5, 7, and 12 days in culture and every week thereafter. P8 bones were labeled with BrdU for 24 h on days 12, 30, 61, and 90 of culture, and the percentage of BrdU positive cells was calculated (D). Data represent means $\pm$ S.E.M.

ex vivo despite formation of a cartilage template, presumably due to the lack of blood supply. These observations also indicate that bone maturation does not occur in the *ex vivo* setting.

Responsiveness to IGF1 in cultured fetal and postnatal rat metatarsal bones

IGF1 (100 ng/ml) stimulated the growth of fetal (E20) and postnatal (P8) metatarsal bones in a similar way (Fig. 3A and B). After 2 days of IGF1 exposure, the lengths of E20 and P8 bones exceeded that of control bones by 84 ± 6 and $83 \pm 8\%$ respectively ($P < 0.001$ versus control). Similarly, when assessed over the first 2 days of exposure, the growth velocities of IGF1 treated bones were identical in the E20 and P8 groups (364 ± 37 and 367 ± 15 $\mu\text{m}/\text{day}$ respectively; Fig. 3C and D). Interestingly, IGF1 promoted only a transient increase in growth velocity. Moreover, growth velocity declined more rapidly in bones isolated from older rats (P8) reaching control values after 5 days while in E20 bones it returned to the control level first after 12 days (Fig. 3C and D). Between days 2 and 5 of culture, a transient drop in growth velocity was recorded in E20 bones (Fig. 3C). However, this we believe is an artifact caused by the early disappearance of loose connective tissue that remained after the microdissection of these small bones. Interestingly, IGF1 was capable of stimulating postnatal

Figure 3 IGF1 stimulates growth of cultured fetal and postnatal rat metatarsal bones. The bones were dissected from fetuses at day 20 of gestation (E20) (A and C) or postnatally from 8-day-old rats (P8) (B and D) and cultured *ex vivo* in DMEM/F12 medium with or without IGF1 (100 ng/ml). IGF1 treatment started either from the beginning of culture (A–D), or at day 75 of culture (B and D). Length increase (A and B) and growth velocity (C and D) were calculated after 2, 5, 7, and 12 days in culture, and thereafter weekly. Data represent means $\pm$ S.E.M. Results were analyzed by ANOVA with the Holm–Sidak test for multiple pair-wise comparisons. ** $P < 0.01$ and *** $P < 0.001$ versus the corresponding control.

Figure 4 Dexamethasone (Dexa) inhibits growth of both fetal and postnatal rat metatarsal bones, while catch-up growth is restricted to postnatal bones. Metatarsal bones were dissected from fetuses at day 20 of gestation (E20) (A and C) or from 8-day-old rats (P8) (B and D), and cultured *ex vivo* in DMEM/F12 with or without Dexa (1 μM). Dexa was added during the first 12 days of culture (Dexa12). Thereafter, the bones were washed in PBS and cultured without Dexa. Length increase (A and B) and growth velocity (C and D) were calculated after 2, 5, 7, and 12 days in culture and thereafter weekly. Data represent means $\pm$ S.E.M. *** $P < 0.001$ versus the corresponding control.

metatarsal bone growth even when added after 75 days in culture suggesting an intact IGF-responsiveness throughout these long-term cultures (Fig. 3B and D).

Dexa inhibits growth of both fetal and postnatal metatarsal bones in culture

Treatment with Dexa (1 μM) for 12 days inhibited the growth of both fetal (E20; Fig. 4A) and postnatal (P8; Fig. 4B) metatarsal bones in culture. Interestingly, P8 bones were more sensitive to Dexa-induced growth retardation than E20 bones. When assessed after 7 days exposure to Dexa, growth velocity was 55 ± 17 and 8 ± 4 $\mu\text{m}/\text{day}$ in E20 and P8 bones respectively (Fig. 4C and D).

Catch-up growth after Dexa-treatment occurs in postnatal but not in fetal bones

After discontinuing Dexa treatment on day 12, a clear catch-up growth (defined as a time period with growth velocity significantly above the corresponding control) was observed in P8 bones peaking on day 26 (growth velocity 82 ± 8 vs 52 ± 5 $\mu\text{m}/\text{day}$ in control, $P < 0.001$, Fig. 4D). The difference in length between control and Dexa-treated P8 bones decreased with time; from 1105 ± 40 μm at day 12 to 593 ± 134 μm at day 110 of culture ($P < 0.001$). In contrast, after cessation of Dexa treatment in E20 bones, growth

velocity only reached the control level and consequently no catch-up growth occurred (Fig. 4A and C). The difference in bone length between control and Dexa-treated E20 bones further increased with time in culture; from $951 \pm 39 \mu\text{m}$ at day 12 to $1587 \pm 134 \mu\text{m}$ at day 110 ($P < 0.001$). Altogether, these data demonstrate that catch-up growth after Dexa treatment occurs in postnatal but not in fetal metatarsal bones.

Catch-up growth is dependent on the duration of previous Dexa exposure

To clarify if the capacity for catch-up growth is dependent on the duration of previous glucocorticoid exposure, P8 bones were divided into the following groups: control (treatment with vehicle (0.1% EtOH) for 12 days, thereafter culture medium); Dexa7 (treatment with Dexa for 7 days, thereafter culture medium); Dexa12 (treatment with Dexa for 12 days, thereafter culture medium); and Dexa19 (treatment with Dexa for 19 days, thereafter culture medium). These bones were monitored throughout a 160-day period of culture. After discontinuing Dexa treatment, a clear catch-up growth was observed in Dexa7 (84 ± 4 vs $52 \pm 5 \mu\text{m/day}$ in control; day 19; $P < 0.001$) and Dexa12 (82 ± 8 vs $52 \pm 5 \mu\text{m/day}$ in control; day 26; $P < 0.001$) but not in Dexa19 bones (Fig. 5A). After 120 days in culture, Dexa7 bones had reached the same length as control bones (9.3 ± 0.2 vs $9.5 \pm 0.2 \text{ mm}$ for control; $P > 0.05$) while Dexa19 bones were significantly shorter still after 160 days in culture (9.1 ± 0.1 vs $9.9 \pm 0.2 \text{ mm}$ for control; $P < 0.001$) (Fig. 5B) albeit the length difference between treated and untreated bones decreased with time ($1657 \pm 71 \mu\text{m}$ for day 26 versus $770 \pm 186 \mu\text{m}$ for day 160; $P < 0.001$). Dexa12 bones exhibited an intermediate growth pattern compared to Dexa7 and Dexa19 bones and the length at day 160 of culture was not statistically different from control bones (9.5 ± 0.3 vs $9.9 \pm 0.2 \text{ mm}$; $P > 0.05$) (Fig. 5B). Remarkably, all bones were still growing after 160 days of culture although the growth velocity was quite low at that time point ($4.3 \pm 1.4 \mu\text{m/day}$ for control; $6.8 \pm 1.7 \mu\text{m/day}$ for Dexa7, $7.9 \pm 2.3 \mu\text{m/day}$ for Dexa12; and $4.9 \pm 1.1 \mu\text{m/day}$ for Dexa19; Fig. 5A).

Cellular mechanisms of catch-up growth in cultured postnatal metatarsal bones

Treatment of P8 bones with Dexa for 7 or 19 days clearly inhibited chondrocyte proliferation when assessed on the last day of exposure ($P < 0.01$ versus control; Fig. 6A). When assessed 5 days after the cessation of 7 days Dexa treatment (Day 7+5), chondrocyte proliferation rate was significantly elevated when compared with corresponding control bones ($P < 0.001$; Fig. 6A). In contrast, the proliferation rate had not increased when determined 5 days after the termination of 19 days of previous exposure to Dexa (Day 19+5; Fig. 6A). These findings

Figure 5 Prolonged exposure to Dexa abolishes catch-up growth. Metatarsal bones were dissected from 8-day-old rats (P8), treated with Dexa (1 μM) for 7 (Dexa7), 12 (Dexa12), or 19 (Dexa19) days and then followed for 160 days in culture. Growth velocity (A) and total bone length (B) were analyzed at days 2, 5, 7, and 12 of culture, and thereafter weekly. Data represent means $\pm$ S.E.M. ** $P < 0.01$ and *** $P < 0.001$ versus the corresponding control.

are in line with the observed catch-up growth in P8 bones exposed to Dexa for 7, but not 19 days (Fig. 5A).

Morphological analysis of the height of the proliferative zone did not reveal any differences between the groups (Fig. 6B and Supplementary Figure 2, see section on supplementary data given at the end of this article). Together with decreased proliferation, this observation suggests that Dexa delayed the chondrocyte transition from flat towards hypertrophic chondrocytes and thereby suppressed growth. In support of this observation, the number of hypertrophic chondrocytes was decreased in Dexa-treated bones (Fig. 6F). Moreover, Dexa-exposure suppressed chondrocyte hypertrophy as determined by collagen type X immunostaining (Fig. 6C), hypertrophic zone length (Fig. 6D) and hypertrophic chondrocytes size (Fig. 6E). In contrast to proliferation rate, none of the hypertrophy parameters did undergo catch-up after the cessation of Dexa treatment (Fig. 6C–E).

Figure 6 Dexa inhibits chondrocyte proliferation and differentiation while catch-up growth is only linked to increased proliferation. Metatarsal bones were dissected from 8-day-old rats, treated with Dexa (1 μ M) for 7 or 19 days and analyzed either immediately after treatment (day 7 and 19) or 5 days later (day 7+5 and day 19+5 respectively). Corresponding controls were cultured in DMEM/F12 medium supplemented with vehicle (0.1% EtOH). Chondrocyte proliferation was analyzed based on BrdU incorporation (A), while differentiation was determined by measuring the height of collagen type X staining (C). The heights of the zone of flat chondrocytes (B), the hypertrophic zone (D) and the size of hypertrophic chondrocytes (E) were determined by histomorphometry. Number of hypertrophic chondrocytes was counted manually (F). Data represent means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus the corresponding control.

As IGF1 is known to stimulate chondrocyte hypertrophy we examined whether the expression of this protein was affected during and after the exposure to Dexa. Indeed, the expression level of IGF1 was reduced by Dexa as early as after 7 days of treatment ($P < 0.001$) but was not restored 5 days after the cessation of the exposure (Supplementary Figure 3, see section on supplementary data given at the end of this article), suggesting that regulation of IGF1 expression by Dexa is not involved in the observed catch-up growth.

As glucocorticoid exposure *in vivo* has been linked to increased chondrocyte apoptosis in the rat growth plate (Chrysis *et al.* 2003), we also performed TUNEL-analysis in postnatal (P8) rat metatarsal bones exposed to Dexa *ex vivo*. We then observed that the level of apoptosis (TUNEL-positive cells) was increased by $18 \pm 55\%$ at day 7 of

continuous Dexa exposure and by $134 \pm 50\%$ at day 19 of continuous Dexa exposure ($P = 0.9$ and $P = 0.069$ versus control respectively). Albeit not statistically significant, these data suggest that apoptosis may be a cause of compromised growth after prolonged exposure to Dexa.

Discussion

Here we present a new protocol allowing long-term studies of fetal as well as postnatal rat metatarsal bone growth *ex vivo*. The strengths of the model include persistent growth over at least 5 months in culture under serum-free conditions, an ability to respond to known suppressors and stimulators of bone growth,

and importantly a possibility to study the phenomenon of catch-up growth *in vitro*. Applying this model, we found that the capacity for catch-up growth after cessation of glucocorticoid exposure is restricted to postnatal bones and negatively correlated to the duration of treatment with Dexamethasone.

We found that both fetal and postnatal rat metatarsal bones have the capacity to grow for up to 5 months *ex vivo* when cultured in a medium free from serum but rich in nutrients (DMEM/F12). It has generally been postulated that postnatal bones do not grow *ex vivo* (Scheven & Hamilton 1991, Coxam *et al.* 1996). Postnatal bones cultured in MEM supplemented with 1% BSA have been reported to grow negligibly when compared to fetal bones (Coxam *et al.* 1996). To our knowledge, so far there is only one recent report of a successful short-term culture of postnatal bones demonstrating that metatarsals from 2-day-old mice can be grown for 8 days in the presence of 10% bovine serum (MacRae *et al.* 2006). When comparing the growth patterns of fetal and postnatal bones, we found that after being transferred to *ex vivo* conditions, growth velocity decreased earlier in postnatal bones, which may be linked to the fact that they are older. The observed decline in growth velocity was associated with a gradual decrease in chondrocyte proliferation. This is in line with the senescence hypothesis proposing that chondrocyte senescence is a function of their proliferative capacity (Baron *et al.* 1994a). Interestingly, metatarsal bone growth velocity never reached zero, not even after 5 months in culture, which most likely reflects the fact that rodents continue to grow even later in life, although at a slow rate.

Here we report that both fetal and postnatal metatarsal bones cultured *ex vivo* have the capacity to respond to Dexamethasone and IGF1, a known suppressor and stimulator of bone growth respectively (van der Eerden *et al.* 2003, Mushtaq *et al.* 2004). We found that IGF1 stimulated the growth of fetal and postnatal bones in a similar way. To our surprise, after 75 days in culture, postnatal bones still had an intact IGF1 responsiveness which strengthens the relevance of this model for long-term mechanistic studies of the regulation of postnatal bone growth. Furthermore, this observation suggests that growth plate chondrocytes are not going through dedifferentiation when cultured under these conditions. When exposed to Dexamethasone, growth velocity almost approached zero in postnatal bones while it was partially preserved in fetal metatarsals suggesting that postnatal growth is more sensitive to Dexamethasone exposure than fetal. In contrast, postnatal bone growth demonstrated a better ability to recover compared to fetal bones. Altogether, our data demonstrate that postnatal bones exhibit a similar growth pattern as fetal bones and respond to hormonal stimuli as expected when kept in long-term cultures *ex vivo*.

The new protocol launched here allows bones to be cultured *ex vivo* for prolonged periods of time, thereby allowing mechanistic studies of the phenomenon of catch-up growth. Here we decided to focus on Dexamethasone-induced growth retardation since the capacity for catch-up growth after

glucocorticoid exposure is of great clinical relevance. Our *ex vivo* finding that catch-up growth occurs after short-term (7 and 12 days) but not long-term (19 days) Dexamethasone exposure mimics what occurs in children known to display catch-up growth after previous short-term glucocorticoid exposure while prolonged exposure results in incomplete catch-up growth and compromised final height (Mosier *et al.* 1972, Strickland *et al.* 1972). Mechanistic studies, showed that upon cessation of Dexamethasone treatment, the level of chondrocyte proliferation rose above control after short-term exposure (7 days) but not long-term (19 days) exposure, whereas differentiation was unchanged in both cases, suggesting that increased proliferation most likely is the primary local underlying mechanism for the phenomenon of catch-up growth. Interestingly, the duration of Dexamethasone exposure was positively correlated to the time period needed for growth to normalize since after prolonged exposure to Dexamethasone (19 days), growth velocity reached control level first 10 days after the cessation of treatment while after short-term exposure (5 days) growth velocity normalized earlier. Accordingly, normalization of cell proliferation in 19 days Dexamethasone-exposed bones was not observed 5 days after cessation of treatment in contrast to bones exposed only for 7 days. Our *ex vivo* data are in line with the previously published *in vivo* observation that growth plate chondrocyte proliferation rate is increased upon cessation of systemic glucocorticoid treatment (Gafni *et al.* 2001), previous tamoxifen treatment (Karimian *et al.* 2008) and hypothyroidism (Marino *et al.* 2008). To our knowledge, it has previously not been demonstrated that elevated proliferation during catch-up growth is attributed to local mechanisms within the growth plate. Previous reports show increased apoptosis in growth plate chondrocytes after high-dose Dexamethasone treatment in rats *in vivo* (Chrysis *et al.* 2003) and in cultured human chondrocytes (Chrysis *et al.* 2005) suggesting that apoptosis may contribute to incomplete catch-up growth after glucocorticoid exposure. Such a mechanism is supported by our present observation of a slight increase in the level of chondrocyte apoptosis after long-term Dexamethasone exposure, although the differences did not reach statistical significance.

It has previously been suggested that glucocorticoid effects on bone growth may be mediated through modulation of IGF1 (van der Eerden *et al.* 2003), a growth factor known to stimulate proliferation and hypertrophy while suppressing apoptosis in growth plate chondrocytes (Chrysis *et al.* 2005). Here we report that Dexamethasone treatment reduced the proportion of growth plate chondrocytes expressing IGF1 and that the expression level remained below control even after cessation of the treatment when catch-up growth occurred. Together with previous findings (Luo & Murphy 1989, Wang *et al.* 1999), our data support a role for IGF1 as a mediator of Dexamethasone-induced growth retardation but not in the subsequent process of catch-up growth. Furthermore, the observation that catch-up growth may occur under serum-free conditions indirectly suggests that catch-up growth is not dependent on systemic factors.

The fact that catch-up growth after Dexamethasone exposure occurred *ex vivo* in cultured postnatal bones strongly suggests that the phenomenon of catch-up growth must, at least partially, be intrinsic to the growth plate. This conclusion is supported by previously published *in vivo* data showing that unilateral injection of Dexamethasone into the rabbit tibia growth plate resulted in unilateral growth restriction followed by partial restoration of bone length upon cessation of the treatment (Baron *et al.* 1992, 1994a). These studies suggested that the intrinsic mechanism of catch-up growth might be attributed to delayed senescence of growth plate chondrocytes (Baron *et al.* 1994a). Despite support from previous observations (Baron *et al.* 1994a,b), we cannot exclude the involvement of systemic mechanisms in the catch-up phenomenon since not only bones display catch-up growth, but also the entire organism (Tanner 1963).

We observed that after Dexamethasone treatment, catch-up growth occurs in postnatal but not in fetal bones. In some studies, treatment of premature infants with Dexamethasone was associated with decreased longitudinal growth (Wood *et al.* 2003, Rijken *et al.* 2007), but this was not confirmed by others (Halliday 2001, Romagnoli *et al.* 2002). Interestingly, premature infants with a relative insensitivity to glucocorticoids, exhibit a complete catch-up growth during the first 2 years of life while infants without this polymorphism remain below the mean height for the normal population up to 19 years of age (Finken *et al.* 2007). This finding is in line with our *in vitro* observation of an absent catch-up growth in fetal bones after the exposure to Dexamethasone. However, rats prenatally exposed to Dexamethasone indeed have been reported to undergo postnatal catch-up growth (O'Regan *et al.* 2004) emphasizing the need for further studies clarifying catch-up growth after exposure to glucocorticoids during fetal life.

In summary, here we present a protocol for the long-term culture of fetal as well as postnatal rat metatarsal bones which allowed us to confirm that catch-up growth may occur *ex vivo* without any influence of systemic factors. We also found that the capacity for catch-up growth after cessation of Dexamethasone exposure is restricted to postnatal bones and negatively correlated to the duration of the treatment. Finally, our data link catch-up growth to increased chondrocyte proliferation rate which most likely is the major local underlying mechanism for this phenomenon.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-09-0307>.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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