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Growth cartilage expression of growth hormone/insulin-like growth factor I axis in spontaneous and growth hormone induced catch-up growth

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ABSTRACT

Introduction: Catch-up growth following the cessation of a growth inhibiting cause occurs in humans and animals. Although its underlying regulatory mechanisms are not well understood, current hypothesis confer an increasing importance to local factors intrinsic to the long bones' growth plate (GP).
Aim: The present study was designed to analyze the growth-hormone (GH)-insulin-like growth factor I (IGF-I) axis in the epiphyseal cartilage of young rats exhibiting catch-up growth as well as to evaluate the effect of GH treatment on this process.
Material and methods: Female Sprague–Dawley rats were randomly grouped: controls (group C), 50% diet restriction for 3 days + refeeding (group CR); 50% diet restriction for 3 days + refeeding & GH treatment (group CRGH). Analysis of GH receptor (GHR), IGF-I, IGF-I receptor (IGF-IR) and IGF binding protein 5 (IGFBP5) expressions by real-time PCR was performed in tibial growth plates extracted at the time of catch-up growth, identified by osseous front advance greater than that of C animals.
Results: In the absence of GH treatment, catch-up growth was associated with increased IGF-I and IGFBP5 mRNA levels, without changes in GHR or IGF-IR. GH treatment maintained the overexpression of IGF-I mRNA and induced an important increase in IGF-IR expression.
Conclusions: Catch-up growth that happens after diet restriction might be related with a dual stimulating local effect of IGF-I in growth plate resulting from overexpression and increased bioavailability of IGF-I. GH treatment further enhanced expression of IGF-IR which likely resulted in a potentiation of local IGF-I actions. These findings point out to an important role of growth cartilage GH/IGF-I axis regulation in a rat model of catch-up growth.

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1. Introduction

Longitudinal growth may be impaired in several chronic disorders like inflammatory bowel diseases, juvenile idiopathic arthritis and malnutrition as well as by exposure to some drugs. The term catch-up growth, introduced by Prader et al. [1], describes the phenomenon by which longitudinal growth velocity transiently stands above the statistical limits of normality for age and/or maturity after the removal of a growth-inhibiting condition. Catch-up growth occurs in humans and animals and its underlying pathogenic mechanism is not clear [2,3]. A recent hypothesis proposes an intrinsic essential mechanism for catch-up growth in the long bones' growth plate [4].

Linear bone growth results from proliferation, hypertrophy and matrix production of cartilage cells in growth plate, on whose metaphyseal end, bone replaces the cartilage [5]. Growth plate of endochondral bones is comprised of cells representing various states of differentiation: the resting zone, the proliferative zone with actively dividing cells, and the hypertrophic zone with maturing cells enlarging and ultimately dying [6]. These proliferative and maturation processes are induced by numerous systemic endocrine effectors such as circulating growth hormone (GH). The effects of GH are to a great extent mediated by the actions of insulin-like growth factor-I (IGF-I), a peptide growth factor produced in great abundance by hepatic tissue, source of the circulating plasma IGF-I [7], but also locally produced by chondrocytes. Likewise, growth hormone receptor (GHR) [8–10] have been localized in several regions of the growth cartilage, demonstrating that chondrocytes are capable of responding directly to GH in a paracrine way. Cellular proliferation and apoptosis are carefully coordinated processes within the growth plate. The GH axis is known to play a critical role in proliferation and IGF-I is an identified regulator of apoptosis in many tissues [11,12]. The interplay of proliferation and apoptosis in the developing growth plate has only recently been

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investigated and has focused on the hypertrophic chondrocytes which are known to undergo apoptosis [13–18].

The regulation of longitudinal growth is influenced by numerous systemic and local factors. Although the interplay among these factors is still unclear [6], GH and IGF-I are crucial. Circulating GH can act directly on growth plate stimulating the local IGF-I synthesis, which in turns induces chondrocyte expansion [19]. Additionally, there are some evidences that systemic IGF-I could also stimulate growth plate chondrocytes [20]. However, locally derived IGF-I seems to be more important in longitudinal growth than systemic IGF-I [21]. Abnormalities of the GH/IGF-I axis are one of the major determinants of growth retardation in children with chronic illnesses [22]. There are experimental and clinical evidences demonstrating important alterations in GH/IGF-I axis during chronic diseases, like chronic renal failure [23], chronic bowel inflammatory diseases [24] and juvenile idiopathic arthritis [25]. It is well known that treatment with GH potentiates catch-up growth in several conditions [5,26]. The GH/IGF-I axis in the growth plate at the time of catch-up growth was previously implicated by Gat-Yablonski in a rodent model of food restriction [9]. Nevertheless, how catch-up growth is influenced by GH treatment is largely unknown.

A previous experimental study of our group [5] examined the pattern of catch-up growth in young rats after an inhibitory growth condition. The existence of a growth recovery implies the return to the previous growth path after noxious stimulus suppression. Our experimental model showed that growth rate acceleration inherent to a spontaneous catch-up, was associated with enhancement of proliferative activity, and specially, to an expansion of the hypertrophic zone of the cartilage. GH administration started at the time of noxious stimulus suppression, further accelerated catch-up growth velocity, this effect being likely related to a greater enlargement of the terminal chondrocyte height [5]. The study presented here used the above mentioned animal model to determine if the ability to exhibit catch-up growth and the effect of GH treatment on this process are related to alterations in the growth plate GH-IGF-I axis modulation.

2. Materials and methods

2.1. Experimental protocol

Samples from animals used in a formerly published study were utilized [5]. Briefly, young female Sprague-Dawley rats (Harlam Iberia, Barcelona, Spain) were acclimatized to the experimental area and randomly grouped as follows: controls fed ad libitum (group C), diet restriction for 3 days + refeeding (group CR), diet restriction for 3 days + refeeding & GH treatment (group CRGH). On days 11, 12 and 13 of the protocol, groups CR y CRGH received 50% of the average daily food consumed by the same animals the previous three days. CRGH animals were treated with 3.3 mg/kg/day recombinant human GH (Norditropin, Novo Nordisk Pharm, Madrid-Spain) given by intraperitoneal route from day 14 on. Untreated animals received the same volume of vehicle (saline). Rats were killed at different time points after refeeding (n = 5 rats each time). Catch-up growth, assessed by measurement of osseous front advance by calcein labeling, was found in CR and CRGH groups on day 21 (C: 164.5 ± 7.7 μm; CR: 186.1 ± 6.1 μm; CRGH: 201.1 ± 4.4 μm). Therefore, samples extracted on day 21 were used for the study presented here. The study complied with current legislation on animal experiments in the European Union and was approved by our Institution Ethics Committee for Investigation with animals.

2.2. Samples

The proximal ends of both tibiae were obtained after sacrifice by exsanguination under anesthesia. The dissection of growth plate was performed with magnifying lens and a scalpel to allow removing the adjacent bone zones accurately. The right tibiae were immediately frozen in liquid nitrogen for mRNA studies and the left tibiae were embedded in methylmethacrylate, fixed in 4% paraformaldehyde (PFA) and used for immunohistochemistry and in situ hybridization studies on growth plate as previously described [27–29].

2.3. Immunohistochemistry

Immunohistochemical staining was performed in PFA-fixed frontal sections (5 μm thick) of proximal tibiae, using IGF-I and IGF binding protein 5 (IGFBP5) polyclonal antibodies (Santa Cruz Biotechnology Inc, CA, USA) as described elsewhere [27].

2.4. In situ Hybridization

IGF-I, GHR, IGF-I receptor (IGF-IR) and IGFBP-5 antisense and sense RNA probes were supplied by Genedect (Genedetect.com, Bradenton, FL, USA). The hybridization procedure was carried out as described elsewhere [27]. Two parallel sections served as negative controls. One section hybridized with a labeled sense riboprobe and a second section was incubated adding a negative control probe to the hybridization mixture. No hybridization signal was found in any of these negative controls.

2.5. Real-time PCR

mRNA was extracted from frozen tibiae samples using modified Chomczynski method [30]. RNA purity and integrity were assessed by 260/280 ratio measurement using an ultra violet spectrophotometer (Gene Quant Pro, Amersham Biosciences, GE Healthcare Spain) and an 1.5% agarose gel respectively. Samples were diluted to 1 mg/ml, before reverse transcription, with a commercial kit (Qiagen Iberia, Madrid, Spain).

GHR, IGF-I, IGF-IR and IGFBP5 expressions were quantified by real-time PCR system (One-Step, Applied Biosystems, Foster City, CA, USA) using SYBR Green (ABgene Products, Thermo Fisher Scientific, Rockford, IL, USA) as fluorophore and β-actin as a housekeeping gene. For all the qPCR experiments, melting curve showed a unique product that corresponded with an exclusive temperature of DNA dissociation. Oligonucleotide primer pairs were as follows: for β-actin, forward: ACCAGGGTGTGATGGTGGGTAT and reverse: CGTCCCAGTTGACAATGC; for GHR, forward: AATTAATCCAAGCCTGAGGGAAA and reverse: GGAACGACACTTGGT GAATCG; for IGF-I, forward: TACCAGCTCGGCCACAGC and reverse: GTGGGCTTGTTGAAGTAAAAGC; for IGF-IR, forward: GCTGCTGGACCACAAATCG and reverse: TCGCTTCCCACACACACTTG; for IGFBP5, forward: GACCAAGGCCCCTGCCGCA and reverse: CACAGTTGGGCAGGTACACGGC. Primer sequences were designed with the Primer Express software (Perkin–Elmer Applied Biosystems) and synthesized by Invitrogen (Fisher-Invitrogen, Barcelona, Spain). Standard curves were obtained for each primer pair with efficiency near to 0.99. Afterwards, qPCR analysis was performed by ΔΔCt values.

2.6. Statistical analysis

Results are given as mean ± SEM and groups were compared using one-way ANOVA. When the normality test failed, ANOVA on ranks was used. When treatments were significantly different, Dunn's test and the Holm–Sidak method were used to perform pairwise multiple comparisons. Statistical significance was set at a two-tailed value of $P<0.05$.

3. Results

3.1. C and CR groups

3.1.1. Immunohistochemical analysis

Analysis of IGF-I and IGFBP5 by immunohistochemical technique showed no variations between C and CR groups for the distribution pattern. The positive signals for both peptides were largely restrained to

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