Spatial and temporal regulation of GH–IGF-related gene expression in growth plate cartilage

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Abstract

Previous studies of the GH–IGF system gene expression in growth plate using immunohistochemistry and in situ hybridization have yielded conflicting results. We therefore studied the spatial and temporal patterns of mRNA expression of the GH–IGF system in the rat proximal tibial growth plate quantitatively. Growth plates were microdissected into individual zones. RNA was extracted, reverse transcribed and analyzed by real-time PCR. In 1-week-old animals, IGF-I mRNA expression was minimal in growth plate compared with perichondrium, metaphyseal bone, muscle, and liver (70-, 130-, 215-, and 400-fold less). In contrast, IGF-II mRNA was expressed at higher levels than in bone and liver (65- and 2-fold). IGF-II expression was higher in the proliferative and resting zones compared with the hypertrophic zone ($P<0.001$). GH receptor and type 1 and 2 IGF receptors were expressed throughout the growth plate. Expression of IGF-binding proteins (IGFBPs)-1 through -6 mRNA was low throughout the growth plate compared with perichondrium and bone. With increasing age (3-, 6-, 9-, and 12-week castrated rats), IGF-I mRNA levels increased in the proliferative zone (PZ) but remained at least tenfold lower than levels in perichondrium and bone. IGF-II mRNA decreased dramatically in PZ (780-fold; $P<0.001$) whereas, type 2 IGF receptor and IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 increased significantly with age in growth plate and/or surrounding perichondrium and bone. These data suggest that IGF-I protein in the growth plate is not produced primarily by the chondrocytes themselves. Instead, it derives from surrounding perichondrium and bone. In addition, the decrease in growth velocity that occurs with age may be caused, in part, by decreasing expression of IGF-II and increasing expression of type 2 IGF receptor and multiple IGFBPs.


Introduction

The mammalian growth plate is a specialized cartilaginous structure at which longitudinal bone growth occurs. The growth plate is organized into three zones, the resting zone (RZ), the proliferative zone (PZ), and the hypertrophic zone (HZ). The RZ contains slowly replicating (Schrier et al. 2006) stem-like cells which can generate new clones of proliferative chondrocytes (Abad et al. 2002). Proliferative chondrocytes replicate at a high rate and align themselves in columns oriented parallel to the long axis of the bone (Aszodi et al. 2003). Farther away from the epiphysis, chondrocytes cease replicating, enlarge, and alter the extracellular matrix to form the HZ. Hypertrophic cartilage attracts blood vessels, osteoclasts, and differentiating osteoblasts which remodel the newly formed cartilage into bone tissue (Gerber et al. 1999). This combination of chondrogenesis and ossification results in longitudinal bone growth.

Over time, the growth plate undergoes functional and structural changes collectively referred to as growth plate senescence. This process, which appears to be due to a mechanism intrinsic to the growth plate (Nilsson & Baron 2004), causes longitudinal bone growth to slow with age and eventually cease resulting in attainment of adult body length/height.

The growth hormone–insulin-like growth factor (GH–IGF) system regulates longitudinal bone growth at the growth plate (van der Eerden et al. 2003). Thus, targeted ablation of the GH receptor, IGF-I, IGF-II, or the type 1 IGF receptor impairs bone growth (Baker et al. 2004, Liu et al. 1993, Mohan et al. 2003, Wang et al. 2004). This regulation involves both endocrine and autocrine/paracrine mechanisms. Pituitary GH acts on the liver to generate IGF-I which then acts as an endocrine factor to stimulate longitudinal bone growth (Daughaday 2000, Yakar et al. 2002). In addition, GH appears to act locally on bone to stimulate elongation (Isaksson et al. 1982, Isgaard et al. 1986, Schlechter et al. 1986). This local action of GH appears to be mediated in part by stimulation of local IGF-I production (Nilsson et al. 1986, Salmon & Burkhalter 1997). It has been suggested that GH acts primarily on the RZ, whereas IGF-I acts on the PZ (Gevers et al. 1996). However, other studies have not confirmed this finding (Hunziker et al. 1994, Gevers et al. 2002).
To explore the mechanisms by which the GH–IGF system regulates longitudinal bone growth, multiple previous studies have attempted to define the local expression of components of the GH–IGF system in the growth plate. However, some of the results have been conflicting. For example, controversy has arisen regarding the presence or absence of IGF-I mRNA in growth plate chondrocytes using in situ hybridization (Nilsson et al. 1990, Shinar et al. 1993, Wang et al. 1995, Reinecke et al. 2000).

The controversies concerning local expression of components of the GH–IGF system in the growth plate may arise from limitations in the methods employed. Immunohistochemistry and in situ hybridization provide precise spatial information but are primarily non-quantitative and may lack adequate specificity and sensitivity. Therefore, to complement existing data based on immunohistochemistry and in situ hybridization and to try to resolve the controversies concerning GH–IGF system gene expression in the growth plate, we chose to use real-time PCR, a quantitative method which can provide excellent specificity and sensitivity. To provide anatomic localization, we performed the assay on samples obtained by microdissection of the growth plate and surrounding tissue.

Materials and Methods
Animal procedures and tissue processing
Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2003). All animals received standard rodent chow (Zeigler Bros, Gardners, PA, USA) and water ad libitum. Male rats (n = 5 for 1-week old) and (n = 6), 3-, 6-, 9-, and 12-week-old were killed by carbon dioxide inhalation and proximal tibial epiphyses were rapidly excised, trimmed of cortical bone, embedded in Tissue-Tek OCT Compound (Electron Microscopy Sciences, Hatfield, PA, USA) and stored at −80 °C for subsequent processing. In addition, gastrocnemius muscle and a portion of the largest lobe of the liver were excised, immediately frozen on dry ice, and stored at −80 °C. To study growth plate senescence without the complicating effects of sex steroids, 3-, 6-, 9-, and 12-week-old rats were castrated at 18 days of life. Since sex steroid levels do not rise by 1 week of age, 1-week rats were not castrated. The protocol was approved by the Animal Care and Use Committee, National Institute of Child Health and Human Development, National Institutes of Health.

Growth plate microdissection
Frozen sections (60 μm) of proximal tibial epiphyses were mounted on Superfrost Plus slides (Fisher Scientific, Chicago, IL, USA). Slides were fixed, stained, and dehydrated in the following solutions for 1 min each at room temperature: 70% ethanol, 100% methanol, 95% ethanol, eosin, 70% ethanol, and 100% ethanol, xylene. Sections were microdissected under xylene. Standard precautions against RNases were employed. Using an inverted microscope, hypodermic needles, and razor blades, longitudinal sections of proximal tibiae from 1-week-old rats were separated into epiphyseal cartilage, RZ, PZ, proliferative hypertrophic transition zone, and HZ (Fig. 1). In addition, perichondrium and metaphyseal bone were collected from the slides. To avoid cross-contamination between RZ and PZ, a segment of cartilage containing the uppermost part of the proliferative columns and the lowest part of RZ was discarded. Due to decreasing growth plate height, the growth plates from older animals could not be microdissected into four individual zones, and therefore only PZ, metaphyseal bone and perichondrium were collected from 3-, 6-, 9-, and 12-week-old animals. For each zone, microdissected tissue from 15–35 sections from both epiphyses, from a single animal was pooled prior to RNA isolation. RNA isolation was performed as previously described except that we used one-tenth of every volume (Heinrichs et al. 1994). For liver and muscle samples, the volumes were not reduced. The final pellet was suspended in 9 μl DEPC-treated water. Approximately, 30–200 ng total RNA were extracted from each zone of the growth plate in individual 1-week-old animals and at least 200 ng from proliferative cartilage of 3-, 6-, 9-, and 12-week-old animals. The 28S/18S ratio was typically between 1.7 and 2.0 as assessed by a Bioanalyzer 2100 using RNA Pico Chips and version A.02.12 of the Bio Sizing software according to manufacturer’s instructions (Agilent Biotechnologies Inc., Palo Alto, CA, USA).

Figure 1 Growth plate microdissection. Representative photomicrograph of a microdissected proximal tibial epiphysis from a 7-day-old rat. The section (60 μm) has been cut with a blade to separate the sample into epiphyseal cartilage (EC), resting zone (RZ), proliferative zone (PZ), proliferative hypertrophic transition zone (TZ), and hypertrophic zone (HZ). To minimize cross-contamination between the resting zone and the proliferative zone, a segment of cartilage containing the transition from resting to proliferative zone was discarded.
Validation of microdissection

Levels of collagen type X and alkaline phosphatase, both markers of HZ chondrocytes, were previously determined using real-time PCR. A 250-fold higher expression level of collagen type X and a 100-fold higher expression level of alkaline phosphatase in the HZ versus the PZ confirmed the accuracy of our microdissection technique (Nilsson et al. 2007).

Real-time quantitative RT-PCR

For real-time PCR, 30–200 ng total RNA were reverse transcribed using 200 U Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA solution was diluted 10–25 times and stored at −20°C until used for real-time PCR. Quantitative real-time PCR was performed using the following pre-prepared assays containing primers and specific intron-spanning FAM- or VIC-labelled (18S rRNA) TaqMan probes provided by Applied Biosystems (Foster City, CA, USA): IGF-I, Rn00710306 m1; IGF-II, Rn01454518 m1; GH receptor, Rn00567298 m1; type 1 IGF receptor, Rn00583837 m1; type 2 IGF receptor, Rn00562622 m1; IGF–binding protein (IGFBP)-1, Rn00565713 m1; IGFBP-2, Rn00565473 m1; IGFBP-3, Rn00561416 m1; IGFBP-5, Rn00563116 m1; IGFBP-6, Rn00567035 m1; and 18S rRNA, 4319413E. Alternatively, intron-spanning primers were designed using Primer Express 2.0 (Applied Biosystems): IGFBP-4 forward, CGTCCGGTGGCCAGGGTCTCT; IGFBP-4 reverse, GAAGCTTCACCCCTGTCTTCG. These primers and SYBR green (Applied Biosystems) were used for the PCRs. The SYBR green assay was confirmed by generating a single dissociation curve analysis during the study. Reactions were performed on each animal’s cDNA in triplicate using 1 μl cDNA solution, 2× TaqMan Universal PCR Master Mix or SYBR green Master Mix (Applied Biosystems), primers and probes (Applied Biosystems) in a 24 μl final reaction volume, using the ABI prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. The cDNA was amplified using the following thermal cycling conditions: one cycle at 50°C for 2 min and 95°C for 10 min, followed by 42 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantity of each mRNA was calculated relative to the amount of starting cDNA (using the calibrator gene 18S rRNA) taking into account the efficiency of the respective PCRs, using the formula: Relative Expression, \( E_{i}^{r} = \frac{(E_{i}^{r})_{CT}}{(E_{i}^{c})_{CT}} \times 10^{6} \), where \( r \) represents 18S rRNA, \( i \) represents the gene of interest, \( E \) represents efficiency of the PCR, and CT represents the threshold cycle (equation modified from Pfaffl 2001). Relative expression values were multiplied by 10^6 for convenience. Serial tenfold dilutions of bone, liver, or spleen cDNA were used to determine the efficiencies of the PCRs. In order to determine intra- and interassay variability, IGF-II mRNA concentration of one cDNA sample was assessed twice in five independent real-time PCR experiments. In these experiments, the intra- and interassay coefficients of variation were found to be 4-3 and 11% respectively.

Statistical analysis

The data are presented as mean ± S.E.M. All expression data were log-transformed before analysis to obtain a normal distribution. The SigmaStat 3.1 statistical program was used to perform all statistical measures. Statistical analysis was performed on the paired data (several zones were dissected from each animal) of 1-week-old rats using a paired t-test comparing RZ, PZ, and HZ. The Holm method was employed to correct for multiple comparisons. Comparison of expression levels in samples collected from rats of different ages was performed by one-way ANOVA on the log-transformed relative expression data with age as the independent variable. Age comparisons were made for PZ, perichondrium, and metaphyseal bone independently. P values < 0.05 were considered significant.

Results

Spatial mRNA expression in 1-week-old rat growth plate, perichondrium, and metaphyseal bone

IGF-I mRNA expression was minimal in all zones of the growth plate in 1-week-old animals. In particular, expression of IGF-I mRNA in the growth plate was ~70-fold less than in perichondrium, 130-fold less than in metaphyseal bone, 215-fold less than in muscle, and 400-fold less than in liver (Fig. 2A). In contrast, IGF-II mRNA expression in the RZ and PZ of the growth plate was ~3-fold higher than in perichondrium, 2-fold higher than in liver, 65-fold higher than in bone, and similar to levels in muscle. Within the growth plate, expression of IGF-II mRNA was approximately fivefold lower in HZ than in PZ and RZ (\( P < 0.01 \), Fig. 2B).

GH receptor mRNA was expressed throughout the growth plate at levels similar to surrounding perichondrium and metaphyseal bone but at substantially lower levels than in muscle and liver (~10- and 15-fold respectively; Fig. 3A). Expression of type 2 IGF receptor mRNA was present in all growth plate zones with levels higher in RZ than in PZ and HZ (~1.5- and 2.2-fold respectively; both \( P < 0.05 \); Fig. 3C). Type 1 IGF receptor mRNA was expressed throughout the growth plate with levels in PZ approximately threefold higher than in HZ (\( P < 0.05 \); Fig. 3B).

In 1-week-old animals, IGFBP-2 through IGFBP-6 showed minimal mRNA expression in the growth plate when compared with levels in liver and muscle (Fig. 4). IGFBP-1 was undetectable in all zones of the growth plate but was expressed in perichondrium and bone (data not shown). With the exception of IGFBP-2, mRNA expression of all IGFBPs was significantly higher in perichondrium than in the growth plate zones (Fig. 4B–E). Within the growth plate,
IGFBP-3 and IGFBP-5 mRNA expression was significantly lower in PZ versus HZ (nine- and sixfold respectively, \(P<0.01\); Fig. 4B and D).

**Temporal changes in mRNA expression in the growth plate and surrounding tissues**

Expression of IGF-II mRNA decreased significantly with age from 1 to 12 weeks in growth plate PZ (780-fold), perichondrium (45-fold), and metaphyseal bone (14-fold; all \(P<0.001\); Fig. 5B). In contrast, IGF-I mRNA was expressed at similar levels throughout development in
Figure 4 Expression of IGFBP-2 through IGFBP-6 mRNA in growth plate (black bars) compared with surrounding tissues, muscle, liver, and epiphyseal cartilage (hatched bars). Proximal tibial growth plates from 1-week rats (n= 5) were microdissected into individual zones. The relative amount of each mRNA was determined using real-time PCR, normalized to 18S rRNA, and multiplied by $10^6$. 

perichondrium and metaphyseal bone. In growth plate PZ, IGF-I mRNA expression increased 25-fold with age ($P<0.001$; Fig. 5A). Despite this increase, IGF-I mRNA expression remained much lower in the growth plate than in perichondrium and bone.

We did not detect any age-dependent changes in the mRNA expression of GH receptor or type 1 IGF receptor in the growth plate (Fig. 6A and B). In contrast, type 2 IGF receptor mRNA expression increased with age in growth plate PZ (fourfold; $P<0.001$), decreased with age in metaphyseal bone (threefold; $P<0.05$), and was detected at a similar level throughout postnatal development in perichondrium (Fig. 6C).

Though undetectable in the growth plate of 1-week-old rats, IGFBP-1 mRNA expression was detected in PZ of 3-, 6-, 9-, and 12-week-old rats. In perichondrium, there was increased expression with age of IGFBP-1 (100-fold), IGFBP-2 (22-fold), IGFBP-3 (4-fold), and IGFBP-4 (2-fold; Fig. 7). This pattern was also seen in the metaphyseal bone for expression of IGFBP-3 (12-fold) and IGFBP-4 (2-fold; Fig. 7C and D).

Discussion

Using manual microdissection and quantitative real-time PCR, we found that IGF-II, not IGF-I, is the predominantly expressed IGF in growth plate chondrocytes of young rapidly growing rats. In addition, in 1-week-old rats, GH receptor and type 1 IGF receptor are expressed in all zones of the growth plate and mRNA expression of the IGFBPs is very low when compared with other tissues studied. With age, as the rat's growth velocity decreases, IGF-II mRNA expression decreases dramatically. Furthermore, expression of many of the IGFBPs and the type 2 IGF receptor increases with age, suggesting a decline in the availability of IGFs in growth plate cartilage. These findings represent expression of components of the GH–IGF system in rat epiphyses without the influence of sex steroids.

Manual microdissection provides a simple and reliable method that allows for RNA extraction from distinct zones of the growth plate and surrounding tissue. In order to minimize cross-contamination between zones, a buffer zone was discarded between RZ and PZ and a transition zone containing lower PZ and upper HZ was collected. However, a low-grade contamination between bordering zones cannot be excluded and differences in mRNA expression between bordering zones may thus represent an underestimation.

We found that IGF-I mRNA was expressed at far lower levels in growth plate than in any other tissue studied, suggesting that local production of IGF-I by growth plate chondrocytes may not be biologically important. These findings help resolve the long-standing controversy regarding IGF expression in growth plate. Some previous studies using in situ hybridization found that IGF-I is not expressed in growth plate chondrocytes (Shinar et al. 1993, Wang et al. 1995), whereas other studies did report IGF-I expression (Nilsson et al. 1990, Reincke et al. 2000). Our study helps resolve this controversy using a new independent approach that has several advantages. First, real-time PCR is highly sensitive because it exponentially amplifies the signal. Secondly, real-time PCR provides high specificity for IGF-I because a signal is only generated if both of the primers and the probe hybridize to the cDNA. The completely different expression patterns for IGF-I and IGF-II seen in our study exclude the possibility of cross-reactivity which might confuse in situ hybridization and immunohistochemical studies. Thirdly, we quantitatively compared expression levels with those in other tissues to gauge whether the levels detected in growth plate are likely to be biologically important. Additionally, the conflicting
results regarding IGF-I expression by growth plate chondrocytes may reflect differences in the animal models studied.

Previous studies demonstrate that IGF-I has an important effect on growth plate chondrocytes in vivo (Liu et al. 1993, Wang et al. 2004). Although our data do not exclude an effect of local IGF-I production by chondrocytes, the very low levels of IGF-I expression instead suggest that IGF-I protein in the growth plate primarily arrives by diffusion from local sources in perichondrium and/or adjacent bone and from liver and other systemic sources via the circulation.

In contrast to IGF-I, IGF-II was highly expressed in the growth plate cartilage when compared with other tissues studied. For example, growth plate cartilage expressed IGF-II at levels similar to those found in muscle, which is known to express IGF-II at high levels (Brown et al. 1986). These findings confirm previous studies of IGF-II in growth plate using in situ hybridization (Shinar et al. 1993, Wang et al. 1995). In addition, we found that expression of IGF-II mRNA is higher in RZ and PZ than in HZ, suggesting that IGF-II may regulate cell proliferation of both the stem-like cells of RZ and the highly proliferative cells of PZ.

GH receptor was detected at similar levels in all zones of the growth plate, whereas type 1 IGF receptor was expressed at approximately threefold higher levels in PZ than in HZ. These data are consistent with the findings reported by Hunziker et al. (1994) that GH acts not only on RZ chondrocytes, but also in PZ and HZ. The type 1 IGF receptor was also expressed throughout the growth plate, which is consistent with the concept that IGFs regulate all zones of the growth plate (Hunziker et al. 1994). The particularly high levels of type 1 IGF receptor expression in PZ may help support the high proliferation rate in that zone. The presence of GH receptor mRNA and minimal amounts of type 2 IGF receptor expression in growth plate chondrocytes raises the possibility that GH may act on growth plate chondrocytes by an IGF-independent mechanism. In contrast, both GH receptor and IGF-I mRNA were expressed in the perichondrium suggesting that GH may also exert effects by stimulating IGF-I production in the perichondrium.

Expression of all IGFBPs was low in the growth plate of 1-week-old rats. Expression of IGFBP-1 through IGFBP-6 has been demonstrated in many tissues (Schuller et al. 1994) and as a group, these proteins tend to inhibit the action of IGFs. The low level of IGFBP expression in growth plate of rapidly growing bones may enhance the biological effect of IGFs present in the growth plate, regardless of their source. This hypothesis is further supported by the fact that within the growth plate, IGFBP-3, IGFBP-5, and IGFBP-6 mRNA showed the lowest level of expression in PZ, the area of greatest cell proliferation.

IGF-II mRNA expression in PZ decreased ~1000-fold from 1 to 6 weeks of age. IGF-II mRNA expression also declined markedly with age in perichondrium and metaphyseal bone. This age-dependent loss of IGF-II expression may contribute to the age-dependent decline in growth rate. This concept is consistent with the observation
that IGF-II deficiency in mice impairs longitudinal bone growth primarily early in life (Mohan et al. 2003). To our knowledge, these are the first data that quantify the dramatic age-dependent decline in IGF-II mRNA expression in growth plate chondrocytes. Others have shown an analogous decline of IGF-II mRNA in non-skeletal tissues of the rat, including muscle, heart, intestine, and liver from prenatal to adult life (Brown et al. 1986, Lund et al. 1986). This decline in multiple tissues during postnatal development suggests a regulatory mechanism governing IGF-II expression that is common to multiple cell types.

The type 2 IGF receptor, also known as the mannose-6-phosphate receptor, acts to eliminate IGFs from the extracellular fluid by receptor-mediated endocytosis (Oka et al. 1985, Morgan et al. 1987). We found a significant increase in expression of type 2 IGF receptor mRNA with age. This change may limit the availability of IGFs, also contributing to the decline in growth rate that occurs with age. It is possible that, in the setting of a significant decrease in IGF-II, as occurs in the rat with age, fewer of the type 2 IGF receptors will be occupied by IGF-II and thus more of the type 2 receptors will be available to bind IGF-I. A similar hypothesis is suggested by Ludwig et al. (1996) to explain their observation that mice lacking both the IGF-II and the type 2 IGF receptor are heavier than those which lack only IGF-II.

In the tissues studied, we found that mRNA expression of IGFBPs tends to increase with age. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 increased in perichondrium, and IGFBP-3 and IGFBP-4 increased in metaphyseal bone. This pattern of increased expression of many of the IGFBPs with age in the growth plate, perichondrium, and bone further supports the idea that growth plate senescence may be due in part to a decreased availability of IGFs. Interestingly, the most consistent increase in IGFBP expression is seen in the perichondrium where IGF-I mRNA expression is the highest, suggesting an inhibitory effect on IGF-I action.
with age. Increased expression of IGFBPs might also have IGF-independent actions on growth plate chondrocytes, as reported in other cell types (Leal et al. 1997, Rajah et al. 1997, Miyakoshi et al. 2001, Salih et al. 2004).

In conclusion, we used manual microdissection and real-time PCR to study expression of the GH–IGF system in growth plate cartilage, including both the spatial and temporal regulation of expression. We found that in the young, rapidly growing rat, IGF-I mRNA is expressed in perichondrium but is minimally expressed in growth plate chondrocytes themselves. In contrast, IGF-II is highly expressed by growth plate chondrocytes. These findings suggest that IGF-II is the predominant IGF expressed by growth plate chondrocytes during early postnatal growth and that IGF-I protein in the growth plate is primarily derived from plasma, surrounding perichondrium and/or bone. With age, as the rat growth velocity decreases, there is a dramatic decline in IGF-II mRNA expression in the growth plate, an increase in expression of type 2 IGF receptor mRNA in growth plate cartilage, and an increase in many of the mRNAs for IGFBPs in growth plate, perichondrium, and metaphyseal bone, all of which may contribute to the fundamental biological mechanism that causes the age-dependent decline in longitudinal bone growth, and thus limit the overall size of the organism.

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