Original Article

Lengthening of mouse hind limbs with local administration of insulin-like growth factor 2

Ping Zhang a,b,c,*, Chang Jiang a, Hiroki Yokota a,c

a Biomedical Engineering, Indiana University Purdue University, Indianapolis, Indiana, USA
b School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China
c Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana, USA

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Abstract

Background: For devising potential clinical approaches for limb length discrepancies, we examined local administration of insulin-like growth factor 2 (IGF2).

Methods: C57/B6 mice (~ 8 weeks old) were used in this study, and the mice were separated into two groups: an IGF2-treated group and a placebo group. In the IGF2-treated group, IGF2 was locally administered into the distal epiphysis of the left femur, and the right femur was used as a contralateral control. In the placebo group, saline was administered to the left femur as a vehicle control. The left and right tibiae, without any direct intervention, were employed as negative controls. The dosage of IGF2 was 100 μg/kg/day for 5 consecutive days, and bone samples were harvested on Day 14. Microcomputed tomography images did not show any anomaly at the IGF2 or saline injection sites.

Results: In comparison with the vehicle control as well as the contralateral control, the results revealed that IGF2 significantly lengthened the treated femur, with an elevation of bone mineral density (BMD) as well as bone mineral content (BMC). The increase in the femoral length of the IGF2-treated left limb was 1.6% (p < 0.05) to the vehicle control, and 1.7% (p < 0.05) to the contralateral control. However, the length, BMD, and BMC of the tibiae were not affected by administration of IGF2 or saline. Western blotting analysis demonstrated that this administration of IGF2 upregulated phosphorylation of an extracellular signal-regulated kinase in the treated femur.

Conclusion: The current study supports for the first time the potential effectiveness of administration of IGF2 in adjusting limb length discrepancy.

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Keywords: bone length; femur; insulin-like growth factor; mouse

1. Introduction

Limb length discrepancy occurs for many reasons, including bone injury, bone infection, inflammatory diseases, and neurologic disorders.1,2 In the general population, approximately 25% of the populace exhibit a length difference of 1 cm or more in their lower limbs.3 Some of these differences are congenital (from birth), develop from childhood diseases, or may affect the elderly due to posttraumatic incidents and osteoporosis.4,5 Although the effects of such a discrepancy in limb length differ depending on the individual’s age and stature, treatments are usually recommended when the difference exceeds 2 cm.6 With the exception of the use of shoe lifts, the currently available treatments are both surgical and invasive.7 Bones do have the capacity to remodel when the surgical treatment is not excessively disassembling. However, there are risks associated with the surgical options, including premature consolidation, delayed union, and infection. As a potentially noninvasive treatment, this study was designed to evaluate the use of locally administered insulin-like growth factor 2 (IGF2) and assess its impact on discrepancy in limb length.
In the regulation of bone formation and remodeling, a growth hormone (GH) insulin-like growth factor (IGF) axis is known to play a key role, and it is important for longitudinal bone growth. In the GH-IGF axis, two known IGFs are IGF1 and IGF2, which are polypeptides with high sequence similarity to insulin. GH regulates synthesis of IGF1 primarily in the liver. It has been reported that administration of IGF1 is effective in lengthening the tibia in rabbits, although an increased incidence of adrenal medullary hyperplasia and pheochromocytoma was reported. IGF2 is an imprinted gene in both mice and humans, producing a 7.5 kD mature protein consisting of 67 amino acid residues. It is considered to be active primarily at the early stage of skeletal growth and its knockout mice are smaller and lighter. However, little is known about its role in the mature skeleton, particularly in longitudinal bone growth.

In the present study we addressed a question: does local administration of IGF2 to a femur lengthen it compared with a contralateral femur or a vehicle-treated femur without affecting the length of a tibia? It was hypothesized that IGF2, if locally administered to the femur, would stimulate longitudinal growth only in the treated femur. To lengthen the mouse hind limb, we previously developed a knee loading modality in which gentle mechanical loads (5 minutes/day at 0.5 N) were applied sideways to the knee. This loading modality was able to stimulate longitudinal bone growth of the hind limb, but it altered not only the length of the femur but also that of the tibia. In this study, we aimed to lengthen the treated femur without affecting the tibia.

Our previous studies, as well as earlier investigations by other researchers, indicated that IGF2 activates transforming growth factor beta (TGFβ) and extracellular signal-regulated kinase (ERK) signaling. To evaluate a potential mechanism linked to the ERK pathway, the level of phosphorylation of ERK1/2 was examined in response to administration of IGF2 and saline.

2. Methods

2.1. General preparations

Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Forty C57/BL/6 female mice (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were used. In the current project, we used ~8-week-old mice, which are roughly equivalent to people 12 years of age. Four to five mice were housed per cage, and they were provided with mice feed and water ad libitum. The animals were allowed to acclimate for 2 weeks before experimentation.

The four limb samples in this study included: (1) two vehicle limbs (left, saline injected limb, right, contralateral saline limb), and (2) two IGF2 limbs (left, IGF2 injected limb; right, contralateral IGF2 limb). Using C57/BL/6 mice (~8 weeks), we examined the hypothesis by measuring bone length, bone weight, bone mineral density (BMD), and bone mineral content (BMC) of the femora and tibiae. Since clinical data indicate an increase in body weight following the administration of IGF1, we monitored total body weight before and after the administration of IGF2.

2.2. Administration of IGF2

Thirty mice were divided equally between the IGF2 treated group (n=15) and the vehicle control group (n=15). All animals were weighed prior to initial injection of either IGF2 or saline and at the time of sacrifice. After the mice were given general anesthesia with 1.5% isoflurane, their knees were shaved with scissors and a small incision was made in the skin at the lateral part of the knee joint. In the IGF2 group, a total volume of 5 μl phosphate buffered saline (PBS) containing 0.4 μg/μl recombinant IGF2 (R & D systems, Inc., Minneapolis, MN, USA; 792-MG) was injected into the distal epiphyseal region of the left femur using a microsyringe (Hamilton Company, Reno, Nevada, USA) with a #30 gauge needle. In the placebo group, the left epiphysis of the distal femur received a similar amount of saline. Local administration of IGF2 was performed daily for 5 consecutive days with a dosage of 100 μg/kg/day. After administration of IGF2 or PBS, the animals were allowed to move freely in the cage and they were sacrificed on Day 14 (Fig. 1).

2.3. Microcomputed tomography imaging

Microcomputed tomography imaging was performed to evaluate the extent of bone damage, if any, due to administration of IGF2 or PBS, using a desktop μCT-20 (Scanco Medical AG, Bruttisellen, Switzerland; Fig. 1). The bone sample was centered in the gantry of the machine in a plastic tube filled with 70% ethanol. A series of cross-sectional images were captured at 30-μm resolution. Three-dimensional reconstruction was performed and the images were analyzed using Scanco Medical software (μCT v.1.4).21

2.4. Measurements of bone length, bone weight, BMD, and BMC

Femora and tibiae were harvested. These samples were cleaned of soft tissue and fixed in 10% neutral buffered formalin. After 48 hours in the fixative, they were transferred to a 70% alcohol solution for storage. The lengths of the femur and the tibia were determined using a digital caliper at 10-mm resolution (Mitutoyo Co., Aurora, IL, USA). Femoral length was defined as the maximum distance from the distolateral condyle to the most medial and proximal position on the femoral head. Tibial length was defined from the most proximal position of the tibial plateau to the most distal position of the medial malleolus. Bone weight was determined immediately after length measurements with an electronic balance. Using the procedure described previously, BMD and BMC of the entire femur and tibia were determined with a PIXImus densitometer (version 1.4, GE Medical System Lunar, Lunar Corp., Madison, WI, USA). Note that...
the “percent alterations” were defined as differences between the treated (T) and the control (C) hind limbs such as \((\frac{|T - C|}{C} \times 100\%\)).

2.5. Western blot analysis

Ten mice were used for protein analysis. IGF2-treated and control femora were isolated 3 hours after the administration of IGF2 or saline on Day 5. Soft surrounding tissues were dissected out from the samples, which were then ground with a mortar and pestle in a radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, San Diego, CA, USA). The samples were sonicated using a sonic dismembrator (Model 100, Fisher Scientific, Hanover Park, IL, USA). Proteins were fractionated using 10% sodium dodecyl sulfate (SDS) gels, and electro-transferred to Immobilon-P membranes (Millipore). The membrane was incubated for 1 hour with primary antibodies followed by 45 minutes of incubation with goat antirabbit immunoglobulin (Ig) G (Cell Signaling Technology, Danvers, MA, USA) or goat antimouse IgG conjugated horseradish peroxidase (Amersham Biosciences, Piscataway, NJ, USA). We used antibodies against ERK1/2, p-ERK1/2 (Thr202/Tyr204), and b-actin (Sigma, St. Louis, MO, USA). Protein levels were assayed using an enhanced chemiluminescence (ECL) advance Western blotting detection kit (Amersham Biosciences), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film Manufacturing USA, Inc., Greenwood, SC, USA).

2.6. Statistical analysis

The data were expressed as mean ± standard deviation. One-way analysis of variance was employed to evaluate statistical significance among the groups. For pair-wise comparisons, a post-hoc test was conducted using Fisher’s protected least significant difference (PLSD) test. A paired t-test was used to evaluate statistical significance between the treated and control samples. All comparisons were two-tailed and statistical significance was assumed for \(p < 0.05\).

3. Results

3.1. Mouse characteristics

During administration of IGF2 or saline no apparent damage was detected at the site of injection. Furthermore, microcomputed tomography images did not show any apparent bone damage at the injection site at harvest time (Fig. 1). After the treatments no mice showed a weight loss or a diminished food intake.
3.2. Body weight gain by administration of IGF2

At harvest after 2 weeks of treatment, the body weight of the vehicle control group was slightly elevated (17.54 ± 0.83 g to 17.82 ± 0.63 g). In the IGF2 treated group the weight increase was larger (17.62 ± 1.06 g to 18.65 ± 0.93 g) than the vehicle control group (Table 1). Specifically, the weight gain of the IGF2 treated group (1.03 ± 0.26 g) during 2 weeks was significantly greater than weight gain in the vehicle control group (0.28 ± 0.26 g; *p* < 0.001).

3.3. IGF2-driven increase in bone length and bone weight in the femur

Administration of IGF2 increased bone length and bone weight in the femur. Compared with the vehicle control, the increase in the femoral length over the 2-week period was 1.6% (13.77 ± 0.27 mm in vehicle and 13.99 ± 0.28 mm in IGF2; *p* < 0.05). This is shown in Fig. 2. Compared with the contralateral control, the IGF2 injected femur increased bone length by 1.7% (13.75 ± 0.25 mm in the contralateral femur and 13.99 ± 0.28 mm in the IGF2-treated femur; *p* < 0.05). Furthermore, the gain of the femoral weight was (48.54 ± 2.33 mg in the vehicle injected femur and 51.08 ± 2.41 mg in the IGF2-treated femur; *p* < 0.01). This is shown in Fig. 3. The femoral weight increased in 2 weeks by 6.5% (47.97 ± 2.05 mg in contralateral control and 51.08 ± 2.41 mg in IGF2; *p* < 0.001). However, in the tibia, no statistical difference was detected between the IGF2 and saline groups in bone length (*p* = 0.293) and bone weight (*p* = 0.505). No difference was observed in the tibiae between the IGF2-treated and the contralateral hindlimbs in bone length (*p* = 0.362) or bone weight (*p* = 0.157).

3.4. IGF2-driven increase in BMD and BMC in the femur

Local administration of IGF2 increased BMD and BMC in the femur. Compared with the IGF2 and the vehicle groups, IGF2-treated group increased BMD (0.0434 ± 0.0020 g/cm² in vehicle; and 0.0450 ± 0.0020 g/cm² in IGF2; *p* < 0.05). This is shown in Fig. 4. Compared with the contralateral control, the IGF2 injected femur increased BMD (0.0427 ± 0.0017 g/cm² in contralateral control; and 0.0450 ± 0.0020 g/cm² in IGF2; *p* < 0.01) and BMC (0.0157 ± 0.0014 g in contralateral control; and 0.0172 ± 0.0011 g in IGF2; *p* < 0.01). This is shown in Fig. 5. However, no statistically significant difference after administration of IGF2 or saline was observed in BMD (*p* = 0.813~0.957) or BMC (*p* = 0.622~0.733) in the tibia.

3.5. Activation of ERK phosphorylation

As a part of signaling mechanism with administration of IGF2, we examined the phosphorylation level of ERK1/2. The Western blot analysis revealed that, compared to the vehicle control, phosphorylation of ERK1/2 in the IGF2 treated femur was significantly activated (Fig. 6). No differences were observed in the tibia (data not shown).

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**Table 1**

Body weight in the vehicle control and IGF2-treated groups (n = 15/group).

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Control (PBS)</th>
<th>Administration of IGF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 (mean ± SD) (g)</td>
<td>17.54 ± 0.83</td>
<td>17.62 ± 1.06</td>
</tr>
<tr>
<td>Day 14 (mean ± SD) (g)</td>
<td>17.82 ± 0.63</td>
<td>18.65 ± 0.93</td>
</tr>
<tr>
<td>Gain from Days 1 to 14 (mean ± SD) (g)</td>
<td>0.28 ± 0.26</td>
<td>1.03 ± 0.25</td>
</tr>
</tbody>
</table>

IGF2 = insulin-like growth factor 2; PBS = phosphate buffered saline; SD = standard deviation.

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**Fig. 2.** IGF2-driven alterations in bone length. (A) Alterations in the femur length; (B) alterations in the tibia length. *p* < 0.05 (n = 15/group). IGF2 = insulin-like growth factor 2.
4. Discussion

This study demonstrated that local administration of IGF2 to the distal femur increased the treated femur length without altering the contralateral femur length or any of the tibia length. Unlike knee loading that alters both femur length and tibia length, this study presented a possibility of lengthening the length of femur alone with a minimally invasive method. For lengthening bone in a clinical practice, distraction osteogenesis is a widely used surgical procedure. In the procedure, a surgical cut is made in the bone to be lengthened and two sections of the bone are spread and fixed with an internal metal device and an external fixator. In spite of its efficacy, one of the potential complications of the procedure is delayed union resulting from slow healing and premature consolidation, where two sections fuse prematurely without effective lengthening, and the bone needs to be cut again. Thus, the data presented herein with local administration of IGF2 support a possibility of noninvasive treatments to limb length discrepancy.

IGFs (IGF1 and IGF2) are known to be key regulators in bone growth, remodeling, and repair. It is also well understood that IGFs can be synthesized and stored by bone tissue. IGF1 is a primary mediator of GH and stimulates systemic body growth including skeletal muscle, cartilage, and bone throughout the human lifetime, whereas IGF2 most efficaciously promotes growth during gestation. Various IGF-binding proteins and specific proteases have been identified, indicating that fine-
tuning of the availability of free IGF1 or IGF2 is of crucial importance for bone formation and remodeling. Both IGF1 and IGF2 bind to IGF receptor 1, but only IGF receptor 2 can be bound by IGF2 and not by IGF1. They are reported to be able to recruit primary osteoblastic cells, and regulate proliferation and differentiation of chondrocytes in the growth plate.9 Our results support the notion that therapeutic administration of IGF2 can enhance longitudinal bone growth in a mature skeletal system in which IGF2 is not considered to be actively utilized.

Note that administration of IGF1 is reported to cause adverse effects including lymphoid hyperplasia, and an increase in body fat.9,24 Although the current study reveals that administration of IGF2 also elevates body weight, it is yet to be investigated whether any other side effects associated with IGF2 exist and whether a combinatory administration of IGF1 and IGF2 (and other factors) may minimize adverse effects.9

We have previously shown that a C28/I2 chondrocyte cell line that administration of IGF2 (5 hours at 10–100 ng/ml) activates TGFβ signaling,19 which stimulates osteoblast development through activation of ERK.9 Although other pathways such as phosphoinositide 3-kinase signaling are also involved,25 we evaluated any linkage between administration of IGF2 and the phosphorylation of ERK. The results show that the level of ERK phosphorylation is locally elevated in the treated femur without significantly affecting its level in the tibia in the same hind limb. Studies on gene regulation of IGF2 are mostly conducted in the area of the early stage of skeletal development and imprinting, and further analysis of the mechanisms and responses to IGF2 in the mature skeleton is warranted to enhance our understanding of bone lengthening. A better comprehension of the effects of longer treatments (e.g., 1–2 months) might be needed to develop a potential therapy for treating limb length discrepancy.

In conclusion, bone-lengthening effects are observed with local administration of IGF2 in the femur. Unlike knee loading that stimulates lengthening of the femur and the tibia, the effects of IGF administration are restricted to the femur without altering the length of the tibia. The weight, BMD, and BMC of the treated femur are also elevated.

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Fig. 5. IGF2-driven alterations in BMC. (A) Alterations in BMC of the femur; (B) alterations in BMC of the tibia. *p < 0.01 (n = 15/group). BMC = bone mineral content; IGF2 = insulin-like growth factor 2.

Fig. 6. Phosphorylation levels of extracellular signal-regulated kinase (ERK) in the femur in response to administration of IGF2 (n = 10). IGF2 = insulin-like growth factor 2.

References


