Cysteine induces longitudinal bone growth in mice by upregulating IGF-I

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Abstract. Cysteine (Cys) is known to exert various effects, such as antioxidant, antipancreatic and antiadipatic effects. However, the effects of Cys on longitudinal bone growth have not been elucidate to date. Thus, the aim of the present study was to evaluate the effects of Cys on bone growth. Growth-plate thickness and bone parameters, such as bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), connectivity density (Conn.D) and total porosity were analyzed by means of micro-computed tomography (μCT). The levels of serum insulin-like growth factor-I (IGF-I) were measured by enzyme-linked immunosorbent assay (ELISA). Hepatic IGF-I mRNA expression was analyzed by quantitative polymerase chain reaction (qPCR). The phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5) was investigated by western blot analysis. Our results revealed that Cys increased IGF-I mRNA expression in HepG2 cells. The thickness of the growth plates was increased following treatment with Cys. Moreover, BV/TV, Tb.Th, Tb.N, Conn.D and total porosity were improved following treatment with Cys. Hepatic IGF-I mRNA expression and serum IGF-I levels were increased by Cys. The levels of phosphorylated JAK2 and STAT5 were elevated by Cys. The findings of our study indicate that Cys increases the thickness of growth plates through the upregulation of IGF-I, which results from the phosphorylation of JAK2-STAT5. Thus, our data suggest that Cys may have potential for use as a growth-promoting agent.

Introduction

Longitudinal bone growth is a results of the proliferation, hypertrophy and matrix production of cartilage cells in growth plates (1). The growth plate is a relatively thin layer of cartilage which is found in growing long bones during longitudinal skeleton growth (2,3). The growth of the long bone at the growth plate is regulated by an endocrine signaling system that involves glucocorticoids, growth hormone (GH) and insulin-like growth factor-I (IGF-I) (4). If a child is afflicted by poor nutrition, the levels of thyroid hormones and IGF-I decline, and during puberty, malnutrition also causes the downregulation of sex steroids. These endocrine changes decrease the elongation of bones (4). In order to reproduce this malnutrition, mice are fed a low-protein diet. Protein-energy malnutrition (PEM) is an important form of undernourishment and is a condition caused by an inequality between food intake (protein and energy) and the amount that the body requires for optimal growth and function (5).

GH is a pivotal factor in development and growth (6). GH begins to exert its effects when it binds to its receptor, GH receptor (GHR), which is present on the cell surface (7). After GH binds to GHR, several signaling cascades are activated, including the Janus kinase 2 (JAK2)-signal transducer and activator of transcription (STAT)5 signaling pathway, and the phospholipase C/protein kinase C/Ca2+ pathway (8,9). The most well characterized signaling cascade is the JAK2-STAT5 pathway, which mediates the effects of GH on gene transcription in the liver (10,11). This pathway is responsible for the transcription of the GH target gene IGF-I (12). IGF-I is considered essential for longitudinal bone growth and plays an important role in cell growth and survival. In general, the majority of plasma IGF-I is produced in the liver (13-15).

Cysteine (Cys) (Fig. 1) is a semi-essential amino acid that is known to exert antioxidant, antipancreatic and antiadipatic effects (16-18). However, the effects of Cys on bone growth have not been elucidate to date. Thus, in this study, we aimed to investigate the effects of Cys on longitudinal bone growth and to elucidate the underlying mechanisms.

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Key words: cysteine, growth plate, bone parameters, insulin-like growth factor-I, Janus kinase 2, signal transducer and activator of transcription 5
Materials and methods

Reagents. Cys, avidin-peroxidase and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-mouse IGF-I antibody (Cat. no. MAB791), biotinylated anti-mouse IGF-I antibody (Cat. no. BAF791) and recombinant mouse IGF-I (Cat. no. 791-MG-050) were purchased from R&D Systems (Minneapolis, MN, USA). The phosphorylated (p-)JAK2 (Cat. no. 44-426G) and STAT5 (Cat. no. 71-6900) antibodies were purchased from Invitrogen Life Technologies (Camarillo, CA, USA). JAK2 (Cat. no. sc-294) and STAT5 (Cat. no. sc-836) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment. The human hepatocellular carcinoma cell line, HepG2 (from the Korean Cell Line Bank), was grown in DMEM (Gibco, Grand Island, NY, USA) containing penicillin (100 units/ml; Gibco), streptomycin (100 µg/ml; Gibco) and 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in an incubator with 95% humidified air/5% CO₂. The cells were treated with 0.5-50 µg/ml of Cys for 12 h. The control group represented cells treated with the vehicle (distilled water).

Animals and diet. Male Institute of Cancer Research (ICR) mice (4 weeks old) and food were purchased from Dae-Han Experimental Animal Center (Eumsung, Korea). The mice were allowed to acclimatize for 7 days and were then randomly assigned to an adequate-protein diet group (control (CON) group, 20% protein) or a low-protein diet group (PEM group, 4% protein), as previously described (19). The protein source used was casein. Aside from the protein content, the 2 diets were identical and isocaloric (Table I). After 2 weeks, the mice were divided into 3 groups (5 mice per group) as follows: i) the CON group: mice were fed an adequate-protein diet + distilled water (DW); ii) the PEM group: mice were fed a low-protein diet + DW; and iii) the Cys group: mice were fed a low-protein diet and administered Cys. The mice were fed an adequate-protein diet or a low-protein diet and were administered orally with DW or Cys (50 mg/kg) using a stomach sonde twice a week for 12 weeks, as previously described in the study by Farombi et al. (20). It is widely accepted that bone growth is associated with a gain in body weight. Thus, the mice were weighed at the end of the experiment. The mice were housed in a laminar air-flow room, with a constant temperature of 22±1°C and a relative humidity of 55±1%, throughout the study. All experiments were conducted in accordance with internationally accepted principles for laboratory animal use and care, as found in US guidelines (NIH publication no. 85-23, revised in 1985).

Table I. Composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Standard chow diet (g/kg diet)</th>
<th>PEM diet (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (&gt;85% protein)</td>
<td>200</td>
<td>40</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fiber</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Corn oil</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>L-methionine</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>556.5</td>
<td>716.5</td>
</tr>
</tbody>
</table>

Isocaloric diets providing 1716.3 kJ/100 g. Mineral and vitamin mixtures were prepared according to the 1993 recommendations of the American Institute of Nutrition for adult mice (19). PEM, protein-energy malnutrition.

Enzyme-linked immunosorbent assay (ELISA). The levels of IGF-I were measured by a modified ELISA, as previously described (21-24). A sandwich ELISA for IGF-I was carried out in a duplicate in a 96-well ELISA plate. The plate was coated with anti-mouse IGF-I antibody and incubated overnight at 4°C. The plate was washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% bovine serum albumin, 5% sucrose and 0.05% NaN₃ for 1 h. After additional washes, the culture supernatant and IGF-I standards were added and incubated at room temperature for 2 h. After 2 h incubation at room temperature, the plate was washed and biotinylated anti-mouse IGF-I was then added and again incubated at room temperature for 2 h. After washing the wells, avidin-peroxidase was added and the plate was incubated for 30 min at 37°C. The wells were again washed and TMB substrate (Pharmingen, San Diego, CA, USA) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on the plate using recombinant mouse IGF-I in serial dilutions.

Quantitative polymerase chain reaction (qPCR). qPCR was performed using SYBR-Green Master Mix, and the detection of mRNA was carried out using an ABI StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA), as previously described (25-27). PCR was performed with the following primers: human IGF-I forward, 5’-TGC CCA AGA CCC AGA AGT-3’ and reverse, 5’-CTG CTG TCC CCT CCT TCT GTT-3’; human GAPDH forward, 5’-TCC ACA GTC AGC CGC ATC TTC TTT-3’ and reverse, 5’-ACC AAA TCC GTT GAC TCC GAC CTT-3’; mouse IGF-I forward, 5’-CCG GAC CAG AGA CCC TTT G-3’ and reverse, 5’-CTT GTG GGC TGG TTG TTA AGG TAA A-3’; and mouse GAPDH forward, 5’-GGA AAA TCC AAC GGC ACA-3’ and reverse, 5’-GTT AGT GGG TTC CTC TTC C-3’. Typical profile times used were an initial step, 95°C for 10 min followed by a second step at 95°C for 15 sec and 60°C for 30 sec for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of GAPDH and compared with the control. Data were analyzed using the ΔΔCT method.
Micro-computed tomography (µCT). The 2D and 3D information on bone geometry was attained by high-resolution µCT, as previously described (31,32). The mice were sacrificed; the femora and tibiae were dissected, cleaned of soft tissue, and fixed in 4% formaldehyde before storage. The source of the open tube type and the minimum focal spot size was 8 µm. Reconstruction was carried out using a modified Feldkamp algorithm using SkyScan NRecon software (SkyScan, Kontich, Belgium). The x-ray source was set at 75 kV and 100 mA. Four hundred projections were acquired over an angle of 180°. The image slices were reconstructed using cone-beam reconstruction software based on the Feldkamp algorithm (Dataviewer; SkyScan). The trabecular bone was extracted by drawing ellipsoid contours with CT analyzer software. Trabecular bone volume (BV/TV; percentage) and trabecular number (Tb.N) of femur epiphysis and proximal tibial metaphysis were calculated by the mean intercept length method. Trabecular thickness (Tb.Th; mm) was calculated according to the study of Ngueguim et al (31). 3D parameters were based on analysis of a Marching cubes-type model with a rendered surface. To analyze 3D parameters in tibias, whole bone was scanned, and 600 slices of 8 µm in thickness were placed through the former area. Bone mineral density (BMD), bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), connectivity density (Conn.D), and total porosity were recorded. The thickness of excised bone growth plate was determined using SkyScan 1076 software (SkyScan) as described in a previously published protocol (32).

Statistical analysis. Statistical analysis was performed using SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA). All results are expressed as the means ± SEM. The statistical evaluation of the results was performed by an independent t-test and an ANOVA with Tukey’s post hoc test. P values <0.05 were considered to indicate statistically significant differences.

Results

Effect of Cys on IGF-I mRNA in HepG2 cells. IGF-I plays an important role in linear bone growth (33). Thus, to determine whether Cys increases IGF-I mRNA expression in the liver, we used a hepatocyte cell line, HepG2. When we treated the HepG2 cells with Cys (0.5-50 mg/ml), IGF-I mRNA expression was elevated in a dose-dependent manner (Fig. 2). Treatment with 50 mg/ml of Cys had the most prominent effect, and thus we evaluated the effects of treatment with 50 mg/kg of Cys in the next set of experiments, i.e., in vivo mouse models.

Effect of Cys on growth plates in mice. The thickness of the growth plate in the proximal tibia was assessed (Fig. 3A), and we also investigated linear bone growth using µCT. The thickness of the growth plates in the proximal tibias in the CON and PEM groups were 124.0±2.9 and 90.1±3.1, respectively; the growth-plate thickness in the Cys group was 117.0±4.3 (P<0.05; Fig. 3B). Thus, it is clear that Cys significantly increased linear bone growth, compared to the PEM group. In addition, treatment with Cys significantly increased the body weight of the mice compared to the PEM group.

Effect of Cys on trabecular bone parameters. We used µCT measurements to determine whether Cys improves longitudinal bone growth. The administration of Cys increased BMD (Fig. 4A). The results of the 3D µCT reconstruction of the trabecular bone images, which were converted into parameters representing trabecular connectivity, demonstrated that the BV/TV, Tb.Th, Tb.N and Conn.D in the tibia were increased by treatment with Cys, and that total porosity was decreased (Fig. 4B-F).

Effect of Cys on the regulation of IGF-I. To investigate whether Cys increases serum IGF-I levels, we measured the IGF-I levels by ELISA. The serum IGF-I levels were significantly increased by Cys, compared to those of the PEM group (P<0.05; Fig. 5A). We then examined the mRNA expression of IGF-I in the liver, as the majority of plasma IGF-I is produced in the liver. The mRNA expression of IGF-I in the liver was shown to be significantly upregulated in the mice administered Cys, compared to the mice in the PEM group (P<0.05; Fig. 5B).

Effect of Cys on levels of p-JAK2 and p-STAT5. To examine whether the linear bone growth caused by Cys is regulated by JAK2-STAT5 phosphorylation, we performed western blot analysis. As depicted in Fig. 6, the levels of p-JAK2 and
Figure 3. Effect of cysteine (Cys) on the thickness of the tibial growth plate. (A) The thickness of excised bone from the growth plate was measured at 5 points using the SkyScan 1076. (B) Growth-plate thickness in the mice in each group. (C) Body weight of the mice in each group. Data represent the means ± SEM of 3 independent experiments. *P<0.05 denotes a significant difference compared to the control (CON). #P<0.05 denotes a significant difference compared to the protein-energy malnutrition (PEM) group. CON, group administered adequate-protein diet + distilled water (DW); PEM, group administered low-protein diet + DW; Cys, group administered low-protein diet + Cys.

Figure 4. Effect of cysteine (Cys) on trabecular bone parameters. Bone mineral density (BMD) and trabecular parameters of excised bone at the tibia were measured. (A) BMD levels were measured using 2D micro-computed tomography (µCT). (B-F) Quantified parameters (BV/TV, Tb.Th, Tb.N, Conn.D and total porosity) of proximal tibia were measured using 3D µCT. Data represent the means ± SEM of 3 independent experiments. *P<0.05 denotes a significant difference compared to the CON. #P<0.05 denotes a significant difference compared to the protein-energy malnutrition (PEM) group. BV/TV, bone volume/tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Conn.D, connectivity density; CON, group administered adequate-protein diet + distilled water (DW); PEM, group administered low-protein diet + DW; Cys, group administered low-protein diet + Cys.

Figure 5. Effect of cysteine (Cys) on insulin-like growth factor I (IGF-I) levels. (A) Serum IGF-I levels were measured by ELISA. (B) The mRNA expression of IGF-I was measured by qPCR. Data represent the means ± SEM of 3 independent experiments. *P<0.05 denotes a significant difference compared to the control (CON). #P<0.05 denotes a significant difference compared to the protein-energy malnutrition (PEM) group. CON, group administered adequate-protein diet + distilled water (DW); PEM, group administered low-protein diet + DW; Cys, group administered low-protein diet + Cys.
The role of the resting zone in growth - Growth cartilage expression of IGF-I in rabbits. It is fortunate that IGF-1 possesses anti-apoptotic properties and may, therefore, have the ability to prevent dexamethasone-induced chondrocyte apoptosis. Chrysis et al (44) demonstrated that IGF-I prevented dexamethasone-induced apoptosis, as well as the suppression of chondrocyte proliferation. In the present study, Cys elevated the level of serum IGF-I (Fig. 5). Thus, we can speculate that Cys partially counteracts the dexamethasone-induced growth retardation through the upregulation of IGF-I.

GH has long been known to stimulate linear growth and regulate metabolism. The binding of GH to GHR promotes the phosphorylation of JAK2 and STAT5 (45,46). GH-induced IGF-I gene transcription is mediated by STAT5B (12). In our study, we noted that Cys increased the levels of p-JAK2 and p-STAT5 (Fig. 6). Thus, our findings suggest that Cys promotes linear bone growth through the phosphorylation of JAK2-STAT5.

In conclusion, in the present study, we demonstrated that Cys increased growth-plate thickness and improved bone parameters in mice. In addition, Cys upregulated the IGF-I levels through the phosphorylation of JAK2-STAT5. Overall, these results suggest that Cys may prove to be helpful in children whose growth is retarded, as it induces longitudinal bone growth through the upregulation of IGF-I.

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