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, antipancreatitic and antidiabetic 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, TbN, 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.

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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 systems 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/Ca^{2+} 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, antipancreatitic and antidiabetic 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 mechansims.

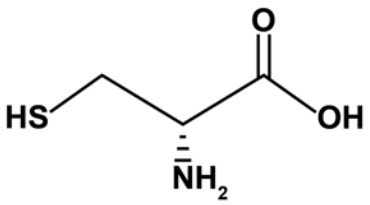

Figure 1. Chemical structure of cysteine (Cys).

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% CO_2. 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 epxeriments 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.

Ingredients	Standard chow diet (g/kg diet)	PEM diet (g/kg diet)
Casein (>85% protein)	200	40
Sucrose	100	100
Fiber	10	10
Corn oil	80	80
Mineral mixture	40	40
Vitamin mixture	10	10
L-methionine	1.5	1.5
Choline bitartrate	2.5	2.5
Cornstarch	556.5	716.5

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% NaN_3 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'-CTC CTG TCC CCT CCT TCT GTT-3'; human GAPDH forward, 5'-TCG 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'-CCT GTG GGC TTG TTG AAG TAA AA-3'; and mouse GAPDH forward, 5'-GGC AAA TTC AAC GGC ACA-3' and reverse, 5'-GTT AGT GGG GTC TCG CTC CTG-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 $\Delta\Delta$CT method.

Western blot analysis. The supernatants of the homogenized liver tissues were prepared in a sample buffer [62.5 mM Tris hydrochloride (Tris-HCl), pH 6.8, 2% sodium dodecyl sulphate (SDS), 20% glycerol and 10% 2-mercaptoethanol], as previously described (28-30). The samples were heated at 95˚C for 5 min and briefly cooled on ice. Following centrifugation at 15,000 x g for 5 min, the proteins in the lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-tween-20 for 1 h at room temperature and then incubated with primary (p-JAK2, 1:500 dilution; p-STAT5, 1:500 dilution; JAK2, 1:1,000 dilution; STAT5, 1:1,000 dilution) and secondary (mouse anti-rabbit IgG-HRP, 1:5,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Finally, the protein bands were visualized by an enhanced chemiluminesence solution purchased from Amersham Co. (Newark, NJ, USA) following the manufacturer's instructions.

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.

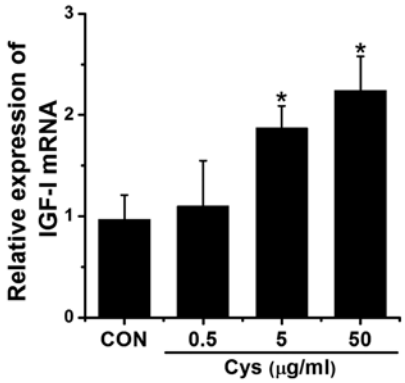

Figure 2. Effect of cysteine (Cys) on insulin-like growth factor-I (IGF-I) mRNA expression in HepG2 cells. HepG2 cells ($5x10^5$) were treated with 0.5-50 μg/ml of Cys for 12 h. Data represent the means ± SEM of 3 independent experiments. *P<0.05 denotes a significant difference compared to the control (CON, vehicle-treated cells).

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 increase 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

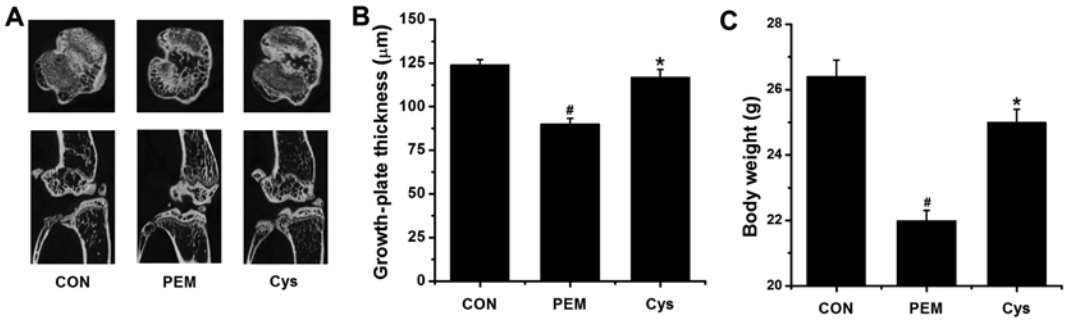

Figure 3. Effect of cysteine (Cys) on the thickness of the tibial growth plate. (A) The thickness of excised bone from the growth plate was measured at 5 points using the SkyScan 1076. (B) Growth-plate thickness in the mice in each group. (C) Body weight of the mice in each group.Data represent the means ± 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.

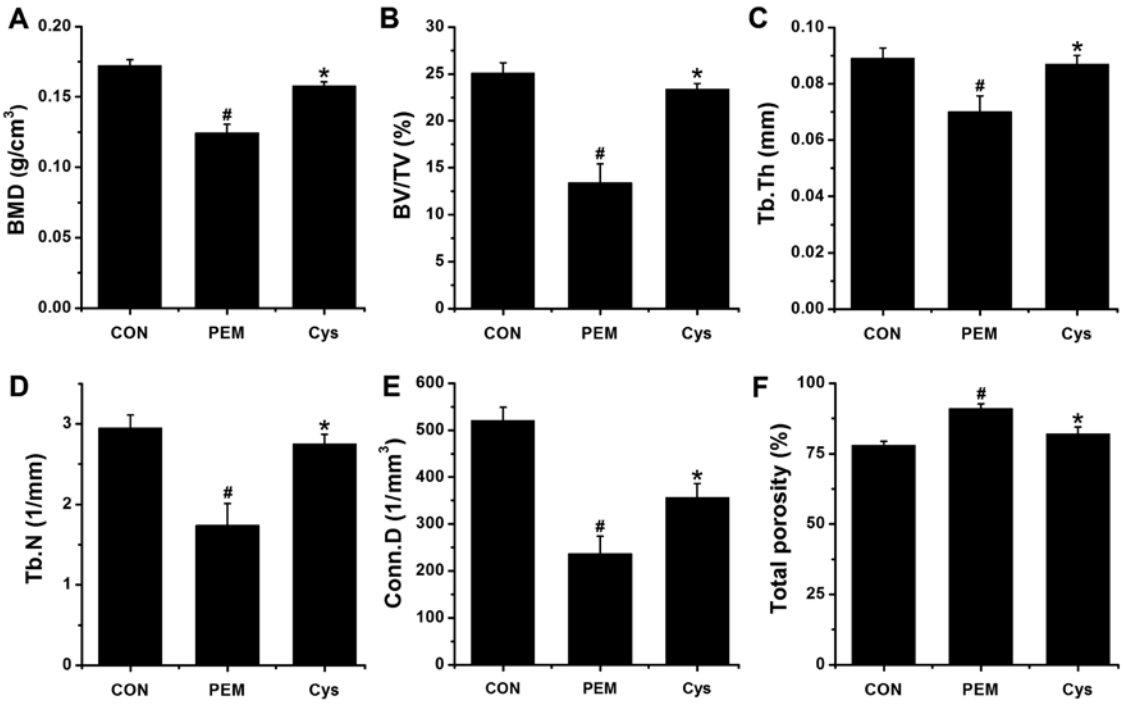

Figure 4. Effect of cysteine (Cys) on trabecular bone parameters. Bone mineral density (BMD) and trabecular parameters of excised bone at the tibia were measured. (A) BMD levels were measured using 2D micro-computed tomography (μCT). (B-F) Quantified parameters (BV/TV, Tb.Th, Tb.N, Con.D and total porosity) of proximal tibia were measured using 3D μCT. Data represent the means ± SEM of 3 independent experiments. #P<0.05 denotes a significan 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.

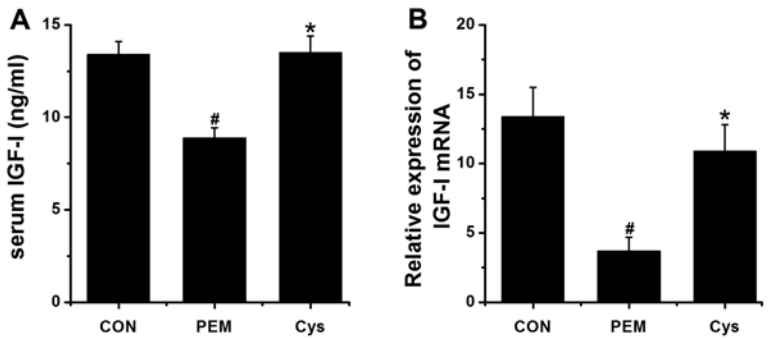

Figure 5. Effect of cysteine (Cys) on insulin-like growth factor I (IGF-I) levels. (A) Serum IGF-I levels were measured by ELISA. (B) The mRNA expression of IGF-I was measured by qPCR. Data represent the means ± SEM of 3 independent experiments. #P<0.05 denotes a significant difference compared to the 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.

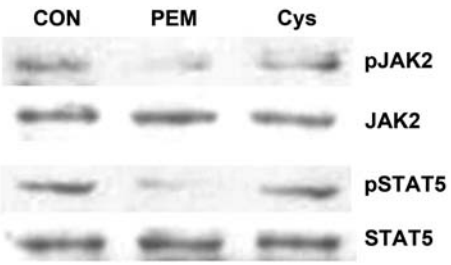

Figure 6. Effect of cysteine (Cys) on the phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5 (STAT5). Liver tissues of each mouse were homogenized and analyzed for the expression of phosphorylated (p-)JAK2, JAK2, p-STAT5 and STAT5. CON, group administered adequate-protein diet + distilled water (DW); PEM, group administered low-protein diet + DW; Cys, group administered low-protein diet + Cys. PEM, protein-energy malnutrition.

p-STAT5 in the liver were decreased due to malnutrition. However, the decrease in the level sof p-JAK2 and p-STAT5 was reversed by treatment with Cys (Fig. 6).

Discussion

In the present study, we demonstrated that Cys increased the mRNA expression of IGF-I, increased the thickness of growth plates, and increased the BV/TV, Tb.Th, Tb.N and Conn.D; Cys decreased total porosity. In addition, Cys increased the serum IGF-I levels through JAK2-STAT5 phosphorylation.

In general, endochondral cell proliferation in the growth plate results in bone growth. Thus, growth-plate thickness is a direct indicator of linear bone growth (34). Our results demonstrated that Cys increased growth-plate thickness (Fig. 3). From this result, it is suggested that Cys may prove to be helpful to children whose growth is retarded.

Cys is a sulfur-containing amino acid (35). A deficiency of sulfur amino acid has been shown to decrease neonatal pig growth (36). McLean *et al* (37) reported that Cys restored growth which was inhibited by paracetamol. In addition, Cys has been shown to induce the growth of human peritoneal mesothelial cells (38). In the present study, the sulfur-containing amino acid, Cys, increased growth-plate thickness in mice fed a PEM diet (Fig. 3). Thus, we can presume that the effect of Cys on bone growth, in part, is due to sulfur.

Baines *et al* (39) reported that subjects with low BMD had a significantly lower plasma Cys concentration, suggesting that there is an association between Cys and bone mass. In the present study, we demonstrated that Cys elevated tibial BMD, in ways consistent with a previous report (39). Furthermore, we noted that Cys improved bone parameters (it increased BV/TV, Tb.Th, Tb.N, Conn.D and decreased total porosity; Fig. 4B-F). Thus, Cys may have the potential to improve not only growth retardation, but may also alleviate osteoporosis.

Growth retardation is a major concern in children with chronic inflammatory diseases, uncontrolled juvenile idiopathic arthritis and chronic kidney disease (40-42). Thus, many children are administered the anti-inflammatory drug, dexamethasone. However, Baron *et al* (43) reported that dexamethasone acts locally to suppress linear bone growth 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 demonsrated 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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