Studies on Gene Expression in Calvaria and Serum Levels of Insulin-Like Growth Factor-I and Bone Gla Protein in the Methimazole-Induced Congenital Hypothyroid Rat

HIROMI TAKEUCHI, YUICHI NAKAGAWA, AND YOSHIO IGARASHI

Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu 431–31, Japan

Abstract. In this study, we determined gene expression of both insulin-like growth factor (IGF)-I and bone Gla protein (BGP; osteocalcin) in calvaria in comparison with their serum levels in methimazole (MMI)-induced congenital hypothyroid (CHT) rats during the first 4 weeks of life. Pups from the MMI-treated dams revealed congenital hypothyroidism with cretinoid physical appearance and showed significant growth retardation compared to the controls. The expression of mRNA for IGF-I in the CHT pups lacked the age-associated increase with a little spurt in their somatic growth rate, although the expression in the controls increased steeply (from 1.86-fold on postnatal day 21 to 3.52-fold on day 28 compared to the day 7 value; $P < 0.01$) according to the spurt in their growth. Moreover, serum IGF-I levels in the CHT rats were significantly lower than those in the controls on postnatal day 28 (63.0 ± 8.0 ng/ml vs. 285.0 ± 33.2 ng/ml, respectively; $P < 0.01$). Both BGP gene expression in calvaria and serum intact molecular BGP levels determined by a newly developed ELISA (164.4 ± 15.5 ng/ml in the CHT rat vs. 238.6 ± 17.8 ng/ml in the control on postnatal day 28; $P < 0.01$) correlated well with the somatic growth in the two groups and clearly demonstrated impaired osteogenesis in the CHT rats. Further studies are needed to clarify how hypothyroidism affects somatic growth and bone metabolism; it is particularly important to understand the autocrine/paracrine mechanisms of action of IGFs in the bone matrix turnover, *in vivo*.

Key words: Gene expression, Calvaria, Insulin-like growth factor-I, Bone Gla protein, Congenital hypothyroidism. (Endocrine Journal 40: 351–362, 1993)

THYROID hormone is essential for somatic growth and developmental maturation during the perinatal and postnatal periods [1–5]. Somatic growth retardation is one of the features of congenital hypothyroidism, and it is well known that low serum insulin-like growth factor (IGF)-I levels in congenital hypothyroidism [6, 7] may be either a result of diminished growth hormone (GH) secretion or due to a direct effect of hypothyroidism on IGF-I production [8]. In previous studies with the methimazole (MMI)-induced hypothyroid rat treated from either the

fetal or neonatal period [4, 9, 10], it has been reported that thyroid hormone is essential for the neonatal changes in IGF and IGF-binding protein (IGFBP) profiles and a critical period for these changes is during the perinatal development of the rat. On the one hand, in the rat hypophysectomized during the early postnatal period [11, 12], it has been demonstrated that systemically administered IGFs were poor promoters of somatic growth. Other studies also suggested that the role of IGFs as somatic growth promoters could be achieved via autocrine/paracrine mechanisms and that an increase in tissue IGF content was required for significant promotion of somatic growth [13].

Prior studies on bone metabolism *in vitro* have shown that IGF-I stimulates osteoblasts [14], which synthesize bone Gla protein (BGP: osteocalcin)

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Correspondence to: Dr. Hiromi TAKEUCHI, Department of Pediatrics, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431–31, Japan

during osteogenesis. BGP binds to both calcium and hydroxyapatite during mineralization of the bone tissue, and serum BGP levels have been reported as a specific marker of bone formation [15–19] and a helpful predictor of the growth response to GH therapy in humans [20–22]. But, to our knowledge, there is no report on the gene expression of either IGF-I or BGP in the bone tissue in comparison with their simultaneous serum mature protein levels in congenital hypothyroidism *in vivo*.

In this study, we studied these gene expression in calvaria with their serum levels in the MMI-induced congenital hypothyroid (CHT) rats during the first 4 weeks of life.

Materials and Methods

Animals

Timed pregnant Sprague-Dawley rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Since there is no sexual dimorphism in the somatic growth of the rat within the first 30 to 35 days after birth [23], both females and males were included in the study. Litter size was adjusted to 8–10 pups and each dam was placed in a separate cage. The animals were housed in 12-h light (between 0700 and 1900 h) 12-h dark animal facilities, with the ambient temperature adjusted to 22–26°C. The dams of suckling pups and the weaning pups were allowed to drink tap water and to eat standard block feed *ad libitum*.

Treatments

Dams were divided into two groups, the control group and the hypothyroid group. The latter were rendered hypothyroid by administering methimazole (1-methyl-2-mercaptoimidazole: MMI; purchased from CHUGAI PHARMACEUTICAL CO., LTD., Tokyo, Japan) in the drinking water (0.025%) from day 14 of pregnancy until the end of the study [24]. MMI readily crosses the placenta barrier in pregnant rats and is secreted into the milk in lactating dams, inducing hypothyroidism in the fetuses and maintaining it in the suckling pups [25].

Growth parameters

At 3- to 4-day intervals, body weight was measured on a digital scale (model FX-320 A and D Co., Tokyo, Japan), and tail length from the anus to the tip of the tail was measured as an index of skeletal growth [11].

Biological samples

Serum samples were collected at 1, 7, 14, 21 and 28 days of age for hormonal and/or peptide assays. In case of samples from day 1, blood from three or four animals was pooled. The animals were killed by decapitation, and blood was collected from the cervical stumps. It was then allowed to clot at 4°C for 15 min, and was centrifuged at 8,000 g for 15 min to obtain serum. The serum samples were stored at –80°C before analysis. Calvaria, collected from the same pups for serum samples except for day 1, were quickly removed, frozen in liquid nitrogen within 10 min of decapitation, and subsequently stored at –80°C.

Serum total thyroxine determination

Serum total thyroxine (T₄) levels were measured with a RIA kit (Ciba Corning Diagnostics Co., Ltd., Tokyo, Japan), with a detection limit of 1.0 µg/dl.

Serum IGF-I determination

Serum IGF-I levels were measured with a RIA kit (Ciba Corning Diagnostics Co., Ltd., Tokyo, Japan), with a detection limit of 0.5 ng/ml for the human serum IGF-I concentration, and the cross-reactivity with human IGF-II was 0.11% [26]. In this kit, polyclonal antibodies to human IGF-I have wide species cross-reactivity and have been successfully used in experimental studies on rats [27].

Serum intact molecular BGP determination

Serum intact molecular BGP levels were measured by a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for rat newly developed by Dr. K. Hosoda (Institute for Biomedical Research, Teijin Ltd., Tokyo, Japan) and co-workers [28]. This system is a modification of the method which was previously used for human intact molecular BGP [22, 29, 30].

Total cellular RNA extraction (modified AGPC method)

Total cellular RNA was isolated from the calvaria by a modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method [31]. Approximately 0.5 g tissue was minced on ice and homogenized at room temperature (RT) with 4 ml denaturing solution [4 M guanidine isothiocyanate (GIBCO BRL, Gaithersburg, MD, USA), 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol] in a glass-Teflon homogenizer and subsequently transferred to a polypropylene tube. Then 0.4 ml of 2 M sodium acetate, pH 4.0; 4 ml of phenol (saturated with 0.1 M Tris buffer, pH 8.0), and 0.8 ml of a chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing and were shaken vigorously for 15 sec after the addition of each reagent and cooled on ice for 20 min. Samples were centrifuged at 8,000 g for 20 min at 4 °C. After centrifugation, the aqueous phase was transferred to a fresh tube followed by precipitation with 1 vol of isopropanol, and then placed at -80°C for more than 12 h. Sedimentation at 10,000 g for 20 min was again performed and the resulting RNA pellet was dried in vacuum at RT. The dried pellet was transferred to a 1.5 ml Eppendorf tube and dissolved in 0.7 ml protein digesting solution [0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl, 10 mM EDTA-Na, 1% sodium dodecyl sulfate (SDS), 20 mg/ml proteinase K (Boehringer Mannheim GmbH, Germany)] at 37–40°C. After dissolving, RNA was deproteinized by phenol-chloroform (saturated with water) extraction followed by chloroform-isoamylalcohol extraction, and precipitated with 1 vol of isopropanol at -80°C for more than 12 h. After centrifugation at 10,000 g for 20 min at 4°C, the RNA pellet was rinsed with 70% ethanol, vacuum dried at RT for more than 15 min, and dissolved in about 30–50 µl of 0.05% SDS at 65°C for 10 min. RNA was then quantified spectrophotometrically (model UV-2200, SHIMADZU, Kyoto, Japan), and stored at -80°C before analysis.

Northern blot and dot blot analysis

RNA samples were denatured with glyoxal for northern blot and dot blot analysis [32]. For northern blot analysis, denatured RNAs were electrophoresed through 1.4% agarose gel and

transferred onto nylon membrane (RPN. 3050N, Amersham International plc, Amersham, Bucks, U.K.). Dot blot analysis was examined with a dot blotting apparatus (IMMUNODOT® model AE-6190, ATTO Co., LTD., Tokyo, Japan). Membranes were incubated in a prehybridizing solution (100 µg/ml salmon sperm DNA, 6 × SSC, 10 × Denhardt solution, and 1% SDS; 50 ml/filter) at 42°C for more than 6 h, followed by hybridization with a radioactively-labeled probe in a hybridizing solution (100 µg/ml salmon sperm DNA, 6 × SSC, 5% dextran sulfate, 50% formamide, and 1% SDS; 10 ml/filter) at 42°C on a rotating bath for more than 18 h. The probe for rat IGF-I, coding Exon 3 region, was a 956-base-pair (bp) BamHI-EcoRI restriction fragment of a series of overlapping clones from two rat chromosomal libraries with the 3' end next to the T7 promoter [33]. The probe for rat BGP was a 480-bp EcoRI-restriction fragment from a rat osteosarcoma cell line (ROS 17/2) [34]. These probes were generously donated by Dr. P. Rotwein (probe for IGF-I) and Dr. J. M. Wozney (probe for BGP), respectively. Equivalent amounts of RNA/lane or dot were assessed by monitoring β-actin RNA expression hybridized with a probe purchased from the American Type Culture Collection (Rockville, Maryland, USA). The probe for IGF-I was labeled with [³²P]UTP (NEN Research Products/Du Pont, Boston, MA, USA; specific activity 800 Ci/mmol) with a T7 promoter (TransProbe T Kit, Pharmacia LKB Biotechnology AB, Sweden) [35]. The probes for BGP and β-actin were labeled with [³²P]dCTP (NEN Research Products/Du Pont; specific activity 3,000 Ci/mmol) with a nick translation (Nick Translation System, BRL LIFE TECHNOLOGIES, INC., MD, USA) [36]. After hybridization, membranes were rinsed twice in washing solution I (2 × SSC and 0.1% SDS) at 42°C for 10 min, followed by three washes of 20 min each at 42°C in washing solution II (0.1 × SSC and 0.5% × SDS). Autoradiography was performed by exposing to New-RX X-ray film (FUJI PHOTO FILM CO., LTD., Tokyo, Japan) with an intensifying screen (Sigma, St. Louis, MO, USA) at -80°C overnight.

Densitometry

Autoradiographs were densitometrically scanned and the absorbance units were measured for lanes and dots.

Statistical analysis

The significance of the differences between hypothyroid animals and controls in body weight, tail length, and serum T_4 , IGF-I and BGP levels was analyzed by Student's *t*-test. The densitometric data from northern and dot blots for the two groups were also analyzed by Student's *t*-test.

Results

Serum T_4 concentrations and somatic growth

Serum T_4 levels in the employed animals are shown in Fig. 1. In the MMI-treated dams and their pups (CHT in figures), these levels decreased below the detection limit throughout the study. On the other hand, control pups had the normal rat perinatal thyroid hormone serum pattern reported previously [4]. Serum T_4 levels in the control dams were within normal limits (2–4 $\mu\text{g/dl}$) throughout the study, which were slightly lower than those of pups at the age of 28 days.

The somatic growth of the CHT pups was remarkably impaired both in body weight gain and in tail length gain (Fig. 2a and 2b, respectively). At the age of 28 days, both body weight and tail length were significantly lower than those in the control group ($P < 0.01$). The CHT pups had a slightly cretinoid physical appearance, already reported in the previous report [4], with blunt snouts, unfolded ears, and rounded bodies, and 3–4 day delay in eye opening, which normally occurs on postnatal day 14.

Serum IGF-I concentrations

Serum IGF-I levels in the CHT pups (63.0 ± 8.0 ng/ml, mean $\pm$ SD) were significantly lower than those in the controls (285.0 ± 33.2 ng/ml) on postnatal day 28 ($P < 0.01$). We could not determine serum IGF-I levels except on postnatal day 28 because of the limited samples available. In the present study, we could not obtain purified rat IGF-I, and a RIA kit developed for human samples was employed. Regrettably, the standards and the scales were therefore human IGF-I levels.

Fig. 1. Serum total T_4 levels, determined by radioimmunoassay, from serum of control (open circles) and hypothyroid (CHT; solid circles) pups as a function of postnatal age. The levels in the serum of control (open triangles) and MMI-treated (CHT; solid triangles) dams are added. Each symbol represents the mean $\pm$ SD (day 1 samples are plural serum pools from three or four pups). Undetectable levels are shown as a dotted area.

Serum intact molecular BGP concentrations

As shown in Fig. 3, serum intact molecular BGP concentrations in the control group were increased significantly from low level of 96.0 ± 14.3 ng/ml (mean and SD) on postnatal day 1 up to a level of 192.4 ± 5.2 ng/ml on day 14 ($P < 0.01$ vs. day 1) and 238.5 ± 17.8 ng/ml on day 28 ($P < 0.01$ vs. day 1 and day 14). On the other hand, CHT pups showed a slow rise in serum intact molecular BGP levels from 91.2 ± 12.8 ng/ml on postnatal day 1 up to a level of 103.5 ± 6.9 ng/ml on postnatal day 14 [not significant ($P > 0.05$) vs. day 1, $P < 0.01$ vs. control] and 164.4 ± 15.5 ng/ml ($P < 0.01$ vs. day 1 and vs. day 14, $P < 0.01$ vs. control), reaching approximately 69% of the control values on postnatal day 28, respectively.

Fig. 2. Effect of MMI treatment on somatic growth. Growth patterns are shown by body weight (left panel) and tail length (right panel) of control (open circles) and hypothyroid (CHT; solid circles) rats. Each symbol represents the mean $\pm$ SD. Statistical significance of the differences between two groups at individual time points is estimated by Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Northern blot and dot blot analysis for IGF-I gene expression

Fig. 4 shows the result of northern blot analysis performed on pooled total cellular RNA obtained from calvaria. Two different sizes of RNA were present: 4.0–4.4 and 1.6–2.0 kb. Although three sizes (7.8, 1.6–2.0 and 1.0–1.4 kb) were reported in the previous study [33], 7.8 and 1.0–1.4 kb sizes were not detected but 4.0–4.4 kb size was faintly present in this study. In the present study, there was no difference between the two groups in the northern blotting pattern.

To facilitate comparisons of gene expression among the sampling points in the two groups, dot blot analysis and densitometric analysis of the auto-radiographs are shown in Fig. 5a and 5b, respectively. IGF-I gene expression in the CHT rats was 2.4 times that in the control on postnatal day 7 and 1.2 times on postnatal day 28, despite low serum IGF-I levels in the CHT rats. To avoid

this complexity, relative absorbancy is expressed as a function of the percentage of the value on postnatal day 7 in each group individually and the results of the densitometry are expressed as the mean $\pm$ SD of five dots from the pooled RNA examined at each point. In the control rats, the expression of IGF-I increased steeply from postnatal day 21 to day 28 similar to the spurt in somatic growth shown in Fig. 2, although the expression in the CHT rats remained a small increase compared to the value for postnatal day 7 and there was no clear spurt in their somatic growth.

Northern blot analysis for BGP gene expression

In Fig. 6, the result of northern blot analysis with the rat BGP cDNA showed a single band which corresponded to a mRNA of 0.4–0.8 kb reported previously [34]. To clarify the difference between the two groups in gene expression and

Fig. 3. Serum intact molecular BGP levels, determined by enzyme-linked immunosorbent assay, from serum of control (open circles) and hypothyroid (CHT; solid circles) rats. Each symbol represents the mean $\pm$ SD (day 1 samples are plural serum pools from three or four pups). Statistical significance of the differences between two groups at individual time points are estimated by Student's *t*-test (**, $P < 0.01$).

among the sampling points, the results of northern blot analysis performed on pooled total cellular RNA obtained from calvaria are shown (Fig. 7a), and relative absorbancy is expressed as a function of the percentage of the value on postnatal day 7 in the control pups (Fig. 7b). BGP gene expression in the CHT rats was lower than in the controls during the experimental period from postnatal day 7 to day 28. On postnatal days 14 and 28, the relative decrease in BGP gene expression in the calvaria of the CHT pups was very similar to that in serum intact molecular BGP levels shown in Fig. 3.

Discussion

Our results demonstrated that the expression of mRNA for IGF-I in the MMI-induced CHT rat calvaria lacked the age-associated increase with a

Fig. 4. Northern blot analysis of IGF-I mRNA in rat calvaria. Total cellular RNA (20 μ g/lane) pooled from rat calvaria was electrophoresed, transferred to a nylon membrane and hybridized with 32 P-labeled probe. The probe for IGF-I derived from rat chromosomal libraries hybridized to mRNA characteristic for rat IGF-I: 1.6–2.0 kb. Mobility of molecular size markers (28S and 18S rRNA) is indicated on the right. Autoradiographic exposure was 18 h.

little spurt in their somatic growth rate, although the expression in the control rat calvaria increased steeply accompanying the spurt in their growth from postnatal day 21 to day 28. Moreover, the serum IGF-I levels in the CHT rats were significantly lower than those in the controls on postnatal day 28. The decrease in the serum IGF-I concentration was recognized as a result of the reduced IGF-I gene expression in the liver [37].

In this study, the probe for IGF-I derived from rat chromosome libraries hybridized to only one of the mRNAs characteristic for rat IGF-I (1.6–2.0 kb), but not to two other sizes (7.8 kb and 1.0–1.4 kb) detected in the liver [33]. In the rat, it is well known that the processing of the primary IGF-I gene transcript appears to be very complicated and various alternative mRNA splicings occur at both the 5'- and 3'-ends of the gene [13]. In a previous report [37], 7.5 kb IGF-I mRNA expression in the liver with a cDNA encoding the entire coding sequence for rat IGF-I did not correlate

Fig. 5. Gene expression of mRNA for IGF-I in rat calvaria visualized by dot blot analysis (upper panel, a) and densitometric analysis of the autoradiographs (lower panel, b). a, Dot blot analysis of control (dots 1–4) and hypothyroid (CHT; dots 5–8) samples. Total cellular RNA (2 μ g/dot) pooled from rat calvaria was blotted onto nylon membrane and hybridized with 32 P-labeled probes for IGF-I and β -actin, individually. b, Densitometric analysis of the auto-radiographs, showing the mean $\pm$ SD absorbance units in the control (open circles) and hypothyroid (solid circles) samples (five dots were examined at individual time points). Relative absorbancy is expressed as a function of the percentage of the value on postnatal day 7 in each group, individually. Differences between the groups were evaluated at individual time points by Student's *t*-test (**, $P < 0.01$).

with either serum IGF-I levels or somatic growth under the dietary protein restriction, and the authors speculated that unknown mechanisms might regulate the expression of a specific IGF-I mRNA species. Besides, 1.0–1.4 kb and 4.0 kb mRNAs characteristic of rat IGF-I were not evaluated even though these sizes could be detected strongly (1.0–1.4 kb) or weakly (4.0 kb) with a rat IGF-I cDNA probe [13]. Therefore, the physiological significance of these mRNAs is unknown. Additionally, the detectable pattern of

IGF-I mRNA sizes might be different in the liver and in bone tissue, because, to our knowledge, there is no report of the detection of IGF-I mRNA in bone tissue by means of this probe. We therefore came to the conclusion that 1.6 kb mRNA could be evaluated as a representative IGF-I mRNA in calvaria in this study.

In previous studies, Isgaard *et al.* reported that the expression of mRNA for IGF-I in the growth plate of costae was diminished by hypophysectomy [38] and D'Ercole *et al.* demonstrated that

Fig. 6. Northern blot analysis of BGP mRNA in rat calvaria. Total cellular RNA (20 μ g/lane) pooled from rat calvaria was electrophoresed, transferred to a nylon membrane and hybridized with 32 P-labeled probe. The probe for BGP derived from rat cDNA clone hybridized to mRNA characteristic for rat BGP: 0.4–0.8 kb. Mobility of molecular size markers (28S and 18S rRNA) is indicated on the right. Autoradiographic exposure was 18 h.

hypophysectomy causes a decrease in the IGF-I concentration in cartilage [39]. But in our dot blot analysis, the IGF-I gene expression signal in the CHT rat was stronger than that in the control. Although we did not determine the content of mature IGF-I in the tissues, the higher gene expression of IGF-I in the CHT rat calvaria may reflect the feedback mechanism related to the decrease in locally produced IGF-I, which activates gene expression through autocrine and/or paracrine mechanisms. But further studies are needed

to find the precise answer.

In the present study, impaired osteogenesis in the CHT pups was confirmed by the decrease in both BGP gene expression in the calvaria and in serum intact molecular BGP levels. BGP has been reported to be a vitamin K-dependent bone-specific peptide produced by osteoblasts and evaluated as a marker of bone matrix turnover [40, 41]. In the rat, it has been speculated that BGP gene expression is regulated by the thyroid hormone through thyroid hormone responsive element (TRE)-like sequences in the BGP gene [42]. These TRE-like sequences are the same as those of the established TRE sites in the rat GH gene [43], and it has been reported, although in abstract form, that the expression of mRNA for BGP is regulated by the thyroid hormone status [44]. The BGP molecules, after cleavage of the propeptide, are partly released into the peripheral circulation and can be detected in serum or plasma by RIA [45–47]. But previous reports have demonstrated that the majority of their fragments, which derive from the processing during bone resorption, are also released into the circulation and that former RIAs for BGP cross-react with the fragments [48–50]. Consequently, osteoblastic bone formation could be evaluated by using a specific assay system to detect intact molecular BGP. In the present study, we employed a cloned cDNA which encodes rat BGP and a newly developed ELISA specific to rat intact molecular BGP and determined the osteogenesis in the CHT rat calvaria *in vivo*. Our northern blot analysis showed that the expression of mRNA for BGP in the CHT rat calvaria was lower than that in the control throughout the experimental period, from postnatal day 7 to day 28. The serum intact molecular BGP levels in the CHT pups were also significantly lower than those in the controls on postnatal day 14 and day 28, although there was little difference between the two groups on postnatal day 1. Besides, as shown in Fig. 2, the difference between the two groups in somatic growth became obvious from postnatal day 14. To be brief, BGP gene expression and serum intact molecular BGP levels were well correlated with somatic growth in the two groups and confirmed the diminished osteogenesis in the CHT rat.

During this study, maternal thyroid function was also completely suppressed. As for body weight, food consumption, water intake and

Fig. 7. Gene expression of mRNA for BGP in rat calvaria visualized by northern blot analysis (upper panel, a) and densitometric analysis of the autoradiographs (lower panel, b). a, Northern blot analysis of control (lanes 1–4) and hypothyroid (CHT; lanes 5–8) samples. Total cellular RNA (20 μ g/lane) pooled from rat calvaria was hybridized with 32 P-labeled probes for BGP and β -actin, individually. b, Densitometric analysis of the autoradiograph in a: relative absorbancy is expressed as a function of the percentage of the value on postnatal day 7 in the control. Control (open bars) and hypothyroid (CHT; hatched bars) samples are shown.

maternal behavior, there was no difference between the MMI-induced hypothyroid dams and the controls (data not shown). But it would be more reliable that the maternal thyroid function of the CHT pups could be kept euthyroid by simultaneous administration of both thyroxine and

MMI subcutaneously [4, 10].

Finally, no age-associated increase in IGF-I gene expression was demonstrated in the CHT rat, and BGP gene expression in calvaria and serum intact molecular BGP levels were diminished in this model. All these facts were well correlated with the

somatic growth retardation in the CHT rat during the first 4 weeks of life. But the mechanism of the strong IGF-I gene expression in the CHT rat is still unknown. We are currently investigating IGF-II and IGFBP-3 gene expressions in the CHT rat. Further studies are needed to clarify how hypothyroidism affects somatic growth and bone metabolism. It is particularly important to understand the autocrine/paracrine mechanisms of action of IGFs in the bone matrix, *in vivo*.

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