Growth of long bones in renal failure: Roles of hyperparathyroidism, growth hormone and calcitriol

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Background. The treatment of secondary hyperparathyroidism (2°HPT) associated with chronic renal failure adversely affects skeletal growth.

Methods. We assessed epiphyseal growth plate morphology by quantitative histology and measured mRNA levels for selected markers of chondrocyte proliferation and differentiation by in situ hybridization in the growth plate cartilage of subtotally nephrectomized rats with either mild or advanced 2°HPT.

Results. The width of the growth plate cartilage in the proximal tibia and mRNA levels for PTH/PTHrP receptor were unchanged in rats with mild 2°HPT, however, they were markedly less in nephrectomized rats with advanced 2°HPT than in intact controls. Treatment with growth hormone 10 IU/kg/day increased growth plate thickness both in mild and in advanced 2°HPT and raised mRNA levels for type II and type X collagen in rats with advanced 2°HPT. The administration of calcitriol 50 ng/kg/day attenuated these responses in animals with advanced 2°HPT. Overall, PTH/PTHrP receptor mRNA levels did not correspond to the serum levels of PTH indicating that PTH/PTHrP receptor expression is down-regulated in renal failure by a PTH-independent mechanism.

Conclusion. Calcitriol counteracts the trophic actions of growth hormone on epiphyseal growth plate cartilage and modifies chondrocyte differentiation in vivo, and these mechanisms may contribute to disturbances in longitudinal bone growth in renal failure.

Growth retardation is a major consequence of renal failure in children. Several factors have been implicated in the pathogenesis of this disorder, but tissue resistance to the actions of growth hormone and/or insulin-like growth factor I (IGF-I) and renal osteodystrophy due to secondary hyperparathyroidism are generally considered to be the most important contributors to impaired linear growth in pediatric patients with established kidney disease [1, 2]. As such, recombinant human growth hormone (rhGH) and calcitriol, or 1,25-dihydroxyvitamin D₃, are widely used to prevent and/or treat growth retardation in children with chronic renal failure [3, 4].

Growth hormone increases the rate of cell proliferation both in chondrocytes and in osteoblast-like cells, enhances collagen synthesis in vitro, promotes longitudinal bone growth in experimental animals, and increases the thickness of growth plate cartilage in normal and in uremic rats [5–7]. Accordingly, growth velocity increases during treatment with rhGH in children with chronic renal failure and in those who have undergone kidney transplantation [8]. In contrast to these findings, calcitriol diminishes chondrocyte proliferation in vitro, and it influences the differentiation of chondrocytes within growth plate cartilage [9, 10]. Thus, reductions in chondrocyte number and decreases in cellular organization within epiphyseal cartilage have been reported in neonatal mice given large doses of 1,25-dihydroxyvitamin D [10]. The importance of these findings is underscored by the observation that treatment with large intermittent doses of calcitriol diminishes linear growth in pre-pubertal children undergoing regular dialysis [11].

Recent work has markedly increased our understanding of the molecular mechanisms that regulate endochondral bone formation during skeletal development. Parathyroid hormone-related peptide (PTHrP), the receptor for PTH/PTHrP and Indian hedgehog are integrally involved in the control of chondrocyte differentiation within epiphyseal cartilage [12], and as such, the PTH/PTHrP receptor plays an important role in bone elongation and longitudinal growth. Expression of the receptor for PTH/PTHrP in vitro is affected by several hormonal agents including growth hormone and 1,25-dihydroxyvitamin D, and it is reduced in experimental animals with renal failure [13]. It is not known, however, whether this abnormality contributes to the growth retardation that characterizes chronic renal disease. To investigate this issue, we assessed the separate

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and combined effects of growth hormone and calcitriol on epiphyseal growth plate morphology, and measured mRNA levels for selected molecular markers of endochondral bone formation in the growth plate cartilage in rats with renal failure and either mild or severe secondary hyperparathyroidism.

**METHODS**

Two separate experiments were done to assess the responses to growth hormone and calcitriol in rats with renal failure and different degrees of secondary hyperparathyroidism.

**Experiment #1 - Mild secondary hyperparathyroidism**

Forty-five weanling male Sprague-Dawley rats (Harlan-Sprague Dawley Laboratories, Indianapolis, IN, USA) weighing 60 to 80 grams were studied. Animals were housed in cages at constant temperature with a 12-hour light-dark cycle; all had free access to food and drinking water. After one week of acclimatization, 35 rats underwent a two-stage 5/6 nephrectomy to induce renal failure as previously described [14]. Anesthesia was achieved using ketamine and xylazine. During the first stage nephrectomy, approximately 2/3 of the left kidney was removed after ligating both the upper and lower poles [14]. Seven days later, the entire right kidney was removed after ligating the vascular pedicle. Ten rats underwent sham nephrectomy in two stages on the same days that actual nephrectomy procedures were done. Sham nephrectomy involved a flank incision with gentle manipulation of the kidney, but no tissue was removed.

After both surgical procedures were completed, animals were fed standard rat chow containing 23.4% protein, 0.6% calcium and 0.68% phosphorus (Purina Mills, Indianapolis, IN, USA). Rats with normal renal function were pair-fed with animals who had undergone subtotal nephrectomy by providing the amount of chow each day to intact rats that had been consumed the previous day by nephrectomized animals. Animals were weighed each week throughout the experiment, and body length was determined weekly by measuring from the tip of the nose to the end of the tail while animals were sedated.

Four weeks after the second stage nephrectomy was done, rats were begun on daily intraperitoneal (i.p.) injections of saline vehicle or hormonal agents; the duration of treatment was 10 days. Ten intact control rats (C) and six nephrectomized control rats (Nx-C) received vehicle only. Five nephrectomized rats were given daily i.p. injections of rhGH (Nx-GH), 10 IU/kg/day (Pharmacia, Stockholm, Sweden), whereas six nephrectomized rats received daily i.p. injections of calcitriol (Nx-D), 50 ng/kg/day (Abbott Laboratories, Chicago, IL, USA). An additional six nephrectomized animals received daily doses of both rhGH and calcitriol (Nx-GH+D).

After 10 days of treatment, rats were anesthetized with ketamine and xylazine, and the animals were killed by exsanguination by cardiac puncture; blood was saved for subsequent biochemical determinations. Immediately after death, tissues were fixed by transcardiac perfusion with 4% paraformaldehyde in phosphate buffered saline (PFA/PBS). Tibiae were excised, freed of adherent soft tissue and immersed in 4% PFA/PBS for 48 hours. Bones were then decalcified in 15% ethylenediamine tetra-acetic acid (EDTA) in PBS, pH 7.0, at 4°C for two weeks. After decalcification was complete, tibiae were washed in 10 mM PBS for 24 hours, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Blocks were carefully positioned to obtain frontal sections of tissue from the proximal tibia with the plane of section oriented parallel to the longitudinal axis of the bone. Separate five micrometer sections of bone for morphometric analysis and for in situ hybridization were obtained using a Jung-Reichert Model 1140 microtome (Warner Lambert, Buffalo, NY, USA), and these were mounted on Superfrost Plus slides (Fisher Scientific Co, Springfield, NJ, USA).

**Experiment #2 - Severe secondary hyperparathyroidism**

A total of fifty weanling male Sprague-Dawley rats weighing 60 to 80 grams were prepared for study as outlined in detail for Experiment #1. Ten rats underwent sham nephrectomy and 40 rats underwent a two-stage 5/6 nephrectomy as previously described [14]. Rats with normal renal function were given standard rat chow containing 23.4% protein, 0.6% calcium and 0.68% phosphorus (Purina Mills) as described in Experiment #1. Nephrectomized animals received the same amounts of protein and calcium, but the amount of phosphorus in the diet was raised to 1.2% to increase the severity of secondary hyperparathyroidism [13]. Again, nephrectomized and intact control rats were pair-fed and given free access to drinking water throughout the experiment.

Four weeks after completing two-stage nephrectomies, 10 intact control rats (C) were started on daily i.p. injections of saline vehicle (C), and seven nephrectomized animals also received daily vehicle injections (Nx-C). Seven nephrectomized rats were treated with daily i.p. injections of rhGH (Nx-GH), 10 IU/kg/day, seven nephrectomized animals were given daily i.p. injections of calcitriol (Nx-D), 50 ng/kg/day and five nephrectomized rats received daily injections of both hormones (Nx-GH+D). The doses of growth hormone and calcitriol were the same as those used in Experiment #1. Nephrectomized animals were assigned at random to each treatment group.

After 10 days of treatment with either hormonal agents or vehicle, the rats were killed as previously described. Blood was obtained by cardiac puncture for subsequent biochemical determinations, and tibiae were harvested after perfusing the animals with 4% PFA/PBS.
Serum biochemical determinations

After blood samples were obtained, serum was separated by centrifugation, and these were stored at −70°C until biochemical or hormonal assays were begun. Blood ionized calcium levels were determined at the time of sacrifice using a calcium sensitive electrode (ICA Radiometer, Copenhagen, Denmark). Serum phosphorus, creatinine and urea nitrogen levels were measured by standard laboratory methods. Serum parathyroid hormone (PTH) levels were measured using an immunoradiometric assay for rat PTH (Immunotopics Inc, San Clemente, CA, USA).

Growth plate cartilage measurements

For morphometric analysis, four 5 μm sections of bone were obtained from each tibia. These were mounted on glass slides, stained with hematoxylin and eosin and counterstained with azure A. The total width of the growth plate cartilage at the proximal end of each tibia was measured at equally spaced intervals using a digitizer tablet (Summagraphics, Seymour, CT, USA) connected to a microcomputer. A minimum of four width measurements was obtained from each epiphyseal growth plate, and final width determinations represent the average of these values. The width of the zone occupied by hypertrophic chondrocytes was measured by the same method. In addition to the average width of the hypertrophic zone, the percentage of the total width of the growth plate comprised of hypertrophic chondrocytes was also calculated. The extent of marrow fibrosis was graded on a scale ranging from 0 to 3+ by a single observer who had no knowledge about the experimental procedures done on animals from whom specimens had been collected.

In situ hybridization

In situ hybridization in sections of bone was done using methods described in detail elsewhere [15]. Three specimens from separate treatment groups were placed on single glass slides to minimize slide-to-slide variation during processing and development. Approximately 80 slides were included in each hybridization using 35S-labeled riboprobes encoding rat type II collagen, type X collagen and PTH/PTHrP receptor. Riboprobes were generated using the Gemini transcription kit (Promega, Madison, WI, USA). Sense mRNA probes were used as negative controls. In situ hybridization was completed using 1 × 106 cpm of 35S-labeled (Amersham, Arlington Heights, IL, USA) type II collagen mRNA, type X collagen mRNA and PTH/PTHrP receptor mRNAs at 55°C overnight. After hybridization, sections were washed with 5 × SSC at 50°C, 50% formamide and 2 × SSC at 50°C, 10 mM Tris-HCl with 500 mM NaCl and 1 mM EDTA at 37°C, 2 × SSC, 0.2 × SSC and increasing concentrations of ethanol. Slides were exposed to X-ray film overnight (Fujifilm, Tokyo, Japan), and emulsion autoradiography was done using NTB-2 (Eastman Kodak, New Haven, CT, USA) at 4°C. The length of exposure was estimated from the intensity of the signals on the X-ray film. The slides were developed and stained with hematoxylin and eosin.

Quantitation of in situ hybridization signals

Slides were viewed at ×100 by bright field microscopy. The images were captured using a CCD camera control unit (Hamamatsu Photonics, Hamamatsu-City, Japan), and these were displayed on a computer monitor. The number of silver grains overlying each chondrocyte profile was counted using an image analysis system (Kontron 200; Kontron Elektronik, Germany). Twenty to 30 cell profiles were assessed in each bone specimen, and the results for individual bone samples represent the average of these measurements. Data are expressed as the number of silver grains/1000 μm² of cell profile.

Statistical analysis

All results are expressed as means ± one sd. Data were evaluated using one-way analysis of variance with contrasts [16]. Chi-square analysis was used to compare the extent of marrow fibrosis among groups.

RESULTS

Experiment #1 - Mild secondary hyperparathyroidism

Neither total body wt at the end of study nor weight gain during the 10-day experimental period differed among groups (Table 1). Body length and the increase in length during study also did not differ among groups (Table 1).

As expected, serum creatinine and blood urea nitrogen (BUN) levels were higher in each group of nephrectomized rats than in control animals with normal renal function, whereas serum phosphorus values did not differ among groups (Table 2). Serum PTH levels were moderately higher in Nx-C than in C, but values were substantially elevated in Nx-GH (Table 2). However, blood ionized calcium concentrations did not differ among these three groups. In contrast, serum PTH levels were substantially lower and blood ionized calcium levels were higher both in

<p>| Table 1. Anthropometric measurements in rats with normal renal function (Control) and in four groups of subtotally nephrectomized rats with mild secondary hyperparathyroidism |</p>
<table>
<thead>
<tr>
<th>Control</th>
<th>N = 10</th>
<th>Nx-C</th>
<th>N = 6</th>
<th>Nx-GH</th>
<th>N = 5</th>
<th>Nx-D</th>
<th>N = 6</th>
<th>Nx-GH+D</th>
<th>N = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight a g</td>
<td>241 ± 25</td>
<td>247 ± 41</td>
<td>259 ± 33</td>
<td>227 ± 18</td>
<td>243 ± 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain in weight b g</td>
<td>11 ± 25</td>
<td>17 ± 18</td>
<td>25 ± 8</td>
<td>−10 ± 30</td>
<td>6 ± 34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length c cm</td>
<td>38 ± 0.1</td>
<td>38 ± 1.6</td>
<td>38 ± 1.9</td>
<td>37 ± 0.6</td>
<td>38 ± 1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gain in length b cm</td>
<td>7.9 ± 0.9</td>
<td>8.1 ± 1.8</td>
<td>8.0 ± 1.7</td>
<td>7.5 ± 0.5</td>
<td>8.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nephrectomized animals received daily intraperitoneal injections of saline vehicle (Nx-C), growth hormone (Nx-GH), calcitriol (Nx-D) or both hormonal agents together (Nx-GH+D) for 10 days.

| a Obtained at the time of sacrifice
| b Difference before and after 10 days of treatment

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The width of the growth plate cartilage did not differ from control values in nephrectomized rats given vehicle (Nx-C), growth hormone (Nx-GH), calcitriol (Nx-D) or both hormonal agents together (Nx-GH+D) for 10 days (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nx-C</th>
<th>Nx-GH</th>
<th>Nx-D</th>
<th>Nx-GH+D</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH pg/ml</td>
<td>82±24</td>
<td>111±69</td>
<td>157±58</td>
<td>6.0±2.0</td>
<td>7.8±2.0d</td>
</tr>
<tr>
<td>Ionized calcium</td>
<td>1.3±0.02</td>
<td>1.3±0.03</td>
<td>1.3±0.04</td>
<td>1.5±0.09d</td>
<td>1.5±0.07d</td>
</tr>
<tr>
<td>Phosphorus mg/dl</td>
<td>9±1.0</td>
<td>9±0.6</td>
<td>9±0.7</td>
<td>9±1.2</td>
<td>8±0.9</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>0.4±0.04</td>
<td>1.1±0.4</td>
<td>1.1±0.34</td>
<td>1.0±0.1</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Urea nitrogen mg/dl</td>
<td>15±1</td>
<td>57±25</td>
<td>57±22</td>
<td>60±12</td>
<td>61±18</td>
</tr>
</tbody>
</table>

* P < 0.001 vs. Control
b P < 0.05 vs. Nx-C
c P < 0.001 vs. Nx-C

d P < 0.001 vs. Nx-GH

The amount of marrow fibrosis was greatest in Nx-GH, whereas less fibrosis was seen in Nx-C, Nx-D and Nx-GH+D (Table 4). Serum phosphorus levels were also elevated in all four groups of nephrectomized rats reflecting the higher dietary phosphorus content utilized in Experiment #2 (Table 4).

Overall, body weight at the end of study and the increase in body weight during each 10 day experiment were similar in intact control animals in Experiments #1 and #2. In nephrectomized rats with severe secondary hyperparathyroidism, the weight gain was less in Nx-C than in C (Table 3). Weight gain was somewhat greater in Nx-GH than in Nx-C, but nephrectomized rats given calcitriol, either alone or together with growth hormone, failed to gain weight during the study. Neither total body length at the end of the experiment nor the increase in body length differed among groups (Table 3).

Experiment #2 - Severe secondary hyperparathyroidism

Serum biochemical determinations at the end of study in rats with normal renal function (Control) and in subtotally nephrectomized animals with mild secondary hyperparathyroidism given daily injections of saline vehicle (Nx-C), growth hormone (Nx-GH), calcitriol (Nx-D) or both hormonal agents together (Nx-GH+D) for 10 days are shown in Table 2. Serum creatinine, calcium and PTH values were substantially lower and blood ionized calcium concentrations were substantially lower in Nx-C and in Nx-GH than in C; however, the mean values did not differ between Nx-C and Nx-GH (Table 4). Serum PTH values were also elevated in both groups of nephrectomized rats given calcitriol, but PTH values were substantially lower and blood ionized calcium concentrations were higher in Nx-D and Nx-GH+D than in either Nx-C or Nx-GH (Table 4).

The width of the growth plate cartilage was moderately reduced in Nx-C compared to C, and there was a decrease in the organization of linear columns of proliferating and hypertrophic chondrocytes in Nx-C compared to that seen in intact control animals (Fig. 3). Treatment with rhGH not only restored the micro-architecture of the growth plate, but also increased its width. As such, the growth plate thickness in Nx-GH exceed that in Nx-C, and values in Nx-GH did not differ from those in C (Fig. 3). The thickness of the growth plate cartilage did not differ between Nx-D and Nx-C, but values were less in Nx-GH+D than in C. Thus, the growth plate width did not differ between Nx-GH+D and Nx-C (Fig. 3). The width of the zone of hypertrophic chondrocytes did not differ among groups.

The amount of marrow fibrosis was greatest in Nx-GH, whereas less marrow fibrosis was seen in Nx-C, Nx-D and Nx-GH+D. Scores for marrow fibrosis, on a scale ranging from 0 to 3, were 2.5, 1.0, 1.5 and 2.0, respectively, in Nx-GH, Nx-C, Nx-D and Nx-GH+D (χ² = 16.6, P < 0.05).

In contrast to rats with mild secondary hyperparathyroidism, PTH/PTHrP receptor expression was less in Nx-C than in C; however, transcripts for PTH/PTHrP receptor were again localized to chondrocytes in the lower proliferating zone and in the upper hypertrophic region. Type X collagen mRNA expression in each of these two groups did not differ from that in C (Fig. 2). Combined treatment with growth hormone and calcitriol did not further increase type X collagen mRNA expression from levels seen in Nx-GH or Nx-D.
zone and in the upper hypertrophic region (Fig. 4). Expression of mRNA for PTH/PTHrP receptor increased both in Nx-GH and in Nx-D compared to Nx-C, and there were no differences in PTH/PTHrP receptor mRNA expression between Nx-GH and Nx-GH-D (Fig. 5). Neither type X collagen nor type II collagen mRNA expression differed between Nx-C and C, but mRNA transcripts for both type X collagen and type II collagen were more abundant in the growth plate cartilage in Nx-GH (Fig. 5). Expression of mRNAs for both type X and type II collagens did not differ in the Nx-GH-D from values determined in Nx-C.

**DISCUSSION**

The PTH/PTHrP receptor mediates the actions of two distinct hormones: parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) [12]. Its prominent expression in bone and kidney accounts for the well-established systemic calcium-regulating actions of PTH, whereas its abundance in epiphyseal growth plate cartilage mediates the localized actions of PTHrP on chondrocyte proliferation and differentiation, thereby serving as an important regulator of skeletal modeling and bone elongation [12]. Because PTH/PTHrP receptor expression is reduced in renal failure [13], the autocrine/paracrine functions of PTHrP may be altered in this disorder, and such changes could contribute to impairments in linear growth. Moreover, since both calcitriol and growth hormone modify PTH/PTHrP receptor expression in vitro and because both hormonal agents are widely used in the clinical management of children with growth retardation due to chronic renal failure, we chose to examine the impact of calcitriol and/or growth hormone administration on markers of chondrocyte differentiation in the epiphyseal growth plate cartilage of rats with renal failure and either mild or advanced secondary hyperparathyroidism.

The results of the current investigation indicate that differences in the severity of secondary hyperparathyroidism affect not only epiphyseal growth plate morphology, but also the expression of several molecular markers of endochondral bone formation in experimental animals with...
renal failure. Growth plate thickness was reduced andPTH/PTHrP receptor mRNA levels were diminished in nephrectomized rats with advanced secondary hyperparathyroidism, but neither change was evident in animals with mild secondary hyperparathyroidism. Calcitriol blunted the trophic effect of growth hormone on epiphyseal growth plate thickness in rats with advanced secondary hyperparathyroidism, and this disturbance was associated with reductions in growth hormone-induced increases in mRNAs for both type X and type II collagen. In contrast, calcitriol did not attenuate the increase in growth plate thickness associated with growth hormone administration in rats with mild secondary hyperparathyroidism. Calcitriol also did not affect the rise in PTH/PTHrP receptor mRNA expression following growth hormone administration in nephrectomized rats with either mild or advanced secondary hyperparathyroidism despite substantial differences in basal PTH/PTHrP receptor mRNA levels in growth plate chondrocytes. Although the changes need to be confirmed by immunohistochemical techniques, the current results suggest that the severity of secondary hyperparathyroidism can modify the expression of key regulators of endochondral bone formation and longitudinal bone growth in rats with renal failure, and that 1,25-dihydroxyvitamin D influences bone formation and longitudinal bone growth in rats with renal failure. Such findings are consistent with results reported previously by Mehls and colleagues [7]. In contrast, Hanna et al noted that only the width of the zone of hypertrophic chondrocytes increased in rats treated with growth hormone, whereas the overall width of the epiphyseal cartilage did not change [17]. Others have also failed to document increases in growth plate thickness during treatment with growth hormone despite increases in the number of proliferating chondrocytes in epiphyseal cartilage [18, 19]. The explanation for these divergent findings remains uncertain, but the presence and/or the severity of secondary hyperparathyroidism may be a contributing factor. Since the mitogenic actions of parathyroid hormone on growth plate chondrocytes are mediated, at least in part, by IGF-I [20], PTH-dependent changes in basal IGF-I levels locally within epiphyseal cartilage may serve to augment the IGF-I mediated actions of growth hormone in proliferating chondrocytes in rats with markedly elevated serum PTH levels.

Several reports have implicated PTH as a potential modifier of the actions of growth hormone in skeletal tissues [20, 21]. Although serum PTH levels were not measured, Berger and co-workers found that linear growth was less in growth hormone-treated rats given intermittent doses of calcitriol, which would be expected to lower serum PTH levels, than in animals receiving growth hormone alone [22]. More recently, Kuizon et al reported that pre-pubertal children undergoing regular peritoneal dialysis with relatively low serum PTH levels and adynamic renal osteodystrophy grew less well than subjects of the same age and pubertal status with overt secondary hyperparathyroidism [11]. Indeed, changes in Z-scores for height over 12 months correlated directly with serum PTH levels. Although such findings suggest that PTH can modify the skeletal response to growth hormone as judged by changes in linear growth, direct inhibitory effects of calcitriol on chondrocyte proliferation and/or differentiation could also explain these results.

In the current study, mRNA levels for the PTH/PTHrP receptor were reduced in the growth plate chondrocytes of nephrectomized rats with severe secondary hyperparathyroidism but not in those with mild disease, indicating that the severity of secondary hyperparathyroidism may be an important determinant of PTH/PTHrP receptor expression in rats with renal failure. Such findings differ from those reported by Ureña and co-workers, who found that PTH/PTHrP receptor expression in the kidney, as assessed by immunohistochemistry, was similarly reduced both in parathyroidectomized and in intact rats with renal failure [23]. Previous studies have not, however, assessed PTH/PTHrP receptor mRNA levels in the growth plate cartilage of animals with varying degrees of secondary hyperparathyroidism. Given the crucial role of PTHrP and its receptor as regulators of chondrocyte differentiation during endochondral bone growth, differences in basal PTH/PTHrP receptor expression between mild and advanced secondary hyperparathyroidism could account for variations in the trophic effect of growth hormone on epiphyseal growth

| Table 4. Serum biochemical determinations at the end of study in rats with normal renal function (Control) and in subtotally nephrectomized (Nx-C), growth hormone (Nx-GH), calcitriol (Nx-D) or both hormonal agents together (Nx-GH+D) for 10 days |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | Nx-C            | Nx-GH           | Nx-D            | Nx-GH+D         |
| PTH pg/ml        | 84 ± 22         | 557 ± 157*     | 619 ± 84        | 342 ± 289*      | 348 ± 264*      |
| Ionized calcium mmol/L | 1.3 ± 0.01    | 0.9 ± 0.18     | 1.0 ± 0.18      | 1.3 ± 0.05*     | 1.2 ± 0.17*     |
| Phosphorous mg/dl | 8 ± 0.3         | 15 ± 5.0*      | 16 ± 3.0*       | 13 ± 4.5*       | 16 ± 8.6*       |
| Creatinine mg/dl | 0.3 ± 0.5       | 1.3 ± 0.51*    | 1.0 ± 0.33*     | 0.9 ± 0.34*     | 1.0 ± 0.32*     |
| Urea nitrogen mg/dl | 14 ± 2.0       | 103 ± 57*      | 81 ± 42*        | 74 ± 44*        | 96 ± 56*        |

* P < 0.01 vs. Control  
* P < 0.005 vs. Nx-C  
* P < 0.001 vs. Nx-GH

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plate cartilage in these two disorders. Indeed, the effect of calcitriol to blunt growth hormone-induced increases in growth plate thickness and mRNA levels for both type II and type X collagen in rats with advanced secondary hyperparathyroidism without modifying the growth hormone-associated rise in PTH/PTHrP receptor levels suggests that calcitriol can directly affect chondrocyte proliferation and/or differentiation by mechanisms separate from those mediated by PTHrP and its receptor.

Previous in vitro studies have demonstrated dose-dependent inhibitory effects of calcitriol on cell proliferation and DNA synthesis in chondrocytes [9, 10]. Silbermann and co-workers reported that doses of calcitriol exceeding 50 ng/kg/day in the rat reduced the heights of the zones of proliferating and hypertrophic chondrocytes in epiphyseal cartilage, and growth retardation was evident after only seven days of treatment with large doses of calcitriol [10]. The mechanisms responsible for these inhibitory effects of calcitriol remain uncertain, but Scharla et al suggested that the blunted response to growth hormone during calcitriol therapy might be explained by reductions in local IGF-I.
Production and/or by increases in the amount of the inhibitory IGF binding protein IGF-BP-4 [24]. In this regard, neither the addition of growth hormone nor IGF-I to the culture medium could overcome the suppressive effect of calcitriol on cell proliferation in chondrocytes and osteoblast-like cells [25]. Additional work will be required to determine whether similar mechanisms explain the \textit{in vivo} inhibitory actions of calcitriol on epiphyseal growth thickness in rats with severe secondary hyperparathyroidism.

PTH/PTHrP receptor mRNA levels failed to correspond to the prevailing concentration of PTH in serum in the current series of experiments. Therefore, PTH/PTHrP receptor mRNA levels were markedly greater in rats given growth hormone than in untreated nephrectomized animals with advanced secondary hyperparathyroidism, whereas serum PTH levels did not differ between these two experimental groups. Similarly, the concurrent administration of calcitriol and growth hormone to rats with severe secondary hyperparathyroidism did not alter PTH/PTHrP receptor mRNA expression compared to values seen in rats given growth hormone alone, but serum PTH levels were markedly lower in calcitriol-treated animals. Such findings indicate that growth hormone and calcitriol can each affect PTH/PTHrP receptor message expression in growth plate chondrocytes \textit{in vivo} by mechanisms that are not dependent on the concentration of PTH in serum. The current results are consistent, therefore, with previous work indicating that elevated serum PTH levels are not required for down-regulation of PTH/PTHrP receptor expression in the kidney of rats with renal failure [23].

Considerable evidence indicates that PTHrP and its receptor together with at least one of the hedgehog proteins, that is, Indian hedgehog, participate in the regulation of chondrocyte differentiation in epiphyseal cartilage during endochondral bone formation [12]. Mice in which both alleles of the gene encoding PTHrP have been deleted exhibit marked skeletal abnormalities characterized by shortened limbs, premature skeletal mineralization and early closure of the epiphyses of long bones [26]. Such changes result from the premature differentiation of proliferating chondrocytes into hypertrophic chondrocytes leading to early cessation of linear growth, and they are associated with increases in type X collagen expression which is generally considered to be a phenotypic marker for hypertrophic chondrocytes.

In the current study, the administration of growth hormone to nephrectomized rats with severe secondary hyperparathyroidism increased mRNA expression for type X collagen, but mRNA levels for type II collagen also increased. Calcitriol diminished the magnitude of these responses in association with lesser changes in growth plate thickness during growth hormone treatment. Such findings suggest that growth hormone increases the rate of cell proliferation in proliferating chondrocytes and that it may also promote the expression of a more differentiated phenotype in hypertrophic chondrocytes. In contrast, calcitriol appears to offset the trophic effects of growth hormone on chondrocyte proliferation in epiphyseal cartilage as judged by measurements of growth plate thickness, and this mode of action may contribute to reductions in growth plate thickness and in longitudinal bone growth.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_5.png}
\caption{Type X collagen mRNA, type II collagen mRNA and PTH/PTHrP receptor mRNA expression in the growth plate cartilage in rats with normal renal function (Control; □) and in four groups of subtotally nephrectomized rats with advanced secondary hyperparathyroidism. Nephrectomized animals received daily intraperitoneal injections of saline vehicle (Nx-C; ■), growth hormone (Nx-GH; □), calcitriol (Nx-D; □), or both hormonal agents together (Nx-GH+D; □) for 10 days. \textit{a} \( P < 0.05 \) vs. Control; \textit{b} \( P < 0.001 \) vs. Nx-C; \textit{c} \( P < 0.05 \) vs. Nx-C; \textit{d} \( P < 0.05 \) vs. Nx-GH; \textit{e} \( P < 0.001 \) vs. Nx-GH.}
\end{figure}

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during treatment with calcitriol in rats with advanced secondary hyperparathyroidism due to chronic renal failure. The precise effect of calcitriol on the differentiation of proliferating cells into hypertrophic chondrocytes in vivo remains less certain, however, since the widely recognized actions of calcitriol to promote cellular differentiation would have been expected to increase rather than decrease type X collagen expression in the current experiment. Additional work will be required to clarify this matter.

In summary, the results of the current investigation indicate that both growth hormone and calcitriol influence the proliferation and differentiation of epiphyseal growth plate chondrocytes in rats with secondary hyperparathyroidism due to chronic renal failure. Calcitriol blunts the trophic actions of growth hormone on epiphyseal growth plate thickness and on type X and type II collagen expression by chondrocytes without modifying mRNA levels for PTH/PTHrP receptor. Such actions may contribute to impaired skeletal growth in pre-pubertal children with end-stage renal disease who are treated with large doses of calcitriol, and they may also account for the variable clinical response to growth hormone administration in pediatric patients with chronic renal disease.

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