Catch-up growth follows an abnormal pattern in experimental renal insufficiency and growth hormone treatment normalizes it

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The primary goal of this study was to determine if the ability to undergo catch-up growth following a transient injury is preserved in an experimental model of moderate chronic renal failure (CRF) and the effect of growth hormone (GH) administration on such phenomenon. Young rats were subtotally nephrectomized (days 0 and 4) (Nx). From days 11 to 13, food intake was restricted in subgroups of Nx and control (C) rats (NxR and CR). After refeeding, subgroups of NxR and CR rats received GH from days 14 to 20 (NxRGH and CRGH). Rats were killed on days 14 (C, CR, Nx, NxR), 17 and 21 (C, CR, CRGH, Nx, NxR, NxRGH), and 36 (C, CR, Nx, NxR). Longitudinal growth rate was measured by osseous front advance in the proximal tibiae. With refeeding, growth rate of CR, NxR, and NXrGH rats became significantly greater than that of C, indicating catch-up growth. This occurred later and with lower growth rate in NxR than in CR rats, whereas the characteristics of catch-up growth in CR and NxRGH animals were similar. Changes in growth rate were associated with modifications in the morphology and proliferative activity of growth cartilage. We conclude that catch-up growth occurs in renal insufficiency but follows a different pattern from that observed with normal renal function. GH treatment normalizes the pattern of catch-up growth in CRF. Changes in growth velocity are associated to modifications in the structure and dynamics of growth cartilage.

Kidney International (2006) **70,** 1955–1961. doi:10.1038/sj.ki.5001949; published online 11 October 2006

KEYWORDS: chronic renal failure; growth; growth hormone; rats; growth plate; nutrition

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Received 15 May 2006; revised 2 August 2006; accepted 29 August 2006; published online 11 October 2006

Growth retardation is still a major manifestation of children with chronic renal failure (CRF). The 2005 Annual Report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS)[1] indicates that 36% of 5827 children younger than 21 years with CRF have height *Z*-score worse than −1.88. Except in the group of infants younger than 1 year of age, the height *Z*-score does not improve after a follow-up period of 24 months. As also shown by the NAPRTCS report,[1] the vast majority of children with CRF have a height below the 50th percentile. This suggests a loss of potential to achieve the genetically determined final height that occurs not only in patients with advanced stages of CRF but also in children with mild–moderate degrees of CRF.

The typical growth pattern of a child with congenital CRF whose adult height becomes stunted comprises two periods, infancy and puberty, during which the height deficit worsens, that is, there is a further deviation from the normal percentiles, and the mid-childhood period during which the growth rate parallels the normal growth channel.[2,3] This pattern suggests that the ability to accelerate growth velocity is reduced in individuals with CRF.

The subtotally nephrectomized young rat is commonly used as experimental model of growth retardation in CRF.[4] Whereas longitudinal growth rate of rats with severe degrees of CRF is sustainedly lower than that of sham-operated animals fed *ad libitum*, rats with milder reductions of renal function exhibit reduced growth rate only during few days following the second stage nephrectomy.[5] Thereafter, their growth rate parallels that of control animals. In these rats with mild–moderate CRF, decreased length gain coexists with longitudinal growth velocity not different from that of controls.

The term catch-up growth, introduced by Prader *et al.*,[6] describes the phenomenon by which longitudinal growth velocity transiently stands above the statistical limits of normality for age and/or maturity after the removal of a growth-inhibiting condition. It has been observed in humans and animals and its underlying pathogenic mechanism is not clear.[7,8]

The study presented here was designed to determine if the ability to exhibit catch-up growth is preserved in CRF as well as to know its characteristics, associated changes in long bone growth plate and the effect of growth hormone (GH) treatment. It was hypothesized that individuals with CRF are able to keep a 'normal' longitudinal growth rate but have a reduced potential to increase growth velocity following a growth-inhibiting condition. In addition, GH treatment, which has been shown to accelerate growth velocity of subjects with CRF,[9,10] might likely modify the pattern of catch-up growth in CRF.

Table 1 | Renal function and growth of animals (*n*=5 per group) killed at the different time points of the protocol

	SUN (mg/dl)	Creatinine (mg/dl)	Weight (g)	Length gain (cm)
Day 14				
C	12±0	0.4±0.0	130.7±3.4	6.1±0.3
CR	9±0	0.4±0.0	108.2±2.9[a]	5.1±0.2[a]
Nx	28±2[a,b]	0.7±0.1[a,b]	117.6±5.2	4.8±0.2[a]
NxR	30±2[a,b]	0.7±0.1[a,b]	91.8±3.4[a,b,c]	3.7±0.2[a,b,c]
Day 17				
C	11±1	0.3±0.0	136.3±1.8	6.7±0.3
CR	11±1	0.3±0.0	127.6±1.2	6.2±0.3
CRGH	13±0	0.3±0.0	137.0±3.5	6.4±0.2
Nx	32±3[a,b,d]	0.7±0.1[a,b,d]	131.0±4.2	6.0±0.4
NxR	27±2[a,b,d]	0.5±0.0[a,b,d]	120.3±3.4	5.1±0.3[a]
NxRGH	29±1[a,b,d]	0.5±0.0[a,b,d]	129.2±5.1	5.3±0.4
Day 21				
C	14±1	0.3±0.0	145.1±3.1	8.1±0.3
CR	11±1	0.3±0.0	146.0±2.2	7.8±0.2
CRGH	14±1	0.4±0.0	164.4±6,5[a,b]	8.2±0.3
Nx	31±1[a,b,d]	0.6±0.0[a,b,d]	144.4±3.5[d]	7.7±0.2
NxR	30±1[a,b,d]	0.5±0.0[a,b,d]	134.6±2.4[d]	6.8±0.2[a,d]
NxRGH	24±0[a,b,c,d,e]	0.6±0.0[a,b,d]	155.2±3.5[e]	7.6±0.2
Day 36				
C	11±0	0.4±0.0	188.8±5.4	11.7±0.4
CR	11±1	0.4±0.0	191.0±5.9	11.8±0.1
Nx	26±2[a,b]	0.7±0.0[a,b]	183.5±2.9	11.1±0.3
NxR	25±2[a,b]	0.7±0.0[a,b]	185.0±4.7	11.1±0.3

GH, growth hormone.
SUN means serum urea nitrogen. C means control group. CR means normal renal function group undergoing diet restriction from days 11 to 13. CRGH means normal renal function group undergoing diet restriction from days 11 to 13 and treated with GH from day 14 until killing. Nx means chronic renal failure group. NxR means chronic renal failure group undergoing diet restriction from days 11 to 13. NxRGH means chronic renal failure group undergoing diet restriction from days 11 to 13 and treated with GH from day 14 until killing. Length gain means body length increment between day 4 and the corresponding day of killing.
Data are mean±s.e.m.
[a]Significantly different from C.
[b]Significantly different from CR.
[c]Significantly different from Nx.
[d]Significantly different from CRGH.
[e]Significantly different from NxR.

RESULTS

On day 4, weight and length of animals were not different among groups: C (74.4±0.9 g, 22.6±0.2 cm), CR (73.2±1.0 g, 22.8±0.1 cm), CRGH (77.0±1.6 g, 22.8±0.3 cm), Nx (71.4±1.1 g, 22.6±0.2 cm), NxR (71.7±0.9 g, 22.8±0.2 cm), NxRGH (73.7±0.7 g, 23.1±0.2 cm). Data on serum urea nitrogen (SUN), serum creatinine, body weight, and length gain in animals killed at different time points of the protocol are given in Table 1. Renal function was significantly and similarly reduced in the three groups of nephrectomized rats. Food restriction decreased length gain on day 14. Length gain of animals subjected to diet restriction became practically identical to their corresponding control groups on day 21 for those groups treated with GH and on day 36 for the untreated groups.

Longitudinal bone growth rates assessed by daily osseous front advances at the different time points of the study are shown in Table 2. Catch-up growth was demonstrated on day 21 in CR, CRGH, and NxRGH groups and on day 36 in NxR rats. Representative images of calcein labeling to illustrate greater osseous front advance in NxRGH and NxR groups than C rats are shown in Figure 1a.

Histomorphometry of growth cartilage and index of proliferating chondrocytes on days 21 and 36 of the protocol are summarized in Tables 3 and 4. Representative images are shown in Figure 1b and c. In addition, no differences were found in the pattern of expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1) or vascular endothelial growth factor (VEGF), as revealed by immunohistochemistry, among the groups. PECAM-I was expressed in the hypertrophic zone with a clear intracellular signal that was mildly weaker in the area next to the primary spongiosa (data not shown). By contrast, VEGF was mainly expressed in the most distal chondrocytes (data not shown). *In situ* hybridization showed signal for collagen X mRNA confined to the hyper-

Table 2 | Osseous front advance (μm/day) in the different groups of animals (*n*=5 per group) according to the moment of killing

	C	CR	CRGH	Nx	NxR	NxRGH
Day 14	250.4±6.2	229.0±3.5	NA	243.8±3.8	202.5±9.9	NA
Day 17	223.8±4.1	172.9±4.2	182.2±6.3	191.7±6.5	146.7±10.5	144.5±9.5
Day 21	164.5±7.7	186.1±6.1[a]	201.1±4.4[a]	193.0±7.5	173.3±3.9	201.2±6.0[a]
Day 36	91.5±1.1	95.6±3.0	NA	97.6±2.0	118.8±2.7[a]	NA

GH, growth hormone; NA, not available.
Catch-up growth was observed on day 21 in CR, CRGH, and NxRGH groups and on day 36 in NxR animals.
C means control group. CR means normal renal function group undergoing diet restriction from days 11 to 13. CRGH means normal renal function group undergoing diet restriction from days 11 to 13 and treated with GH from day 14 until killing. Nx means chronic renal failure group. NxR means chronic renal failure group undergoing diet restriction from days 11 to 13. NxRGH means chronic renal failure group undergoing diet restriction from days 11 to 13 and treated with GH from day 14 until killing.
Data are mean±s.e.m.
[a]Significantly greater than C.

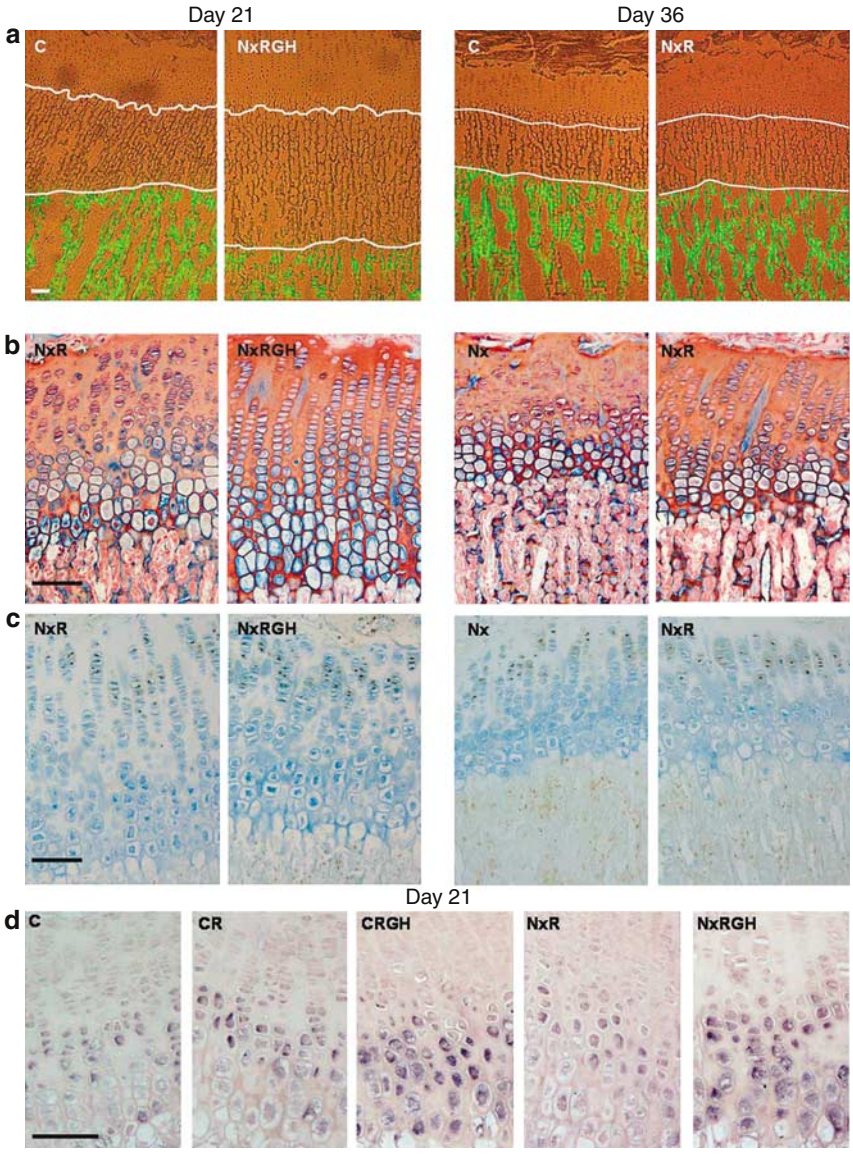

Figure 1 | Representative histological images of the tibial growth plate in the different groups of animals. (**a**) Longitudinal bone growth rate, assessed by the measurement of osseous front advance, in the two moments of the study when catch-up growth was observed in chronic renal failure rats subjected to diet restriction between days 11 and 13 of the protocol. In growth hormone treated (NxRGH) and untreated (NxR) animals, the distance between the calcein front and the metaphyseal end of growth cartilage, limits indicated by the two horizontal lines, was greater than in control (C) animals, indicating the occurrence of catch-up growth, on days 21 and 36 of the protocol, respectively. Photographs taken from methylmethacrylate-embedded sections of proximal tibia. Bar = 100 μm. (**b**) Morphological aspect of growth cartilage of chronic renal failure rats subjected to diet restriction between days 11 and 13 when catch-up growth was observed in growth hormone-treated (NxRGH, day 21) or untreated (NxR, day 36) groups. In both time points, catch-up growth was associated with expansion of cartilage height and its hypertrophic stratum, the differences being more apparent in growth hormone-treated rats. Nx means chronic renal failure group not subjected to diet restriction. Photographs taken from methylmethacrylate-embedded sections of proximal tibia stained with alzian blue and safranine. Bar = 100 μm. (**c**) Proliferative activity assessed by immunohistochemical detection of bromodesoxiuridine labeled nuclei – brown nuclei – in growth cartilage of animals when catch-up growth was observed in chronic renal failure rats subjected to diet restriction between days 11 and 13 of the protocol and treated (NxRGH, day 21) or untreated (NxR, day 36) with growth hormone. Differences in the index of proliferating chondrocytes were not detected. Nx means chronic renal failure group. Photographs taken from methylmethacrylate-embedded sections of proximal tibia. Bar = 100 μm. (**d**) *In situ* hybridization for collagen X mRNA in growth cartilage of the different groups of rats on day 21 of the protocol. The pattern of expression was essentially the same in all groups although in rats treated with GH the signal seemed to be more evident in the most distal rows of chondrocytes. C means control group. CR means normal renal function group subjected to diet restriction from days 11 to 13. CRGH means normal renal function group subjected to diet restriction from days 11 to 13 and treated with growth hormone from days 14 to 20. NxR means chronic renal failure group subjected to diet restriction from days 11 to 13. NxRGH means chronic renal failure group subjected to diet restriction from days 11 to 13 and treated with growth hormone from days 14 to 20. Photographs taken from methylmethacrylate-embedded sections of proximal tibia. Bar = 100 μm.

Table 3 | Characteristics of growth cartilage in the animals (*n*=5 per group) killed on day 21 of the protocol

	C	CR	CRGH	Nx	NxR	NxRGH
Growth cartilage height (μm)	381±11	404±16[a]	451±13[a,b]	406±8	415±7	473±9[c,d]
Hypertrophic zone height (μm)	230±7	244±10[a]	268±13[a]	239±6	233±6	284±10[c,d]
Terminal chondrocyte height (μm)	30±0	30±1	32±1[a,b]	30±1	30±1	33±1[c,d]
Proliferating chondrocytes (cells/100 columns)	181±14	282±15[a]	296±12[a]	233±8	252±11	230±14

GH, growth hormone.
Catch-up growth (groups CR, CRGH, and NxRGH) was associated with changes in cartilage morphology and cell proliferation.
C means control group. CR means normal renal function group undergoing diet restriction from days 11 to 13. CRGH means normal renal function group undergoing diet restriction from days 11 to 13 and treated with GH from days 14 to 20. Nx means chronic renal failure group. NxR means chronic renal failure group undergoing diet restriction from days 11 to 13. NxRGH means chronic renal failure group undergoing diet restriction from days 11 to 13 and treated with GH from days 14 to 20.
Data are mean±s.e.m. Comparisons between groups were performed within normal renal function or nephrectomized groups.
[a]Significantly different from C.
[b]Significantly different from CR.
[c]Significantly different from Nx.
[d]Significantly different from NxR.

Table 4 | Characteristics of growth cartilage in the animals (*n*=5 per group) killed on day 36 of the protocol

	C	CR	Nx	NxR
Growth cartilage height (μm)	240±11	252±9	268±6	292±9[a]
Hypertrophic zone height (μm)	116±8	120±6	125±3	148±5[a]
Terminal chondrocyte height (μm)	24±1	24±0	24±1	25±1
Proliferating chondrocytes (cells/100 columns)	152±14	188±10	202±10	180±23

Catch-up growth (groups NxR) was associated with changes in cartilage morphology.
C means control group. CR means normal renal function group undergoing diet restriction from days 11 to 13. Nx means chronic renal failure group. NxR means chronic renal failure group undergoing diet restriction from days 11 to 13.
Data are mean±s.e.m. ANOVA was below or equal to 0.001 for each parameter. Comparisons between groups were performed within normal renal function or nephrectomized groups.
[a]Significantly different from Nx.

trophic stratum with attenuation or disappearance of the signal in the two o three rows of chondrocytes adjacent to the metaphyseal bone. In rats treated with GH, the collagen X mRNA signal was homogenous along the hypertrophic zone, the signal being clearly appreciable in the row of cells contiguous to bone (Figure 1d).

DISCUSSION

An outstanding finding of this study was that the two groups of animals undergoing diet restriction, either with normal or reduced renal function, showed complete catch-up growth in body length returning precisely to the length of their corresponding control groups. Full catch-up longitudinal growth to the small size of non-fasted individuals, has been observed in rats and rabbits treated with corticosteroids[11,12] and in head-irradiated[13] rats following fasting. Our study indicates that rats with mild–moderate renal failure retain the ability to accelerate longitudinal growth velocity and recover their former growth curve after removal of an additional growth-inhibiting condition. The mechanisms controlling the phenomenon of catch-up growth are still unknown. Tanner[14] proposed the hypothesis that catch-up growth is regulated by a central nervous system mechanism that compares the individual's actual size to an age-appropriate set point and then adjusts the growth rate accordingly. In CRF, the set point would be reset to a lower level explaining why the animals of the present study reassumed the growth curve that they were following before the injury. It has alternatively been proposed that the mechanism governing catch-up growth is intrinsic to the growth plate[15] and that catch-up growth is due, at least in large part, to a delay in growth plate senescence.[15,16] According to this hypothesis, growth plate chondrocytes have a limited and programmed proliferative capacity that determines the growth rate and, when the number of replications is exhausted, the end of longitudinal growth. A growth inhibiting condition transiently interrupts or slows chondrocytes' proliferative activity, which is reassumed when the aggression ceases resulting in a greater growth rate than expected for age. In the light of this hypothesis, the inability of the nephrectomized rats to achieve a normal length after removal of food restriction could be justified by the persistence of CRF-related growth-inhibiting factors.[7]

It is also of note that the pattern of catch-up growth was markedly different in CRF and normal renal function rats. Firstly, acceleration of growth rate occurred much later in the nephrectomized rats. Osseous front advance of these animals became higher than that of control rats on day 36 of the protocol, whereas in rats without renal insufficiency catch-up growth was demonstrated on day 21 of the protocol. Secondly, catch-up growth took place at much higher growth velocity in rats with normal renal function. The differences in chronology and maximum osseous front advance suggest that CRF limits the potential to reach a marked increment of growth rate and catch-up growth is delayed until periods where the longitudinal growth velocity is physiologically slow. This reduced potential to maximally accelerate growth might be present in children with early-onset chronic renal insufficiency and explain why height retardation of these patients worsens in infancy and puberty, periods of life where growth velocity is normally very high.[2]

The phenomenon of catch-up growth was accompanied by expansion of growth cartilage and its hypertrophic zone in rats with normal renal function as well as in rats with CRF. The height of growth cartilage columns is the result of the

dynamic equilibrium between two vectors. One goes from diaphysis to epiphysis and represents bone apposition. The other one goes in opposite sense and represents cartilage formation and progression.[17] The increased size of the columns of chondrocytes suggests a transient disequilibrium between these two vectors so that the vector representing chondrogenesis increased more than that of new bone apposition. In normal renal function rats, catch-up growth was associated with a significant increment of chondrocyte proliferating activity, which could not be demonstrated in renal failure rats. This is consistent with the assumption that in the setting of renal failure, the process of catch-up growth is delayed and occurs with slower growth velocities. Likewise, the less marked increase of cartilage height associated to catch-up growth in nephrectomized animals also suggests that the process of catch-up growth occurs more gradually in CRF.

The effect of GH was evident in rats killed on day 21 of the protocol, after 1 week of treatment. GH accelerated growth rate in rats with normal renal function and with renal failure as well. This finding agrees with previous clinical and experimental studies demonstrating the growth promoting effect of GH in CRF.[9,10] The effect of GH treatment was associated with improved food efficiency (0.33 ± 0.01 g of weight gained/g of food ingested from days 14 to 21 in NxRGH vs 0.28 ± 0.01 g in NxR, $P < 0.05$) but not stimulation of food intake (135.0 ± 3.9 vs 127.7 ± 4.7 g in both groups over the same period of time). It is interesting to note that GH treatment normalized the pattern of catch-up growth in nephrectomized rats so that catch-up occurred earlier, at the same time as rats with normal renal function, and with much greater growth rate than the group of untreated diet restricted CRF rats. It should be pointed out that catch-up growth induced by GH was associated with increased height of growth cartilage and its hypertrophic zone as well as that of the distal chondrocytes. The expansion of growth cartilage has formerly been reported in uremic rats treated with GH[10,18] and may reflect a transiently greater stimulus of GH on chondrogenesis than on new bone apposition. In normal renal function GH-treated rats, acceleration of growth rate was accompanied by an increase in the proliferative activity of chondrocytes that could not be demonstrated in GH-treated rats with renal insufficiency. However, in this group, GH administration seemed to exert a beneficial effect on the process of maturation and hypertrophy of the chondrocytes as shown by enlargement of most distal chondrocytes and extension of collagen X mRNA expression up to the terminal rows of chondrocytes. Former *in situ* hybridization studies have reported unchanged[19,20] and decreased[21,22] collagen X mRNA expression in the hypertrophic zones of uremic rat growth plates as well as increased expression following GH treatment.[21] The patterns of expression in the hypertrophic chondrocytes of PECAM-1, peptide with potent antiapoptotic properties,[23] and VEGF, angiogenic factor that is supposed to play an important role in the process of endochondral bone formation,[24] were essentially unchanged in the different groups of animals.

Finally, the characteristics of the experimental model obtained in this study deserve some comments. Female rats were chosen because it has been shown that respond better to GH treatment.[10] The rats with CRF had similar degree of renal function reduction as reflected by the lack of difference in SUN and serum creatinine concentrations among the three groups of nephrectomized rats. On day 21, NxRGH rats had lower SUN values, this difference being likely related to the well-known anabolic effect of GH therapy. Although renal failure was not severe, nephrectomized rats were growth retarded in comparison with the control group, as confirmed by lower length gains in Nx rats than in rats with normal renal function on day 14 of the protocol. Importantly, growth retardation coexisted with a growth rate at the moment of killing not different from that of controls. This agrees with former studies showing that, after nephrectomy, growth retardation worsens only in young nephrectomized rats with SUN concentrations greater than 3–4 times normal values, whereas animals with milder degrees of renal failure become stunted as a result of the surgical stress and subsequent acute renal failure but their further growth velocity is not different from controls.[5] In these animals, the adverse effect of mild CRF on growth becomes manifest by the inability to return to the normal growth percentiles. Achieving a model of growth retardation with normal growth rate was a goal of the experimental design because it cannot be expected that animals unable to maintain a normal basal growth rate are capable of undergoing catch-up growth. Diet restriction slowed growth rate as shown by reductions of osseous front advance of 17 and 9% in NxR and CR groups, respectively, in comparison with their corresponding diet unrestricted groups. Thus, identical percentage of food intake reduction caused a greater adverse effect on growth velocity in animals with CRF.

In summary, the study presented here shows that the ability to undergo catch-up growth after a transient growth-inhibiting condition is preserved in an experimental model of moderate CRF. However, the pattern of catch-up growth differs markedly from that observed in individuals with normal renal function. In renal failure, catch-up growth occurs later and with slower growth rates. Regardless renal function, marked modifications in the structure and dynamics of growth plate are associated with the catch-up growth phenomenon. Administration of GH normalizes the pattern of catch-up growth in rats with CRF and, at the same time, seems to exert a beneficial effect on the process of maturation and hypertrophy of chondrocytes.

MATERIALS AND METHODS

Animals

The experiments conducted complied with current legislation on animal experiments in the European Union and were approved by our Institution's Ethics Committee for Investigation with Animals.

Female Sprague–Dawley rats of 25 days of age and weight of 56 ± 6 g were purchased (Harlan Interfauna Ibérica SL, Barcelona, Spain) and housed in individual cages at room temperature between 22 and 24°C and 12-h light–dark cycle. Rats received standard diet containing 17.2% of proteins and 3100 kcal/kg (PANLAB SA, Barcelona, Spain) and had free access to tap water. After 4 days of acclimation to the experimental area the animals were grouped as follows: normal renal function + no diet restriction (C); normal renal function + diet restriction (CR); normal renal function + diet restriction + GH treatment (CRGH); CRF + no diet restriction (Nx); CRF + diet restriction (NxR); CRF + diet restriction + GH treatment (NxRGH).

Animals were killed ($n = 5$) on different days of the protocol. Day 14: groups C, CR, Nx, and NxR; day 17: groups C, CR, CRGH, Nx, NxR, and NxRGH; day 21: groups C, CR, CRGH, Nx, NxR, and NxRGH; day 36: groups C, CR, Nx, and NxR.

Experimental protocol

Days 0 and 4: two stage subtotal nephrectomy or sham operation.[9]

From day 4 until killing: daily measurement of body weight and food consumption from days 11 to 13: 3-day diet restriction to the appropriate groups which received 50% of the average daily food consumed by the same animals the 3 previous days (from days 8 to 10).

From day 14 onward: removal of diet restriction.

From days 14 to 16 (protocol of 17 days) or from days 14 to 20 (protocol of 21 days), 10 IU/kg/day of intraperitoneal recombinant human GH (Norditropin' Novo Nordisk Pharma, Madrid, Spain) was administered at 0009 and 1700 hours, approximately, to CRGH and NxRGH groups. Untreated animals received the same volume of solvent (saline).

Rats received 2 mg/100 g body weight of calcein (Sigma-Aldrich Spain, Tres Cantos, Madrid, Spain) solved in saline and sodium bicarbonate by intraperitoneal route 72 h before killing.

Rats received three intraperitoneal doses of 5-bromo-2′-desoxiuridine (BrdU) at 10 mg/100 g body weight (Sigma-Aldrich Spain) 60 min, 9, and 17 h before killing.

Days 0, 4, and immediately before killing: Measurement of snout to tail tip length with a ruler. The gain in body length was calculated for each time of the study.

Tissue samples were obtained at killing, which was carried out by exsanguination under anesthesia.

Samples

Serum samples were stored at −20°C until measurement of SUN and creatinine with a Kodak Ektachem DT60 analyzer (Rochester, NY, USA). Tibiae were removed and processed as follows. Immediately after killing, the proximal end of the tibiae, including the growth plate, was dissected out. The right tibia was fixed in 40% ethanol for analysis of calcein labeling and BrdU immunohistochemistry. The left tibia was fixed in 4% neutral formalin at 4°C, for morphometric analysis, VEGF, and PECAM-1 immunohistochemistry as well as for collagen X *in situ* hybridization. Then, both tibiae were dehydrated in graded solutions of ethanol and embedded in methylmethacrylate as described formerly.[25,26]

Measurement of longitudinal growth rate

Frontal 10-μm thick sections of proximal end of tibiae were obtained using a rotary microtome (HM355S, Microm, Barcelona, Spain) fitted with tungsten carbide blades. Sections were examined under an Olympus incident light fluorescence microscope (Olympus BX41) coupled to a digital camera (Olympus DP11, Olympus Optical España, Barcelona, Spain) to detect calcein label. Images were captured and the distance between the chondro-osseous junction and the calcein label was measured using an image analysis system (Scion Image, Scion Corporation, Frederick, MD, USA). The average value of these measurements divided by 3 (days) was considered the osseous front advance per day, representing the daily longitudinal bone growth rate in each animal. Catch-up growth was considered to occur in a group of rats when their longitudinal bone growth rate, assessed by osseous front advance as detailed above, was significantly greater than that of control animals with normal renal function and not subjected to former diet restriction (group C). Measurement of proliferative activity of chondrocytes, histomorphometry, immunohistochemistry, and *in situ* hybridization studies were performed in growth cartilage sections obtained at those times of the protocol when catch-up growth was detected.

Index of proliferating chondrocytes

Frontal 5-μm thick sections of proximal end of tibiae were incubated for 15 min in fresh 100% acetone to remove methylmethacrylate. After hydration, sections were incubated in HCl (2 N, 60 min, 37°C), thoroughly washed in water, rinsed in 0.1 M Tris-hydrochloric buffer, and treated with trypsin (1 mg/ml, 1% $CaCl_2$; 60 min, 37°C). After several washes in Tris-hydrochloric buffer, endogenous peroxidase activity was inactivated by 30-min treatment in 0.6% hydrogen peroxide. Samples were incubated with horse serum (1:50, 75 min; Sigma-Aldrich), followed by 48-h incubation with monoclonal antibody to BrdU (1:20; Dako Diagnosticos SA, Barcelona, Spain) in moist chamber at 4°C. Then, sections were incubated at room temperature with an anti-mouse IgG biotin conjugate (1:200, 60 min; Sigma-Aldrich) and ExtrAvidin (3:100, 45 min; Sigma-Aldrich). The final reaction product was revealed with 3-3′-diaminobenzidine (Sigma-Aldrich), 10 mg of 3-3′-diaminobenzidine in 50 ml of 0.05 M Tris-hydrochloric buffer plus 50 μl of 0.3% hydrogen peroxide. Preparations were lightly counterstained with alzian blue. The number of BrdU-positive cells per column of chondrocytes was counted as well as the number of columns per field. Six fields per section and two sections per animal were measured. For each animal, proliferative activity was expressed as the mean number of BrdU-labeled cells per 100 columns.

Morphometric analysis of growth plate

Heights of growth cartilage and its hypertrophic zone as well as height and area of the three most distal hypertrophic chondrocytes were measured in 5 μm thick sections stained with alzian blue and safranine at 20 randomly selected locations on each section, using the above-mentioned image analysis system.

Immunohistochemical analysis for PECAM-1 and VEGF

Immunohistochemical staining for PCAM-1 or VEGF was performed in formalin-fixed sections. After deplastination in acetone and rehydration as before, the sections for PCAM-1 analysis were heated in buffer citrate (pH 6.0, 80°C, 45 min). Then, all sections were treated with 3% hydrogen peroxide and 25% goat serum and incubated overnight (18 h) at 4°C with 1/100 solution of PECAM-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or 6/100 polyclonal anti-VEGF (Neo Markers, Westinghouse, CA, USA). After 30-min incubation with anti-rabbit secondary conjugated antibody (En Vision + TM; Dako Diagnosticos), the final reaction product was revealed with 3-3′-diaminobenzidine (Sigma-Aldrich); 20 mg of 3-3′-diaminobenzidine in 50 ml of 0.05 M

Tris-hydrochloric buffer plus 50 μl of 30% hydrogen peroxide. Sections were counterstained with alzian blue.

In situ hybridization for collagen X

The collagen X antisense and sense RNA probes were supplied by Genedect (Genedetect.com, Bradenton, FL, USA). For the hybridization procedure, guidelines of the supplier were followed. After hydration, sections were treated with triethanolamine (0.1 M) and acetic anhydride for 16 min and 300 μg/ml proteinase K for 30 min at 50°C, fixed in 4% paraformaldehyde, and washed in phosphate-buffered saline for 5 min twice. Prehybridation was performed for 2 h at 40°C. Hybridization was performed overnight at 40°C in moist chamber. After washing in 1 × standard sodium citrate at room temperature, and 1 × standard sodium citrate and 0.5 × standard sodium citrate at 55°C, nonspecific binding was blocked by 1% blocking buffer (Blocking Reagent, Boehringer, Manheim, GmbH, Germany) in Tris buffered saline for 30 min. Slides were placed in a moist chamber and incubated overnight with alkaline phosphatase-conjugated anti-digoxigenine (1:1000; Boehringer) in Tris buffered saline at 4°C. After three washes (5 min each) in Tris buffered saline, alkaline phosphatase was then revealed with a substrate solution containing 0.16 mg/ml 5-bromo-4-chloro-3-indolylphosphatase (Sigma-Aldrich) and 0.33 mg/ml nitroblue tetrazolium (Sigma-Aldrich) in 1 M Tris buffer. The enzymatic reaction was stopped with distilled water for several minutes and mounted with glycergel (Dako Diagnosticos). Two parallel sections served as negative controls. One section hybridized with a labeled-sense riboprobe and a second section incubated without adding any probe to the hybridization mixture. No hybridization signal was found in any of these negative controls.

Statistical analysis

Values for each group are given as mean ± s.e.m. For comparisons, the four time points of the protocol (14, 17, 21, and 36 days) were considered as independent studies. Comparisons among groups were performed by analysis of variance after confirming the normal distribution of the values by means of the Kolmogorov–Smirnov and Shapiro–Wilks test. Comparisons between two groups were performed by the Bonferroni corrected Student's *t*-test when appropriate. A *P*-value above 0.05 was considered as indicative of significant difference. Calculations were carried out by using SPSS 11.0 software (SPSS Inc. Headquarters, Chicago, IL, USA).

ACKNOWLEDGMENTS

This work was supported by the Fund of Health Research of the Spanish Ministry of Health (PI 02/1050), by the Institute of Health Carlos III Network of Centres on Molecular and Clinical Genetics (C3/07), by the Growth and Nutrition Foundation (FNYC), and by the University Cancer Institute of Asturias (IUOPA). We thank Dr Rafael Muñoz for technical assistance.

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