Chronic kidney disease induced by adenine: a suitable model of growth retardation in uremia

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Chronic kidney disease induced by adenine: a suitable model of growth retardation in uremia. Am J Physiol Renal Physiol 309: F57–F62, 2015. First published May 13, 2015; doi:10.1152/ajprenal.00051.2015.—Growth retardation is a major manifestation of chronic kidney disease (CKD) in pediatric patients. The involvement of the various pathogenic factors is difficult to evaluate in clinical studies. Here, we present an experimental model of adenine-induced CKD for the study of growth failure. Three groups (n = 10) of weaning female rats were studied: normal diet (control), 0.5% adenine diet (AD), and normal diet pair fed with AD (PF). After 21 days, serum urea nitrogen, creatinine, parathyroid hormone (PTH), weight and length gains, femur osseous front advance as an index of longitudinal growth rate, growth plate histomorphometry, chondrocyte proliferative activity, bone structure, aorta calcifications, and kidney histology were analyzed. Results are means ± SE. AD rats developed renal failure (serum urea nitrogen: 70 ± 6 mg/dl and creatinine: 0.6 ± 0.1 mg/dl) and secondary hyperparathyroidism (PTH: 480 ± 31 pg/ml). Growth retardation of AD rats was demonstrated by lower weight (AD rats: 63.3 ± 4.8 g, control rats: 112.6 ± 4.7 g, and PF rats: 60.0 ± 3.8 g) and length (AD rats: 7.2 ± 0.2 cm, control rats: 11.1 ± 0.3 cm, and PF rats: 8.1 ± 0.3 cm) gains as well as lower osseous front advances (AD rats: 141 ± 13 μm/day, control rats: 293 ± 16 μm/day, and PF rats: 251 ± 10 μm/day). The processes of chondrocyte maturation and proliferation were impaired in AD rats, as shown by lower growth plate terminal chondrocyte height (21.7 ± 2.3 vs. 26.2 ± 1.9 and 23.9 ± 1.3 μm in control and PF rats) and proliferative activity index (AD rats: 30 ± 2%, control rats: 38 ± 2%, and PF rats: 42 ± 3%). The bone primary spongiosa structure of AD rats was markedly disorganized. In conclusion, adenine-induced CKD in young rats is associated with growth retardation and disturbed endochondral ossification. This animal protocol may be a useful new experimental model to study growth in CKD.

GROWTH IMPAIRMENT is a major manifestation of chronic kidney disease (CKD) in the pediatric population. Although advances in the management of these patients have led to improvements in their growth rate and final height over the last two decades (7), it is of note that 45–60% of adults having CKD since infancy or childhood still have a height below normal reference values.

The pathogenesis of growth retardation in CKD is multifactorial, with several factors being involved to a variable extent: small birth size, metabolic acidosis, salt and water deficits, anorexia, malnutrition and cachexia, anemia, abnormal osseous metabolism, resistance to both growth hormone and IGF-I, repeated infections and surgical interventions, and use of glucocorticoids (10, 13, 14). Aggressive nutritional intervention, appropriate control of CKD-induced metabolic and bone mineral disorders, use of erythropoietin and iron supplements, administration of growth hormone, early kidney transplantation, and avoidance of inflammation are all measures able to exert a favorable effect on growth of children with CKD (18, 19).

Longitudinal bone growth, responsible for the increase in height, resides in the growth plate and results from the progressive replacement of growth plate cartilage by osseous tissue at the metaphyseal end of long bones. Physiologically, the rate and extent of growth for a given growth plate are determined by the combination of chondrocyte proliferation, matrix production, and enlargement of chondrocytes (8). The balance between two vectors, one of apposition of new bone at the metaphyseal cartilage-bone junction and another determined by the production and progression of new cartilage cells from epiphysis to metaphysis, is essential to maintain the adequate dynamics of endochondral growth (20).

The anatomic characteristics of the growth plate make its study extremely difficult in the clinical setting and justify the use of animal models for a better understanding of the mechanisms interfering with normal growth in CKD patients. In 1974, Chantler and Holliday (3) first described the use of an experimental model of uremia induced by two-stage 5/6th nephrectomy in prepubertal rats to investigate growth failure in CKD. From then on, this rat model has been extensively used to this end despite some important limitations and technical difficulties such as high animal mortality, variability in the degree of renal function reduction, need of surgery, nonreversibility of renal lesion, and postsurgical period of acute renal failure.

In 1982, Yokowaza (23) proposed a new model of CKD based on the intake of adenine. Oral administration of adenine is immediately metabolized to 2,8-dihydroxyadenine, which precipitates and forms crystals in the microvilli and apical epithelial region of the proximal tubule (12), causing degenerative changes in renal tubules and the interstitium. Adult animals with adenine-induced CKD have been shown to develop hyperphosphatemia, secondary hyperparathyroidism, bone disease, and vascular calcification (11). However, to the best of our knowledge, no studies aimed at the study of growth failure in young uremic rats are available. Therefore, in the present study, we report a protocol of CKD induced by adenine in young rats, describing the characteristics of the growth failure as well as the underlying alterations in the growth plate and bone.
MATERIALS AND METHODS

Animals and experimental protocol. Female Sprague-Dawley rats aged 21 ± 2 days (Charles River Laboratories, L’Arbresle, France) were housed in individual cages at constant room temperature (21–22°C) with a 12:12-h light-dark cycle. After 3 days of adaption, animals were classified into the following three groups of similar weight (65 ± 5 g): normal diet ad libitum (control group), 0.5% adrene diet ad libitum (AD group), and normal diet pair fed with the AD group (PF group). Chow was purchased from Smiff Spezialitäten (reference no. V1534, Soest, Germany). The diet contained 1% calcium, 0.7% phosphorus, 19% protein, and 1 IU/g vitamin D. All animals had free access to tap water. On day 16 of the protocol, animals received 30 mg/kg ip calcein (Sigma, St. Louis, MO). 5-Bromo-2′-deoxy-uridine (BrdU; Sigma-Aldrich, Madrid, Spain) at intraperitoneal doses of 100 mg/kg were injected 17, 9, and 1 h before euthanization by exsanguination under anesthesia on day 21. The present study complied with current legislation on animal experiments in the European Union and was approved by the Ethical Committee on Animal Research of our institution.

Samples taken at euthanization. Serum samples were collected at −20°C for subsequent biochemical analysis. Abdominal aortas were removed to evaluate calcification. Femurs were removed, and the proximal ends were fixed and embedded in methyl-methacrylate, as previously described (15). Left femurs were used for measurements of calcine labeling, BrdU immunohistochemistry, and microscanning analysis. Right femurs were used for histomorphometry. Right kidneys were removed and used for histological analysis.

Biochemical analysis. In serum samples, creatinine, serum urea nitrogen (SUN), and calcium and phosphate concentrations were measured using an autoanalyzer (Kodak Ektachem, Eastman Kodak, Rochester, NY). Serum intact parathormone (PTH) levels were determined by ELISA (Rat BioActive Intact ELISA kit, Immunotopics, San Clemente, CA). Serum FGF-23 levels were measured in Dr. Manuel Clemente, CA). Serum FGF-23 levels were measured in Dr. Manuel Clemente’s research laboratory (Córdoba, Spain) by ELISA (Kainos Laboratories, Tokyo, Japan).

Growth and nutrition. Food intake and body weight were measured daily using an electronic balance (Ohaus GT 2100, Florham Park, NJ). Nose to tail tip length was measured under anesthesia on days 0 and 21. Food efficiency was calculated as the ratio between weight gained and food consumed (in g/g) by each animal between days 0 and 21 of the protocol. The longitudinal growth rate was measured in 10-μm-thick frontal sections of the proximal end of femurs obtained using a rotary microtome (HM355S, Microm, Barcelona, Spain) fitted with tungsten carbide blades. Sections were cut for histological analysis with three paraffin. Sections were cut at 5 μm for histological analysis with three stains: hematoxylin-eosin, PAS, and Masson trichrome. The two first stains were used to evaluate cell morphology and architecture of the glomeruli, tubules, interstitium, and arteries. Masson trichrome staining was used to assess fibrosis. Histological renal damage was descriptively assessed by a single observer.

Histology and histomorphometry. Frontal sections (5 μm thick) of proximal femurs fixed in parafomaldehyde (PFA) were stained by the following methods: alcian blue-safranine for morphometric analysis, von Kossa staining for mineralization and osteoid analysis (osteoid volume/bone volume, in %), tartrate-resistant acid phosphatase for osteoclast identification, periodic acid-Schiff (PAS) reaction for glycoprotein deposits, alkaline phosphatase stain for chondrocyte maturation, and picrosiris red-alcan blue-hematoxylin for analysis of the bone and cartilage extracellular matrix. Heights of growth cartilage and proliferative and hypertrophic zones were identified following morphological criteria and measured at regular intervals using an image-analysis system described elsewhere (15). The height of the three most distal hypertrophic chondrocytes was measured in alternate columns using the same procedure.

RESULTS

Renal failure. As shown in Table 1, the AD group had SUN and serum creatinine concentrations higher than those of control and PF groups. Renal failure induced by AD treatment was associated with secondary hyperparathyroidism (serum PTH: 480 ± 31 vs. 84 ± 17 and 67 ± 22 pg/ml in control and PF rats, P < 0.05), elevated serum phosphate (10.7 ± 0.7 vs.
were fewer PAS-positive chondrocytes in AD rats (1.0 ± 0.0 vs. 0.2 ± 0.0 in control, <0.2 ± 0.0 in PF group). The transition from the proliferative to hypertrophic zone was abrupt (Fig. 1B). These alterations resulted in macroscopic changes denoting differences between control and PF groups from the AD group, as shown in Fig. 2B.

Growth and renal function in the three groups of rats.

Table 1. Growth and renal function in the three groups of rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Group</th>
<th>AD Group</th>
<th>PF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum urea nitrogen, mg/dl</td>
<td>14 ± 1</td>
<td>70 ± 6*</td>
<td>16 ± 1†</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>&lt;0.2 ± 0.0</td>
<td>0.6 ± 0.1*</td>
<td>&lt;0.2 ± 0.0</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>112.6 ± 4.7</td>
<td>63.3 ± 4.8*</td>
<td>60 ± 3.8*</td>
</tr>
<tr>
<td>Length gain, cm</td>
<td>11.1 ± 0.3</td>
<td>7.2 ± 0.2*</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>Food intake, g</td>
<td>406.5 ± 10.5</td>
<td>256.6 ± 9.0*</td>
<td>256.6 ± 9.0*</td>
</tr>
<tr>
<td>Water intake, ml/100 g</td>
<td>22 ± 1</td>
<td>47 ± 2*</td>
<td>23 ± 1†</td>
</tr>
<tr>
<td>Water body wt</td>
<td>0.26 ± 0.01</td>
<td>0.23 ± 0.01*</td>
<td>0.22 ± 0.01*</td>
</tr>
<tr>
<td>Food efficiency, g/g</td>
<td>293 ± 16</td>
<td>141 ± 13*</td>
<td>251 ± 10*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Rats in the control group received normal diet ad libitum, rats in the AD group received diet containing 0.5% adenine, and rats in the PF group were control rats that received normal diet pair fed with AD rats. *Significantly different from control rats; †significantly different from AD rats.

8.6 ± 0.2 and 8.0 ± 0.4 mg/dl in control and PF rats, P < 0.05), and normocalcaemia (10.9 ± 0.3 vs. 10.8 ± 0.3 and 11.4 ± 0.5 mg/dl in control and PF rats). Serum FGF-23 levels in the PF group (170 ± 35 pg/ml) were lower (P < 0.05) than those of control (317 ± 30 pg/ml) and AD (361 ± 60 pg/ml) rats.

Growth and nutrition. As shown in Table 1, cumulative food intake of AD and PF rats was ~56% of that of control animals. Water intake corrected by body weight was higher in the AD group. AD animals were growth retarded, as shown by lower body length gain and osseous front advance than control and PF animals (Fig. 1A). Food efficiency of AD and PF rats was similarly decreased compared with that of control rats.

Growth plate. Growth plate height of the AD group was smaller than that of the control group but greater than that of the PF group. The hypertrophic zone, expressed as proportion of the entire growth plate, was similar in the three groups (Fig. 1B). In addition, the proliferative layer was higher in control rats. The height of the terminal chondrocytes was lower in AD animals than in control and PF animals (Table 2).

Even in a context of evident morphological variability, some histological features were consistently found in AD animals. Growth cartilage height was irregular, and the architecture was clearly disorganized, with loss of the normal arrangement of cells in parallel columns in large areas. Groups of flattened hypertrophic chondrocytes, with their long axis oriented perpendicular to the longitudinal axis of the bone, were found near the metaphyseal end of cartilage. The transition from the proliferative to hypertrophic zone was abrupt (Fig. 1B). There were fewer PAS-positive chondrocytes in AD rats (1.0 ± 0.2 chondrocytes/100 μm²) than in PF rats (2.4 ± 0.3 chondrocytes/100 μm²) and control rats (1.6 ± 0.1 chondrocytes/100 μm²), but the pattern of distribution throughout the hypertrophic zone with attenuation of the signal in the area adjacent to the cartilage-metaphyseal bone junction remained essentially unchanged (Fig. 1C).

Vascular invasion front and primary spongiosa. Tartrateresistant acid phosphatase-positive cells (chondroclasts/osteoclasts) were found in septa of the primary spongiosa and along the vascular invasion front in the three groups of rats. These longitudinal trabeculae, nuclei of cartilage matrix blue covered by a thin layer of bone matrix red, marked the path of invading capillary sprouts. The longitudinal arrangement of septa and vessels was distorted in AD samples due to the presence of transverse unresorbed septa made of cartilage and/or bone matrix. As opposed to the slender structure of primary trabeculae found in control and PF animals, thin trabeculae were often seen in the primary spongiosa of AD animals (Fig. 2A). Picrosirius red-alcan blue staining highlighted morphological and structural differences in the primary spongiosa of AD rats. Thick longitudinal septa, parallel to the long axis of bone, defined the primary spongiosa in control and PF animals. These longitudinal trabeculae, nuclei of cartilage matrix blue covered by a thin layer of bone matrix red, marked the path of invading capillary sprouts. The longitudinal arrangement of septa and vessels was distorted in AD samples due to the presence of transverse unresorbed septa made of cartilage and/or bone matrix. As opposed to the slender structure of primary trabeculae found in control and PF animals, thin trabeculae were often seen in the primary spongiosa of AD animals (Fig. 2A). Alterations resulted in macroscopic changes denoting differences between control and PF groups from the AD group, as shown in Fig. 2B.

Kidney histology. Kidney weight and length were greater in the AD group (20.0 ± 0.3 g and 1.7 ± 0.1 cm) than in the control (16.4 ± 0.3 g and 0.8 ± 0.0 cm) and PF (16.0 ± 0.3 g and 0.6 ± 0.0 cm) groups. Microscopic examination did not disclose vascular or glomerular injury in any group. AD rats had tubulointerstitial lesions consisting in inflammatory interstitial infiltrate and acid uric crystals deposits causing tubular lumen dilation and granulomatous reactions. Other findings, including cellular atrophy at proximal and distal renal tubules, were also found in AD animals.

Vascular calcification. No abdominal aorta calcifications were detected by micro-CT analysis or von Kossa staining in any group.

DISCUSSION

Our study describes, for the first time, an experimental model of CKD caused by intake of adenine in young rats and puts special emphasis on the analysis of growth retardation and growth plate and bone abnormalities.

The experimental protocol resulted in moderate renal failure, as reflected by SUN and serum creatinine concentrations about five and four times higher in the AD group than in control and PF groups, respectively. In a preliminary study, SUN and serum creatinine concentrations measured in a blood sample taken in the AD group on day 11 of the protocol were 49.0 ± 3.2 and 0.3 ± 0.0 mg/dl, respectively, indicating that CKD of this animal model follows a progressive course. The protocol was well tolerated by the young rats, and none of the rats died before the scheduled euthanization. The degree of renal function reduction was quite homogeneous, as shown by the small variability of SUN and serum creatinine values in AD rats. Interestingly, CKD was associated with secondary hyperparathyroidism despite the normal phosphorus content of the diet. These findings account for an advantage of this model over the
classically used 5/6th nephrectomy protocol, which entails significant mortality, variable intensity of the achieved renal failure, and difficulty in inducing hyperparathyroidism unless rats are given a high-phosphorus diet or the period of CKD extends for a long period of time. Circulating levels of FGF-23 were higher in AD rats than in PF rats, indicating that, for the same amount of phosphorus intake, CKD increases FGF-23.

In agreement with the tubulointerstitial lesions found in the kidneys of AD rats, this model of CKD was polyuric, and, accordingly, AD rats were secondarily polydipsic. Renal histological findings in the AD group corresponded to those already described in adult rats (9, 24).

This model of adenine-induced CKD caused growth retardation. Although other factors potentially associated with growth retardation, such as electrolyte disturbances, metabolic acidosis, or inflammatory markers, were not measured in this study, the animals behaved normally and looked apparently healthy. Moreover, in rats with severe renal failure induced by adenine, bone abnormalities could not be explained by the blood levels of pH and CO2 (5). In addition, a previous study (2) from our group demonstrated that the alterations induced by metabolic acidosis in the rat growth plate are markedly different from those caused by uremia and described in the present study.

It is of note that AD rats gained less length than control and PF rats, and their growth velocity, as assessed by the osseous front advance, was also decreased compared with the two normal renal function groups. This pattern of growth impair-

Fig. 1. Animals were classified into the following three groups: normal diet ad libitum [control (C) group], 0.5% adenine diet ad libitum (AD group), and normal diet pair fed with the AD group (PF group). A: osseous front advance in the proximal femurs of the three groups of animals. The distance between white lines, which indicates the metaphyseal end of growth cartilage and the proximal end of the calcine label, represents the osseous front advance over a 3-day interval. Scale bars: 500 μm. B: heights of growth cartilage and its hypertrophic stratum in the proximal femurs of the three groups of animals. Scale bars: 100 μm. C: periodic acid-Schiff (PAS) reaction in the proximal femurs of the three groups of animals. Cytoplasmic glycogen deposits are shown (arrow). Scale bars: 100 μm. D: proliferative activity in growth cartilage of the proximal femurs of the three groups of animals, as assessed by bromodeoxyuridine labeling. Scale bars: 100 μm. E: osteoclastic activity in the primary spongiosa of the proximal femurs of the three groups of animals, as assessed by the tartrate-resistant acid phosphatase technique (arrow). Scale bars: 100 μm. C, control
Table 2. Histomorphometry of the proximal femur growth plate in the three groups of rats

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>AD Group</th>
<th>PF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth plate height, μm</td>
<td>438.0 ± 20.1</td>
<td>366.0 ± 19.6*</td>
<td>310.0 ± 19.5*†</td>
</tr>
<tr>
<td>Proliferative zone height, μm</td>
<td>156.6 ± 10.3</td>
<td>113.8 ± 6.6*</td>
<td>121.8 ± 8.5*</td>
</tr>
<tr>
<td>Hypertrophic zone height, μm</td>
<td>220.9 ± 16.4</td>
<td>191.4 ± 19</td>
<td>149.5 ± 7.7†</td>
</tr>
<tr>
<td>Hypertrophic zone height, %</td>
<td>50 ± 1.5</td>
<td>52 ± 2.8</td>
<td>48 ± 1.6</td>
</tr>
<tr>
<td>Height of most distal chondrocytes, μm</td>
<td>26.2 ± 1.9</td>
<td>21.7 ± 2.3*</td>
<td>23.9 ± 1.3†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *Significantly different from control rats; †significantly different from AD rats.

Table 3. Bone structure in the three groups of rats

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>AD Group</th>
<th>PF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume/tissue volume, %</td>
<td>25.1 ± 1.3</td>
<td>27.1 ± 1.5</td>
<td>27.7 ± 0.8</td>
</tr>
<tr>
<td>Trabecular number, 1/mm</td>
<td>6.3 ± 0.3</td>
<td>7.1 ± 0.4</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>Trabecular separation, mm</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Trabecular thickness, mm</td>
<td>0.040 ± 0.001</td>
<td>0.038 ± 0.001*</td>
<td>0.041 ± 0.001†</td>
</tr>
<tr>
<td>Bone surface-to-volume ratio, 1/mm</td>
<td>90 ± 1.3</td>
<td>94.8 ± 1.7*</td>
<td>85.7 ± 0.7†</td>
</tr>
<tr>
<td>Osteoid volume/bone volume, %</td>
<td>4.5 ± 1.3</td>
<td>15.3 ± 2.1*</td>
<td>5.5 ± 1.5†</td>
</tr>
<tr>
<td>Total area inside the periosteum, mm²</td>
<td>32.4 ± 2.2</td>
<td>28.6 ± 1.7</td>
<td>31.0 ± 1.9</td>
</tr>
<tr>
<td>Cortical area, mm²</td>
<td>3.7 ± 0.3</td>
<td>3.2 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Cortical area/total area, %</td>
<td>10.8 ± 0.7</td>
<td>11.3 ± 0.5</td>
<td>11.5 ± 0.6</td>
</tr>
<tr>
<td>Cortical thickness, mm</td>
<td>0.51 ± 0.07</td>
<td>0.40 ± 0.01*</td>
<td>0.44 ± 0.01†</td>
</tr>
<tr>
<td>Cortical porosity, %</td>
<td>1.1 ± 0.2</td>
<td>3.53 ± 0.3*</td>
<td>1.31 ± 0.2†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *Significantly different from control rats; †significantly different from AD rats.
primary spongiosa. Normal endochondral growth requires the coupled replacement of cartilage by the apposition of new bone. The abnormal disposition of osseous trabeculae reflects the disarrangement of the new bone formation. In addition, the increased osteoclast activity in association with the lower trabecular thickness in primary spongiosa and the increased cortical porosity point to an effect of secondary hyperparathyroidism on bone structure and dynamics (22) in this model of CKD despite the fact that the animal protocol lasted only a few weeks. As mentioned above and in agreement with the explanation given to the histological alterations found in bone, AD rats had high serum PTH concentration, elevated serum phosphate concentration, and normal calcemia. Thus, the defective mineralization cannot be attributed to low circulating levels of calcium and phosphate. Unfortunately, vitamin D metabolites were not measured in our study.

No aorta calcifications were found in any of the AD rats. In adult rats treated with adenine, the presence of vascular calcifications varies as a function of how long adenine was given, the adenine content of the diet, the amount of protein in the diet, and the methodology used to detect calcifications (6, 16, 17, 21). Usually, calcifications are found in protocols in which adenine was administered for longer than 5 wk. Data in young rats were not available. The lack of calcifications found in our study is interesting because it probably agrees with the clinical situation in children with moderate degrees of renal failure and suggests that this animal model can be used to analyze growth retardation and bone impairment in prepuberal individuals with CKD in the absence of prolonged inflammation and noticeable systemic involvement.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


