Severe hyperparathyroidism with bone abnormalities and metastatic calcification in rats with adenine-induced uraemia

Keiichi Tamagaki, Qunsheng Yuan, Hiroyuki Ohkawa, Ikuo Imazeki, Yoshiyuki Moriguchi, Nobuo Imai, Susumu Sasaki, Kazuo Takeda and Masafumi Fukagawa

Abstract

Background. Marked parathyroid hyperplasia with bone diseases and vascular calcification are unsolved issues in dialysis patients. In this study, we made azotemic model rats by adenine feeding and analyzed the development and progression of the abnormalities.

Methods. Renal failure was induced in 8-week-old male Wistar rats by feeding 0.75% adenine-containing diet for 6 weeks. Serum parameters, parathyroid hyperplasia, bone changes and metastatic calcification were examined at 2, 4 and 6 weeks.

Results. Progressive increase of serum creatinine and inorganic phosphate, and decreased levels of serum calcium and 1,25(OH)\(_2\)D\(_3\) were confirmed. Markedly enlarged parathyroid glands and extremely high PTH levels were observed in all adenine-fed rats compared with the control (PTH: 199.3±58.0 vs 10.5±3.0 pmol/l, \(P<0.01\), respectively, at 6 weeks).

In cortical bone of the femur, the morphometric parameters showed increased bone resorption with increased fibrosis, whereas in the trabecular bone, bone resorption decreased and bone volume increased with a larger amount of osteoid compared with the control. Metastatic calcification in aorta, coronary artery and other soft tissues were also found in adenine-fed rats.

Conclusions. Uraemic rats made by adenine diet developed severe abnormalities of calcium metabolism in a relatively short period and therefore they may serve as a useful model for the analysis of parathyroid hyperplasia and vascular calcification in chronic renal failure.

Keywords: adenine; calcification; chronic renal failure; hyperparathyroidism; renal osteodystrophy

Introduction

A number of experimental models of renal failure with secondary hyperparathyroidism have been developed by nephrectomy and diet [1,2], and also by chemicals [3]. In the models such as 5/6 nephrectomy and chemical-induced renal involvement, it took 4–6 months for the secondary hyperparathyroidism to be formed. A single injection of nephritogenoside provoked a slowly progressive renal failure with almost the entire range of complications, which are thought to be compatible with the clinical findings. However, it took more than 8 months until these abnormalities became evident [4,5].

Recently, the rats with adenine-induced renal failure were used as a disease-model for evaluation of drug efficacy [6]; however, a detailed analysis of their pathophysiology was insufficient. Orally administered adenine is immediately metabolized to 2,8-dihydroxyadenine, which precipitated and formed crystals in the microvilli and the apical region of the proximal tubular epithelia only 2 days after the adenine administration [7]. Increased crystals induced degenerative changes in the cells of these tissues and caused renal dysfunction with increased levels of serum creatinine and inorganic phosphate, and decreased levels of serum calcium [8]. The deteriorated renal biochemical parameters had recovered partially to the normal level, if the 0.75% adenine diet was ended within two weeks. However, if the adenine feeding period was continued for four weeks or more, irreversible renal failure was induced and deteriorating renal biochemical parameters did not recover [9]. Therefore, rats to which...
the adenine administration had been continued for more than 4 weeks are considered to be a model of rapidly progressive type of chronic renal failure.

In this study, we examined the development in an adenine-fed model of abnormal calcium metabolism, including secondary hyperparathyroidism, bone diseases and metastatic calcification, which have been only partially described in previous studies. Our data suggest that adenine model is one of the most rapid, simple and reliable methods of inducing severe hyperparathyroidism and calcification associated with chronic renal failure, which are more compatible with the clinical findings in dialysis patients.

Materials and methods

Model rats made by adenine diet

Eight-week-old Wistar male rats (Charles River Japan Inc., Yokohama, Japan) were pair-fed standard chow, either CE-2 containing 1.2% calcium and 0.6% phosphorus (Nihon Clea Inc., Tokyo, Japan) for the control group \((n = 15)\), or CE-2 containing 0.75% adenine (Sigma, St Louis, MO, USA) for the adenine-fed rats \((n = 30)\). Five control and ten adenine-fed rats each were sacrificed at 2, 4 and 6 weeks.

Biochemical parameters

Blood samples were taken for biochemical analysis by puncture of the abdominal aorta under diethyl ether anesthesia at sacrifice. The serum levels of urea nitrogen, creatinine, calcium and inorganic phosphate were measured by an autoanalyzer (Hitachi 7070, Hitachi Co. Ltd, Tokyo, Japan). The serum PTH levels were measured by rat intact PTH enzyme-linked immunosorbent assay (ELISA) (Immutopics, Inc., San Clemente, CA, USA). The serum levels of 1,25(OH)\(_2\)D\(_3\) were measured by radioresort binding assay using vitamin D receptors derived from calf thymocytes (Yamasa, Tokyo, Japan).

Histopathological examinations

For histopathological examination, all resected tissues were fixed with 10% buffered formalin and 5 µm-thick sections were prepared for histomorphometry. Histomorphometry was performed using image analyzing computer (Cosmozone 1SA, Nikon, Tokyo, Japan). The following histomorphometric parameters were measured: osteoclast surface/bone surface, Oc.S/BS (%); porosity area/cortical bone area, Po.Ar/Ct.Ar (%); osteoid volume (intra cortical bone)/cortical bone area, OV(In)/Ct.Ar (%); mineralizing surface/osteoid surface, MS/BS (%); bone formation rate/cortical bone area, BFR/Ct.Ar (%/year); fibrosis tissue volume/cortical bone area, Fb.V/Ct.Ar (%); trabecular thickness, Tb.Th (µm); osteoid volume/bone volume, OV/BV (%); bone formation rate/bone volume, BFR/BV (%/year); and fibrosis tissue volume/tissue volume, Fb.V/TV (%). The histomorphometric parameters were defined and named according to the nomenclature proposed by the ASBMR [10].

Statistical analysis

The results are presented as the mean ± SE. Statistical analysis was performed with statistical analysis software (SAS v. 5.00, SAS institute, Japan). An F-test was used for the comparison of the variances between the two groups. If the variances were equal, the statistical significance was determined by Student’s unpaired two-tailed t-test. If the variances were unequal, the statistical significance was determined by Welch’s t-test. P values less than 0.05 were considered significant.

Results

Renal function and biochemical parameters

Serum urea nitrogen and creatinine concentrations at 2, 4 and 6 weeks increased significantly (\(P < 0.001\)) in all adenine-fed rats compared with those of control rats (Table 1). Kidneys displayed tubular dilatation, epithelial proliferation with giant cells and spaces of crystal of adenine (Figure 1A and B), calcification of the
basement membrane of tubules (Figure 1C), and inflammatory cell infiltration in the interstitium (Figure 1A), although only minimal changes were observed in the glomeruli (Figure 1C and D). These results are totally comparable to those of previous reports [8].

Serum calcium levels decreased and phosphate levels increased significantly at 4 and 6 weeks in all adenine-fed rats (Table 1). Serum levels of 1,25(OH)2D3 decreased significantly at 2, 4 and 6 weeks.

**Parathyroid function and hyperplasia**

Serum PTH levels increased progressively in adenine-fed rats and reached extremely high levels compared with those of control rats at 4 weeks (162.1±41.9 vs 7.0±2.5 pmol/l, \(P < 0.01\), respectively) and at 6 weeks (199.3±58.0 vs 10.5±3.0 pmol/l, \(P < 0.01\), respectively), and also were higher than those seen in other models of chronic renal failure within 6 weeks (Table 1). Parathyroid glands from the adenine-fed rats were markedly enlarged and their size was remarkably increased compared with those of control rats at 4 weeks (0.51±0.05 vs 0.24±0.04 mm2, \(P < 0.01\), respectively) and at 6 weeks (0.72±0.06 vs 0.24±0.04 mm2, \(P < 0.01\), respectively).

### Table 1. Serum biochemical parameters in adenine-induced renal failure rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Adenine-administered period</th>
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<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td>Urea nitrogen (mmol/l)</td>
<td></td>
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<tr>
<td>Control</td>
<td>8.4±0.4</td>
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<tr>
<td>Adenine</td>
<td>40.14±1.1c</td>
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<tr>
<td>Creatinine (μmol/l)</td>
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<td>Control</td>
<td>43.3±1.7</td>
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<tr>
<td>Adenine</td>
<td>152.4±6.7c</td>
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<tr>
<td>Calcium (mmol/l)</td>
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<tr>
<td>Control</td>
<td>2.63±0.03</td>
</tr>
<tr>
<td>Adenine</td>
<td>2.49±0.03a</td>
</tr>
<tr>
<td>Phosphorus (mmol/l)</td>
<td></td>
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<tr>
<td>Control</td>
<td>2.89±0.03</td>
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<tr>
<td>Adenine</td>
<td>3.19±0.09a</td>
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<tr>
<td>Intact PTH (pmol/l)</td>
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</tr>
<tr>
<td>Control</td>
<td>0.2±0.2</td>
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<tr>
<td>Adenine</td>
<td>29.7±5.5c</td>
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<tr>
<td>1,25(OH)2D3 (pmol/l)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>272.4±23.9</td>
</tr>
<tr>
<td>Adenine</td>
<td>85.5±9.1c</td>
</tr>
</tbody>
</table>

Data are presented as mean±SE. 

\(a P < 0.05; \ b P < 0.01; \ c P < 0.001\): significance of difference vs corresponding control group (control group: \(n = 5\); adenine-fed group: \(n = 10\)).

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Fig. 1. Histopathological findings of kidney in adenine-fed rats. (A) Crystals of adenine in proximal tubules (arrows), tubular dilatation, epithelial hyperplasia, calcification of the basement membrane of tubules, and inflammatory cell infiltration and giant cells (arrowhead) in the interstitium of the kidney of adenine-fed rat at 4 weeks, HE, 80×. (B) Giant cells with crystals of adenine in the interstitium of the kidney of adenine-fed rat at 4 weeks, HE, 80×. (C) Calcification of the basement membrane of tubules in the kidney of adenine-fed rat at 6 weeks, HE, 160×. (D) Minimal changes were observed in the glomeruli in the kidney of adenine-fed rat 4 weeks, HE, 320×.
These parathyroid glands consisted of increased numbers of hypertrophic chief cells (clear chief cells) with the normal lobular configuration obliterated and compressed by adjacent thyroid follicles (Figure 2A and B). Increasing numbers of mitosis (Figure 2C) and PCNA positive cells (Figure 2D) were also confirmed in the parathyroid glands in all adenine-fed rats at 4 and 6 weeks compared with those of control rats (Figure 3).

**Metastatic Calcification**

Medial calcification was found in the aorta and coronary arteries in adenine-fed rats at the 4 and 6 weeks (Figure 4A–C). Calcification of the aorta was localized in the media of vessel wall, simulating the typical calcification pattern of arteries in chronic dialysis patients (Figure 4B). Also, in glandular stomach of these rats, metastatic calcification was observed in lamina propria mucosae, mainly in glandular body and muscular layer (Figure 4D).

**Table 2.** The size of the parathyroid gland in adenine-induced renal failure rats

<table>
<thead>
<tr>
<th>Adenine-administered period</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum cross-sectional area (mm²)</td>
<td>Control</td>
<td>0.24±0.02</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td></td>
<td>Adenine</td>
<td>0.26±0.03</td>
<td>0.51±0.05a</td>
</tr>
</tbody>
</table>

Data are presented as mean±SE.

*a*P < 0.01; *b*P < 0.001: significance of difference vs corresponding control group (control group: n = 5; adenine-fed group: n = 10).

**Bone histology and histomorphometry**

Very slight bone changes with resorption cavities in cortical bone were observed in adenine-fed rats at 2 weeks. Such changes developed markedly in all adenine-fed rats at 4 and 6 weeks in a time-dependent manner as shown by contact microradiography (Figure 5A and B) and by HE staining (Figure 5C and D). Bone changes were characterized by an increase of osteoid and resorption cavities with osteoclasts, osteoblasts and fibrosis (Figure 5C and D) in the cortical bone. Increase of osteoid was observed in the trabecular bone at the metaphysis of the distal femur (Figure 5E and F).

By bone histomorphometry of the cortical bone of diaphysis in all adenine-fed rats compared with those of control rats, increases in the bone resorption parameter (Oc.S/BS: 3.80±0.59 vs 0.54±0.33%, *P* < 0.01, respectively, at 6 weeks) (Figure 6A) and porosity ratio (Po.Ar/Ct.Ar: 12.05±1.95 vs 0.45±0.10%, *P* < 0.001, respectively, at 6 weeks) (Figure 6B) were observed. Conversely, the bone...
formation parameter of osteoid volume significantly increased (OV(In)/Ct.Ar: 5.07 ± 1.38 vs 0.10 ± 0.01%, \( P < 0.01 \), respectively, at 6 weeks) (Figure 6C) accompanied by the decrease of mineralization (MS/BS: 4.9 ± 1.4 vs 32.9 ± 2.8%, \( P < 0.001 \), respectively, at 6 weeks) (Figure 6D) and bone formation (BFR/Ct.Ar: 49.4 ± 12.8 vs 176.0 ± 10.0%/year, \( P < 0.001 \), respectively, at 6 weeks) (Figure 6E) compared with those of control rats.

In contrast, osteopetrotic changes were observed in the decalcified sections with HE staining of the trabecular bones at the metaphysis of the adenine-diet rats (Figure 5E and F). Accordingly, the bone histomorphometry of trabecular bone showed the marked decrease of resorption parameter (Oc.S/BS: 4.72 ± 1.78%, \( P < 0.05 \), respectively, at 6 weeks) (Figure 6G) and increased bone volume (Tb.Th: 114.9 ± 17.2 vs 60.2 ± 3.4 μm, \( P < 0.05 \), respectively, at 6 weeks) (Figure 6H) compared with those of control rats. Also, the bone histomorphometry of trabecular bone of adenine-fed rats showed increased osteoid (OV/BV: 47.8 ± 9.1 vs 2.9 ± 0.3%, \( P < 0.001 \), respectively, at 6 weeks) (Figure 6I), and inhibited mineralization (MS/BS: 8.6 ± 2.9 vs 35.4 ± 0.6%, \( P < 0.001 \), respectively, at 6 weeks) (Figure 6J) and bone formation (BFR/BV, 89.8 ± 80.2 vs 824.0 ± 36.7%/year, respectively, at 6 weeks) (Figure 6K) compared with those of control rats.

Increase of fibrosis compared to the control was also observed both in the cortical bone (Fb.V/Ct.Ar: 0.61 ± 0.14 vs 0.00 ± 0.00%, respectively, at 6 weeks) (Figure 6F) and in the trabecular bone (Fb.V/TV: 0.77 ± 0.47 vs 0.00 ± 0.00%, respectively, at 6 weeks) (Figure 6L).

Fig. 3. Number of PCNA positive cells in parathyroid glands in control and adenine-fed rats at 4 and 6 weeks. Number of PCNA positive cells counted on largest cross-sectional surface of parathyroid gland at 200×. \( *P < 0.05 \), \( **P < 0.01 \): significance of difference vs corresponding control group (control group: \( n = 5 \); adenine-fed group: \( n = 10 \)).

Fig. 4. Metastatic calcification in adenine-fed rats at 4 weeks. (A) von Kossa stains of thoracic aorta of control rat, 80×. (B) von Kossa stains of thoracic aorta of adenine-fed rat at 4 weeks, 80×. (C) Coronary artery, 160×. (D) Lamina propria mucosae and muscular layer of glandular stomach, 32×.
Discussion

In this study, we examined the progression of adenine-induced renal failure and subsequent pathology of secondary hyperparathyroidism, bone diseases and metastatic calcification. In particular, we discuss the bone morphometric evaluation in greater detail than previously reported.

It is remarkable that the time necessary to form an advanced secondary hyperparathyroidism and marked deterioration of the renal osteodystrophy was only four weeks in the rats of the adenine administration compared with other models of renal failure. In a study using 5/6 nephrectomized rats, serum intact PTH levels rose almost 33 pmol/l (300 pg/ml) four weeks after surgery, and if the nephrectomized rats were fed a high phosphorus diet for 4 weeks, PTH levels reached over 110 pmol/l (1000 pg/ml) [11]. On the contrary, the 4-week adenine-fed rats in our study provided extremely high serum PTH levels, nearly 165 pmol/l (1500 pg/ml), and increasing numbers of mitosis and PCNA positive cells in parathyroid glands without a modified diet. The extent of hyperplasia in the parathyroid glands from 6-week adenine-fed rats was two times or more than that of normal rats, and almost equal to that of nephrectomized rats on high-phosphorus diet for 12–14 weeks [12]. In adenine-fed rats, it was expected that hypocalcaemia, hyperphosphataemia and low levels of 1,25(OH)₂D₃

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Fig. 5. Histopathological findings of bone in adenine-fed rats. Resorption cavities in the cortical bone increased time dependently in adenine-fed rat at (A) 4 weeks and (B) 6 weeks, femur, contact microradiography (CMR), 16×. Resorption cavities with increased osteoclasts, osteoblasts and marrow fibrosis (arrowhead) in adenine-fed rat, at (C) 4 weeks and (D) 6 weeks, femur, HE, 160×. Increase of osteoid and bone volume was observed in the trabecular at metaphysis of distal femur in adenine-fed rat at (E) 4 weeks and (F) 6 weeks, HE, 32×.
stimulated PTH secretion and parathyroid cell proliferation.

The bone lesion was provided in subtotal nephrectomized rats by a high-phosphorus diet for 12–14 weeks [12]. In adriamycin-induced chronic renal failure rats, it took over 14 weeks to form low-turnover bone lesion [3]. Also in the glycopeptide-induced nephritis rats, it was more than 8 months before severe bone disease became evident [4,5]. In contrast, adenine-fed rats presented severe bone lesion within 4 weeks. And these bone abnormalities persisted even after conversion from an adenine diet to a normal diet after 4 weeks [9]. Our model provides the advantage of obtaining severe irreversible bone disease in a short amount of time.

Furthermore, there are no concurrent studies available that compare changes seen in cortical and trabecular bone in kidney failure models. Here, our bone morphometric findings provide evidence of the heterogeneity of the cortical and trabecular bones, which had become obvious in slightly more than 4 weeks. Increase of fibrosis compared to the control was also observed both in the cortical and trabecular bone.

In the cortex, the morphometric parameters showed increased bone resorption with increased fibrosis, which might result from high level of serum PTH. In contrast, the trabecular bone presented decreased bone resorption and increased bone volume with a large amount of osteoid and increased fibrosis. Due to

![Fig. 6. Bone histomorphometry of cortical and trabecular bone from adenine-fed rats.](image-url)

*P < 0.05, **P < 0.01, ***P < 0.001: significance of difference vs corresponding control group (control group: n = 5; adenine-fed group: n = 10). Data are presented as mean ± SE. The explanation of the abbreviations in Figure 6 was described in the Materials and methods section.
decreased serum levels of 1,25(OH)₂D₃ in our model, osteodystrophy in adenine-fed rats characterized by an increase of osteoid with defective bone formation was thought to result from diminished 1α-hydroxylase in the kidneys and a reduction of 1,25(OH)₂D₃ [13,14], and also result from the change in an internal environment such as latent hypocalcaemia and the direct influence of the uraemic toxin.

Heterogeneity of the cortical and trabecular bones was attributed to the different responses to PTH stimuli between cortical and trabecular bone on bone resorption, but the precise mechanisms need to be clarified. The contrasting responsiveness of PTH on cortical and trabecular bone was also demonstrated in dialysis patients [15,16]. Dialysis patients frequently receive vitamin D treatment, but the adenine-fed rats of this study received no vitamin D medication, and so the internal environment of adenine-fed rats differed from those of dialysis patients in the presence or absence of vitamin D treatment. Therefore, bone lesion in adenine-fed rats might be the model of bone disease in those renal failure patients who have had little or no influence from vitamin D treatment.

Vascular calcification is one of the most important risk factors for mortality in dialysis patients. Calcification of the media, one of the characteristics of vascular calcification in dialysis patients, was confirmed in our model. Metastatic calcification in soft tissue was induced only by adenine diet within 4 weeks, in sharp contrast to other models that required longer time and either load of calcium, phosphate and vitamin D, or a genetically engineered background [17]. In previous studies, no significant ectopic calcification in either aorta or kidney was found in 5/6 nephrectomized rats at 3 months after surgery [18], and if ectopic calcification was required, feeding of high phosphorus diet for 6 months was necessary [2].

High Ca × P products as well as very high PTH levels may have contributed to the development of such rapid vascular calcification in adenine-fed rats; however, precise mechanisms remain to be elucidated. It also remains to be clarified whether adenine has a direct effect on the formation of ectopic calcification. Adenine crystals were found in the proximal tubules, but were not found in any organs other than the kidneys. Characteristically, inflammatory cell infiltration around adenine crystals was also found in the kidneys; however, neither adenine crystals nor inflammatory cell infiltration was seen in the lesion of the vascular calcification. Adenine phosphoribosyltransferase (APRT) knockout mice are characterized by urinary excretion of adenine and the 2,8-dihydroxyadenine that can produce kidney stones and lead to renal failure [19]. Although the pathologic condition of APRT knockout mice generated by defective adenine metabolism was almost the same as that of adenine-fed rats, APRT knockout mice show no vascular calcification. From the above, it can be surmised that the ectopic calcification on soft tissue observed in adenine-fed rats was not attributed to the direct effect of the adenine nor the deposition of 2,8-dihydroxyadenine.

In summary, we have established and analyzed deranged calcium and bone metabolism in uraemic rats fed on adenine diet. Since these rats develop severe abnormalities in a relatively short period, they may serve as a useful model for the manifestations of parathyroid hyperplasia and vascular calcification in chronic renal failure.

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References

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