Role of Low Protein and Low Phosphorus Diet in the Progression of Chronic Kidney Disease in Uremic Rats

Kenichiro Kusano1,2,* Hiroko Segawa2, Ritsuko Ohnishi2, Naoshi Fukushima1 and Ken-ichi Miyamoto2

1 Pharmaceutical Research Department I, Chugai Pharmaceutical Co., Ltd., Shizuoka 412–8513, Japan
2 Department of Molecular Nutrition, Institute of Health Bioscience, University of Tokushima Graduate School, Tokushima 770–8503, Japan

(Received November 21, 2007)

Summary Restriction of dietary protein is useful for chronic kidney disease (CKD) patients to protect residual renal function. However, the mechanism by which a low protein diet confers a beneficial effect in CKD patients remains unknown. One possibility is that the benefit from a low protein diet is associated with phosphorus restriction. The aim of this study is to compare the effect of protein and phosphorus on the progression of renal insufficiency using irreversible Thy1 rats, which histopathologically resemble IgA nephropathy. Irreversible Thy1 rats were fed six types of isocaloric diets consisting of three levels of protein (16.9, 12.6, and 8.4%) and two levels of phosphorus (0.5 and 0.3%) for 13 wk. Renal function was assessed biochemically and histopathologically. The low phosphorus (0.3%) diets showed protection of residual renal function regardless of dietary protein content in uremic rats. With the normal phosphorus (0.5%) diets, however, only the very low protein (8.4%) diet showed a beneficial effect, indicating that dietary phosphorus is a more important factor that affects the progression of renal insufficiency than dietary protein in this model. Furthermore, the low phosphorus diet also prevented an increase in serum parathyroid hormone, indicating that a low phosphorus diet might have beneficial effects not only for residual renal function but also for renal osteodystrophy, a typical complication of patients with CKD.

Key Words phosphorus, protein, irreversible Thy1 rats, chronic kidney disease

Restriction of dietary protein is useful for the protection of residual renal function in chronic kidney disease (CKD) patients. In 2000, the National Kidney Foundation Dialysis Outcomes Quality Initiatives (K/DOQI) released nutrition guidelines in which they recommend a low protein diet for the care of non-dialyzed patients with CKD (1). According to the guidelines, 0.6–0.75 g/kg/d of protein is recommended for patients with CKD stages 1 to 4. However, the mechanism by which a low protein diet is beneficial for CKD patients remains unknown. Because protein sources in food are usually linked to phosphorus, one possibility is that the beneficial effect depends on phosphorus restriction (2) and, therefore, phosphorus is thought to be one of the factors that influence the progression of CKD (3). However, we encounter difficulties when attempting to investigate the individual roles of protein and phosphorus in the progression of CKD in patients because it is difficult to separate phosphorus from protein in food.

There are many animal models of renal disease. The subtotal (5/6) nephrectomy model is most commonly used to study the mechanisms underlying CKD and to assess pharmacological agents. However, it has some drawbacks such as the necessity of technical skills and large interindividual variability in the severity of induced lesions (4). Recently, the irreversible Thy1 rat, induced by injection of Anti-Thy1 antibody to uninephrectomized rats, has been introduced (5, 6). This model is characterized by mesangial cell proliferation and matrix expansion and is accompanied by persistent proteinuria, hypertension, and a moderate decline in renal function. Histopathological analysis indicates that these models resemble IgA nephropathy. Many pharmacological agents have been assessed using irreversible Thy1 rats, indicating that this is a useful model of progressive renal failure (4).

The present study was designed to clarify whether the beneficial effect of a low protein diet is associated with phosphorus content using irreversible Thy1 rats fed six different isocaloric diets, including three levels of protein and two levels of phosphorus, for 13 wk, and renal function was assessed.

MATERIALS AND METHODS

Animals. Irreversible Thy1 rats were established using male Wistar rats, 7 wk old, purchased from Japan SLC, Inc. (Shizuoka, Japan) according to the method of Cheng with minor modification (5). Briefly, unilateral
nephrectomy and sham operation were performed under ether anesthesia. Uninphrectomized rats were injected intravenously with 1 mg/mL/kg anti-rat CD90 (Thy-1.1) monoclonal antibody, clone MRC OX-7 (Cedarlane Laboratories Ltd., Ontario, Canada) and sham-operated rats were injected with 1 mL/kg of vehicle (phosphate buffered saline). They were housed in cages and allowed unlimited access to normal rodent food (CE-2; phosphorus 1.0%, CLEA Japan, Inc.) and tap water until 2 wk after operation.

Normal Sprague Dawley (SD) rats, 6 wk old, were purchased from Japan SLC, housed in cages, and allowed unlimited access to the experimental diets and tap water for 4 d.

The care of the animals and the present protocols complied with the "General Consideration for Animal Experiments" and was approved by Chugai Pharmaceutical's Ethics Committee for the Treatment of Laboratory Animals.

Experimental design. Two weeks after the operation, the experimental rats were matched with respect to serum creatinine concentration and then assigned randomly to a group (8/group). Each group was fed one of six experimental diets for 13 wk. In order to measure biochemical parameters, serum was collected from the jugular vein under ether anesthesia at 2, 6, 10, and 13 wk from start of the test diets. Concurrently, urine was collected every 24 h from rats housed in individual metabolic cages with free access to water and diet. At 13 wk, animals were anesthetized and the right kidneys were removed from the test animals and subjected to histopathological analysis. In order to confirm the establishment of renal insufficiency models, sham-operated control rats (n=3) were fed normal (0.5%) phosphorus diet with normal (16.9%) protein, and serum creatinine was measured at 2, 6, 10, and 13 wk.

Normal SD rats were individually housed in cages and given either normal, low, or very low protein diets of 16.9, 12.6, and 8.4% protein, respectively. Serum was collected every 24 h from day 0.

Diets. The diets were modifications of the AIN-93G diet with adjusted amounts of protein and phosphorus. To confirm whether the dietary protein content affects

<table>
<thead>
<tr>
<th>Protein</th>
<th>16.9%</th>
<th>12.6%</th>
<th>8.4%</th>
<th>16.9%</th>
<th>12.6%</th>
<th>8.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein Phosphorus</td>
<td>20.0</td>
<td>15.0</td>
<td>10.0</td>
<td>20.0</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.849</td>
<td>1.014</td>
<td>1.178</td>
<td>0.165</td>
<td>0.329</td>
<td></td>
</tr>
<tr>
<td>Cornstarch</td>
<td>52.0996</td>
<td>56.9346</td>
<td>61.7706</td>
<td>52.9486</td>
<td>57.7836</td>
<td>62.6196</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

Fig. 1. Low protein and very low protein diets decreased serum urine nitrogen (A) and serum phosphorus (B). Values are expressed as the means±SE (n=4). Circle, normal protein (16.9%); triangle, low protein (12.6%); square, very low protein (8.4%). In this experiment, the phosphorus content of the diet was not adjusted. *p<0.05, significantly different from normal protein group at each day (Student's t-test).
Low Protein and Phosphorus Diet in Uremic Rats

phosphorus metabolism, we prepared three different diets with normal (16.9%), low (12.6%) and very low (8.4%) levels of protein. To assess the effects of protein and phosphorus on the progression of CKD, we prepared six diets using the same percentages of protein combined with normal (0.5%) and low (0.3%) phosphorus as follows: Group 1 (protein 16.9%/phosphorus 0.5), Group 2 (protein 12.6%/phosphorus 0.5), Group 3 (protein 8.4%/phosphorus 0.5), Group 4 (protein 16.9%/phosphorus 0.3), Group 5 (protein 12.6%/phosphorus 0.3), and Group 6 (protein 8.4%/phosphorus 0.3). These diets were isonitrogenous and contained equal amounts of fat, mineral, and vitamin supplements. The rats were allowed unlimited access to the test diets. Composition of the diets is shown in Table 1.

Biochemical parameters. Serum and urine samples were analyzed in a Hitachi 7170E automatic analyzer (Hitachi Co. Ltd., Tokyo, Japan) for phosphorus, creatinine and urea nitrogen. Urinary albumin was also analyzed in a Hitachi 7170E automatic analyzer using Test-Rat urinary ALB reagent (BL Co. Ltd., Numazu, Japan). Serum parathyroid hormone (PTH) levels were measured using a Rat Intact PTH ELISA Kit (Immutopics, CA, USA).

Kidney histopathology. Kidneys were fixed with 20% neutral buffered formalin, embedded in paraffin, cut in 4 μm sections and stained with periodic acid-Schiff (PAS) and hematoxylin-eosin. These samples were examined histopathologically. Kidney lesions including glomerulosclerosis, degeneration of tubules, interstitial fibrosis, were graded into five stages, namely –, not remarkable; ±, very slight; +, slight; ++, moderate; and ++++, severe. Blind analysis was done on all sections by the same observer.

Statistical analysis. All values are expressed as the mean±SE. Statistical analysis was performed using two-way analysis of variance, Student’s t-test or Wilcoxon’s rank test by the SAS system (SAS Institute, Inc., NC, USA). p<0.05 was considered statistically significant.

RESULTS

Effects of low protein diet on phosphorus metabolism

To analyze the effect of dietary protein on phosphorus metabolism, normal SD rats were housed in cages for 4 d and allowed unlimited access to normal, low, or very low protein diets containing 20, 15, and 10% casein, equal to 16.9, 12.6, and 8.4% protein, respectively. There was no significant difference in food intake among the groups (data not shown). Figure 1 shows the time courses for serum urea nitrogen (SUN) (Fig. 1A) and serum phosphorus (Fig. 1B). The level of SUN, which reflects the amount of dietary protein intake, increased significantly in rats fed 12.6 and 8.4% protein dose-dependently. The serum concentration of phosphorus also decreased significantly in rats fed 12.6 and 8.4% protein.

These results indicate that a low protein diet not
phosphorus-adjusted will inevitably lead to a low phosphorus diet.

**Weight gain, fractional excretion of phosphorus (FEPi) and urinary urea nitrogen (UUN)**

To investigate the individual roles of protein and phosphorus in the progression of renal insufficiency, we compared the results from the six isocaloric diets described in the methods (Groups 1 to 6). The animals in Group 1 died at 11 wk, probably due to end-stage renal disease. Body weight gain was not significantly different among the groups until 10 wk after the start of the experiment. However, animals in Groups 1 and 2 gained less weight because of the feed intake reduction due to the progression of renal insufficiency (Fig. 2). Figure 3A and B shows the time course of the FEPi and UUN, respectively. Because the amount of phosphorus content in the diet was adjusted, FEPi was not affected by the dietary protein content. The level of FEPi in rats fed with normal phosphorus (Groups 1, 2, and 3) was higher than those in rats fed with low phosphorus (Groups 4, 5, and 6) throughout the experimental period. The level of UUN, a marker of protein intake, was regulated by dietary protein concentration. At weeks 10 and 13, the UUN level observed in Groups 1 and 2 was lower than that of Groups 4 and 5. These decreases were due to the food intake reduction according to the progression of renal insufficiency.

**Renal function**

The time course of serum creatinine is shown in Fig. 4A. The level of serum creatinine in Groups 1 and 2 increased progressively until 13 wk after dietary treatment. In Groups 3, 4, 5, and 6, serum creatinine also began to increase at week 6, although the amount was less than that in Group 1 or 2. According to two-way analysis of variance, low intake of phosphorus showed a significantly beneficial effect against progression of
The Histological findings

Table 2. Incidence of histopathological change in kidney.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein 16.9/Pi 0.5</th>
<th>Protein 8.4/Pi 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Gr. 2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Gr. 4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Gr. 5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Gr. 6</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

- = not remarkable; ±, very slight; +, slight; ++, moderate; ++++, severe.
*p<0.05, significantly different from Gr. 1 (Wilcoxon’s rank test).

renal insufficiency. Figure 4B shows the time course of creatinine clearance. At week 13, the level of creatinine clearance was also lower in Groups 1 and 2 than in the other groups.

Urinary albumin excretion
The time course of urinary albumin excretion is shown in Fig. 5. The level of urinary albumin excretion in all groups increased progressively until 6 wk after treatment. In the normal phosphorus diet groups, the level of urinary albumin excretion in the very low protein group (Group 3) was lower than that in the normal or low protein groups (Groups 1 and 2).

Serum PTH
The time course of serum PTH is shown in Fig. 6. In all groups, serum PTH began to increase at week 2 and increased progressively until 13 wk after treatment. The levels of PTH in the normal phosphorus groups (Groups 1–3) were higher than those in low the phosphorus groups (Groups 4–6) throughout the experimental period.

Histological findings
Kidneys were examined histopathologically at week 13 (Fig. 7, Table 2). Severe or moderate kidney lesions including glomerulosclerosis, degeneration of tubules, and interstitial fibrosis, were observed in uremic rats in Groups 1 and 2. This damage was significantly ameliorated in Groups 3, 5 and 6.

Discussi

In patients with CKD, nutritional therapy, especially a low protein diet, may help to protect residual renal function (1). Many protein foods are also rich in phosphorus, so low protein diets often lead to a beneficial decrease in dietary phosphorus intake as well. In the present study, normal rats fed a low protein (12.6%) or very low (8.4%) protein diet without an adjusted amount of phosphorus exhibited not only low serum UN but also low serum phosphorus. Although a large number of studies have shown the beneficial effects of the low protein diet, little is known about how dietary protein and phosphorus affect the progression of renal insufficiency independently. The aim of this study was to compare the effects of protein and phosphorus on the progression of renal insufficiency using irreversible Thy1 rats. In the present study, a low phosphorus diet, regardless of protein content, significantly protected the residual renal function assessed by serum creatinine and creatinine clearance (Fig. 4A and B). The result of histopathological analysis was similar to that of biochemical analysis. These results indicate that phosphorus is an important factor that affects the progression of renal insufficiency in this model. However, rats fed a normal (0.5%) phosphorus diet with very low (8.4%) protein showed beneficial effects biochemically and histopathologically, indicating that low protein diet also has beneficial effects that are independent of the effect of phosphorus.

The mechanism by which a low phosphorus diet may prevent the progression of renal insufficiency remains unknown. Previous experimental data suggests that
nephrocalcinosis, calcification of the kidney, is involved in the mechanism of phosphorus toxicity (2), which appears to be related to the induction of calcium phosphorus precipitation resulting in tubulointerstitial damage. In addition, a direct effect of phosphorus restriction on renal hemodynamics has been reported. Harris et al. (7) reported that glomerular capillary pressure was lower in phosphorus-deprived uninephrectomized diabetic rats than in control animals. Interestingly, Kraus et al. (8) reported that the dietary phosphorus restriction inhibited renal hyperfiltration after an oral protein load. Our data are consistent with these results, since creatinine clearance, which is almost equal to the glomerular filtration rate (GFR), in Groups 2 and 3 was higher than in Groups 5 and 6, respectively at the initial stage (Fig. 4B). Further study would be necessary to clarify the mechanism of the beneficial effects of a low phosphorus diet.

In the normal phosphorus diet groups, only the very low protein showed beneficial effect in the progression of renal insufficiency. The mechanism by which a low protein diet may lead to prevention of the progression of renal insufficiency has not yet been fully clarified. However, renal hemodynamics appears to be related to the protection of residual renal function (9, 10). A high protein diet may acutely increase the GFR and possibly cause intraglomerular hypertension, which would lead to the progressive loss of renal function (11). In the present study, at week 2, creatinine clearance in rats fed very low protein with normal phosphorus was significantly lower compared with low or normal protein. These results indicate that renal hemodynamics lead to a beneficial effect. Furthermore, low protein intake is associated with a reduction in albuminuria. In the present study, urinary albumin excretion in rats fed a very low protein diet with normal phosphorus was significantly lower than in those fed a low or normal protein diet. Since albuminuria is not only a marker of glomerular injury but also a cause of tubulointerstitial injury (12, 13), these results indicate that lower urinary albumin is related to the beneficial effect of a very low protein, normal phosphorus diet.

It is well known that in CKD, hyperphosphatemia increases PTH secretion directly and/or indirectly and induces renal osteodystrophy (ROD), characterized by osteitis fibrosa and the activation of osteoblasts and osteoclasts with elevated rates of bone formation, following hyperparathyroidism (14). We could not demonstrate that a low phosphorus diet prevented ROD. However, the low phosphorus diets, regardless of protein content, prevented the increase of serum PTH. It has been reported that phosphorus restriction or phosphate binder treatment prevents not only renal failure but also ROD by reducing serum PTH (15). These results suggest that a low phosphorus diet might have beneficial effects not only for renal function but also for ROD, a typical complication in CKD patients.

Kikuchi et al. (16) reported that dietary treatment with a combination of low protein and phosphorus restriction is more useful than dietary therapy with either low protein or low phosphorus in the treatment of uremic rats. Our results were inconsistent with their results on the point that a synergic effect of low protein and low phosphorus was not observed. The reason for the difference in results may have been from the dietary protein and phosphorus content. Kikuchi et al. used two levels of protein (24 and 6%) and two levels of phosphorus (0.5 and 0.12%) and the synergic effect may have been a result of the very low percentage of protein.

In conclusion, the data from this study showed that the low phosphorus diet protects the residual renal function in irreversibly Thy1 rats, independently of low protein. However, only a normal phosphorus diet with a very low percentage of protein showed a beneficial effect, indicating that dietary phosphorus is a more important factor than dietary protein that affects the progression of CKD in this model. Furthermore, the low phosphorus diets also prevented the increase in serum PTH, indicating that a diet low in phosphorus might have beneficial effects not only for renal function but also for ROD, a typical complication in CKD patients.

Acknowledgments
We thank Drs. Naoshi Horiba, Michinori Hirata and Takeshi Nihei (Chugai Pharmaceutical Co., Ltd.) for providing technical advice during the course of these studies. We also thank Toshio Mori (Chugai Research Institute for Medical Science, Inc.) for technical assistance with the histopathology. We also thank Ms. Ford (Chugai Pharmaceutical Co., Ltd.) for her assistance with English usage.

REFERENCES


