J Tokyo Wom Med Univ
76 (3) 114~121 (2006)

Effect of Citrate on the Development of Experimental Polycystic Kidney Disease

Chika KOBAYASHI[1], Takashi YAGISAWA[2] and Hiroshi TOMA[1]

[1]Department of Urology, Tokyo Women's Medical University School of Medicine
[2]Division of Renal Surgery and Transplantation, Department of Urology, Jichi Medical School

(Accepted Jan. 20, 2006)

[Background] We investigated the effects of citrate on the development of renal cysts and renal dysfunction in experimental polycystic kidney disease (PKD).

[Methods] Renal cystic disease was chemically induced by orally administered diphenylthiazole (DPT). Sprague-Dawley rats were divided into 3 groups: Group 1, normal rats; Group 2, rats with DPT intake for 4 weeks; Group 3, rats with DPT and citrate intake for 4 weeks. The expression and localization of chloride channel-K (CLC-K(2)) were examined using Northern blotting and immunohistochemistry.

[Results] Cysts were found in the kidneys of all the rats in Group 2 and Group 3, but fewer and smaller cysts were found in Group 3. Glomerular filtration rate (GFR) was lowest in Group 2 and significantly better in Group 3. The expression of CLC-K was weaker in Group 2 than in Group 1 and stronger in Group 3 than in Group 2. CLC-K(2) staining in the epithelial cells weakened with cyst development. CLC-K(2) staining was stronger in Group 3 than in Group 2.

[Conclusion] Citrate retards cyst formation and helps to prevent the deterioration of renal function in experimental PKD. Differences in the expression and localization of CLC-K in the epithelial cells may be attributable to the development of PKD.

Key words: polycystic kidney disease, citrate, chloride channel-K

Introduction

Autosomal dominant polycystic kidney disease (PKD) is often accompanied by the formation of numerous fluid-filled cysts in both kidneys and the development of renal failure[1]. Several treatments have been prospected to retard or prevent cyst formation such as vasopressin V2 receptor antagonist, the major cyclic AMP (cAMP) agonist in the collecting duct[2)3)].

In recent experimental studies, treatment with an alkalizer, such as citrate, has been reported to diminish the enlargement of renal cysts and improve renal function[4)5)]. However, the mechanism of this effect has not yet been elucidated[6)7)].

Intracystic fluid accumulation is an important step in cyst formation[8]. Fluid originates from the net secretion of transepithelial fluid, which is driven by transepithelial chloride secretion involving chloride channels, like chloride channel K and cystic fibrosis transmembrane conductance regulator[9)10)].

Animal models of PKD, either drug-induced or genetically produced, have been developed. The heterozygous Han: SPRD rat[11], which is a useful model for human PKD, has been used to investigate the pathogenesis of cyst formation and the effect of treatment in some laboratories. We have used 2-amino-4,5-diphenylthiazole-HCl (DPT)[12] to induce cysts in the kidneys of rats in experiments examining cystic disease and renal cancer[13].

In the present study, we investigated the effect of citrate on the development of cyst formation and focused on changes in renal CLC-K expression and lo-

calization during the development and retardation of renal cysts in our experimental PKD rat model.

Materials and Methods

1. Animals and PKD models

Sprague Dawley (SD) rats, weighing 100 to 200 g, were utilized; renal cysts formation was induced using orally administered 2-amino-4,5-diphenyl-thiazole-HCl (DPT). The DPT dose was 0.5 to 1.0 mg/kg/day. The development of renal cysts depends on the period of DPT intake. After two weeks, small cysts were found mainly in the corticomedullary area. The number and size of the cysts increased over time, and enlarged cysts were widely disseminated throughout the kidney after four weeks (Fig. 1).

The handling and treatment of the experimental animals for this study were approved by the Institutional Committee of Tokyo Women's Medical University.

2. Experimental models

SD rats were divided into three groups according to DPT and citrate intake as follows: Group 1, normal rats; Group 2, rats with DPT intake for 4 weeks; and Group 3, rats with DPT and a mixture of citrate intake for 4 weeks. Each group consisted of 10 rats.

DPT and citrate were administered orally with meals. A mixture of citrate salts comprising citrate sodium, citrate potassium and citric acid were given at a dose of 3.0 to 4.0 g/kg/day.

3. Laboratory and histologic evaluations

At the end of the experiment, 24 hr urine and blood samples were analyzed by the enzymatic method. The kidneys were harvested and examined. A histological examination was performed, and the tubule/cyst lumen areas were measured using NIH-image. The expression and localization of CLC-K were investigated using Northern blot analysis and immunohistochemistry.

Five hundred of the largest tubule/cyst lumen areas were employed for the NIH image analysis. The median and average of the lumen areas were compared among the groups, and the number of tubules/cysts was counted for each group.

4. CLC-K experiments

1) Expression of CLC-K

Total RNA was isolated from the excised kidneys

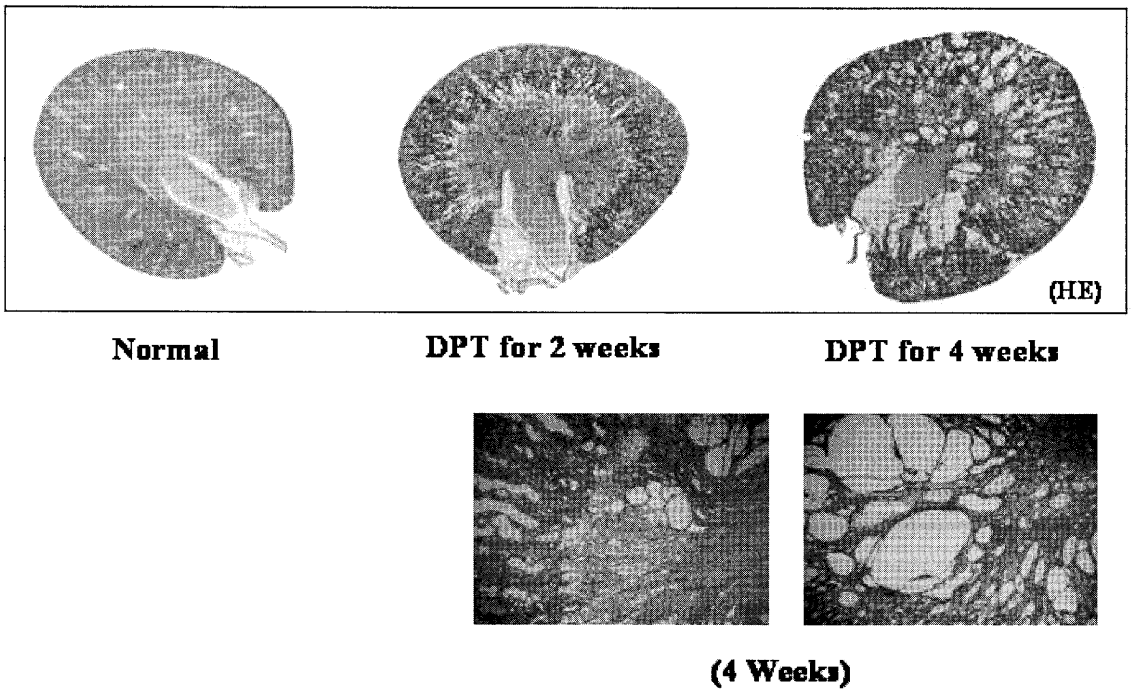

Fig. 1 DPT induced PKD

The number and size of the cysts increased over time. The cysts are spread widely throughout the kidney.

using guanidium isothiocyanate-cesium chloride centrifugation, followed by digestion with RNase-free DNaseI (Takara, Ohtsu, Japan) to remove contaminating genomic DNA. cDNA probes encoding the CLC-K(2) sequence were radiolabeled using the random prime labeling method. The total RNA samples of 20 μg were denatured by formaldehyde, fractionated on 1% agarose gel, and blotted onto nylon membranes (Hybond N).

The membranes were then prehybridized at 65 ℃ for 4 hr in 5× standard saline citrate (SSC) containing 5× Denhaldt's solution, 1% sodium dodecyl sulfate (SDS), and salmon sperm DNA (100 μg/ml). The membranes were subsequently hybridized at 65 ℃ overnight in the same buffer containing a radioactive DNA probe (2×10^6 cpm/ml). The membranes were washed at 65 ℃ with 2× to 0.1× SSC containing 0.1% SDS, and exposed to Kodak XAR-5 films at minus 80 ℃ using an intensifying screen for 3-7 days before development. Glyceraldehydephosphate dehydrogenase (GAPDH) was used as a probe to control RNA loading.

2) Immunohistochemistry

A rabbit polyclonal antibody against rat CLC-K(2) (CHEMICON International Inc, Temecula, California) was used as the primary antibody for the immunohistochemical localization study. The slides were dewaxed by incubation in xylene, a graded ethanol series and water. Endogenous peroxidase activity was removed by 0.3% H_2O_2 in methanol for 30 min. After three rinses in 0.01 M PBS buffer, the slides were treated with 1.5% normal horse serum for 20 min to block nonspecific binding sites. Then the slides were incubated with the primary antibody (a dilution of 1 : 5) for 120 min at room temperature. The slides were rinsed three times in PBS for 3 min and incubated with 1 : 200 biotinylated horse anti-mouse IgG (Vector Laboratory, Burlingame, California) for 30 min. The slides were again rinsed three times in PBS for 3 min and finally incubated with Vectastain ABC reagent for 30 min. Color development was achieved by incubating the tissues with diaminobenzidine tetrahydrochloride substrate for 5 min. After counterstaining with hematoxylin and dehydration, the slides were mounted.

5. Statistical methods

Data are presented as the mean ± standard deviation. The statistical analysis was performed using an ANOVA test, and p values of less than 0.05 were considered significant.

Results

Kidney weight

None of the rats died during the experimental periods (4 weeks). At the end of the experiment, the kidney weights were more increased in Group 2 and 3, compared to that in Group 1. However, the kidney weight/body weight ratio was smaller in Group 3 than in Group 2 (Table).

Table Urine and blood parameters in normal and PKD rats (4 weeks' treatment)

	Normal rats	PKD (DPT) rats		p (G2 vs G3)
	Group 1	Group 2	Group 3	
Body weight (g)	344 ± 30	127 ± 24	173 ± 32	< 0.05
Kidney weight (g)	3.03 ± 0.35	3.69 ± 1.06	3.91 ± 0.71	NS
Kidney/body weight (%)	0.87 ± 0.04	2.83 ± 0.34	2.29 ± 0.58	< 0.05
Ccr (ml/min)	2.18 ± 0.39	0.34 ± 0.12	0.72 ± 0.19	< 0.001
Plasma ammonia (μg/dl)	166 ± 155	236 ± 218	196 ± 197	NS
Urine volume (ml)	14.9 ± 4.5	20.5 ± 2.9	32.6 ± 7.9	< 0.001
Urine volume/BW (ml/kg)	43.4 ± 13.8	163.8 ± 24.4	196.9 ± 69.0	NS
Urine pH	6.87 ± 0.37	6.66 ± 0.29	7.87 ± 0.59	< 0.001
Urine Na (mEq/kg/day)	4.8 ± 0.8	5.1 ± 0.5	16.6 ± 4.6	< 0.001
Urine K (mEq/kg/day)	13.7 ± 0.8	13.3 ± 0.7	26.1 ± 4.7	< 0.001
Urine Cl (mEq/kg/day)	6.8 ± 0.6	8.6 ± 0.6	11.8 ± 2.3	< 0.01
Urine citrate (mg/kg/day)	297 ± 43	80 ± 40	463 ± 157	< 0.001
Urine ammonia (mg/kg/day)	5.32 ± 2.55	4.86 ± 1.29	2.38 ± 1.86	< 0.01

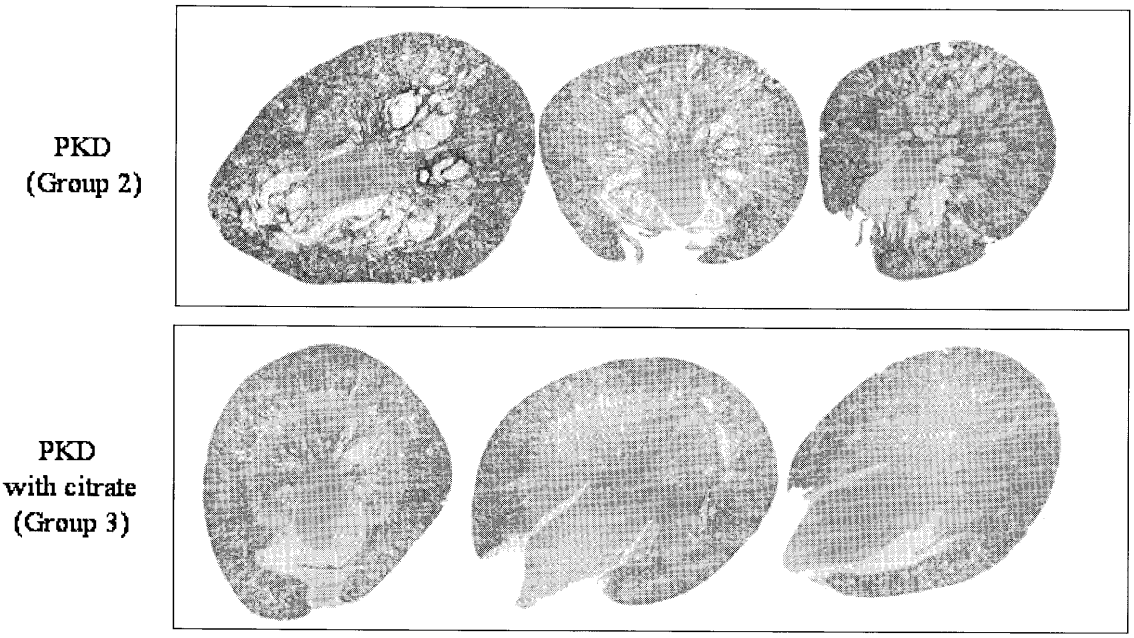

Fig. 2 Specimens from rats with DPT-induced PKD and PKD treated with citrate for 4 weeks
The number and size of the cysts/lumens are smaller in PKD rats treated with citrate than in untreated PKD rats.

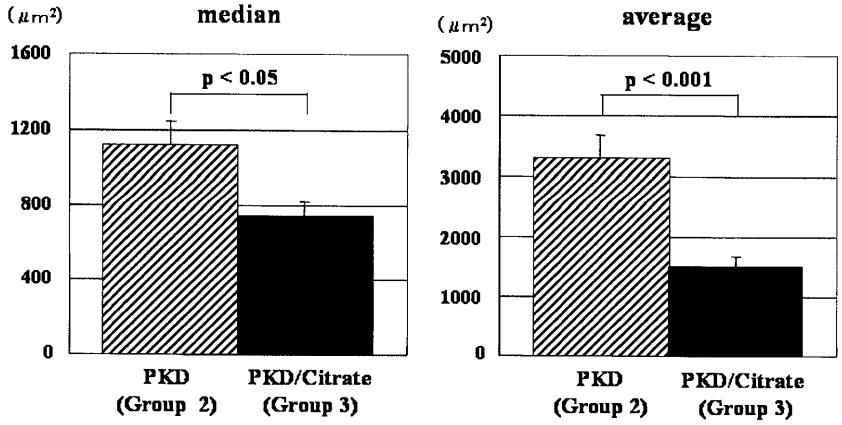

Fig. 3 Lumen area of the 500 largest cysts (PKD vs PKD with citrate)
The lumen area is significantly smaller in the PKD with citrate group than in the PKD group.

Cyst formation

Figure 1 shows macroscopic specimens obtained from Group 2. The number and size of the cysts increased with time, and enlarged cysts were widely spread throughout the kidney after 4 weeks. In Group 3, the number and size of the cysts/lumens were smaller than in Group 2 (Fig. 2). NIH image analysis revealed a significant decrease in the lumen area of the 500 largest cysts (Fig. 3) and in the number of cysts larger than 20,000 μm^2 in Group 3 (Fig. 4). In addition, the number of lumens less than 2,000 μm^2 was significantly greater in Group 3 (Fig. 4).

Renal function

Table shows the laboratory data for each group. Creatinine clearance (Ccr) was significantly lower in Group 2 than in Group 1. Ccr was significantly higher in Group 3 than in Group 2. Urinary citrate excretion was significantly lower in Group 2 than in Group 1. Urinary sodium, potassium and citrate ex-

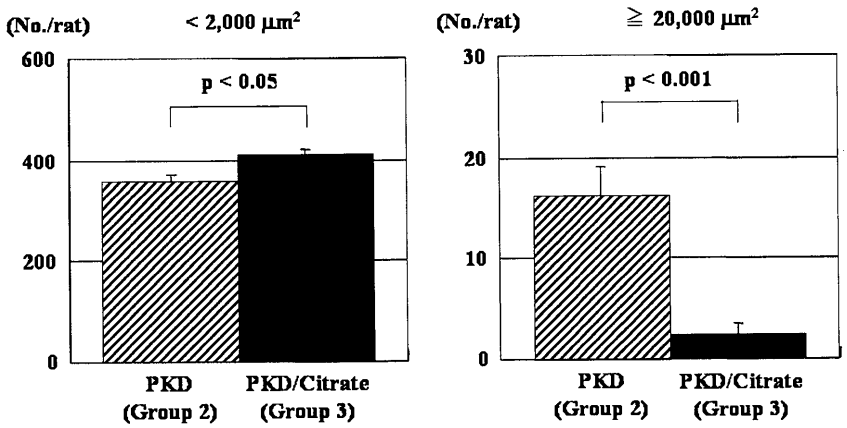

Fig. 4 Number of cysts more than 20,000 μm² and less than 2,000 μm²
The number of cysts more than 20,000 μm² is smaller in the PKD with citrate group than in the PKD group. The number of cysts less than 2,000 μm² is greater in the PKD with citrate group than in the PKD group.

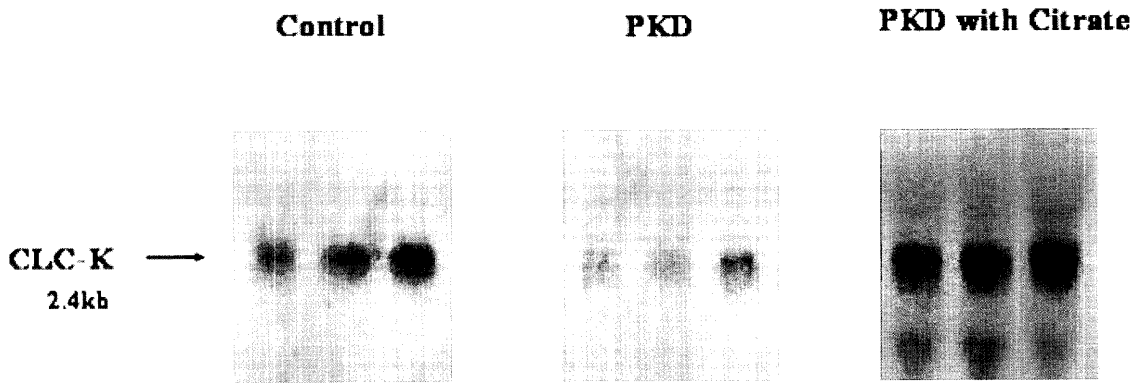

Fig. 5 Northern blot analysis of CLC-K
CLC-K expression is weaker in the PKD group than in normal kidney specimens. CLC-K expression is stronger in the PKD with citrate group than in the PKD group.

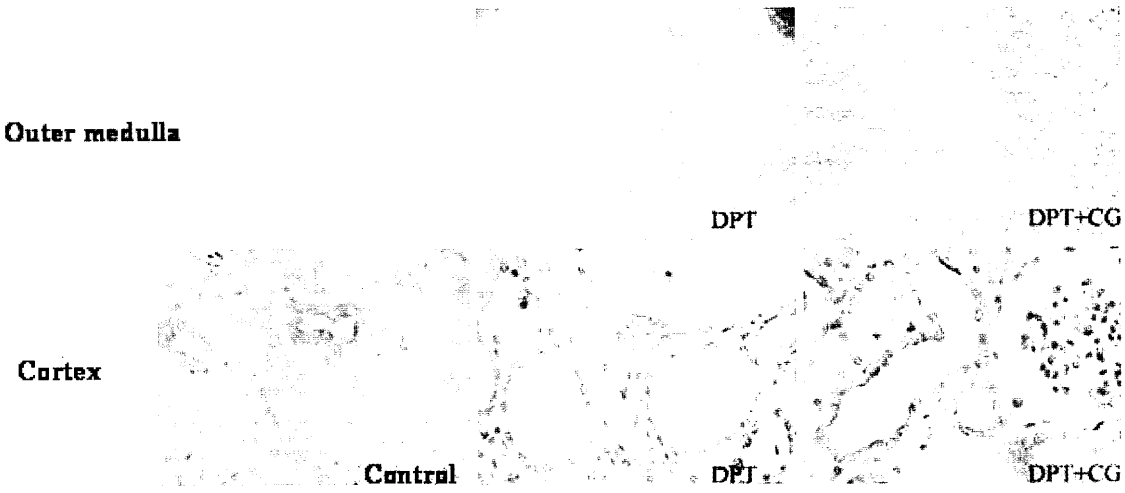

Fig. 6 Immunohistochemistry of CLC-K
CLC-K staining in the epithelial cells is stronger in the PKD with citrate group than in the PKD group. Magnification (400×; outer medulla, 200×; cortex).

cretions were higher in Group 3 than in Group 2.

Expression of CLC-K

CLC-K(2) mRNA was detected in the kidneys of all three groups. The expression was weaker in Group 2 than in Group 1. In Group 3 the expression was stronger than in Group 2 (Fig. 5). CLC-K(2) staining in the epithelial cells weakened with the development of cysts. CLC-K(2) staining in the epithelial cells was stronger in Group 3 than in Group 2 (Fig. 6).

Discussion

In the present study, we investigated the development of cyst formation and the effect of citrate on this process, focusing on changes in CLC-K[9)10)14)15)], in an experimental model for renal cysts. A chemically (DPT)-induced PKD model was used in the present study because we have had a lot of experience using this PKD model[13)]. In this model, the development of renal cysts depends on the period of DPT intake. Small cysts appear mainly in the corticomedullary area of the kidney after two weeks. The number and size of the cysts increase with time, and enlarged cysts are spread widely throughout the kidney at the end of four weeks.

Citrate intake suppressed the development of cyst formation and improved GFR in the DPT-induced PKD rats, similar to results reported by Torres et al[4)] and Tanner et al[5)~7)]. Torres et al[4)] reported the effect of acidosis and alkalosis on renal function and cyst development in genetic PKD (Hans: SPRD) rat models. They found that acidosis resulted in a diminished GFR and a larger kidney size, le alkalosis diminished the enlargement of the cystic kidney without any improvement in GFR. They hypothesized that ammoniagenesis or other metabolic processes played a role in the pathogenesis of PKD and that alkalosis offers a protective effect against the development of cystic disease. Tanner et al[5)~7)] also reported the effects of potassium citrate/citric acid intake on renal structure and renal function in several papers. They discovered that the intake of K citrate/citric acid solution (KCit) prevents the decline in GFR found in untreated rats with PKD. While KCit did not affect kidney size, it did lead to smaller cysts in cystic kidneys[5)6)]. The alkalinizing effect of citrate might result in a reduction in renal ammonia synthesis, and a reduction in ammonia might in turn prevent ammonia-induced complement activation and inflammation in the renal interstitium, resulting in the suppression of abnormal growth and/or fluid secretion by the tubular epithelium[4)6)].

There are many factors that influence cyst formation in PKD. Hyperplasia of epithelial cells[1)2)], transepithelial fluid secretion leading to the net accumulation of intratubular fluid[1)8)~10)], and extracellular matrix abnormalities[8)] are considered the three major processes in renal cyst formation. Recent studies have shown that cAMP also stimulated cyst enlargement and fluid secretion in PKD cells[10)].

Intracystic fluid accumulation is an important step in cyst formation. The fluid originates from a net transepithelial fluid secretion, which was recently shown to be driven by transepithelial chloride secretion involving chloride channels, like chloride channel K (CLC-K)[9)14)] and cystic fibrosis transmembrane conductance regulator (CFTR)[10)]. Chloride secretion through CLC-K and CFTR in epithelial cells has been implicated in the pathway of fluid secretion in PKD epithelia.

In the present study, we focused on CLC-K-which has been implicated in fluid excretion by the epithelial cells, leading to the development or suppression of cyst formation.

Chloride channels that regulate cell volume and transepithelial chloride transports were identified in the kidney in 1994[14)]. Of these channels, CLC-K (2)[14)16)] is widely expressed in the tubules of the kidney, especially in the corticomedullary area where cyst formation first occurs in PKD rat kidneys. Therefore, CLC-K(2) is thought to play a significant role in cyst formation.

The expression of CLC-K(2) was weaker in PKD rats than in normal rats. This result suggests that a dysfunction of CLC-K(2) suppresses a transepithelial chloride secretion, therefore fluid accumulation develops cyst formation in the kidney.

Cyst formation was significantly suppressed and CLC-K(2) expression was higher in PKD rats treated with citrate than in untreated PKD rats.

These findings suggest that citrate increases CLC-K(2) expression in the epithelia, resulting in a decrease in cyst formation. This mechanism is in part assumed to be direct effect by citrate, as there were not any significant histological changes in interstitium. The retardation of cyst formation and alkalization by citrate administration may be linked to an improvement in GFR.

In conclusion, citrate retards cyst formation and helps to prevent the deterioration of renal function in experimental PKD. Differences in the expression and localization of CLC-K in the epithelial cells may be attributable to the development of PKD.

References

1) **Grantham JJ**: The etiology, pathogenesis, and treatment of autosomal dominant polycystic kidney disease: recent advances. Am J Kid Dis **28**: 788–803, 1996
2) **Qian Qi, Harris PC, Torres VE**: Treatment prospects for autosomal-dominant polycystic kidney disease. Kidney Int **59**: 2005–2022, 2001
3) **Torres VE, Wang X, Qian Q et al**: Effective treatment of an orthologous model of autosomal dominant polycystic kidney disease. Nature Med **10**: 363–364, 2004
4) **Torres VE, Mujwid DK, Wilson DM et al**: Renal cystic disease and ammoniagenesis in Han: SPRD rats. J Am Soc Nephrol **5**: 1193–1200, 1994
5) **Tanner GA**: Potassium citrate/citric acid intake improves renal function in rats with polycystic kidney disease. J Am Soc Nephrol **9**: 1242–1248, 1998
6) **Tanner GA, Tanner JA**: Citrate therapy for polycystic kidney disease in rats. Kidney Int **58**: 1859–1869, 2000
7) **Tanner GA, Tanner JA**: Dietary citrate treatment of polycystic kidney disease in rats. Nephron Physiol **93**: 14–20, 2003
8) **Murcia NS, Sweeney Jr WE, Avner ED**: New insights into the molecular pathophysiology of polycystic kidney disease. Kidney Int **55**: 1187–1197, 1999
9) **Sullivan LP, Wallace DP, Grantham JJ**: Chloride and fluid secretion in polycystic kidney disease. J Am Soc Nephrol **9**: 903–916, 1998
10) **Hanaoka K, Guggino WB**: cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells. J Am Soc Nephrol **11**: 1179–1187, 2000
11) **Cowley Jr BD, Gudapaty S, Kraybill AL et al**: Autosomal-dominant polycystic kidney disease in the rat. Kidney Int **43**: 522–534, 1993
12) **Carone FA, Ozono S, Samma S et al**: Renal functional changes in experimental cystic disease are tubular in origin. Kidney Int **33**: 8–13, 1988
13) **Ito F, Toma H, Yamaguchi Y et al**: A rat model of chemical-induced polycystic kidney disease with multistage tumors. Nephron **79**: 73–79, 1998
14) **Adachi S, Uchida S, Ito H et al**: Two isoforms of a chloride channel predominantly expressed in thick ascending limb of Henle's loop and collecting ducts of rat kidney. J Biol Chem **269**: 17677–17683, 1994
15) **Uchida S**: In vivo role of CLC chloride channels in the kidney. Am J Physiol Renal Physiol **279**: F802–F808, 2000
16) **Vandewalle A, Cluzeaud F, Bens M et al**: Localization and induction by dehydration of CLC-K chloride channels in the rat kidney. Am J Physiol **272**: F678–F688, 1997

実験嚢胞腎における嚢胞形成とクエン酸の効果に関する検討

[1]東京女子医科大学　医学部　泌尿器科学（主任：東間　紘教授）
[2]自治医科大学　腎泌尿器外科学　腎臓外科学部門

小林　千佳[1]・八木澤　隆[2]・東間　紘[1]

〔目的〕多発性嚢胞腎では，嚢胞の増加，増大に伴い，進行性に腎機能が低下し腎不全に至るが，嚢胞形成，進展に関与する因子はまだ充分に解明されていない．今回，我々は実験的嚢胞形成ラットを用い，腎嚢胞形成および腎機能悪化に対するクエン酸の影響を検討した．

〔対象および方法〕Sprague-Dawley 種ラットを 3 群に分け，グループ 1 は通常の飼料のみを，グループ 2 はジエチルサイアゾール（DPT）を 4 週間，グループ 3 は DPT とクエン酸を 4 週間与えた．4 週後，24 時間蓄尿と血液検査を行い，摘出腎臓を組織学的に検討した．また，クロライドチャネル-K（CLC-K）の発現をノーザンブロッティングおよび免疫組織学的に確認した．

〔結果〕嚢胞形成は，DPT を摂取したグループ 2 と 3 のすべてのラットに認められたが，DPT とクエン酸をともに摂取したグループ 3 では嚢胞面積は有意に小さかった．GFR はグループ 2 が最も低く，明らかにグループ 3 に比べて低下していた．CLC-K は，グループ 2 でグループ 1 より発現が弱かったが，グループ 3 はグループ 2 より発現が強かった．尿細管上皮細胞での CLC-K (2) はグループ 3 でグループ 2 よりも強く染色され，嚢胞の増大に伴い染色性が低下していた．

〔結語〕クエン酸は，実験的嚢胞形成ラットにおいて，嚢胞形成を阻害し，腎機能悪化を抑制した．尿細管上皮細胞での CLC-K の発現や局在の違いが，嚢胞増大に関与していると考えられた．