

## FKBP-12 Exhibits an Inhibitory Activity on Calcium Oxalate Crystal Growth in Vitro

Urolithiasis and calcium oxalate crystal deposition diseases are still significant medical problems. In the course of *nephrocalcin* cDNA cloning, we have identified FKBP-12 as an inhibitory molecule of calcium oxalate crystal growth.  $\lambda$ gt 11 cDNA libraries were constructed from renal carcinoma tissues and screened for *nephrocalcin* cDNA clones using anti-nephrocalcin antibody as a probe. Clones expressing recombinant proteins, which appeared to be antigenically cross-reactive to nephrocalcin, were isolated and their DNA sequences and inhibitory activities on the calcium oxalate crystal growth were determined. One of the clone  $\lambda$ gt 11 #31-1 had a partial fragment (80 bp) of FKBP-12 cDNA as an insert. Therefore, a full-length FKBP-12 cDNA was PCR-cloned from the  $\lambda$ gt 11 renal carcinoma cDNA library and was subcloned into an expression vector. The resultant recombinant FKBP-12 exhibited an inhibitory activity on the calcium oxalate crystal growth ( $K_d=10^{-7}$  M). Physiological effect of the extracellular FKBP-12 was investigated in terms of macrophage activation and proinflammatory cytokine gene induction. Extracellular FKBP-12 failed to activate macrophages even at high concentrations. FKBP-12 seems an anti-stone molecule for the oxalate crystal deposition disease and recurrent stone diseases.

**Key Words :** Tacrolimus Binding Protein 1A; Calcium Oxalate; Growth Inhibitor; Nephrocalcin; Anti-stone agent

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ABSTRACT

## INTRODUCTION

Deposition of calcium oxalate microcrystal in human body can be a significant problem and it is recognized that 70-80% of kidney stones contain calcium oxalate (1). Deposition of calcium oxalate microcrystal in soft tissues has been observed in primary hyperoxaluria, chronic renal failure, and certain small bowel diseases (2, 3, 20). Although pathologic conditions may arise occasionally when the body fluid is supersaturated with calcium oxalate, its precipitation is normally inhibited by a number of low-molecular weight inhibitors and macromolecular inhibitors (3). Nephrocalcin was discovered by Nakagawa and his collaborators as a 14 kDa urinary protein, accounting for over 80% of calcium oxalate crystal and having a growth inhibitory activity in the normal urine (4). Its molecular characteristics have been extensively studied (5, 7) and the functionally and biochemically equivalent polypeptides have been isolated from the urine of various vertebrates (8, 9). Nephrocalcin is, accordingly, considered to be the primary inhibitory protein of urinary stone formation (3). Nephrocalcin has attracted our

attention due to its potential as a stone-inhibitor agent, and therefore *nephrocalcin* cDNA cloning has been attempted in our laboratory for several years. For this purpose, we have prepared polyclonal antibodies (10) and used it as a probe in the screening of human kidney cDNA library. We have identified a clone whose recombinant peptide was recognized by the antiserum. The clone encoded a fragment of FK506-binding protein (FKBP-12) cDNA (11). We report here the growth inhibitory activity of recombinant FKBP-12 protein on the formation of calcium oxalate crystal by an in vitro assay. Furthermore, we evaluated the potential of recombinant FKBP-12 as an anti-urinary stone agent.

## MATERIALS AND METHODS

### Tissues

Surgical specimens of adenocarcinoma of the kidney obtained at the Department of Urology, Medical Center, University of Chicago were used for the preparation of cDNA libraries.

### Anti-nephrocalcin antiserum

Nephrocalcin was isolated from human urine by Y. Nakagawa at the University of Chicago and rabbit anti-human nephrocalcin antisera were prepared using purified nephrocalcin preparation. Specificity of antisera was reported previously (10). This antiserum (R3) has been used for the phage screening, immunoblotting and immunohistology as a primary antibody.

### Screening and sequencing of *nephrocalcin* cDNA

Total RNA was prepared according to the method of Chomczynski and Sacchi (12) from renal carcinoma.  $\lambda$ gt 11 cDNA libraries were constructed according to the method of Young and Davis (13). *E. coli* strain Y1090 was transfected with the library, and total  $2 \times 10^4$  transfectants replica-plated on nylon-filters were screened for nephrocalcin-like peptides using anti-nephrocalcin antiserum. Phage DNA from a positive clone was extracted by the standard method (15). The insert was cloned by polymerase chain reaction (PCR) using  $\lambda$ gt 11 forward and reverse primers and sub-cloned into pTA (Invitrogen, U.S.A.) for the DNA sequencing by the dideoxy method of Sanger *et al.* (14).

### Recombinant FKBP-12

As the clone designated as #31-1 was consistently positive by the anti-nephrocalcin antibody screening and the 80 bp insert DNA sequence was identical to FKBP-12 cDNA, a full length FKBP-12 cDNA was PCR-cloned from a  $\lambda$ gt 11 human renal carcinoma cDNA library using the PCR primers listed in Table 1. The FKBP-12 cDNA was sub-cloned into pET28a (Novagen, U.S.A.) and *E. coli* BL21 (DE3) was transfected by this pET28a-FKBP-12. The BL21 (DE3) transfectant was induced by IPTG and the resultant recombinant FKBP-12 was purified by Ni-NTA affinity chromatography specific for His-Tag protein. The molecular weight of the recombinant FKBP-12 was estimated by

electrophoresis on a sodium dodecylsulfate-polyacrylamide gel (SDS/PAGE). The electrophoresed protein was further electroblotted onto a nitrocellulose membrane and probed by the anti-human nephrocalcin antibody to confirm the initial antigenic cross-reactivity of FKBP-12 with nephrocalcin.

### Determination of crystal growth inhibitory activity

Affinities of nephrocalcin and the recombinant FKBP-12 to calcium oxalate microcrystal were determined by the spectrophotometric method described by Nakagawa *et al.* (4). The dissociation constant was determined by the Langmuir-type isotherm plot with a least-square fit of  $1/[I]$  vs  $V_0/(V_0 - V)$ , where  $V_0$  and  $V$  are the initial velocities with and without nephrocalcin or the recombinant FKBP-12  $[I]$ , respectively.

### Macrophage activation assay

To determine whether the high concentration of extracellular FKBP-12 triggers the macrophage activation, the macrophage activation assay (16) was carried out. Mouse peritoneal macrophages were collected and contaminating T cells were removed using an anti-CD3 antibody (Becton & Dickinson, U.S.A.), magnetized secondary antibody, and Mac column (Miltenvi Biotec, U.S.A.). Macrophages were cultured for 24 hr, and then various concentrations of the recombinant FKBP-12 (1 to 500  $\mu$ g/mL) were added to each of the cultures. After 6 hr and 16 hr incubation, total RNA were prepared from each culture and the expressions of proinflammatory genes (IL-1, TNF- $\alpha$ , IL-6, IL-12, and NOS) were analyzed by reverse transcriptase-PCR (RT-PCR). The primer sequences used for RT-PCR were obtained from the NCBI GenBank Database (NIH, U.S.A.) and are shown in Table 1.

cDNA profiles on agarose gel electrophoresis were quantified by the BIO-1D V.96 image analysis software (Vilber Lourmat, Cedex, France) and data were expressed as the means  $\pm$  SEM of the percentage of the control level. The statistical significance of the changes was analyzed and a difference from the control value of 100 was considered to be significant when  $p < 0.05$  by Student's *t* test.

### Expression of FKBP-12 mRNA and protein in the transfectants

FKBP-12 cDNA was subcloned into a mammalian expression vector, pcDNA3 (Invitrogen, CA, U.S.A.), and the clone was designated as pcDNA3-FKBP-12. The pcDNA3-FKBP-12 cDNA was purified using the Endofree Maxi kit (Quagen, CA, U.S.A.). Mouse fibroblasts, 3LL, were plated onto 6-well tissue culture dishes at  $5 \times 10^5$  cells/well and were grown to a 80% confluency. Then they were transfected with pcDNA3-FKBP12 cDNA/lipofectamine complex (1  $\mu$ g DNA/10  $\mu$ L liposome) in a serum-free DMEM at 37°C for 5 hr in a CO<sub>2</sub> incubator. After an additional incubation in a

**Table 1.** Primers used for PCR amplifications

Name (bp)	Sequence (sense primer/antisense primer)
FKBP-12 (329)	5'-CCATGGGAGTGCAGGTGGAAC-3'/ 5'-AGTAAGGTCAAATCTTCGAGGTG-3'
IL-1 (812)	5'-CTATGGCAACTGTTCTGAACTC-3'/ 5'-TTAGGAAGACACAAATTGCATGGT-3'
IL-12 (652)	5'-ACATGTGTCAATCACGCTACCTCC-3'/ 5'-CTTTCAGGCGGAGCTCAGATAGCC-3'
IL-6 (638)	5'-TATGAAGTTCCTCTCTGCAAGA-3'/ 5'-CTAGGTTTGCCGACTAGATCTCAA-3'
TNF- $\alpha$ (710)	5'-CCATGAGCACAGAAAGCATGATC-3'/ 5'-TCACAGAGCAATGACTCTAAA-3'
NOS (440)	5'-TCAAAGGAGGCCGCATGAGCTTG-3'/ 5'-TCAGAGCCTCGTGGCTTTGGGCTC-3'

complete DMEM for 48 hr, the cells were grown in a complete DMEM containing G418 (600  $\mu$ g/mL) (GIBCO, U.S.A.). Stable transfectant clones were obtained by a continuous selection by G418. FKBP-12 mRNA and recombinant FKBP-12 expressions were analyzed by RT-PCR and dot-blot immunoassay.

#### Expression of FKBP-12 mRNA in the cDNA-inoculated mice

A group (n=15) of ICR mice (8W, male) were inoculated with 100  $\mu$ g of FKBP-12 cDNA in the quadriceps muscle or by intraperitoneal injection. Ten to fifty days after the inoculation, the mice were sacrificed and the macrophage activation assays (16) were carried out as described above. Tissues were collected for the in situ evaluation of FKBP-12 transcript expression. In brief, muscle pieces from the FKBP cDNA-inoculation sites, liver, spleen, kidney, and testis were cryosectioned at a 8  $\mu$ m thickness to examine the expression of FKBP-12 mRNA by the in situ RT-PCR described by Yoon et al. (17). Reverse transcription reaction was performed at 42°C for 60 min on the cryosections or macrophage slides that had been fixed in 4% paraformaldehyde, dehydrated in ethanol, and treated with protease and DNase. Further in situ RT-PCR was performed on the same slides using Omnigene DNA Thermal Cycler (Hybaid, U.K.) and the amplified cDNA was detected by the DIG DNA-labeling and detection system (Boehringer Mannheim, Germany).

Possible occurrence of anti-FKBP-12 antibody in the cDNA-inoculated mice was also estimated by enzyme-linked immunosorbent assay (ELISA) using the recombinant FKBP-12 as antigen.

## RESULTS

#### $\lambda$ gt 11-HNC clone #31

As stated in the introduction, the original aim of this investigation was to clone nephrocalcin cDNA from the human kidney cDNA libraries using a polyclonal anti-human nephrocalcin antiserum as a probe. Specificity of the antiserum was previously described (10) and the detection limit of nephrocalcin-like protein was determined using purified human nephrocalcin as the standard in the simulated plaque lifting experiments.

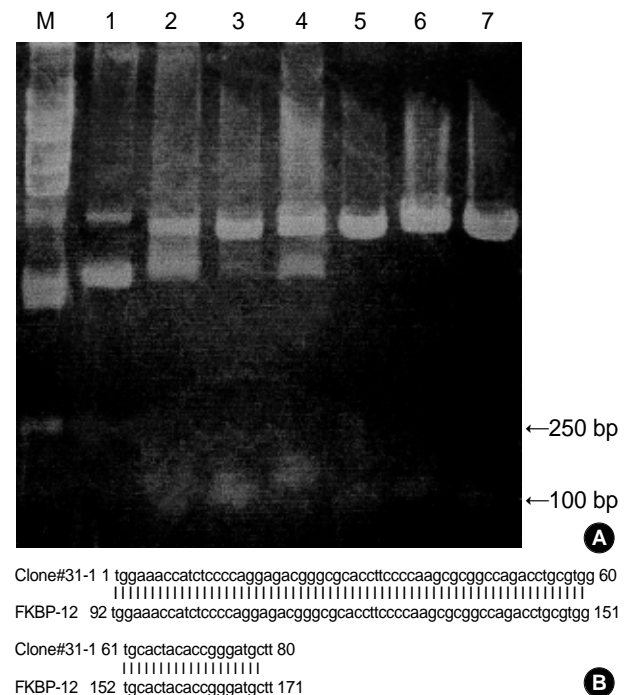
In this approach, approximately  $10^7$  primary clones were screened from human kidney cDNA libraries. Clones that seemed to express recombinant proteins reactive to anti-nephrocalcin antibody were subcloned for rescreening and plaque purification.

Over 50 clones have been scrutinized over years and only one clone designated as #31-1 showed a consistent antigenic cross-reactivity to nephrocalcin (Fig. 1A). DNA sequence

analysis of this clone (#31-1) and a homology search through GenBank database revealed that this clone encodes a partial sequence of FK506-binding protein (FKBP-12) cDNA (Fig. 1B).

#### Recombinant FKBP-12 and its inhibitory activity on calcium oxalate crystal growth

As the clone #31-1 (80 bp) appeared to be identical to the FKBP-12 cDNA in its sequence from #92 to 171 (Fig. 1B), we decided to PCR-clone the full-length FKBP-12 cDNA from a  $\lambda$ gt 11 human kidney cDNA library to confirm whether the recombinant FKBP-12 exhibited an inhibitory activity on the calcium oxalate crystal growth (Fig. 2). Thus, the FKBP-12 cDNA obtained was subcloned, after a sequence verification, into an *E. coli* expression vector, pET 28a (Novagen, U.S.A.). The induced recombinant FKBP-12 was isolated on a Ni-NTA affinity column and the protein was analyzed by a SDS/PAGE (Fig. 3A, B). A large amount of recombinant FKBP-12 was produced in the *E. coli* BL21



**Fig. 1.** Isolation and DNA sequence alignment of the clone #31-1 with FKBP-12. (A) Phage DNA  $\lambda$ gt 11 #1 and #31 subclones were PCR amplified by PCR using  $\lambda$ gt 11 forward and reverse primers and the resultant cDNAs were subcloned into pTA plasmid by the TA cloning system. Lanes: (1) #1-1, (2) #1-2, (3) #31-1, (4) #31-3, (5) #31-5, (6) #31-6, (7) #31-7 plasmid DNAs were digested by *Eco*R I and arrows indicate the position of approximately 100 and 250 bp inserts. The clone #31-1 contained FKBP-12 cDNA partial sequence as shown in (B). (B) A part of cDNA sequence of human FK506-binding protein (FKBP) reported by Maki et al. in 1990 (11) was aligned with cDNA sequence of the clone #31-1.

(DE3) with a tendency to precipitate at high concentrations. FKBP-12 and nephrocalcin share a similar molecular size and their functions are regulated by  $\text{Ca}^{++}$ . However, a comparison of amino acid composition of nephrocalcin (4, 5) and FKBP-12 (11) demonstrated that FKBP-12 is not related to nephrocalcin. Thus, unlike crude preparations, purified the recombinant FKBP-12 exhibited much reduced immunological cross-reactivity to anti-nephrocalcin antibody in a dot-blot immunoassay (Fig. 3C).

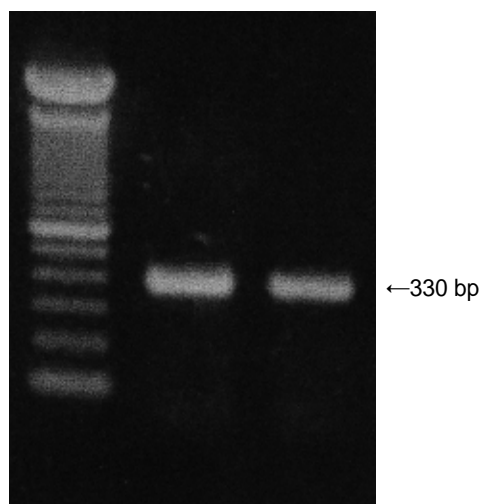


Fig. 2. FKBP-12 cDNA. A full-length FKBP-12 cDNA was cloned by PCR from a  $\lambda$ gt 11 cDNA library prepared from human adenocarcinoma of the kidney.

We compared the inhibitory activity of the recombinant FKBP-12 on the calcium oxalate crystal growth with that of nephrocalcin (Fig. 4). The affinity of nephrocalcin for calcium oxalate crystal has been reported to be between  $10^{-7}$  and  $10^{-8}$  M (4, 5). However, the affinity of nephrocalcin in this study was  $4.4 \times 10^{-7}$  M and that of FKBP-12 was the order of  $10^{-7}$  M. kDa value of uropontin for oxalate crystal was also reported to be in the range of  $\approx 10^{-7}$  (18).

#### Physiological function of extracellular FKBP

FKBP proteins were initially purified from the thymus and spleen, but the subsequent studies at mRNA and protein levels revealed that they are ubiquitously expressed throughout the body (11). Immunohistological study also indicated that nephrocalcin is widely expressed throughout the body (10), even though there is no evidence that it is a secretory protein. Although FKBP-12 is a cytoplasmic protein, it is likely that FKBP-12 is released into the body fluid as a consequence of cell turnover. Therefore it is tempting to speculate that plasma FKBP-12 might serve as an inhibitor of calcium oxalate crystal growth in vivo together with nephrocalcin and others.

We therefore decided to investigate the physiological effect of high concentrations of FKBP-12 on the macrophage activation. Mouse peritoneal the macrophages were cultured in the presence of various concentrations of the recombinant FKBP-12 and the expressions of inflammation-related gene transcripts (IL-1, TNF- $\alpha$ , IL-6, IL-12, and NOS) were examined with total RNAs from the macrophages. In comparison

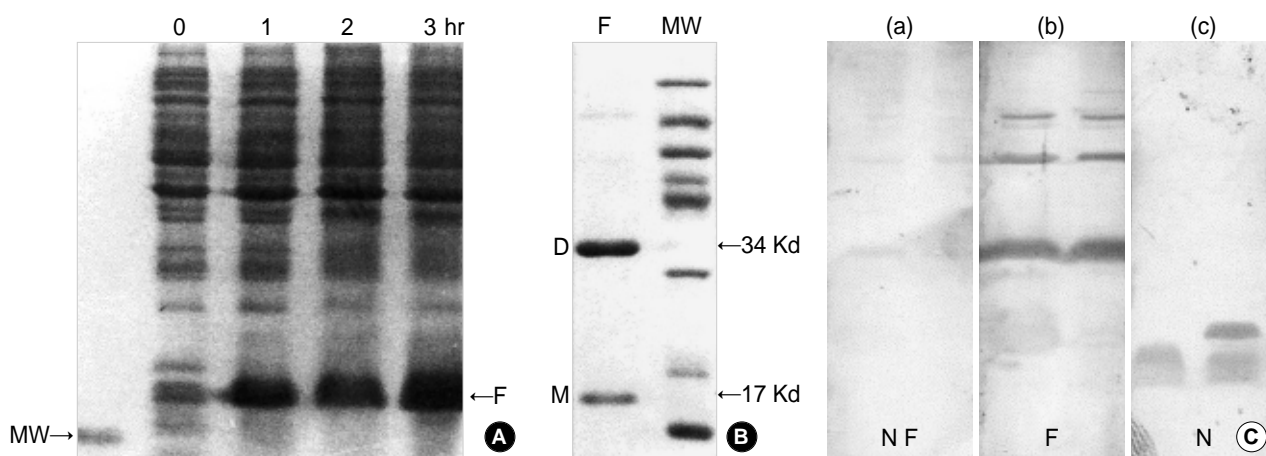


Fig. 3. Characterization of recombinant FKBP-12. (A) FKBP-12 cDNA was subcloned into an *E. coli* expression vector, pET 28a and the recombinant FKBP protein was induced by IPTG for 3 hr (0, 1, 2, and 3 hr). MW: lysozyme (14 kDa), F: recombinant FKBP-12 (17 kDa: the recombinant protein contains an extra peptide sequence that includes His-Tag, T7-Tag, and others). (B) Approximate molecular weight of the recombinant FKBP-12 was estimated by SDS-PAGE. MW: protein molecular weight marker, F: FKBP (M, 17 kDa monomer, D, 34 kDa dimer). Recombinant FKBP-12 with His-Tag sequence was purified by the Ni-NTA affinity column chromatography and the concentrated FKBP-12 exhibited a tendency to aggregate and precipitate. (C) Immunoblot of nephrocalcin (N) and recombinant FKBP-12 (F) by a rabbit anti-nephrocalcin antiserum and normal serum. (a) normal serum control (b) and (c) FKBP-12 (50  $\mu$ g) and purified nephrocalcin (1 ng N & 50 ng F) probed by anti-nephrocalcin antiserum.

with the unstimulated control, the recombinant FKBP-12 did not enhance the expression of proinflammatory cytokine genes (Fig. 5). Therefore, the possibility of macrophage activation by FKBP-12 was ruled out even at high concentrations.

#### Possible application of recombinant FKBP-12 as an anti-stone agent

If extracellular FKBP serves as an physiological inhibitor of oxalate crystal growth, a continuous release of FKBP by

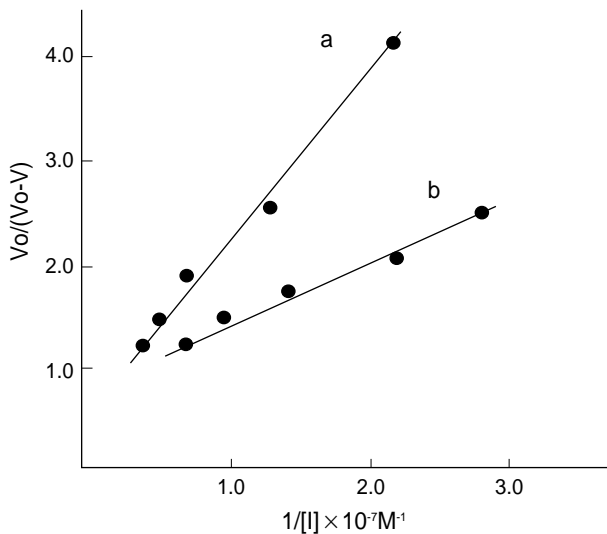


Fig. 4. Langmuir-type isotherm showing an inhibition of calcium oxalate crystal growth with increasing concentrations of (a) recombinant FKBP-12 ( $K_d=1 \times 10^{-7} M$ ) and (b) nephrocalcin ( $K_d=4.4 \times 10^{-7} M$ ).

infusion or by inoculation of FKBP cDNA might be effective in the treatment of oxalate microcrystal deposition disease. Therefore, FKBP-12 cDNA was subcloned into a mammalian expression vector, pcDNA3, to obtain a long-term continuous release model for FKBP-12. The anti-nephrocalcin antiserum used for the initial plaque screening and immunoblotting of the purified recombinant FKBP-12 recognized both nephrocalcin and FKBP-12 in the body fluid as well as in any tissues; an example is shown in Fig. 6A. However, quantitative monitoring of the recombinant FKBP-12 with anti-nephrocalcin antiserum was difficult.

Nevertheless, pcDNA3-FKBP-12-transfected mouse fibroblast culture was established as a simulation model. Releases of FKBP-12 mRNA (Fig. 6B) and the recombinant FKBP-12 (Fig. 6C) into the supernatant of transfectant cultures were demonstrated by RT-PCR and dot-blot immunoassay, respectively.

As a simulation of FKBP-12 gene therapy for the treatment of oxalate crystal deposition disease, pcDNA3-FKBP-12 DNA was inoculated to a group of mice. Ten to fifty days after the inoculation, the expression of FKBP-12 transcripts at the inoculation sites was analyzed by in situ RT-PCR (Fig. 7A). Our previous experience (17) showed that mRNA expression begins on the 10th day and lasts at least several months.

To investigate the macrophage activation by the extracellular FKBP in the pcDNA3-FKBP-12-inoculated mice, the expressions of proinflammatory gene transcripts in the peritoneal macrophages were examined by RT-PCR. As shown in Fig. 7B, FKBP-12 cDNA-inoculated mice did not show any evidence of macrophage activation or histologically abnormal finding in any tissues in this series of experiments (data not presented). Furthermore, anti-FKBP-12 antibody synthesis was not induced. Therefore, we concluded that the con-

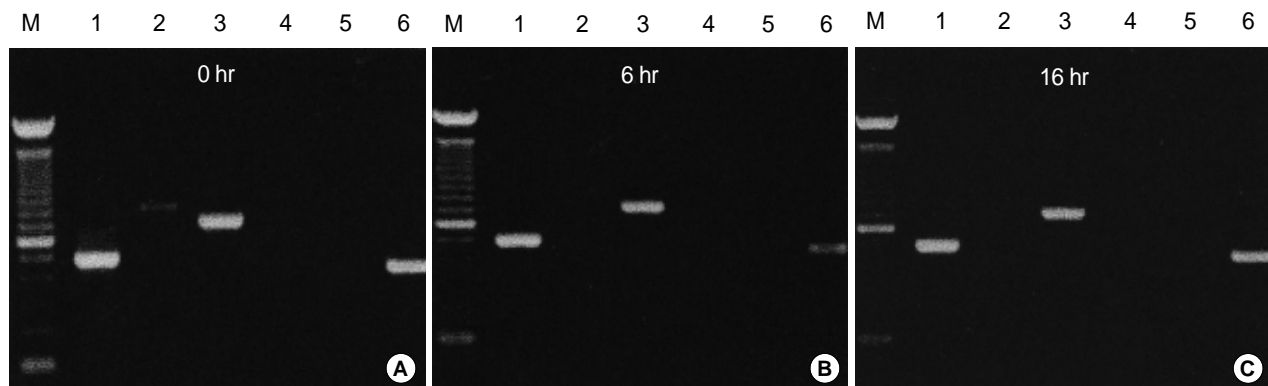


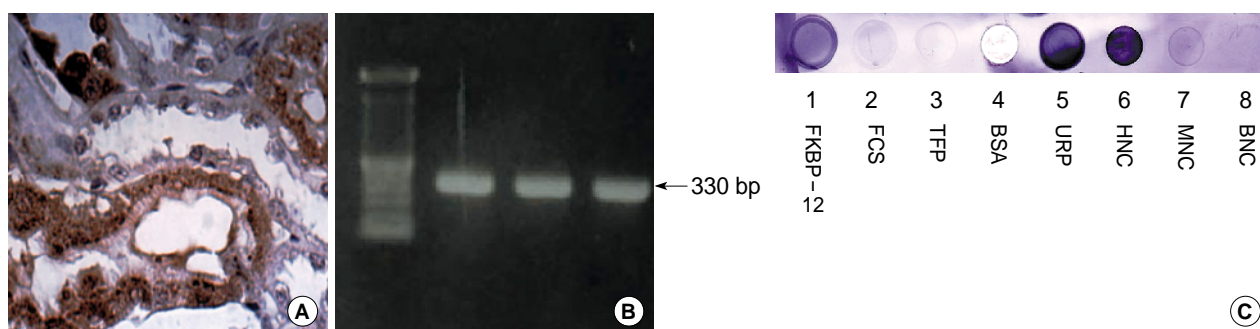
Fig. 5. Effects of extracellular FKBP-12 on the macrophage activation. (A) Mouse peritoneal macrophages were exposed to a various concentrations of the recombinant FKBP-12, and the total RNAs were prepared at the time points of 0, 6 hr and 16 hr incubation. The induction of various cytokine gene transcripts in the macrophages were analyzed by RT-PCR and agarose gel electrophoresis (A, 0 hr, B, 6 hr, C, 16 hr: cultured in the presence of rFKBP-12, M: 100 bp ladder DNA size marker, 1: GAPDH, 2: IL-1, 3: TNF- $\alpha$ , 4: IL-6, 5: IL-12, 6: NOS). cDNA gel images were quantified and compared by cDNA densities of different sampling times and no significant differences were noticed from those of control values. The gel images shown here are from a typical experiment that was repeated three times with nearly identical results.

tinuous release of FKBP-12 by infusion or the FKBP-12 cDNA therapy may be a viable option for the treatment of calcium oxalate microcrystal deposition disease.

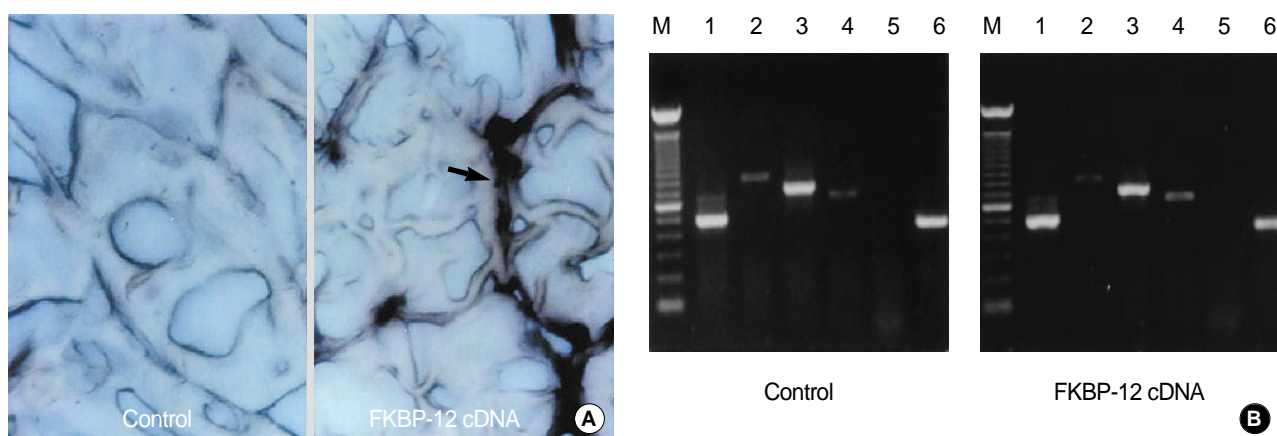
## DISCUSSION

The advent of new technologies for urinary stone removal, such as extracorporeal shock wave lithotripsy, has facilitated stone removal and reduced the morbidity associated with urinary stone diseases. Nevertheless, the recurrence rate of the

disease in the industrialized countries has never been reduced (1), and the incidence of urinary stones in Korea has been steadily increasing since the late 1970s (19). Numerous complications arising from the chronic glomerulonephritis have a common origin of calcium oxalate microcrystal deposition, which seems to trigger inflammation through an in situ complement activation. Blood calcium oxalate supersaturation has been reported in such patients and correlated with the deposition of calcium oxalate microcrystal in kidney, myocardium, blood vessels, and other tissues. The number of patients with chronic glomerulonephritis receiving peritoneal dialysis or



**Fig. 6.** Immunological detection of FKBP-12 by anti-nephrocalcin antiserum. (A) Immunohistological demonstration of FKBP-12 - as well as nephrocalcin-expressing cells in the renal tubules (Immunoperoxidase method,  $\times 400$ ). (B) Demonstration of the FKBP-12 mRNA in the pcDNA3-FKBP-12 - transfected mouse fibroblasts, 3LL, by the RT-PCR technique. (C) Demonstration of the recombinant FKBP-12 in the concentrated culture supernatants from the transfectant 3LL by the dot blot immunoassay. Each spot contained approximately 100 ng of each protein (1) recombinant FKBP-12 100-fold concentrated from the transfectant culture supernatant, (2) fetal calf serum, (3) Tamm-Horsfall protein, (4) bovine serum albumin (5) concentrated human urinary proteins from which human nephrocalcin was purified, (6) human nephrocalcin, (7) mouse nephrocalcin, (8) bovine nephrocalcin.



**Fig. 7.** FKBP-12 cDNA inoculation and macrophage activation. (A) Mice were inoculated with pcDNA3-FKBP-12 cDNA intramuscularly, and the FKBP-12 mRNA expression was demonstrated on day 10 by the in situ RT-PCR technique in the muscular thin sections at the inoculation sites (control: uninoculated muscle/ FKBP-12: 100  $\mu$ g pcDNA3-FKBP-12 cDNA inoculated muscle, arrow indicates the presence of FKBP-12 mRNA). Alkaline phosphatase staining of digoxigenin-labeled cDNA,  $\times 400$ . (B) Mice were inoculated with FKBP-12 cDNA intraperitoneally, and the peritoneal macrophages were collected 10 days after the inoculation. Total RNAs were prepared and the induction of various cytokine gene transcripts was analyzed by RT-PCR and agarose gel electrophoresis (control: macrophages from uninoculated mice, FKBP-12 cDNA: macrophages from FKBP-12 cDNA - inoculated mice, M: 100 bp ladder DNA size marker, 1: GAPDH, 2: IL-1, 3: TNF- $\alpha$ , 4: IL-6, 5: IL-12, 6: NOS). As the quantified cDNA gel image data did not reveal any significant differences from those of control values, the gel images shown here are from a typical experiment that was repeated twice with nearly identical results.

hemodialysis has been increasing (21). Furthermore, both hyperoxalemia resulting from various conditions, such as hereditary oxalate overproduction states (22), or from drugs and toxins that increase oxalate production and hyperoxaluria in various intestinal diseases or following ileal resection or jejunioileal bypass surgery have obvious potential to induce calcium oxalate crystal deposition disease.

The mechanisms of urinary stone formation and microcrystal deposition are complex and have not been fully explained yet. Therefore, efforts should be made to elucidate the mechanisms and to apply the knowledge to the reduction of urinary stone diseases, as well as to the alleviation of various syndromes arising from chronic glomerulonephritis.

According to the currently held viewpoints (3), urinary stone formation and tissue deposition of calcium oxalate microcrystals are directly related to the supersaturation of the body fluid with calcium and oxalate. Furthermore, macromolecular inhibitors of crystal growth and aggregation, such as nephrocalcin (4), osteopontin (18), and mannan-binding lectin (MBL)-associated plasma protein (23), are crucial in the pathogenesis.

Nakagawa and his collaborators' discovery of nephrocalcin (4) and their seminal works on analytical approaches (2, 5-7) stimulated much of academic interests on the urinary stone research. Their detailed analyses on the nephrocalcin isoforms and molecular abnormality of the urinary stones of nephrolithiasis (5, 6) suggested genetic background of nephrolithiasis. Furthermore, application of molecular biological techniques facilitated the identification of new inhibitors of calcium oxalate microcrystals (18, 23). Although nephrocalcin was the prototype and undoubtedly the best-studied inhibitor of calcium oxalate crystal growth under inorganic conditions, it is becoming increasingly apparent that its activity is not unique: many more proteins having similar properties will continue to be identified.

Here we described the inhibitory activity of FKBP-12 on the calcium oxalate crystal growth, which was not significantly different from that reported for nephrocalcin. Even with the best-purified preparations, nephrocalcin exhibited a heterogeneity on a TSK 2000SW HPLC column, and the dissociation constant of each peak fraction ranged from  $4.4 \times 10^{-7}$  M to  $1.0 \times 10^{-8}$  M (4-9). K<sub>d</sub> value of FKBP-12 was  $1 \times 10^{-7}$  M (Fig. 4), as it was in uropontin (18).

FKBP-12 molecule does not share any structural similarity with nephrocalcin in terms of GLA and surface-active property (6, 7), and the structural basis for the calcium oxalate crystal binding of FKBP-12 has not been elucidated yet. FKBP-12 is rich in acidic amino acids (mostly glu) in its C-terminal region, and these residues may provide an ideal electrostatic environment for Ca<sup>++</sup> binding. If the Ca<sup>++</sup> chelation is the only mechanism of calcium oxalate crystal growth inhibitory activity, the K<sub>d</sub> value of FKBP-12 for Ca<sup>++</sup> is too low for the inhibition. Thus, it seems likely that FKBP-12 inhibits the crystal growth by interacting with crystals. FKBP-12 is known

to interact with structurally unrelated diverse molecules, such as FK506, rapamycin, and many other synthetic ligands (24).

To our knowledge, FKBP-12 is the first immunophilin shown to inhibit the calcium oxalate microcrystal formation by in vitro studies. Functional studies (11, 24-28) have demonstrated that FKBP-12 is a rotamase, a receptor for immunosuppressive drugs as well as neurotrophic ligands. Structural basis for the FKBP binding to FK506 and rapamycin was elucidated by Schreiber and his collaborators (27). To explain FKBP-12 as a calcium oxalate microcrystal inhibitory molecule, structural basis for the binding of FKBP-12 to calcium oxalate should be elucidated by crystallographic studies in the future.

Calcium oxalate deposition occurs in tissues of patients with renal failure who are on chronic hemodialysis or peritoneal dialysis (21). FKBP-12 and nephrocalcin are small proteins, which can be lost from those patients through the diseased kidney or during dialysis. An infusion or continuous release of FKBP-12 by gene therapy might be effective in such clinical situations. The extracellular FKBP-12 did not appear to cause macrophage activation (Fig. 5, 7). Therefore, the FKBP-12 infusion therapy or gene therapy could be a viable option for those patients.

In conclusion, we have shown that FKBP-12 exhibited an inhibitory activity on the calcium oxalate crystal growth and that FKBP-12 may be potential therapeutic agent in clinical conditions resulting from the calcium oxalate microcrystal deposition.

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