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ORIGINAL PAPER

cDNA macroarray analysis of genes in renal epithelial cells exposed to calcium oxalate crystals

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Abstract Kidney stone formation is a complex process, and numerous genes participate in this cascade. The binding and internalization of calcium oxalate monohydrate (COM) crystals, the most common crystal in renal stones by renal epithelial cells may be a critical step leading to kidney stone formation. Exposure to COM crystals alters the expression of various genes, but previous studies on gene expression have generally been limited. To obtain more detailed insight into gene expression, we examined gene expression profiles in renal epithelial cells exposed to COM crystals using cDNA macroarray. NRK-52E cells were exposed to COM crystals for 60 and 120 min. Poly $(A)^+$ RNA was isolated and converted into ^{32}P-labeled first-strand cDNA, then the cDNA probe was hybridized to the membrane. Hybridization images were scanned and the signal intensities were quantified. Expression of mRNA of 1,176 genes was analyzed with global sum normalization methods. Exposure to COM crystals altered the expression of some of the genes reported previously. Furthermore, novel genes were also identified. Over 20 genes were found to be regulated at least twofold. We performed a large-scale analysis of gene expression in renal epithelial cells exposed to COM crystals, and identified the genes differentially regulated. cDNA macroarray is a useful tool for evaluating gene expression in urolithiasis research.

Keywords Calcium oxalate · Renal epithelial cell · cDNA array · Gene expression · Nephrolithiasis

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Introduction

Recent studies have indicated that oxalate and calcium oxalate (CaOx) crystals influence the expression of various genes, such as immediate early genes, genes associated with remodeling and repair, and genes that compose the stone matrix in renal tubular cells [1–4]. This alteration influences the attachment, growth and aggregation of calcium oxalate crystals. Kidney stone formation is a complex process and the result of a cascade of events.

Microarray analysis is an emerging technology that is ideally suited for exploring processes where the outcome is known but the mechanism leading to it are not. Gene expression profiles in the kidney of a rat CaOx urolithiasis model using microarray identified for the genes involved in the progression of urolithiasis, such as tubule function and regulation, oxidative damage, inflammation and matrix expansion-related genes [5, 6]. Both hyperoxaluria and CaOx crystal influence to gene expression in rat model, and also renal damage that affects gene expression occurred in rat model.

Crystal-cell interaction is one of the important steps on pathogenesis of urolithiasis. We studied gene expression profiles in renal tubular cells exposed to calcium oxalate monohydrate (COM) crystals in vitro to obtain a better understanding of the molecular mechanism of this step of stone formation,

Materials and methods

Cell culture and crystal preparation

Normal rat epithelial cells, NRK-52E [7], were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Dulbecco's

modified Eagle's medium supplemented with 5% fetal calf serum, 1% non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Rockville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were grown to 80% confluence for experiments.

COM crystals (Nacalai Tesque, Kyoto, Japan) were treated with ultrasound for 15 min to obtain a uniform crystal condition and suspended in serum-free medium at a final concentration of 500 μg/ml [4]. COM crystals were removed from the medium and subsequently rinsed with Hank's balanced salt solution. The cells were then exposed to a suspension of COM crystals for 60 and 120 min. Control cultures were either untreated or treated with 500 μg/ml of latex beads (Sigma, St Louis, MO, USA).

Preparation of DNA target

Total RNA was extracted using acid guanidium thiocyanate–phenol–chloroform method, and treated with an Rnase-free DNase (Qiagen, Hilden, Germany) to eliminate any contaminating DNA. The integrity of the RNA was checked on 1% denaturing formaldehyde/agarose/ethidium bromide gel. Poly A+ RNA was isolated and enriched by Streptavidin Magnetic Bead and Biotinylated Oligo(dT) using an Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA, USA), and converted into ^{32}P-labeled first-strand cDNA using MMLV reverse transcriptase. The labeled cDNA was subjected to column chromatography to exclude unincorporated ^{32}P-labeled nucleotide and small cDNA fragments.

Hybridization and scanning

We used the Atlas™ Rat Toxicology 1.2 Array (Clontech, Palo Alto, CA, USA), which includes 1176 rat cDNAs immobilized in duplicate dots on a nylon membrane. Hybridization was performed according to the manufacturer's instructions. Briefly, test hybridization was performed to check the quality of each probe by hybridizing it to a control (blank) membrane. The membrane was prehybridized for 30 min with continuous agitation at 68°C, subsequently hybridized with the probe overnight with continuous agitation at 68°C. The membrane was washed with sodium citrate and sodium dodecyl sulfate. The membrane was mounted on to Whatman paper (3 mm Chr) and exposed to an imaging plate (BAS-MP 2040S; Fuji, Kanagawa, Japan) at −70°C for 96 h and scanned with a phosphoimager (Bio-Imaging Analyzer, BAS-2500; Fuji).

Data analysis

The images were analyzed by the global sum normalization method using Atlas image analysis software (Clontech, Palo Alto, CA, USA). We performed hybridization twice independently to ensure the optimum quality of the hybridization and then, hybridization was duplicated for each sample and normalized using nine housekeeping genes.

Real-time PCR

Primers and probes were designed by the Primer Express 1.0 software program (Applied Biosystems). Primers having a melting temperature (T_m) between 58 and 60°C (optimal T_m at 60°C) and probes having a T_m 10°C over the T_m of the primers were selected. Primers and probes were designed in GAPDH gene, in osteopontin (OPN) gene and in fibronectin (FN) gene. The probes contained 6FAM as the reporter fluorophore at their 5-end and TAMRA as the quencher at their 3-end. The ABI Prism 7700 sequence detection system (Applied Biosystems) was used. Reaction volumes of 25 μl were prepared in 96-well MicroAmp optical plates with MicroAmp optical caps (Applied Biosystems). GAPDH, OPN and FN amplifications were run in separate wells. Each reaction contained 1× TaqMan buffer A, 5.5 mM $MgCl_2$, 200 M aATP, dCTP, dGTP, 400 M dUTP, 200 nM forward and reverse primers, 100 nM TaqMan probe, 0.01 U/l AmpErase UNG, 0.05 U/l AmpliTaq. Standard wells contained 250, 100, 50, 25, 10, 5 or 2.5 ng of DNA, and test sample wells contained 25 ng of DNA. No template control wells without DNA were included as negative controls. Each test sample and standard was run in triplicate. The runs were monitored via the Sequence Detection Software 1.5. For each reaction, a C_t value, which is the number of cycles necessary to reach the threshold, was identified. The GAPDH gene was used to standardize the level of OPN and FN gene.

Results

Gene expression profiles

The cDNA array is a positively charged membrane on which the DNA fragments representing nine housekeeping genes, and negative control sequences were spotted in duplicate dots. Exposure to COM crystals altered the expression of many genes in the renal tubular cells as shown in Fig. 1.

We selected genes that differed in the expression level by more than twofold. The expression of genes for 35 proteins were altered: annexin V, p53-binding mouse double min 2 homolog, OPN, FN, steroidogenic acute regulatory protein, clusterin, coagulation factor II (thrombin) receptor, mitogen-activated protein kinase 3, cathepsin B, cathepsin L, plasminogen activator inhibitor 1, cytokeratin 8, vimentin, hydrophobic surfactant associated

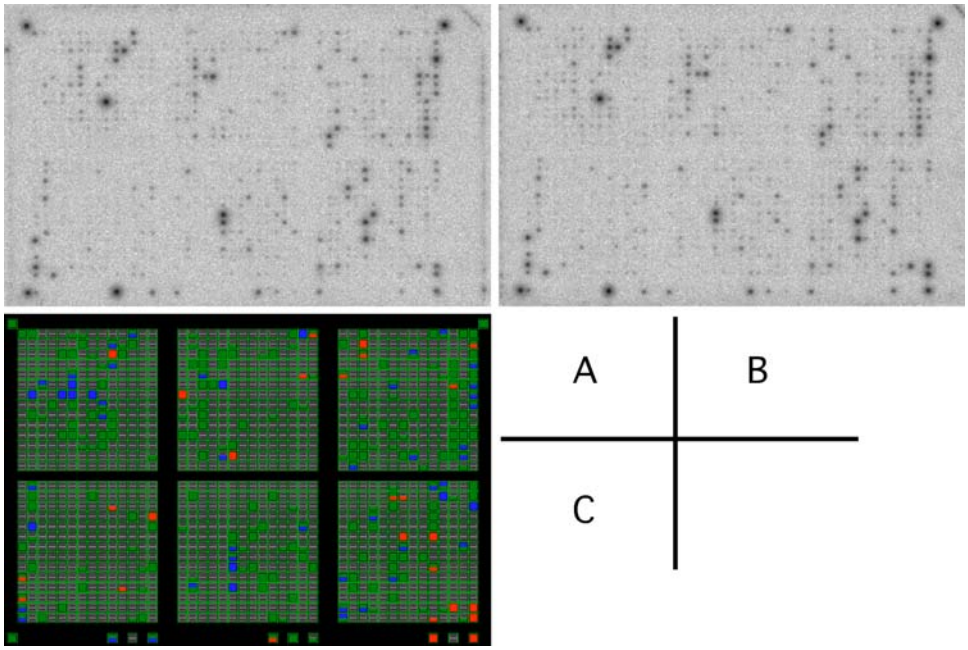

Fig. 1 Expression pattern of genes in control renal epithelial cells (**a**) and renal epithelial cells exposed to COM crystals (**b**) (NRK-52E). Differential cDNA hybridization to Atlas cDNA expression arrays was performed as described in "Materials and methods". Upregulation is shown in *red*, downregulation is in *blue*, equal expression is in *green* and background level is in *gray* on array image (**c**)

protein C, inhibitor of DNA binding 1, 7-dehydrocholesterol reductase, cytochrome P-450 2C23, myeloid cell differentiation protein 1, high mobility group protein 2, 5-hydroxytryptamine receptor 5B receptor, cytochrome c expressed in somatic tissues, connective tissue growth factor, tissue inhibitor of metalloproteinase 1, V(D)J recombination activating protein 1, Tclone15, thymosin beta 10, junD proto-oncogene, inhibitor of DNA binding 2, inhibitor of DNA-binding protein 3, G1/S-specific cyclin D1, collagen, insulin-like growth-factor-binding protein 2, insulin-like growth factor-binding protein 6, profilin 1 and metallothionein 1. Twenty genes had altered expression after a 60-min exposure to COM crystals. Thirteen genes were upregulated and seven genes were downregulated (Table 1). Nineteen genes had altered expression after a 120-min exposure to COM crystals. Nine genes were upregulated and 10 genes were downregulated (Table 2).

Real-time PCR

To validate the differential gene expression revealed by cDNA macroarray-based profiling, real-time PCR analysis was subjected to OPN gene and FN gene. This analysis confirmed the accuracy of the results on transcriptional regulation obtained from cDNA macroarray screening for OPN. Real-time PCR analysis showed that OPN gene was upregulated after 60 and 120 min exposure to COM crystals. FN gene increased both after 60 and 120 min exposure to COM crystals by real-time PCR (Fig. 2); however, macroarray detect upregulation of FN gene only after 60 min exposure.

Discussion

Recent studies have indicated that oxalate and CaOx crystals influence the expression of several genes in renal tubular cells, and that this influences the attachment, growth and aggregation of CaOx crystals. The change in mRNA expression does not always correlate well to the corresponding protein expression owing to in vivo translation efficacy. Displaying the differential mRNA expression is an important approach in kidney stone research. Kidney stone formation is a complex process, which involves many genes. Thus, a systematic approach for simultaneously analyzing large numbers of genes is required.

Katsuma et al. [5] have reported the gene expression profiles in the kidney of a rat CaOx nephrolithiasis model, using cDNA microarrays and identified that major groups of genes involved in inflammatory and fibrotic processes were markedly upregulated in nephrolithiasis. Chen et al. [6] have reported microarray analysis of changes in renal phenotype in the ethylene glycol rat model of urolithiasis. They resulted that changes in the expression of genes associated with tubule function and regulation, oxidative damage, and inflammation were the most common in the functional categories. Changes in the expression of tubule-specific markers indicated that there was damage to the proximal and distal tubules at 2 and 4 weeks. Increased expression of mitochondrial uncoupling protein indicated that there were changes to the mitochondria and oxidative stress at 2 and 4 weeks.

We analyzed the gene expression profile in renal tubular cells exposed to COM crystals to determine the relationship between renal tubular cells and COM crystals, because an

Table 1 List of representative genes differentially expressed in NRK-52E exposed to COM crystals for 60 min

Gene name	Genbank no.	Fold change	
		Up	Down
Annexin V (ANX5); lipocortin 5; placental anticoagulant protein I (PAP-I); Endonexin II; calphobindin I (CBP-I); PP4; thromboplastin inhibitor; vascular anticoagulant alpha (VAC-alpha); anchorin CII	M21730 J03898; J03899	2.1	
p53-binding mouse double minute 2 homolog (MDM2) (rat homolog of human)	Z12020; M92424 (human)	2.3	
Osteopontin	M14656	2.1	
Fibronectin	X05834	2.3	
Steroidogenic acute regulatory protein	AB001349	2	
Clusterin (CLU); testosterone-repressed prostate message 2 (TRPM2); apolipoprotein J (APOJ); sulfated glycoprotein 2 (SGP2); dimeric acid	M64723	2.9	
Coagulation factor II (thrombin) receptor (CF2R); thrombin receptor	M81642	2.2	
Mitogen-activated protein kinase 3 (MAP kinase 3; MAPK3; PRKM3); MAPK1; MNK1; extracellular signal-regulated kinase 1 (ERK1); ERT2; insulin-stimulated microtubule-associated protein 2 kinase (MAP2 kinase)	M61177	2	
Cathepsin B	X82396	2.1	
Cathepsin L	Y00697	2.7	
Plasminogen activator inhibitor 1 (PAll; PLANH1)	M24067	3.9	
Cytokeratin 8 (CK8)	M63482	2.1	
Vimentin (VIM)	X62952	2	
Hydrophobic surfactant associated protein C	Xl4221		2.3
Inhibitor of DNA binding 1 (ID1)	Dl0862		3.1
7-dehydrocholesterol reductase (DHCR7)	AB016800		2
Cytochrome P-450 2C23, arachidonic acid epoxygenase	X55446		2
Myeloid cell differentiation protein 1	AF115380		2.1
High mobility group protein 2 (HMG2)	D84418		2.6
5-hydroxytryptamine receptor SB receptor (HTR5B); serotonin receptor	L10073		2

inflammatory reaction was induced in the renal interstitium of the EG model of CaOx nephrolithiasis [8, 9]. Ebisuno [10] reported that COM crystals adhered to MDCK cells in a time-dependent manner, with plateauing at 2 min. CaOx crystals were attached to the surface of NRK-52E cells [11]. Bikunin has been identified in the organic matrices of kidney stones and calcific crystals produced in the human urine and CaOx crystallization inhibitory activities, and its mRNA expression was significantly increased after 2 h oxalate exposed in MDCK cells [12]. Immediate early genes mRNA increased after 1, 2 and 4 h after oxalate exposure in MDCK cells [13]. Exposure to LLC-PK1 cells to COM crystals stimulated strong phosphorylation and activation of p38 mitogen-activated protein kinase (p38 MAP kinase) and re-initiation of DNA synthesis within 30 min, and inhibition of COM crystal binding to the cells by heparin blocked the effect of COM crystals on p38 MAPK activation [14]. These findings suggest that it is rationale for selecting 60 and 120 min as the exposure times in this study. OPN is the only gene that was upregulated both after 60 and 120 min, and some appear after 60 min but not after 120 min, on the other hand some not appear 60 min but appear after 120 min. It seems upon gene response to time dependence and pitfalls with macroarray. FN did not increase after 120 min in macroarray. In contrast, it increased in real-time PCR. Currently even the most ardent proponents of microarray view the technique as an adjunct rather than as a definitive technique. However, microarray can be used to provide a base for developing hypotheses [6].

In tissue culture studies renal epithelial cells are generally exposed to the synthetic crystals, which are devoid of any organic matrix. However, in vitro crystals produced in the kidneys are coated with macromolecules and contain an organic matrix that block adhesion to renal epithelial cells [15] and recent study has identified naturally produced crystals are less injurious to renal epithelial cells [16]. In the future, it will be of interest to compare the effect of synthetic and naturally produced biocrystals on gene expression in renal epithelial cells.

The genes of the extracellular matrix were upregulated in renal tubular cells after exposure to COM crystals. Involvement of the extracellular matrix in the mechanisms of COM crystal adhesion to the cell surface has been

Table 2 List of representative genes differentially expressed in NRK-52E exposed to COM crystals for 120 min

Gene name	Genbank no.	Fold change	
		Up	Down
Osteopontin	M14656	3.3	
Nucleoside diphosphate kinase A (NDP kinase A; NDKA); tumor	Dl3374	2	
Cytochrome c expressed in somatic tissues (CYCSA)	M20622	2.2	
Connective tissue growth factor (CTGF)	AF120275	2.1	
Tissue inhibitor of metalloproteinase 1 (TIMP1)	L31883	2	
Cytokeratin 8 (CK8)	M63482	2	
V(D)J recombination activating protein 1 (RAG1)	AJ006070	2.2	
Tclonel5	U31867	3.2	
Thymosin beta 10 (TMSB1O; THYB1O); PTMB1O	M17698	3.6	
junD proto-oncogene	D26307		2
Inhibitor of DNA binding 2 (1D2)	Dl0863		5.8
Inhibitor of DNA-binding protein 3 (1D3)	Dl0864		2.8
G1/S-specificcyclinDl (CCND1)	Dl4014		2.3
Collagen, alpha 1, type 3	M21354		6
High mobility group protein 2 (HMG2)	D84418		2.1
Insulin-like growth-factor-binding protein 2 (IGF-binding protein 2)	J04486		2.5
Insulin-like growth factor-binding protein 6 (IGF-binding protein 6)	M69055		2.6
Profilin 1 (PFN1)	X96967		2
Metallothionein 1 (MT1)	J00750		3

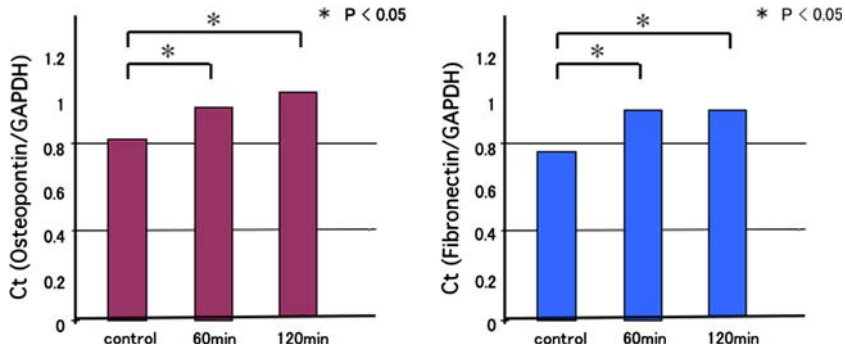

Fig. 2 Real-time PCR of osteopontin and fibronectin ware performed by using ABI Prism 7700 sequence detection system. The Ct ratio of osteopontin and fibronectin were standardized using the GAPDH gene

clearly reported [10]. OPN and FN, which are components of the extracellular matrix are particularly associated with kidney stone formation. Expression of OPN mRNA was increased in the renal distal tubular cells of stone-forming rats [17], and it was also enhanced in monkey renal epithelial cells (BSC-1 line) and in canine renal epithelial cells (MDCK line) after exposure to COM crystals [18]. The production of OPN is gradually increased in response to exposure of renal epithelial cells to oxalate and calcium oxalate crystals [19]. The expression of the OPN gene was increased the most in this study. FN is distributed throughout the extracellular matrix. FN expression is found on the renal tubules in stone-forming rat kidneys, and its content of MDCK cells increase in proportion to the concentration of CaOx crystals added to the culture medium at the protein level [20]. The FN mRNA was also upregulated in this study. It is of interest that any other urinary macromolecules such as Tamm-Horsfall protein [21], bikunin [22], urinary prothrombin fragment 1 (UPTF1) [23] which have already been documented to play some role in calculogenesis and changed their gene expression in rat CaOx nephrolithiasis model identified in microarray. Unfortunately, they do not include in this array.

However, the cDNA expression array technology revealed the altered expression of some novel genes in the renal epithelial cells exposed to COM crystals. The gene encoding annexin V is an upregulated gene. Annexins are Ca^{2+} and phospholipid-binding proteins. The annexin family is composed of two domains: the divergent NH_2-terminal "head" and the conserved COOH-terminal protein core that mediated the Ca^{2+} and membrane-binding properties. Annexins form a highly α-helical and tightly packed disk with a slight curvature and two principle sides. The more convex side contains a Ca^{2+} binding

site, and faces the membrane when an annexin is associated with phospholipids [24], Annexin V blocked COM attachment following exposure of inner medullary collecting duct cells to phosphatidylserine [25]. Kumar et al. [26] isolated potential COM crystal-binding proteins on the surface of renal tubular cells, and identified it as annexin II. Therefore, the annexin family is an interesting molecule.

Expressions of cathepsins B and L mRNA are increased by two- to threefold following exposure of NRK-52E cells to COM crystals in this study. There is considerable evidence that proteases may play an active role in stone formation and experimental nephrocalcinosis and in crystal-cell attachment. The activities of a range of proteases, including cathepsins B, cathepsin L, and cathepsin D, occur in the rat kidney [27] cathepsins B, H, L, and D are also present in human kidney and in confluent primary cultures of epithelia from proximal, collecting, and thick ascending limb tubules [28]. Recent study showed that intracrystalline proteins would increase intracellular proteolytic disruption and dissolution of CaOx crystals, and intracrystalline proteins promote the degradation and dissolution of urinary CaOx crystals in cultured renal epithelial cells in a dose-dependent fashion [29, 30]. These findings suggest that that intracrystalline proteins may defend against stone formation by facilitating the degradation and destruction of crystals retained within the kidney.

We found several genes encoding a diverse class of proteins that were downregulated. There are few reports that prove decreased mRNA expression related to COM crystals and CaOx urolithiasis [21]. This study showed not only upregulated genes but also downregulated genes that have not been observed in previous study. Some of these genes, such as OPN, FN, cathepsins B and L and mitogen-activated protein kinase relate to pathogenesis of stone formation. However, the role is unknown between many genes and calculogenesis. Further studies on their expression will be necessary to confirm their precise role in renal tubular cells exposed to COM crystals and subsequent development of kidney stones.

Conclusions

We demonstrated that the cDNA array technique is an effective tool for monitoring the profile of gene expression in renal tubular cells exposed COM crystals. This method will help to reveal the molecular mechanism of kidney stone formation.

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