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ORIGINAL PAPER

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Effects of oxalate exposure on Madin-Darby canine kidney cells in culture: renal prothrombin fragment-1 mRNA expression

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Abstract It has been suggested that renal tubular cell damage induced by oxalic acid, one of the components of urinary calculi, may be involved in a variety of ways in the development of urolithiasis. During our study on a calculus related protein, renal prothrombin fragment-1 (RPTF-1), we noted that this is an inflammation related substance that mediates an acute inflammatory reaction, one of the original roles of prothrombin. RPTF-1 is a part of prothrombin that is a coagulation factor known to be expressed in the renal tubule. We examined whether oxalic acid may cause cytotoxic effects on tubular epithelial cells and whether such chemical stimulation may promote the translation of RPTF-1 mRNA into RPTF-1 proteins. We used Madin-Darby canine kidney (MDCK) cells derived from the distal tubule of a dog kidney. In this study, the effects of oxalic acid in culture solution at different concentrations on cytotoxicity were assessed using a MTT assay. The location of active oxygen species was identified using dichlorofluorescein diacetate. After the prothrombin sequence of RPTF-1 was confirmed in MDCK cells, RPTF-1 mRNA expression was determined by RT-PCR. The gene sequence of the same promoter area was ligated, and a luciferase sequence was inserted downstream of the vector. The target sequence was transfected into MDCK cells and the relation between oxalic acid and prothrombin promoter was examined. In addition, the variable expression of RPTF-1 mRNA was quantitatively compared depending on oxalic acid concentrations using real-time PCR. When cytotoxicity was investigated, cells were not damaged but, by contrast, were stimulated and activated under oxalic acid below a certain concentration. The relation between cytotoxicity on the cultured MDCK cell membrane and active oxygen species was confirmed. Luminescence in MDCK cells containing the luciferase gene was detected by the addition of oxalic acid, which activated the prothrombin promoter. A part of the prothrombin gene sequence in the MDCK cells was detected and an increase in the expression of RPTF-1 mRNA in MDCK cells by the addition of oxalic acid was confirmed using real-time PCR. Increased expression of prothrombin by adding oxalic acid has already been demonstrated in previous studies. In this study, however, RPTF-1 mRNA was promoted by oxalic acid and a direct association between oxalic acid and RPTF-1 is indicated. This finding shows that increased oxalic acid in urine induces the expression of RPTF-1 in tubular epithelial cells and thereby causes the generation of active oxygen species.

Keyword Prothrombin · RPTF-1 · Urolithiasis · MDCK · CaOx

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Introduction

Recently, certain proteins have been examined for their association with urolithiasis. Much attention has been paid to the fact that the original roles of these proteins are biological functions, such as being involved in a defense mechanisms and inflammation. Recent studies have demonstrated that active oxygen species play important roles as mediators of signal transmission, such as modulation of nuclear factor kappa B (NFκB) activation and activation protein-1 (AP-1) activation, guanylate cyclase activation by nitric oxide (NO), and modulation of oxygen in respiration. The effects of oxalic acid crystals on tubular epithelial cells are likely to act on calculus-related proteins along these signal transmission pathways. There are various environments surrounding tubular epithelial cells. In the process of urinary calculus formation, the state of urine on the surface of tubular epithelial cells is quite different from

the normal state [1]. Therefore, various calculi are formed depending on the various substances present and their concentrations. Above all, oxalic acid is the most common component of urinary calculi and constitutes the majority of clinically detected urinary calculi. In the early stage of urinary calculus formation, the concentration of oxalic acid in the urine reaches saturation, and the cell membrane is in direct contact with this solution. Such chemical stimulation may become a chemical mediator involved in the expression of active oxygen species or other substances. The effects of oxalic acid on rat kidney cells have been reported by Khan and Hackett [2], and the cytotoxicity of oxalic acid and its relation to active oxygen species have been studied using pig tubule epithelial cells by Scheid et al. [3]. Hackett et al. [4] examined the degree of the cytotoxic effects of potassium oxalate on tubule epithelial cells by determining the enzymes released from the cells. To date, a specific relation between the concentration of oxalic acid and cytotoxicity, and urolithiasis or linkage to active oxygen species remains unclear. Our study demonstrates the reaction of tubule epithelial cells with oxalic acid on the cell membrane, concurrent promotion and expression of a calculus-related protein, RPTF-1, and the existence of active oxygen species. This study may provide an important foothold for the identification of RPTF-1 or signal translation of active oxygen species in the process of urinary calculus formation.

Methods and materials

Cell culture

MDCK (CCL-34) cells purchased from the American Type Culture Collection. Antibiotic free D-MEM (Sigma, Mo., USA) supplemented with fetal bovine serum (FBS, Gibco BRL, N.Y., USA) to a final concentration of 5% were used, and the cells were cultured in 5% CO_2 at 37°C. The culture solution was changed to D-MEM without FBS when experiments under various conditions were conducted.

MTT assay

Cells were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl culture medium per well according to the medium needed by the cells, in a humidified atmosphere (37°C 6.5% CO_2). For most experimental setups, the incubation of cells for 24–96 h was appropriate. After the incubation period, 10 µl of the MTT labeling reagent was added (Roche, Mannheim, Germany) to each well. The microplates were incubated for 4 h in a humidified atmosphere (37°C 6.5% CO_2). A total of 100 µl of the solubilization solution was added to each well. The plates were again incubated in a humidified atmosphere (37°C 6.5% CO_2). Absorbance of the samples was determined using a microplate reader (BioRad, USA). The wavelength to measure the absorbance of the formazan product lay between 550 and 600 nm.

Determination of active oxygen species

DCF assay

Cultured cells were washed with colorless D-MEM and 5 µM dichlorofluorescein diacetate (DCF: DCFH-DA, Eastman Kodak) was added to the cells, which were then incubated for 20 min at 37°C. The cells were washed again with colorless D-MEM. Control solutions of 0.5 mM or 5.0 mM oxalic acid were added to the cells, which were incubated for 20 min at 37°C. Reverse fluorescent microscopy (Olympus BX50, Japan) (wavelength: 460–490 nm; filter 515 nm, WIB) was used for observation.

Reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing

The solutions in the culture dishes were removed, 500 µl of TRIZOL (Gibco BRL, N.Y., USA) was added directly to the MDCK cells, and the total RNA was extracted by the acid guanidinium phenol chloroform method. The total RNA was reverse transcribed into cDNA using a RT-PCR kit (Thermo Script RT-PCR System, Gibco BRL). A primer was prepared using a previously reported human prothrombin gene sequence [5], and PCR was then performed using this primer. Thermal cycling conditions were 2 min at 94°C, 30 cycles of 20 s at 94°C, 20 s at 58°C, 20 s at 72°C, followed by 90 s extension at 72°C. MDCK cells were amplified using a primer pair prepared simultaneously with the sequencer, and the prothrombin gene sequence in MDCK cells was confirmed.

Luciferase reporter assay

A 3′ primer containing a start codon from the open reading frame and Hind III site and a primer containing Xho I sites, located 726 bp and 1,346 bp upstream, respectively, were used from a previously reported human prothrombin fragment 1 sequence, and cDNA was prepared and ligated into the promega pGL3 vector containing the luciferase coding region and the multicloning site. The insert orientation was checked in LB + AMP agar medium. The resultant colonies were cultured, and the target gene was purified using cesium chloride density gradient ultracentrifugation and transfected into MDCK cells using Qiagen Effectene Transfection Reagent. Dual-Luciferase Reporter Assay System (Promega) and *Renilla reniformis* luciferase served as an internal control. The intensity of *Photinus pyralis* luciferase, used as the test reporter, was compared between the control group and the oxalic acid group (0.5 mM for 30 min).

Real time PCR

Assays were performed on a Smart Cycler (Cepheid, Sunnyvale, Calif., USA) and analyzed with SmartCycler software (version 1.2b) using the SYBR Green I (TaKaRa, Shiga, Japan). The RPTF-1 forward primer sequence was 5′ cagaacatgttgtcagtgatgcg 3′, and its reverse primer sequence was 5′ ctccagaaccaggtatgagcga 3′ (positions 360–488). The GAPDH forward primer sequence was 5′ aagaaggtggtgaagcaggc 3′, and its reverse primer sequence was 5′ tccaccaccctgttgctgta 3′ (positions 828–1,020). Measurement were made at the end of the 72°C extension step in each cycle, and the second-derivative method was used to calculate the threshold cycle. Melt curve analysis showed a single sharp peak for all samples. The standard lines for GAPDH and the samples were made by a series of five time dilutions (10^0, 10^1, 10^2, 10^3, 10^4 and 10^5) of the cDNA. Samples were exposed to 0.5 mM oxalic acid for 120 min. As for the fluorescence intensity of the series of dilutions for real time PCR reaction, all the samples were assayed in triplicate and simultaneously superimposed on each other.

Results

MTT assay

Cells exposed to oxalic acid (0.1 mM, 0.45 mM, and 1.35 mM) for 1, 4 and 8 h were investigated. In the case of 0.1 mM oxalic acid, no significant decrease in cell activity was observed, although a slight increase in cell activity was shown compared to the control. In the case of 0.45 mM oxalic acid, cell activity decreased to $<95\%$ after 1 h and $<90\%$ after 4 h, as compared with the control group. Similarly, in the case of 1.35 mM oxalic acid, cell activity decreased more significantly with time; a decrease to 90% after 1 h and to $\leq 80\%$ after 4 h (Fig. 1).

DCF assay

After oxalic acid was added to the MDCK cells, the expression of active oxygen species due to the effects of oxalic acid on tubular epithelial cells was examined. As a result, after exposure to 0.5–5.0 mM sodium oxalate for 20 min, DCF in the cells reacted with active oxygen species, and fluorescence was observed under a fluorescence microscope. When the higher concentration of oxalic acid was added, the number of labeled cells within the field of view increased. Based on these results, the expression of active oxygen species in the cell membranes was confirmed. The amount of fluorescence in the presence of active oxygen species increased depending on the concentration of oxalic acid (Fig. 2).

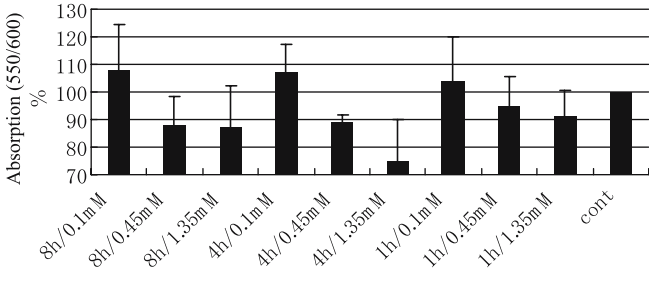

Fig. 1 Examination of the degree of cytotoxicity using an MTT assay: the cells were exposed to oxalic acid (0.1 mM, 0.45 mM, and 1.35 mM) for 1 , 4, and 8 h

Reverse transcriptase polymerase chain reaction and sequencing

The total RNA from the cultured MDCK cells was reverse transcribed into a cDNA fragment using a primer designed from the gene sequence of human prothrombin. The cDNA fragment was amplified using PCR, and the expression of prothrombin was assessed electrophoretically (Fig. 2). MDCK cells are tubular epithelial cells from a dog. The expression of prothrombin in canine tubular epithelial cells, and thereby the existence of a renal prothrombin, RPTF-1, in dogs as well as humans, were shown by this result. This is the first time that a part of the gene sequence of prothrombin (RPTF-1) has been detected in MDCK cells (Fig. 3). Previously reported sequences of prothrombin were compared with that in MDCK cells. As a result, dog prothrombin showed 441/495 (89%) homology to human prothrombin, and 322/376 (85%) homology to rat prothrombin.

Luciferase reporter assay and real-time PCR

After oxalic acid was added to the MDCK cells containing the luciferase gene, fluorescence was detected and prothrombin promoter was activated (Fig. 4). Based on this result, RPTF-1 mRNA expression levels in MDCK cells were measured using real-time PCR. As a result, RPTF-1 mRNA expression was increased in the oxalic acid group compared with the control group (Fig. 5). However, when the oxalic acid concentration exceeded 0.9 mM, the expression decreased. The above result of the MTT assay also shows that oxalic acid may activate cells rather than induce cell damage up to 0.9 mM.

Discussion

The effects of urolithiasis on tubular epithelial cells can be classified broadly into chemical and physical components. Oxalic acid-induced cytotoxicity was revealed in the rat model of hyperoxaluria and cultured MDCK cells by determining enzymes released from the

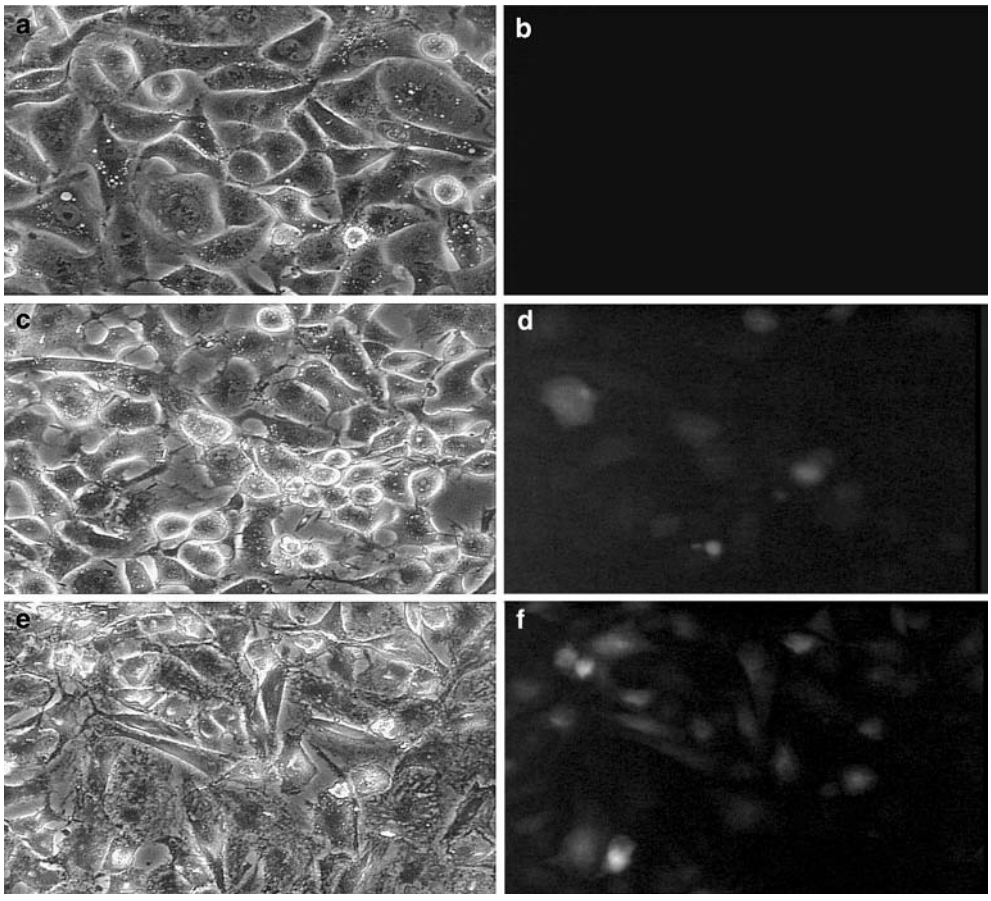

Fig. 2 DCF assay on MDCK cells after the addition of oxalic acid: **A**, **B** Controls; **C**, **D** exposure to 0.5 mM sodium oxalate for 20 min; **E**, **F** 5.0 mM sodium oxalate for 20 min. **B**, **D**, and **F** are inverted fluorescence micrographs. Fluorescence enhancement is observed in the cells of **D** and **F**

cells [2, 6]. When 0.1 mM calcium oxalate crystal was exposed to the cells for approximately 120 min, a significant amount of released enzymes was determined [7]. These cytotoxic effects on the lipid layer in the cell membrane may cause an increase in adhesion molecules, and subsequent crystal adhesion to the cells begins [8]. In other words, the findings show that the chemical effects or cytotoxicity of calcium oxalate, the most common component of urinary calculi, are involved in the formation of such calculi. On the other hand, a study using LLC-PK1 cells and others has shown the generation of active oxygen species, as well as the increased expression of c-jun and FAK in cell membranes [9], indicating that the stimulation is transmitted in both cell membrane and cytoplasm. We initially examined the location of RPTF-1 in the cultured cells. This was extracted from oxalic acid crystals in urine by Suzuki et al. [10]. RPTF-1 is a protein with inhibitory activity on crystal aggregation [11] which has drawn attention as an inhibitor of urinary calculus aggregation. The location of RPTF-1 has been immunohistochemically detected in human renal tubular cells [12], and the expression of RPTF-1 mRNA has also been detected in humans and rats [13]. In fact, the addition of oxalic acid causes an increase in the expression of RPTF-1 mRNA in rat renal tissue [14], as well as an increase in the amount of other calculus-related proteins such as inter-alpha-inhibitor related protein, which is excreted in urine in a rat model of hyperoxaluria [15, 16]. On the other hand, in terms of chemical hypoxic injury, the expression of active oxygen species has been identified in LLC-PK1 cells [17]. These findings suggest that oxalic acid-induced cytotoxic effects occur strongly in cell membranes, leading to calculus formation at the injury site on the lipid layer. In addition, RPTF-1 which is thought be a calculus-related protein playing a central role in calculus formation, is located at the above site, is generated through cellular stimulation prior to oxalic acid-induced cytotoxicity, and is strongly expressed by the addition of oxalic acid. Active oxygen species include singlet oxygen (superoxide, formed from a one-electron reduction of O_2), hydrogen peroxide (H_2O_2) (generated from a two-electron reduction due to the dismutation of superoxide), and the hydroxyl radical (generated by the reaction of hydrogen peroxide). Broadly, others such as the peroxide radical, alkoxy radical, hydroperoxide, and nitric oxide are considered active oxygen species. Involvement of active oxygen species in signal transmission includes the inhibitory effects of thioredoxin on NF-κB and AP-1. Moreover, active oxygen species are generated through mitochondrial respiratory activity, and the neutrophil NADPH oxidase and cytokines are thought to be important factors in the signal transmission system as well as cytotoxic factors [18, 19, 20].

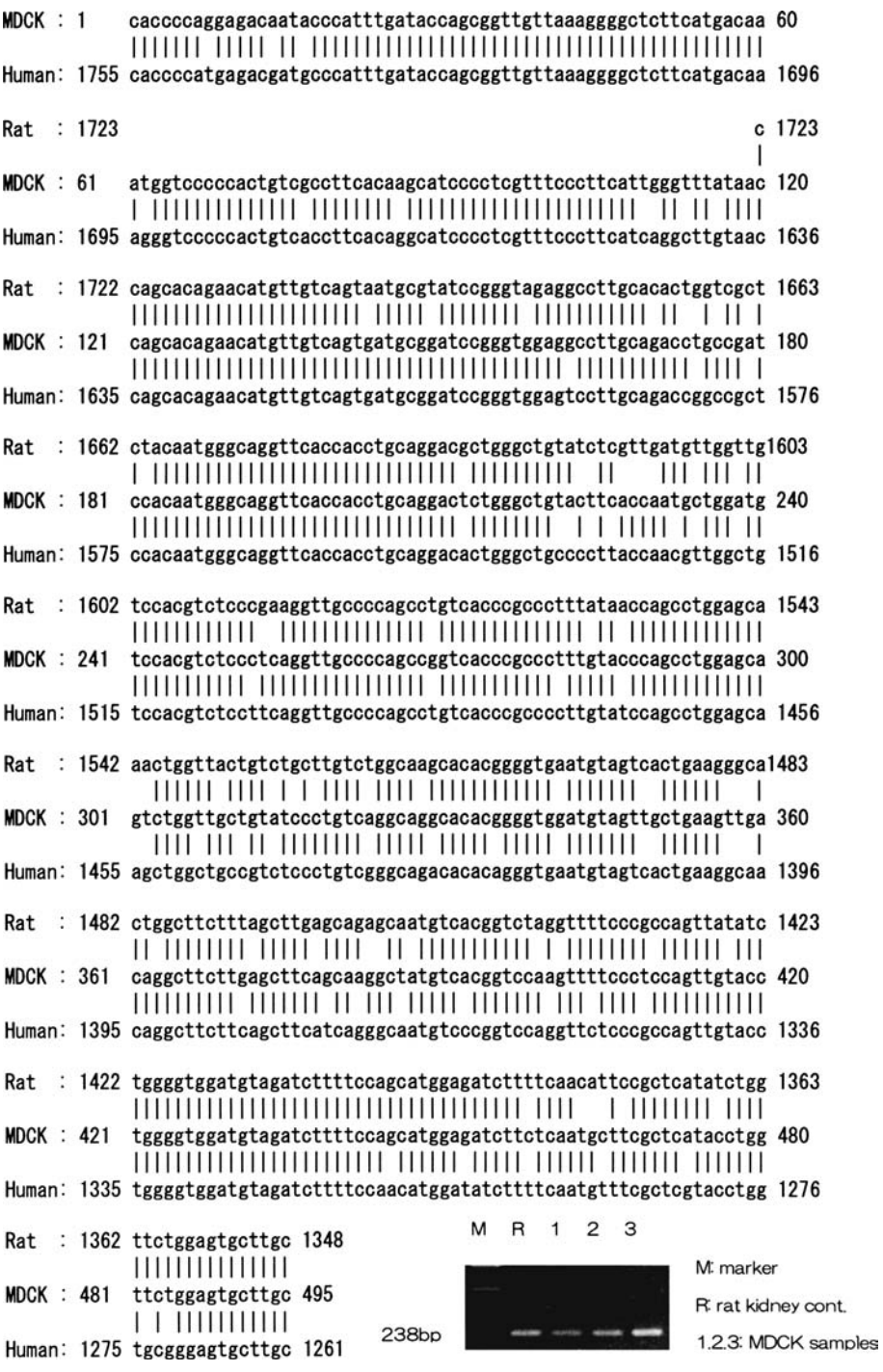

Fig. 3 Prothrombin gene sequence in MDCK cells. Previously reported rat and human gene sequences at the same site are also shown. The image in the *lower right* shows an electropherograph of cDNA

As oxalic acid concentration increases in the tubule lumen in the process of urinary calculus formation, chemical stimulation is produced on the cell membranes and active oxygen species induce cytotoxic effects on these membranes. As a result, ascorbic acid may be produced, transmit signals inside the cells, and the system producing RPTF-1 is started. Now, what is the function of RPTF-1, a calculus-related protein, on cell membranes? RPTF-1 is involved in the successive cascade of cell simulation from a very early phase and remains in crystal, as shown in this study. This protein is involved in the mechanism of urinary calculus formation and the inhibitory mechanism of urinary calculus aggregation after calculus formation. It is thought to be a very important protein for the elucidation of the urinary calculus formation mechanism.

For the purpose of investigating calculus-related proteins in MDCK cells, the expression of prothrombin and bikunin, which is known to exist in cultured rat cells [16] and human renal tubular cells, was examined. In this

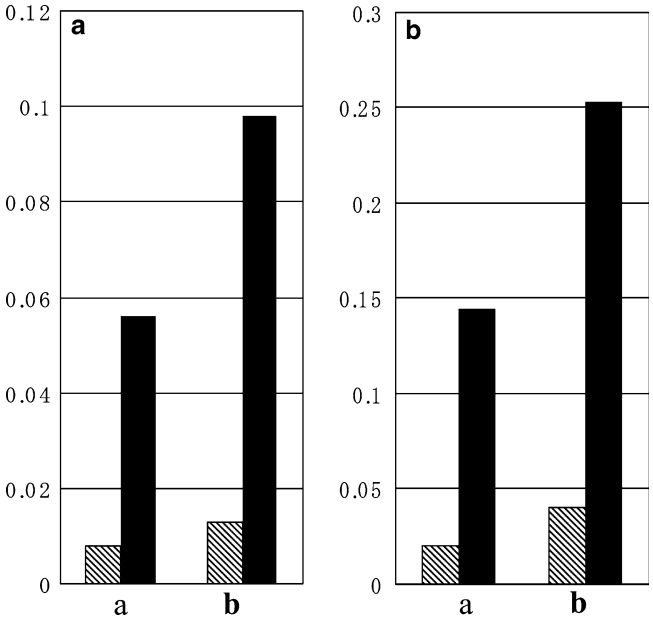

Fig. 4 Comparison of the intensity of luciferase. *a*: Control, *b*: exposure to 0.5 mM sodium oxalate for 30 min. **A** Comparison of the simple amount of luminescence. **B** Comparison of the corrected amount of luminescence under control. *Hatched*: the control group, *black*: the oxalic acid group

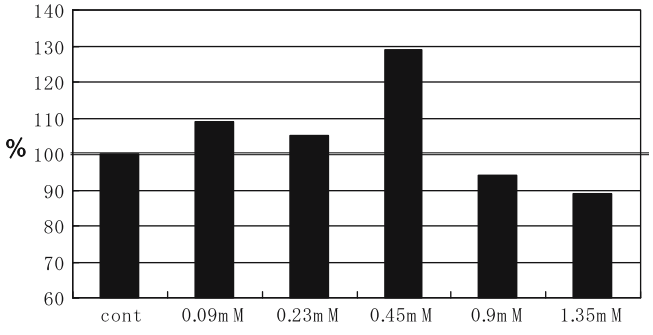

Fig. 5 Determination of prothrombin expression using real-time PCR. The amount of prothrombin expression as compared with the control (100%) was examined depending on the concentration of oxalic acid

study, the expression of bikunin was not detected in MDCK cells as far as we investigated using multiple primers.

The result of this study suggests that MDCK cells may be activated, leading to an increase in the expression of calculus-related proteins and a reduction in calculus formation rather than be damaged at around 0.9 mM oxalic acid. However, as oxalic acid concentration increases and starts to induce cytotoxicity, the expression of calculus-related proteins decreases and inhibitory activity on calculus formation also decreases. To date, a direct association between oxalic acid and RPTF-1 remains unclear. However, the intensity of luciferase luminescence increased significantly in the oxalic acid group as compared with the control group. Therefore, we verified that the addition of oxalic acid in MDCK cells has a close relationship to induction of RPTF-1 protein and generation of active oxygen, that oxalic acid has some involvement in the early process of calculus formation at the stage of stimulation before cytotoxicity, and transmits signals intracellularly to the nucleus using some pathway, which leads to the induction of RPTF-1 mRNA and RPTF-1 protein expression.

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