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ORIGINAL PAPER

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Examination of the anti-oxidative effect in renal tubular cells and apoptosis by oxidative stress

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Abstract The incidence of urolithiasis has increased in the industrialized nations. However, both the pathogenesis and methods for its prevention remain to be clarified. We demonstrate that the antioxidative effect of green tea decreases the formation of calcium oxalate stones, OPN (osteopontin) expression, and apoptosis, and increases SOD (superoxide dismutase) activity in rat kidney tissues. The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to its antioxidative effects. Therefore, we examined oxidative stress in vivo applied to Madin-Darby canine kidney (MDCK) cells, to which catechin, an antioxidant, was added. To evaluate the effects of oxidative stress on MDCK cells, we use a hypoxic condition because hypoxia is known to lead to oxidative stress. Confluent cultures of MDCK cells were exposed to (−)epigallocatechin 3 gallate (EGCG) (0, 0.1, 0.5, 5.0 mg/ml) for 2, 4, 8 or 16 h to determine changes in protein secretion and apoptosis. OPN protein expression was observed in MDCK cells of all 16 groups. The levels of expression of OPN protein were the same among all groups. In all groups, SOD protein expression was observed. In the groups exposed to EGCG 0.5, 5.0 mg/ml, SOD staining was more enhanced than in the EGCG 0 and 0.1 mg/ml groups. No deposits were detected in any of the 16 groups. RT-PCR was performed to detect sequences from OPN (979 bp) and SOD (447 bp). Quantitative analyses showed that SOD activity decreased gradually in all groups. Only in the EGCG 0 mg/ml 16 h group were TUNEL-positive cells observed. In the other groups, TUNEL-positive cells were not detected. EGCG used as an antioxidant protects renal tubular cell from cellular injury caused by oxidative stress through SOD protein expression.

Keywords Urolithiasis · In vitro · MDCK · Catechin · Antioxidant · Apoptosis

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Introduction

Calcium containing urinary stones are a common clinical problem. The recurrence rate is quite high, about 50% at 10 years and 75% at 15 years if untreated [1].

Matrix proteins form 1–5% of urinary stones and several studies have suggested the importance of matrix proteins in stone formation [2]. Previously, we cloned and sequenced the cDNA encoding osteopontin (OPN), an important soluble protein component of calcium oxalate stone proteins. We found a strong expression of OPN mRNA on distal tubular cells in the kidneys of stone forming rats [3].

OPN is a secreted glycoprotein in both phosphorylated and non-phosphorylated forms. It contains an Arg-Gly-Asp cell binding sequence and a thrombin cleavage site. OPN is mainly present in the loops of Henle and distal nephrons in normal kidneys in animals and humans. After renal damage, OPN expression may be significantly up-regulated in all tubule segments and glomeruli [4].

The incidence of urolithiasis has increased in the industrialized nations. However, both the pathogenesis and methods of prevention remain to be clarified. Urolithiasis resembles arteriosclerosis in its mechanism, composition of calcification, and epidemiology. The components of both urolithiasis and arteriosclerosis calcification include calcium, phosphate, OPN, and matrix gla protein, and chemotaxis of macrophages is involved in the pathogenesis [5]. Epidemiologically, urolithiasis and arteriosclerosis frequently develop in middle-aged men and postmenopausal women.

Green tea has been reported to inhibit oxidation/degeneration of LDL, and thus exhibit anti-arteriosclerotic effects [6].

We demonstrated that the antioxidative effect of green tea decreases the formation of calcium oxalate stones, OPN expression, and apoptosis, and increases SOD activity in rat kidney tissues. The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to its anti-oxidative effects [7].

Therefore, we also examined oxidative stress applied to MDCK cells in vitro, with the addition of catechin as an anti-oxidant. This study was carried out to check whether EGCG, which is an antioxidant, can protect renal tubular cells from cellular injury caused by oxidative stress through SOD protein expression.

Materials and methods

Cell culture

Renal epithelial cells of a normal dog kidney line (MDCK: Madin-Darby canine kidney cells; Dainippon Pharmacia, Osaka, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum. High-density cultures were prepared by plating 100,000 cells on Labtek chamber slides (Nunc, Naperville, USA). After semi-confluence of the cells, the medium was aspirated and replaced with fresh medium.

Oxidative stress

To evaluate the effects of oxidative stress on MDCK cells, we used a hypoxic condition because hypoxia is known to lead to oxidative stress. The cells were incubated in DMEM for 24 h before they were subjected to hypoxicia. The hypoxic condition was achieved with BBL gas pack (Becton Dickinson, USA), which releases hydrogen that reacts with oxygen in the container so that the level of oxygen drops to an undetectable level ($< 0.01\%$).

EGCG

Polyphenols are abundant in green tea, mostly in the form of flavonols commonly known as catechins. Green tea leaves contain approximately 13% catechins, and have anti-oxidative effects. Some of the major green tea catechins are (−) epigallocatechin -3-gallate (EGCG), (−) epigallocatechin (EGC), (−) epicatechin-3-gallate (ECG) and (−) epicatechin (EC). Of these, EGCG shows the greatest antioxidant effect. EGCG is a commercial preparation (Ito-en, Japan). The solvent for EGCG is DMEM. Confluent cultures of MDCK cells were exposed to EGCG (0, 0.1, 0.5, 5.0 mg/ml) for 2, 4, 8 or 16 h to determine changes in protein secretion and apoptosis. Therefore, there were 16 groups in this study.

Immunohistochemical staining

Immunohistochemical staining was performed by the streptavidin-biotin method using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan).

Culture tissue specimens were fixed in 4% paraformaldehyde. Sections were deparaffinized and hydrated by processing with xylene and a graded series of alcohol. To block endogenous peroxidase activity, the sections were treated with 0.3% H_2O_2 methanol for 30 min. The primary antibody was diluted with bovine serum albumin in PBS and reacted with the sections at room temperature for 1 h. The Graham-Karnovsky method was used for color reaction. A total of 20 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Dojindo, Kumamoto, Japan) and 0.1 ml of 3% H_2O_2 were dissolved in 100 ml of 0.05 M Tris-HCl buffer solution, pH 7.6, to prepare a reaction solution and this was reacted with the sections at room temperature for 4 min. The color reaction was stopped in running water and the nuclei were stained with 1% methyl green for 10 min. After dehydration with ethanol and penetration with xylene, the sections were mounted using Harleco Synthetic Resin (Matsunami Glass, Osaka, Japan) and kept as permanent preparations. Anti-OPN, anti-SOD antibodies were used as the primary antibodies at 1 μg/ml.

Staining of calcium phosphate deposits

Specific calcification detection was performed with von Kossa staining. The von Kossa section staining converts the crystals into a black precipitate. Culture tissue samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.0), dehydrated in ethanol, and embedded in paraffin.

Reverse transcriptase-polymerase chain reaction

Culture tissue samples for reverse transcriptase-polymerase chain reaction were frozen with liquid nitrogen. Total RNAs were extracted using the method described by Chirgwin et al. [8] from culture tissues. First strand cDNA was synthesized from aliquots of 2 μg of total RNA in 20-μl reaction mixtures containing 0.5 mM dNTP, 25 μg/ml random hexamers, reverse transcriptase, and 4 μl of 5× reverse transcription buffer. The mixtures were incubated at 42°C for 60 min and then 95°C for 5 min to inactivate the enzyme. The primers used for the amplification of OPN were as follows: sense primer 5′-CCATGAGACTGGCAGGGTT-3′, antisense primer 5′-GGAACTGTG GTTTTGCCTCT-3′. The cycling parameters for PCR were as follows: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A total of 30 cycles were performed. The primers used for the amplification of SOD were as follows: sense primer 5′–ATGGTGGCCTTCTTGTT CTGC-3′, antisense primer

5′–GTGCTGTGGGTGCGGCACACC-3′. The cycling parameters for PCR were as follows: denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min. A total of 23 cycles were performed [17]. The primers used for the amplification of β-actin were as follows: sense primer 5′–TCAC CGAGGCC-CCTCTGAACCCTA-3′, antisense primer 5′–GGCA-GTAATCTCCTTCT GCATCCT-3′. Coamplification with β-actin was included to ensure that equal amounts of RNA were reverse transcribed and amplified in each reaction tube. PCR products were visualized by ethidium bromide staining.

SOD activity

SOD activities were determined using the method of Sohal et al. [9]. The supernatant of the cell culture was centrifuged again at 50,000 *g* for 30 min and the pellets discarded. The resulting supernatant was dialyzed against 50 mM PBS containing 0.1 mM H_2O_2 and 0.05 mM 3-amino-1,2,4-triazole at 4°C, and then dialyzed against 50 mM PBS at 4°C for a further 12 h.

The dialysate was added to 2 ml of reaction mixture consisting of 50 mM potassium phosphate buffer with 0.033 mM EDTA, 0.25 mM dianisidine, 9.5 μg of riboflavin, 1.5 mM potassium cyanide and 10 μM sodium azide. The absorbance of the sample at 460 nm was determined before and after illumination using 20 W fluorescent tubes in a box; the change in absorbance observed in the blank was subtracted from each sample. SOD activity was calculated by dividing the change in optical density of the sample by the change in each unit of SOD from the standard curve.

Detection of apoptosis

We examined apoptosis by the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method [10].

In situ end-labeling was performed using an Apoptosis In situ Detection Kit (Wako, Osaka, Japan). Paraffin sections were deparaffinized and hydrated by processing with xylene and a graded series of alcohol. For proteolytic treatment, the sections were treated with diluted protein digestion in an enzyme solution at 37°C for 5 min. To label the 3′ end of DNA, the TdT reaction solution was dripped on the sections and they were incubated in a humid box at 37°C for 10 min. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature. A diluted POD-conjugated antibody solution was then dripped on them, and the sections were reacted for 10 min in a humid box at 37°C. Then, DAB solution was dripped on them, and the sections were incubated for 5 min at room temperature for color reaction.

For evaluation of apoptosis, microscopic fields were selected at random, and TUNEL stain-positive cells per 100 MDCK cells were counted. The frequency of apoptotic cells was compared among the groups.

Statistical analysis

The Mann-Whitney U-test was used to compare the data between groups. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Immunohistochemical studies

OPN protein expression was observed in MDCK cells of all 16 groups. The levels of expression of OPN protein were same among all groups (Fig. 1). In all groups, SOD protein expression was observed. In the groups exposed to EGCG 0.5, 5.0 mg/ml, SOD staining was more enhanced than in the EGCG 0, 0.1 mg/ml groups (Fig. 2).

Histological examination

No deposits were detected in the any of the 16 groups (Fig. 3).

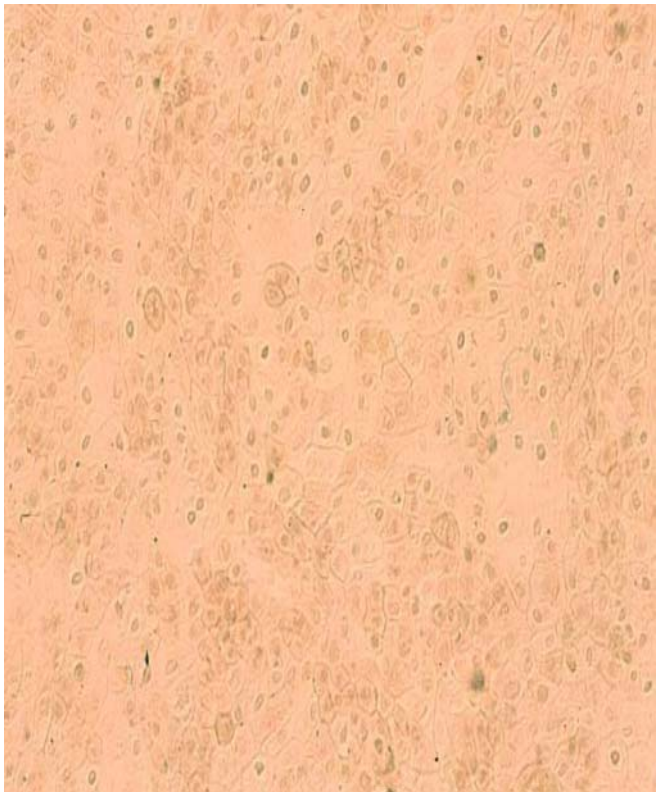

Fig. 1 Immunohistochemical studies of OPN. OPN protein expression was observed in MDCK cells from all 16 groups. The levels of expression of OPN protein were the same among the all groups. ×400

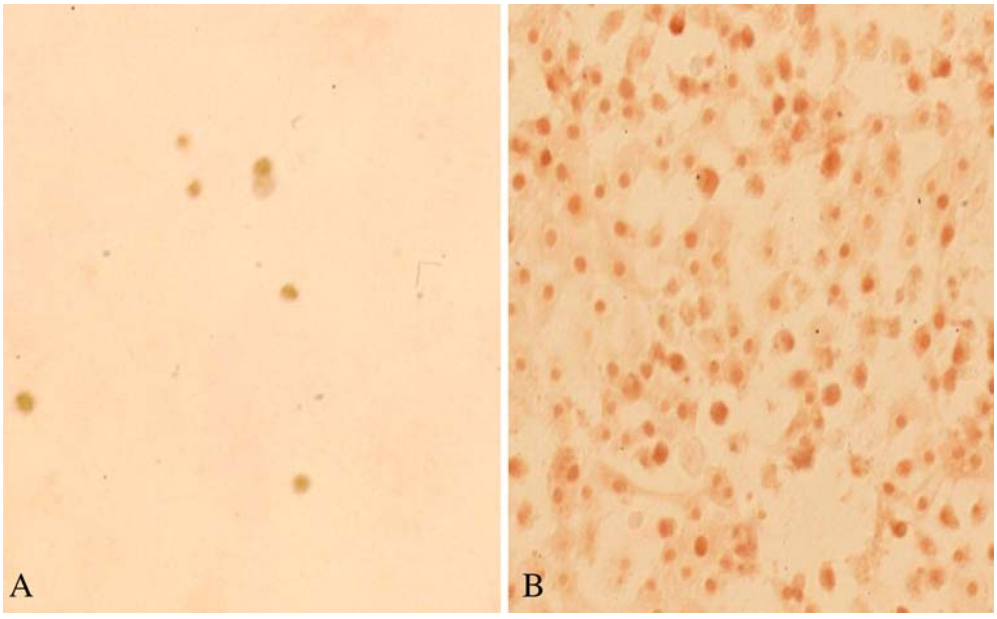

Fig. 2 Immunohistochemical studies of SOD. SOD protein expression was observed in all groups. In the groups exposed to EGCG 0.5, 5.0 mg/ml SOD staining was greater than in the EGCG 0, 0.1 mg/ml groups. **A** EGCG 0 mg/ml, 2 h, **B** EGCG 0.5 mg/ml, 2 h. ×400

RT-PCR

RT-PCR was performed to detect sequences from OPN (979 bp) and SOD (447 bp). Analysis of SOD gene expression using total RNA from MDCK cells showed clear bands in all groups (Fig. 4). Analysis of OPN gene expression in MDCK cells showed clear bands in all groups (data not shown).

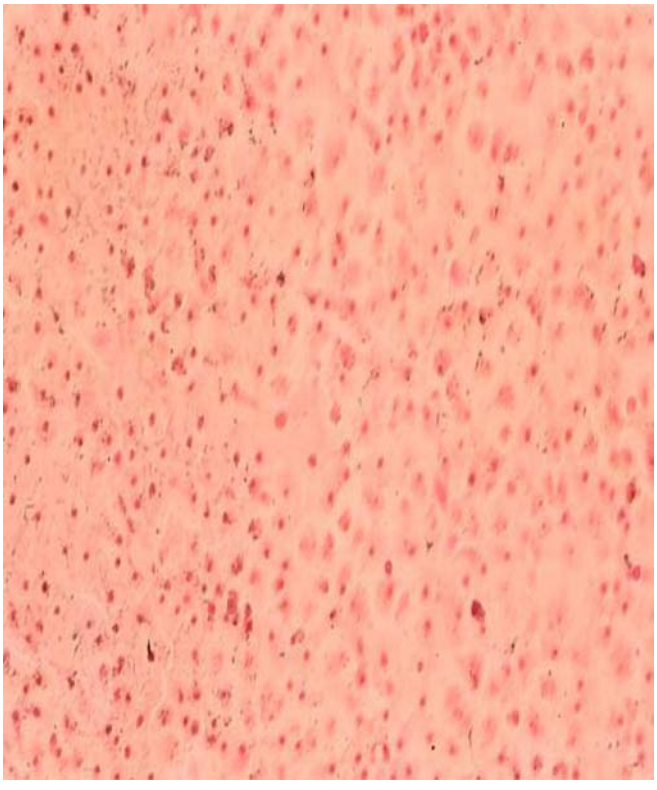

Fig. 3 Specific calcification detection was performed with von Kossa staining. No deposits were detected in any of the 16 groups. ×400

SOD activity

Quantitative analyses showed that SOD activity decreased gradually in all groups (Fig. 5). SOD activity tends to decrease with time.

Detection of apoptosis

Only in the EGCG 0 mg/ml 16 h group, were TUNEL-positive cells observed. In the other groups, TUNEL-positive cells were not detected (Fig. 6).

Discussion

Urolithiasis resembles arteriosclerosis in its mechanism of action, the composition of calcification, epidemiology

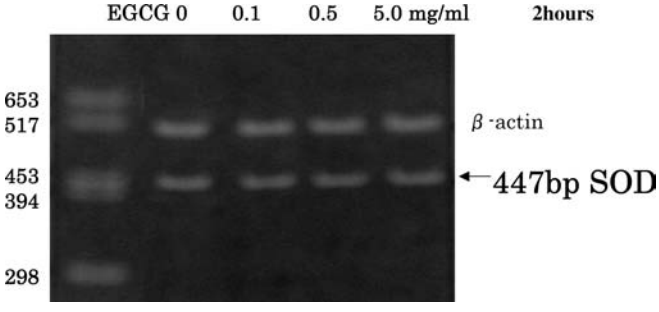

Fig. 4 Analysis of SOD gene expression using total RNA from MDCK cells by RT-PCR amplification. Ethidium bromide-stained gel showed clear bands representing SOD gene expression in all groups

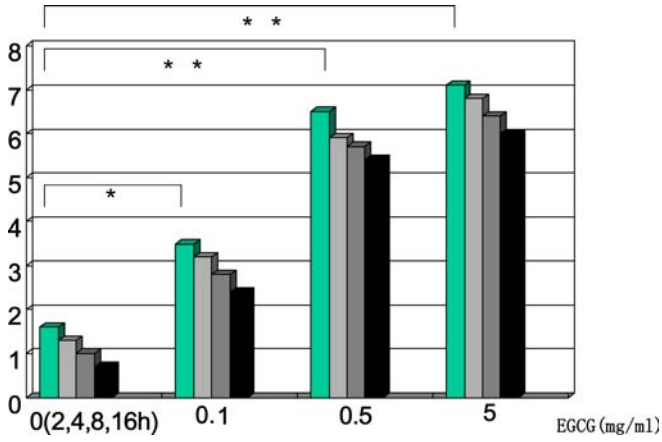

Fig. 5 Quantitative analyses showed that SOD activity (U/ml) decreased gradually in all groups. An *asterisk* indicates $P < 0.05$, a *double asterisk* indicates $P < 0.01$

and gene relationship. It has been reported that the vascular endothelial growth factor gene polymorphism is a suitable genetic marker for urolithiasis [11]. Calcification in arteriosclerosis can be inhibited by antioxidants.

We demonstrated that the antioxidative effect of green tea decreased the formation of calcium oxalate stones, OPN expression, and apoptosis, and increased SOD activity in rat kidney tissues. The inhibitory effect of green tea on calcium oxalate urolithiasis is most likely due to the antioxidative effects [7].

Superoxide radicals are generated as byproducts of metabolic oxidation, and SOD has evolved to inactivate superoxides. In mammalian tissue, SOD is localized in mitochondria and cytosol. Within cells, other potent antioxidants such as glutathione, ascorbate and tocopherol are present, but none of these inactivates superoxide as rapidly or effectively as SOD, which means that SOD is an important initial component in the cellular defense against oxygen toxicity [12].

Antioxidant therapy with vitamin E prevented calcium oxalate precipitation in the rat kidney and reduced urinary oxalate excretion in kidney stone patients [13]. Administration of vitamin E to patients with surgically removed stones rapidly restored the levels of antioxidants in the blood and reduced the urinary excretion of oxalate and calcium [14]. A previous study measured antioxidant enzyme levels in rats with stone formation, and showed that almost all antioxidant enzyme activities were attenuated except that of catalase [15].

Sustained hyperoxaluria in association with calcium oxalate crystals induced apoptosis as well as necrosis [16]. Although cell death by hypoxia is a well known type of oxidative stress, which has generally been believed to be manifested as necrosis, recent biochemical observations suggested the possibility of hypoxia induced apoptosis [17]. Wu and Lozana demonstrated that activation of NF-κB by oxidative stress induced human aortic endothelial cell death and apoptosis through the suppression of bcl-2, translocation of bax and induction of p53 [18]. The apoptosis observed in this experiment was also thought to be dependent on the same mechanism. Blockade of NF-κB activation by antioxidants has been suggested to be an effective strategy for the treatment of urolithiasis and arteriosclerosis [19].

Sarica et al. reported that both calcium oxalate crystals and hyperoxaluria itself may be injurious to renal tubular cells, as indicated by apoptotic changes in a rabbit urolithiasis model [20].

This study revealed the protective potential of EGCG against oxidative stress induced by hypoxia in MDCK cells.

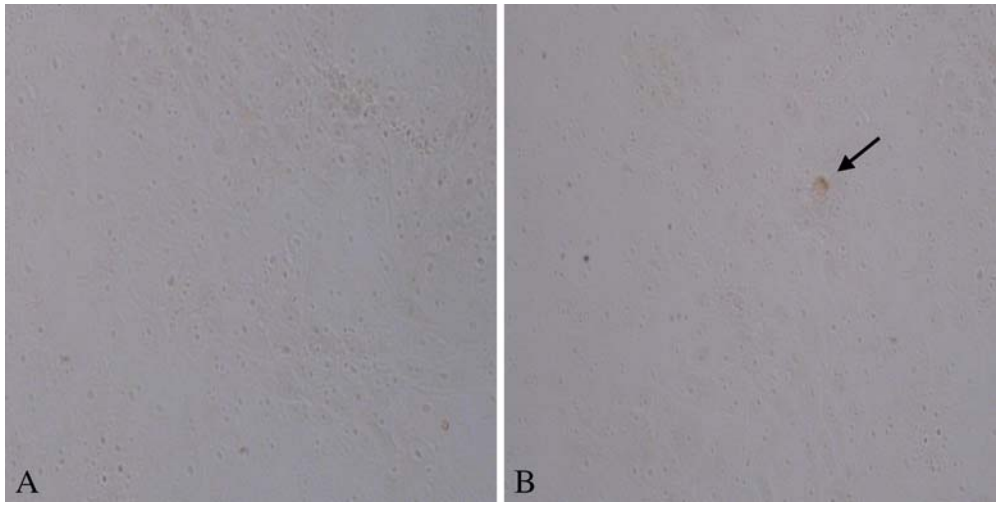

Fig. 6 Detection of apoptosis. **A** EGCG 0.5 mg/ml, 16 h. Only in the EGCG 0 mg/ml, 16 h group (**B**) were TUNEL-positive cells observed. ×400

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