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ORIGINAL PAPER

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Apoptosis induced by oxalate in human renal tubular epithelial HK-2 cells

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Abstracts Oxalate is not only considered to be one of the main constituents of urinary stones, but it also has toxic effects on renal tubular epithelial cells, affecting the pathogenesis of nephrolithiasis. We tried to elucidate the effects of oxalate on human renal tubular epithelial cells (HK-2 cells). In addition, we investigated whether the toxic effect of oxalate occurs by apoptosis, and determined the expression of Bcl-2 family and caspase 9 proteins known as apoptosis-related protein. HK-2 cells were incubated with different concentrations of oxalate, and the effect of oxalate on the growth of the cells was assessed by MTT assay. A caspase-3 activity assay and TUNEL assay were performed on HK-2 cells after oxalate exposure in order to evaluate apoptosis. Immunoblot analysis of Bax, Bcl-2, Bcl-xL, and caspase-9 were performed. Oxalate exposure resulted in significant growth inhibition of HK-2 cells as oxalate concentrations increased. The toxic effect of oxalate on HK-2 cells was considered to occur through apoptosis, as suggested by the increase of caspase-3 activity. The percentage of positive nuclei stained using the TUNEL method was 18 ± 2.3 in oxalate-treated cells and 2.5 ± 0.9 in untreated cells ($P < 0.05$). Bax and caspase-9 protein expression increased significantly as oxalate concentrations increased, but Bcl-2 protein expression decreased. There was no difference in Bcl-xL protein expression among the various concentrations of oxalate. Our results show that oxalate has a toxic effect on HK-2 cells and that this effect is induced by apoptosis, which may be mediated by an intrinsic pathway.

Keywords Oxalate · Apoptosis · Urolithiasis · HK-2 cells

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Introduction

Oxalate, one of main constituents of urinary stone, is an end product of metabolism excreted normally in urine. Recent studies have reported that oxalate was observed to accumulate in renal tubular epithelial cells, having cytotoxic effects [1, 2, 3]. This oxalate-cell reaction is considered to promote crystal attachment and retention, resulting in nephrolithiasis.

Reports from studies using LLC-PK1 and MDCK cells indicate that oxalate has cytotoxic effects by inducing free radicals in renal epithelial cells, while others suggested that cell injury due to oxalate is induced by apoptosis [4, 5, 6].

Apoptosis is a programmed cell death characterized as an active suicide of the cell. It shows characteristic morphological changes such as the condensation of nucleoplasm and cytoplasm, the blebbing of the cytoplasmic membrane, and fragmentation of cells into apoptotic bodies.

One well known apoptotic mechanism is the activation of caspase-9, which in turn activates other caspases. The activation of these caspases results in cell death [7]. The activation of caspase-9 requires an influx of cytochrome C into cytosol from mitochondria. The Bcl-2 family genes, such as Bcl-2, Bax, and Bcl-xL, are known to control the release of cytochrome C [8, 9].

Previously, we observed a concentration-dependent toxic effect of oxalate on LLC-PK1 and MDCK cells [3].

In the present study, we try to elucidate the effect of oxalate on the growth of HK-2 cells originating from normal human renal epithelial cells. In addition, we investigate whether cell injury due to oxalate occurs by apoptosis, and whether caspase-9, Bcl-2, Bax, and Bcl-xL have roles in the apoptosis induced by oxalate in HK-2 cells.

Materials and methods

Cell culture

HK-2 cells (CRL-2190, American Type Culture Collection, USA) of human proximal tubular origin were grown in Dulbecco modified Eagle medium (DMEM) (Gibco Laboratories, New York, USA) supplemented with 10% fetal bovine serum (FBS), 4.5 mM glucose, penicillin 100 IU/ml, and streptomycin 100 μg/ml at 37°C, 95% air and 5% CO_2

MTT assay

HK-2 cells were subcultured in a 96-well plate and grown to 70–80% confluence. Subsequently, the cells were exposed to various concentrations of sodium oxalate (0, 32, 64, 125, 250, 500, 1,000, 2,000, 4,000 μM) for 3 days. At the end of the experimental periods, 0.1 mg (50 μl of 2 mg/ml) of MTT (Sigma, St. Louis, USA) in PBS was added, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved in dimethylsulfoxide. Optical density was determined with a microplate reader (Beckon Dickinson Labware, Lincoln Park, N.J., USA) at 540 nm.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

For the in situ detection of apoptotic cells, the TUNEL assay was performed using the ApoTag peroxidase in situ apoptosis detection kit (Intergen, USA). HK-2 cells were cultured in a 6-well plate. After treatment with oxalate for 24 h, the cells were washed with PBS and fixed by incubating in acetic acid:ethanol (1:2) for 10 min at 4°C. The fixed cells were then incubated with digoxygenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37°C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with antidigoxygenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using 3,3′-diaminobenzidine (DAKO, Denmark) as the substrate for the peroxidase.

Caspase-3 enzyme activity assay

Apoptosis was assessed by measuring the activity of caspase-3 using Apoalert Caspase-3 Assay Kits (Clontech, USA). The number of HK-2 cells was counted after 24 h of the treatment with 0, 125, 250, or 500 μM oxalate. A total of 1×10^6 cells in each group was centrifuged, resuspended in 50 μl of cell lysis buffer, and then cooled on ice for 10 min. The reaction buffer containing DTT and 1 mM conjugated substrates was added to the supernatants after the cell lysates were centrifuged at 12,000 rpm for 3 min and then incubated at 37°C for 1 h. The enzyme activity was estimated by a spectrophotometer at 405 nm.

Western blot analysis of caspase-9, Bcl-2, Bcl-xL, and Bax

The HK-2 cells were washed with PBS and solublized with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.7 mg/ml pepstatin) at 4°C for 30 min after 24 h of the treatment with 0, 250 or 500 μM oxalate. Lysates were centrifuged at 14,000 rpm for 15 min and the supernatants quantified. Samples containing equal amounts of protein (20 μg) were separated on each lane of 12% SDS-polyacrylamide gel and then transferred to 0.45 μm Westran membrane (Amersham Pharmacia Biotech, USA) using standard electroblotting procedures. The membrane was blocked with 5% skim milk in trypsin buffered saline (TBS-T, 1% Tween-20). Blots were immunolabeled with primary antibodies (1:1000) for each of caspase-9, Bax, Bcl-2, and Bcl-xL (NeoMARKERS, Fremont, Calif., USA). Immunoblots were washed and then incubated with secondary antibody (NeoMARKERS). Immunoactivity was detected by a chemiluminescence detection system (Intron, Korea). Immunoblotting of actin with equal amounts of protein was also performed as a control marker for comparative analysis.

Statistical analysis

Each experiment was performed five times. Statistical evaluation was performed using SPSS 10.0 software (SPSS, Chicago, Ill., USA). Values were expressed as mean ± SEM. Statistical significance, defined as $P < 0.05$, was analyzed using one-way ANOVA.

Results

Effect of oxalate on the growth of HK-2 cells

The number of HK-2 cells decreased as oxalate concentration increased. At the concentrations of more

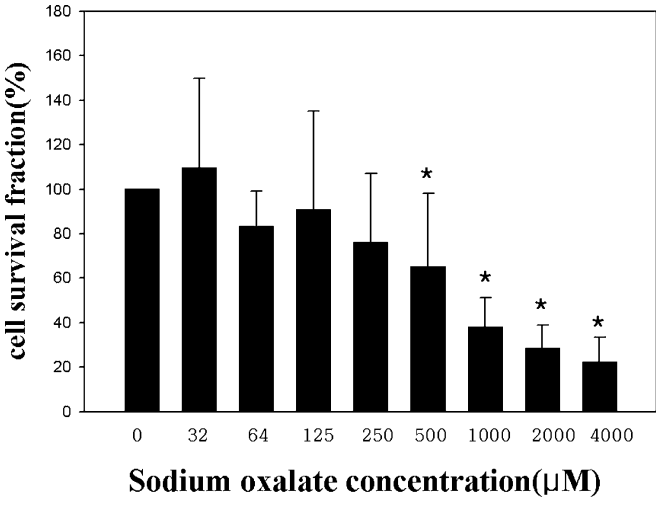

Fig. 1 Effects of various concentrations of oxalate on HK-2 cell survival in DMEM without FBS. Cells were exposed to various concentrations of sodium oxalate (0, 32, 64, 125, 250, 500, 1,000, 2,000, 4,000 μM) for 3 days. The degree of growth inhibition of HK-2 cells was inversely correlated with the concentration of oxalate. An *asterisk* indicates $P < 0.05$

than 500 μM sodium oxalate, the number of cells started to decrease significantly relative to the control without oxalate, resulting in a survival fraction of only 30% at a concentration of 2,000 μM (Fig. 1).

Evaluation of apoptosis

TUNEL staining showed that apoptotic cells with nuclear condensation were observed in HK-2 cells with 500 μM sodium oxalate treatment (Fig. 2A). The percentages of apoptotic cells in all HK-2 cells were respectively 18.1 ± 2.3 and $2.5 \pm 0.9\%$ in oxalate-treated cells and untreated cells ($P < 0.05$). The caspase-3 activities in HK-2 cells after 24 h of the exposure to the respective concentrations of 0, 125, 250, and 500 μM sodium oxalate were assessed. Oxalate was observed to significantly increase the caspase-3 activity in a concentration-dependent fashion. The activity after exposure to 500 μM was shown to be twice as high as that of the control (Fig. 2B).

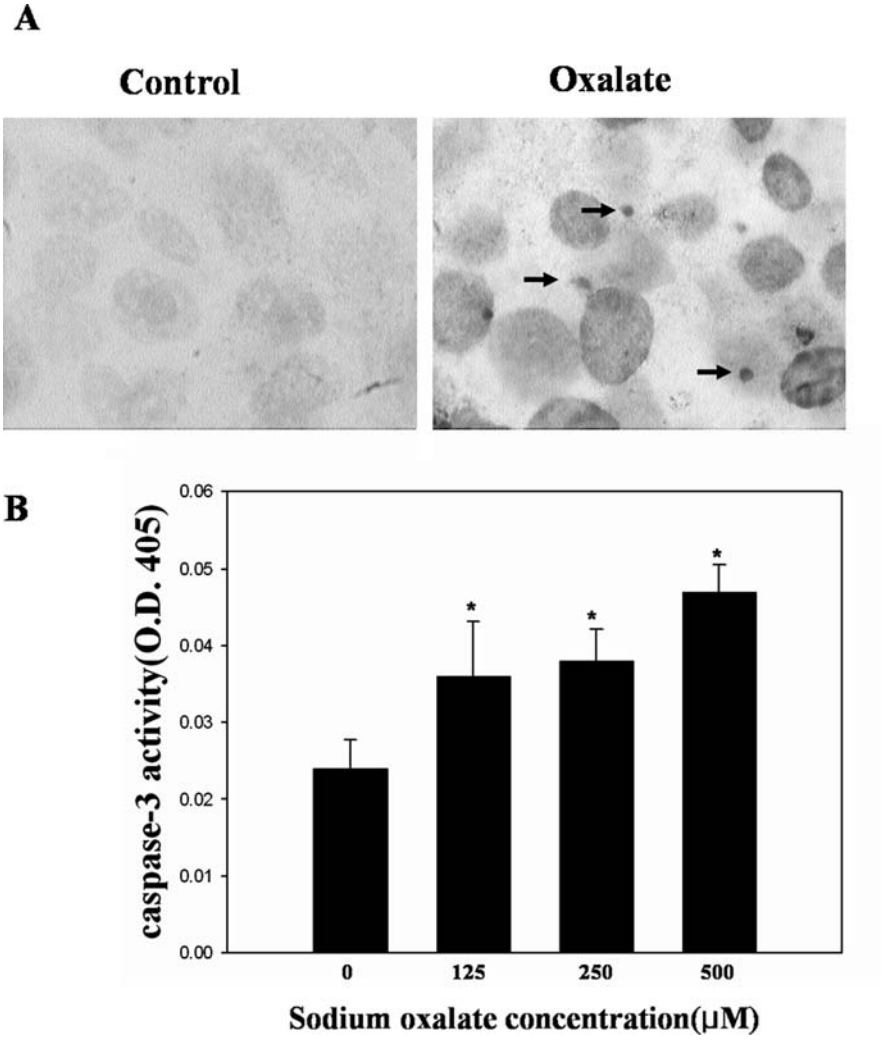

Fig. 2 Apoptosis induced by oxalate in HK-2 cells. **A** HK-2 cells stained using the TUNEL method (control vs 500 μM oxalate treatment). *Black arrows* indicate condensed and marginated chromatin of apoptotic cells, whilst controls (HK-2 cells cultured without oxalate) showed no apoptotic cells. **B** The caspase-3 activity in HK-2 cells after exposure to various concentrations of oxalate. The activity was evaluated after 24 h of the exposure to 0, 125, 250 or 500 μM sodium oxalate. The caspase-3 activity increased significantly in proportion to the concentrations of oxalate. The activity of 500 μM was two times higher than the control. An *asterisk* indicates $P < 0.05$

Expression of the Bcl-2 gene family in apoptosis after oxalate exposure

Bax protein expression

Bax protein expression after exposure to respective concentrations of 0, 250, and 500 μM sodium oxalate in HK-2 cells was also assessed by Western blot. The protein was extracted after 24 h of oxalate exposure. The expression level of Bax protein was demonstrated to be the highest with 500 μM sodium oxalate (Fig. 3A). The OD value of the Bax band at each concentration of oxalate was divided by that of actin to quantify the level of expression exactly. The OD value of Bax was observed to increase significantly with exposure to sodium oxalate; it doubled with 250 μM and tripled with 500 μM compared with the control (Fig. 3A.)

Bcl-2 protein expression

From Western blotting, Bcl-2 protein expression levels were shown to decrease as oxalate concentrations increased. Assessed using OD, the expression level decreased to 50% of the control with 250 μM and to 45% with 500 μM (Fig. 3B).

Bcl-xL protein expression

Bcl-xL protein expression after respective exposures to 0, 250, and 500 μM sodium oxalate showed no significant differences (Fig. 3C).

Caspase-9 expression in apoptosis after oxalate exposure

Caspase-9 protein was highly expressed after exposure to 250 and 500 μM sodium oxalate, but rarely in the control. The expression level of caspase-9 was 2.5 times higher with 250 μM and three times higher with 500 μM, compared with the control (Fig. 4).

Discussion

Oxalate was merely considered as a major constituent of urinary stone. However, oxalate deposits have been observed in benign breast tumor, hyperplastic thyroid, and renal cysts in addition to renal tubular epithelial cells that are associated with cellular proliferation [10, 11, 12]. It would take several hours for crystals to grow large enough to obstruct collecting ducts, but only 3 min for urine to pass from the glomerulus to the renal pelvis [13]. Therefore, it may be assumed that an environment favorable for crystal retention and growth is necessary for stone formation. Several studies have revealed that injury to renal epithelium is associated with the deposition of crystals in renal tubules [1, 2, 3].

Recent in vivo and in vitro studies have indicated that high concentrations of oxalate may have toxic effects on the renal epithelium due to increased oxidant stress within the cells. Oxalate seems to induce cell death, mediated by cellular necrosis, because oxalate exposure induces changes in membrane integrity, the release of cellular enzymes, and membrane lipid peroxidation [1, 4]. Koul et al. showed an increase in oxalate-induced

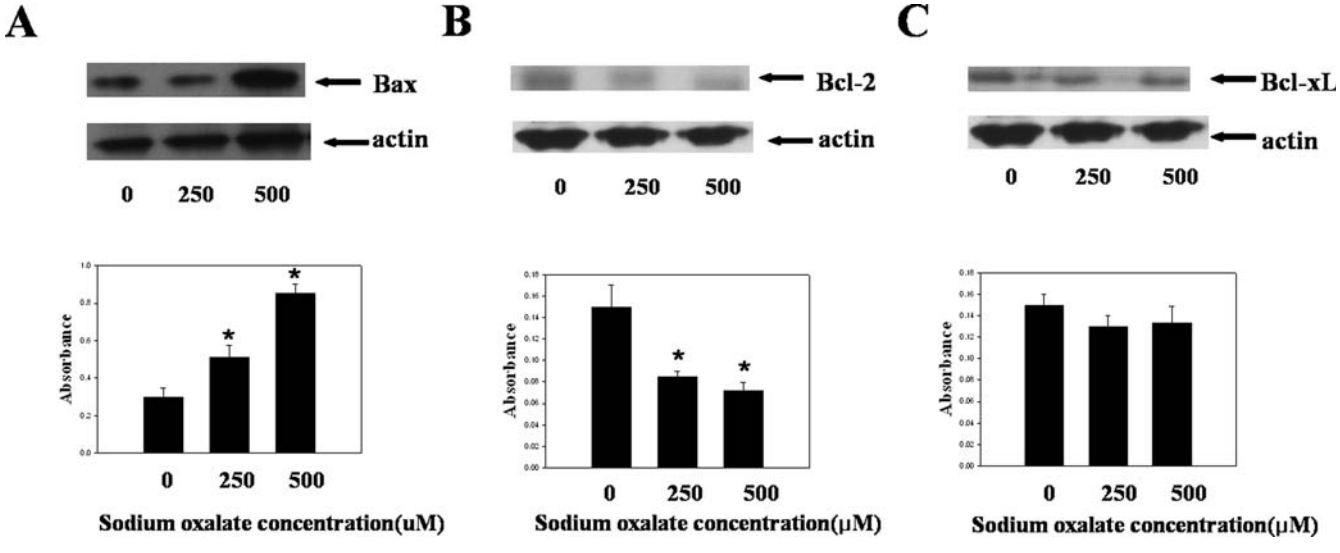

Fig. 3 The protein expression of the Bcl-2 gene family in HK-2 cells after exposure to various concentrations of oxalate by Western blot. The protein was extracted 24 h after the exposure to 0, 250 or 500 μM sodium oxalate. Actin expression served as a control. The OD value of each band was divided by that of actin to quantify the level of expression of Bcl-2 gene family exactly. **A** Shows that the expression level of Bax protein was highest in 500 μM. Bax protein expression became significantly higher as oxalate concentrations increased. **B** Shows that the expression level of Bcl-2 protein was highest in 0 μM. Bcl-2 protein expression was significantly lower in both of 250 and 500 μM than 0 μM. **C** Shows that the expression level of Bcl-xL protein was not significantly different in each concentration of oxalate. An *asterisk* indicates $P < 0.05$

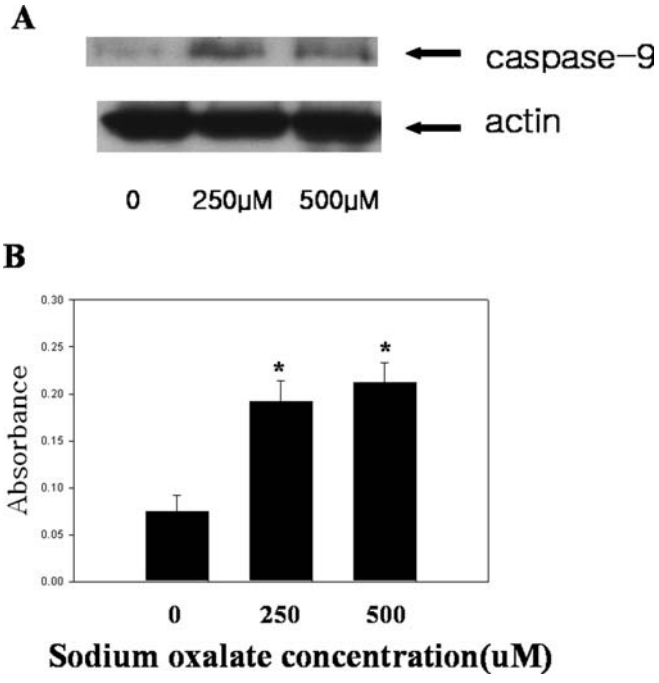

Fig. 4 The caspase-9 protein expression in HK-2 cells after exposure of various concentrations of oxalate by Western blot. **A** The protein was extracted 24 h after the exposure to 0, 250 or 500 μM sodium oxalate. The expression level of caspase-9 protein was higher in 250 and 500 μM than 0 μM. **B** The OD value of caspase-9 band in each concentrations of oxalate was divided by that of actin. There were significant differences between 0 and 250 and 500 μM. An asterisk indicates $P < 0.05$

DNA synthesis and c-myc gene expression [14, 15]. Some studies suggest that oxalate may initiate a programmed cell death in renal epithelial cells [5, 6].

Taken together, apart from the direct cytotoxic effect of oxalate, oxalate interactions with renal epithelial cells may be mediated by an intracellular signaling pathway that results in cell proliferation or death. Therefore, cellular apoptosis has been mentioned as a possible mechanism for oxalate-induced cell death [5, 6].

Our results reveal that oxalate is toxic in a concentration-dependent fashion for HK-2 cell. From our assessment of caspase-3 activity, we know that apoptosis occurred in HK-2 cells due to oxalate exposure. However, oxalate-induced apoptotic death occurred in less than 20% of the HK-2 cells. Apoptosis may be a part of the mechanism behind cellular injury from oxalate exposure in HK-2 cells. Lieberthal et al. have also reported that the type of cell death caused by toxins may vary, depending on the severity of the damage, from apoptosis occurring in response to lesser injury to necrosis in response to more severe injury [16].

Apart from our study, Sarica et al. reported that apoptosis occurs in renal tubular epithelial cells by hyperoxaluria [17]. They observed that crystals were responsible for the induction of apoptotic changes. A recent study reported that after the addition of oxalate to culture medium (DMEM), a wide range of CaOx crystals was found in the equilibrium state [18]. Therefore, in the present study, we should not overlook the effects of spontaneously formed CaOx crystals. But since the toxic effects of oxalate were observed even in the absence of crystalluria, oxalate induced injury was not caused only by CaOx crystals. [19, 20].

The basic pathway of apoptosis has been the subject of study for many researchers, and much has been done to unfold the exact mechanism. Recent work has suggested that mitochondria may play a role in inducing apoptosis by releasing cytochrome C. The process by which cytochrome C and apoptosis-inducing factor (AIF) move from mitochondria into the cytosol or nucleus due to the mitochondrial dysfunction plays an important role in cell apoptosis [21]. In particular, cytochrome C, controlled by a Bcl-2 family gene, is known to drive apoptotic process by activating caspase-9 [22]. Some authors have reported that Bax, one of Bcl-2 family of proteins, increases the release of cytochrome C into cytosol, while others have reported that the anti-apoptotic Bcl-2 family of proteins act as inhibitors in apoptosis by blocking cytochrome C release. In summary, the Bcl-2 family of proteins may have an important effect on cellular apoptosis either as promoters or inhibitors [23, 24].

We performed Western blots for Bax, Bcl-2, and Bcl-xL in order to find out whether these proteins of the Bcl-2 family are related to apoptosis in HK-2 cells after oxalate exposure. In our study, the expression of Bax protein increased, but the expression of Bcl-2 protein decreased as oxalate concentration increased. Also, oxalate was observed to increase the expression of caspase-9 in a concentration-dependent manner. However, the expression of the Bcl-xL protein with such concentrations of oxalate was not significantly changed. Based on the data presented that oxalate exposure resulted in the activation of caspase-9 and Bax and the inactivation of Bcl-2, oxalate-induced apoptosis looks to be mediated by an intrinsic pathway. However, Koul et al. reported that no activation of apoptosis-related proteins, such as Bcl-2 and caspases, was found following oxalate exposure to HK-2 cells [25].

These results make it possible to hypothesize that apoptosis induced by oxalate exposure in HK-2 cells is initiated by an increment of caspase-9 protein resulting from both an increase in Bax protein that elicits cellular apoptosis and a decrease in Bcl-2 protein that inhibits apoptosis. In their study on apoptosis using LLC-PK1 cells, Miller et al. reported that oxalate exposure induced apoptosis, and that the process was blocked in recombinant LLC-PK1 cells transfected with the bcl-2 gene [6]. Our study also showed that expression of the Bcl-2 protein is reduced by increasing oxalate exposure. Therefore, these results support the assumption that oxalate-induced apoptosis is mediated by an intrinsic pathway. In order to confirm that oxalate-induced apoptosis occurs by Bax and Bcl-2 gene control, mitochondrial dysfunction, which leads to cytochrome C release following oxalate exposure, should be identified.

Apoptosis of renal epithelial cells induced by oxalate may play an important role in nephrolithiasis. Apoptotic changes induce the exposure of annexin binding phosphatidylserine at the cell surface. Clusters of negatively charged headgroups of phosphatidylserine may then attract calcium, and calcific crystals attach to the cell surfaces. Such clusters on surfaces of apoptotic bodies and cell debris can promote heterogenous nucleation of calcific salts. Exposure of the basement membrane after detachment of cells may provide sites for crystal attachment [26, 27].

The present study revealed that oxalate has cellular toxicity on HK-2 cells by the process of apoptosis. Apoptosis is considered to be mediated by an intrinsic pathway because of the increased expression of Bax protein and caspase 9, and the decreased expression of Bcl-2 protein.

Conclusions

We observed that oxalate has concentration-dependent toxic effects on HK-2 cells originating from human renal proximal tubular epithelial cells and induces cellular apoptosis that causes injury in these cells. We also investigated how the Bcl-2 family of genes act in apoptosis by oxalate through immunoblotting. Bax and caspase-9 protein expression increased, but Bcl-2 decreased with increasing oxalate concentration. Oxalate exposure had no significant effect on Bcl-xL expression. Thus, it can be concluded that the apoptosis may occur in HK-2 cells exposed to oxalate by the increase of caspase-9 elicited by a process in which Bax increases and Bcl-2 decreases simultaneously.

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