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Inhibition of aquaporin-1 expression by RNAi protects against aristolochic acid I-induced apoptosis in human proximal tubular epithelial (HK-2) cells

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ABSTRACT

The aim of this study was to investigate the protective effect of inhibition of aquaporin-1 (AQP1) expression against aristolochic acid I (AA-I)-induced apoptosis. HK-2 cells impaired by AA-I were used in this study as the cell model of aristolochic acid nephropathy. Apoptosis was studied by different methods, including 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays, flow cytometry, and caspase 3 activity assays. We compared AA-I-mediated apoptosis in HK-2 cells with or without knockdown of AQP1 expression by RNA interference. MTT assays showed that AA-I inhibited the viability of HK-2 cells in a time- and concentration-dependent manner. Apoptosis was evidenced by the results of the Annexin V/propidium iodide assay and the occurrence of a sub-G1 peak in cell-cycle analysis. The activity of caspase 3 was found to have been increased by AA-I in a concentration-dependent manner. However, AQP1 RNA interference provided protection against injury in cells treated with AA-I (40 μM) for 24 h and attenuated the number of apoptotic cells. These results suggested that AQP1 plays an important role in AA-I-induced apoptosis and that inhibition of AQP1 expression may protect HK-2 cells from AA-I-induced apoptotic damage.

1. Introduction

Aristolochic acid I (AA-I) is the active component of herbal drugs derived from plants of the *Aristolochia* species, which have been used for medicinal purposes for many centuries. However, the clinical applications of aristolochic acid have been limited because it can cause aristolochic acid nephropathy [1–3] and induce tumors [4]. Recent studies have demonstrated that AA-I can cause DNA damage and cell-cycle arrest [5], and renal tubular epithelial cells are very sensitive to apoptosis induced by AA-I [1,5,6].

A major hallmark of apoptosis is cell shrinkage, which is termed apoptotic volume decrease (AVD). AVD precedes cytochrome C release, caspase 3 activation, DNA fragmentation, and apoptotic body formation [7]. Cell shrinkage during apoptosis appears to be a rapid phenomenon. Studies have proposed that AQP1 is involved in the movement of water across the plasma membrane in dying cells during AVD, and this is based on the observation that subsequent apoptotic events are prevented when AVD induction is inhibited [8,9].

The human proximal tubular epithelial (HK-2) cell line was established by transduction of the human papilloma virus (HPV 16) E6/E7 genes into primary proximal tubular cells (PTCs) isolated from normal human kidney [10]. In addition, aquaporin-1 (AQP1) is abundantly expressed in the proximal renal tubule [11] and is closely related to water reabsorption [12]. Polyuria is an aristolochic acid-induced renal failure, during which urinary concentration defects can altered regulation of AQP1 water channels in the kidney. Accordingly, the HK-2 cell line was used for evaluating the role of AQP1 in AA-I-mediated apoptosis in vitro.

In this study, we developed a cell model of aristolochic acid nephropathy and used different approaches to evaluate the typical features of apoptosis in HK-2 cells following transfection with an AQP1-specific small interfering RNA (siRNA). We also investigated the potential therapeutic effect of the AQP1 siRNA on AA-I-induced renal damage.

2. Materials and methods

2.1. Cell culture and chemicals

The human HK-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA), and Dulbecco's modified Eagle's medium/nutrient mixture F12, fetal bovine serum (FBS),

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and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). HK-2 cells were grown in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum and HEPES free acid (15 mM) at 37 °C and atmospheric conditions of 95% air and 5% CO_2.

AA-I (98% purity, determined by high-performance liquid chromatography) was purchased from Zhengzhou Tianlin Pharm-Tech (Zhengzhou, Henan, China). AA-I was dissolved in DMSO to obtain stock solutions (15 mM), which were stored at 4 °C. Before being used in the experiments, the stock solution was diluted to concentrations of 20, 40, or 80 μM using the culture medium. During the experiments, the DMSO content in the medium was never greater than 0.5% (v/v).

Propidium iodide (PI); a colorimetric synthetic peptide substrate for caspase 3 proteases (Ac-DEVD-pNA); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT); and ribonuclease A (RNase A) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). An Annexin V/PI apoptosis detection kit was obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

2.2. siRNA transfection

AQP1 siRNA (human) (Cat# sc-29711), control siRNA (Cat# sc-37007), siRNA transfection reagent (Cat# sc-29528), and siRNA transfection medium (Cat# sc-36868) were all purchased from Santa Cruz Biotechnology (Santa-Cruz, CA, USA). Transfection of cells with the siRNAs was performed according to the manufacturer's recommendations. Briefly, prior to transfection, suspensions of cells (2×10^5 cells per transfection per well) were collected by centrifugation at 400 *g* for 5 min. The pellet was washed with the transfection medium, resuspended with the siRNA transfection mixture, and transferred to a 6-well tissue culture plate. After 5 h of incubation, equal volumes of 2× growth medium containing various concentrations of sodium arsenite were added to each well. After 24 h, cytotoxicity was assessed.

2.3. Western bolt analysis

All cells (including nonadherent cells) were harvested after treatment for 24 and 48 h, washed three times with phosphate-buffered saline (PBS), and resuspended in ice-cold buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Cell lysates were centrifuged at 12,000 *g* for 10 min, and the supernatants were sampled. These samples were separated on 12% SDS–polyacrylamide gels and then transferred to nitrocellulose membranes using standard electroblotting procedures. The membranes were blocked with 5% skim milk in Tris–Cl buffered saline (TBS-T, 0.1% Tween-20). The blots were incubated with an affinity-purified anti-rabbit polyclonal AQP1 (1:400; Chemicon International, Temecula, CA, USA) at 4 °C overnight. Immunoblots were washed and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:10000; Chemicon International, Temecula, CA, USA) at room temperature for 1 h and subsequently processed for enhanced chemiluminescence (ECL) detection using SuperSignal Substrate (Pierce Biotechnology, Rockford, IL, USA). Signals were detected using a chemiluminescence detection system (Bio-Rad, Hercules, CA, USA).

2.4. MTT assay

HK-2 cells (5×10^4 cells/ml) were incubated with fresh medium containing different concentrations of AA-I for 24 h. MTT was added to each well, up to a final concentration of 0.5 mg/ml. After the cells were incubated with MTT for 4 h at 37 °C, the formazan crystals derived from MTT were dissolved in DMSO, and the absorbance at 570 nm was measured using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.5. Morphology of the HK-2 cells after treatment with AA-I

After transfection with the AQP1 siRNA (or mock control), the morphologies of the HK-2 cells after exposure to 40 μM AA-I for 24 h were examined using a contrast microscope (1X71S8F-2; Olympus, Tokyo, Japan). After AQP1 siRNA transfection, the HK-2 cells were stained with Hoechst 33258 and visualized to investigate the incidence of apoptosis. To this end, the cells were seeded on coverslips, incubated with 40 μM AA-I for 24 h, and then fixed with 4% paraformaldehyde for 10 min. The nuclear DNA was stained with 5 mg/ml Hoechst 33258 for another 5 min, and these cells were observed under a fluorescence microscope (1X71S8F-2; Olympus, Tokyo, Japan).

2.6. PI staining assay

HK-2 cells were collected at the end of treatment, washed twice with ice-cold PBS, and then fixed in 70% ethanol at 4 °C for 12 h. After fixation, the cells were washed twice with PBS and incubated in PBS containing PI, RNase A, and Triton X-100 (0.5%) at 37 °C for 30 min. The fluorescence emitted from the propidium-DNA complex was measured using FACScan flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells containing hypodiploid DNA were considered apoptotic.

2.7. Annexin V/PI staining assay

The proportion of apoptotic cells was measured using a FACScan flow cytometer according to the instructions provided in the Annexin V/PI kit. HK-2 cells were exposed to AA-I (40 μM) for various time periods. Briefly, after treatment with the AQP1-specific siRNA for 24 h, the treated HK-2 cells (and controls) were harvested and washed twice with precooled PBS and resuspended in a binding buffer containing fluorescein isothiocyanate (FITC)-conjugated Annexin V antibody and PI. After incubation in the dark for 30 min, the cells were analyzed using flow cytometry. The number

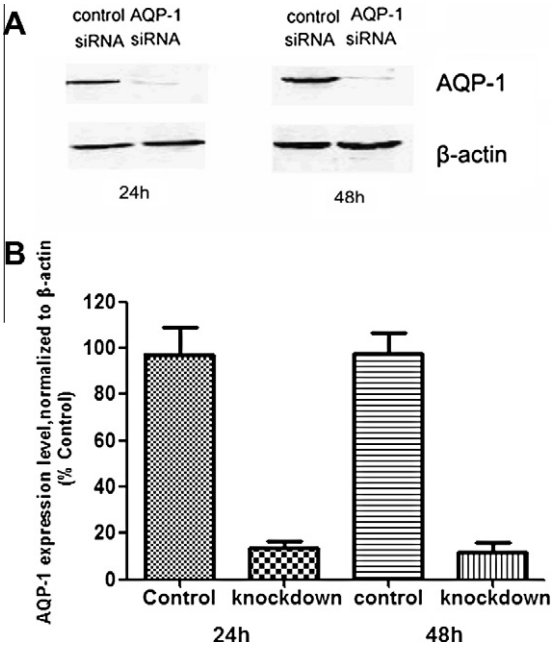

Fig. 1. (A) Knockdown of AQP1 in HK-2 cells for 24 and 48 h, as detected by Western blot analysis. (B) Expression levels were normalized to β-actin.

of cells showing early apoptosis or late apoptosis/necrosis were determined as the percentage of cells that were Annexin V^+/PI^- and Annexin V^+/PI^+, respectively [13].

2.8. Caspase 3 activity assay

The activity of caspase 3 was determined using a previously described method [14]. Briefly, cells (10^6 ml^{-1}) were harvested after treatment, washed three times with PBS, and resuspended in ice-cold buffer containing 50 mM Tris–HCl, 1 mM EDTA, 10 mM ethylene glycol tetraacetic acid (EGTA), and 1 mM DTT. Cell lysates were centrifuged at 12,000 *g* for 5 min, and extracts containing 50 μg of protein were incubated with 100 μM of the enzyme-specific colorimetric substrate Ac-DEVD-pNA at 37 °C for 2 h. The colorimetric release of *p*-nitroaniline from the Ac-DEVD-pNA substrate was measured by determining the absorbance at 405 nm (Safire II microplate reader; Tecan Group, Männedorf, Switzerland).

2.9. Statistical analysis

Each experiment was repeated at least three times, and the data were expressed as the mean ± SD. One-way ANOVA was performed to compare the means between different treatments, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Analysis of AQP1 expression in HK-2 cells after AQP1-specific siRNA transfection

To determine whether AQP1 is a necessary protein for AA-I-induced apoptosis, AQP1-specific siRNA was used to selectively knockdown AQP1 expression in HK-2 cells. Western blot analysis was performed to assess the knockdown efficiency at 24 and 48 h after transfection. Transfection with the AQP1-specific siRNA caused AQP1 expression to decrease to 17% and 15% of the levels in HK-2 cells transfected with the control siRNA at 24 and 48 h, respectively (Fig. 1).

3.2. Effect of reduced levels of AQP1 expression on AA-I-induced HK-2 cell growth

The MTT assay was used to quantify the effects of AA-I on HK-2 cell growth. As shown in Fig. 2, AA-I caused a decrease in cell viability of HK-2 cells in a dose- and time-dependent manner when compared with the control. DMSO alone showed no effect on the viability of HK-2 cells. In contrast, cells incubated with 40 and 80 μM AA-I for 24 h showed a decrease in cell viability by approximately 51.2% and 30.1%, respectively. To investigate the time course of the cytotoxic effects of AA-I, we used 40 μM of AA-I to avoid severe cell loss (the same as the experiments below). A significant effect of AA-I on the viability of the cells was noted at as early as 12 h (Fig. 2B). In contrast, AQP1-specific knockdown alone showed no effect on the viability of the HK-2 cells when compared with the control. However, AQP1 siRNA provided nearly complete protection against cell death in HK-2 cells treated with AA-I (40 μM) for 24 h (Fig. 2C).

3.3. Effect of reduced levels of AQP1 expression on AA-I-induced HK-2 cell morphological changes

After treatment with the AQP1 siRNA for 24 h, HK-2 cells were treated with 40 μM AA-I for a further 24 h. As shown in Fig. 2D,

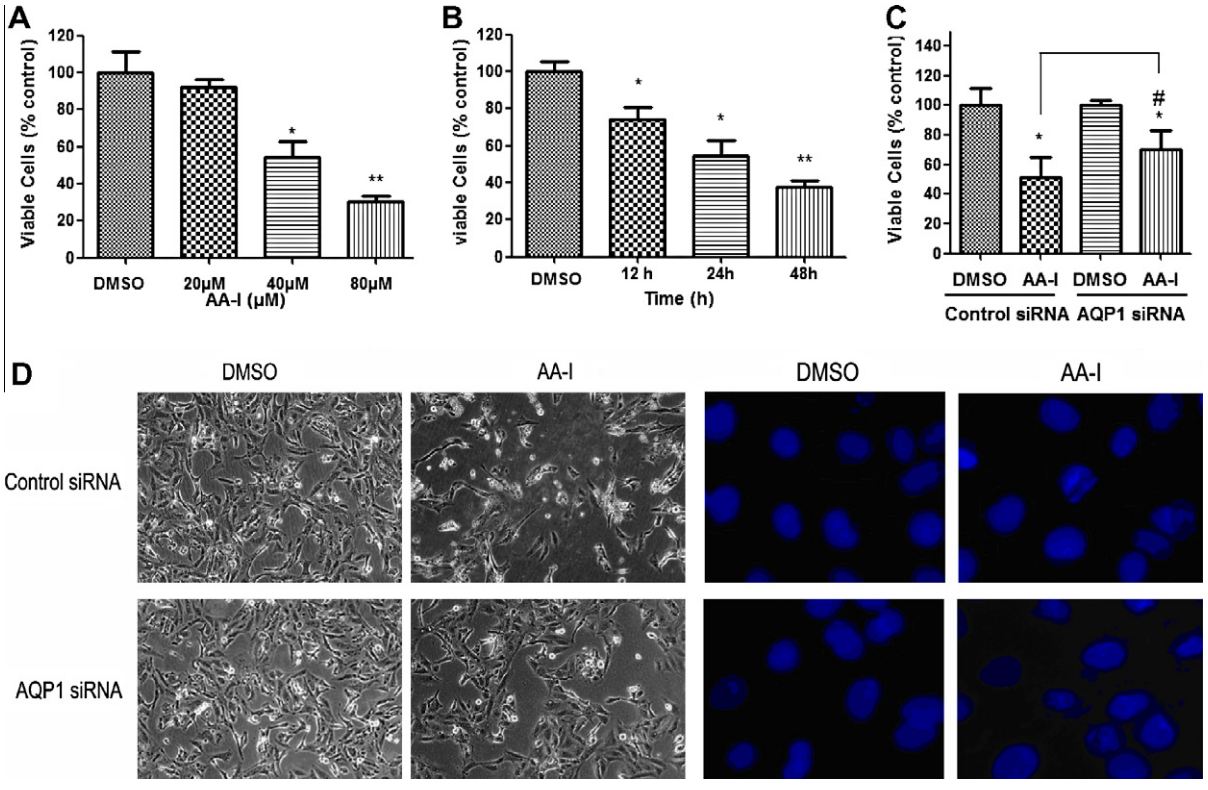

Fig. 2. Effect of AA-I on cell viability and morphology in HK-2 cells. (A) HK-2 cells were treated with AA-I (0, 20, 40, and 80 μM) for 24 h. (B) HK-2 cells were treated with 40 μM AA-I for 0, 12, 24, and 48 h. Cell viability was evaluated using MTT assays as described in Section 2. (C) HK-2 cells treated with AQP1 siRNA and control cells were induced with 40 μM AA-I for 24 h. The viability of cells was investigated using MTT assays. Data represent mean ± SD from three independent experiments. ($^*P < 0.05$, $^{**}P < 0.01$ vs. DMSO group, $^{\#}P < 0.05$ vs. control siRNA group). (D) HK-2 cells were incubated with the AQP1 siRNA for 24 h prior to AA-I (40 μM) treatment for 24 h. They were then visualized under a phase contrast microscope at 10 × 10 magnification and photographed. After the nuclear DNA was stained with Hoechst 33258, HK-2 cells were observed under a fluorescence microscope at 20 × 10 magnification. Apoptotic cells were characterized by nuclear condensation and chromatin margination.

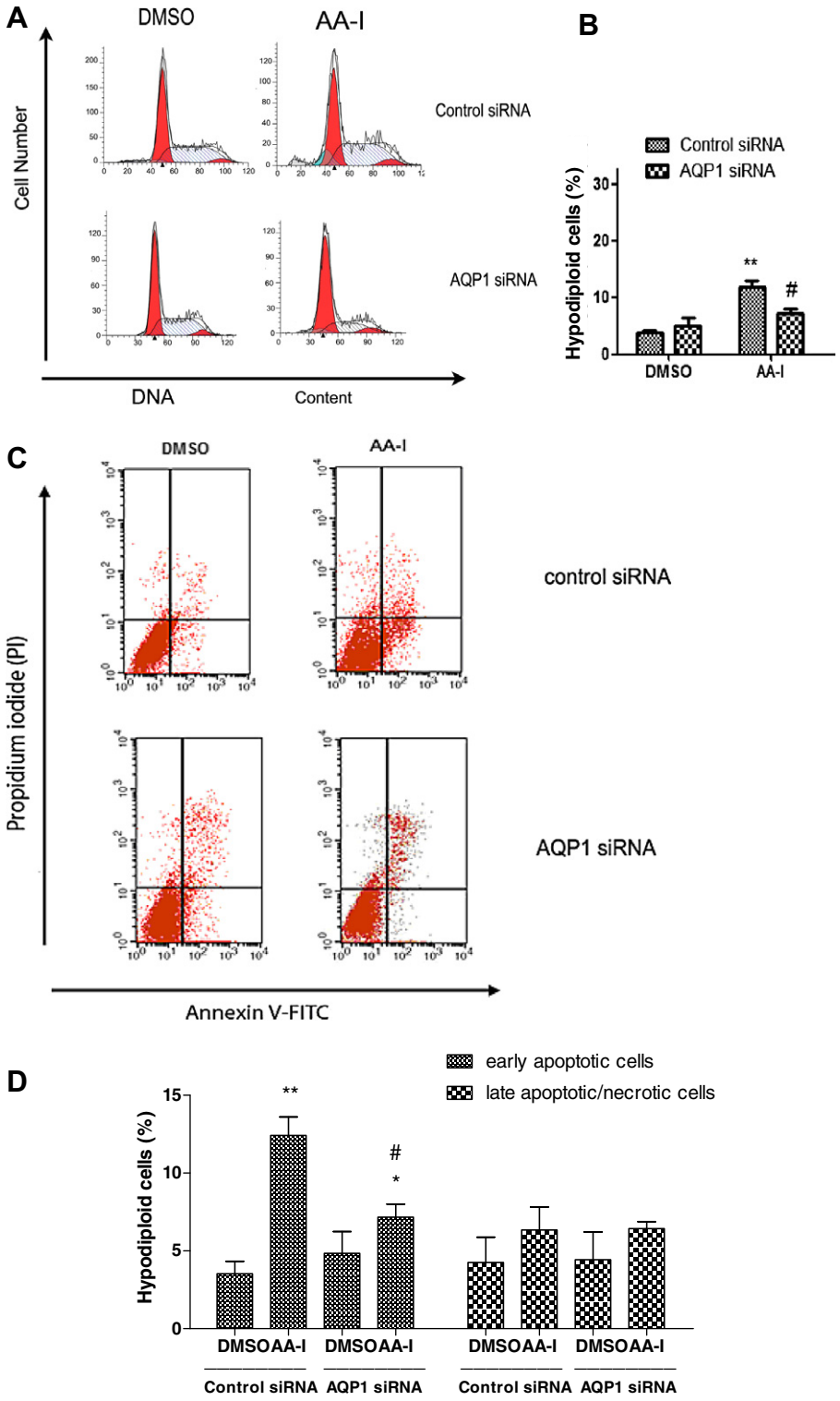

Fig. 3. AA-I-induced apoptosis was determined using cell flow cytometry. AQP1 siRNA transfected HK-2 cells and control cells were induced with 40 μM AA-I for 24 h. (A) Appearance of hypodiploid cells (sub-G1 peak) was detected via PI staining. (B) Quantitative analysis of the percentage of the cell population that contained hypodiploid DNA. (C) AA-I-induced apoptosis was determined using Annexin V/PI dual staining. The results of Annexin V/PI dual staining are showed in (D). ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. DMSO group; $^{\#}P < 0.05$ vs. control siRNA group).

untreated cells appeared to proliferate in spindle-like shapes within the wells, and cells treated with AA-I (40 μM) contracted, became rounded, and were often characterized by chromatin condensation and nuclear fragmentation. The AQP1 siRNA transfected HK-2 cells exposed to AL-I (40 μM) for 24 h maintained their normal cellular morphology, and only a limited portion of the cells displayed some slight damage, such as cellular shrinkage and chromatin condensation.

3.4. Effects of AA-I on PI staining

To determine the effect of AA-I on PI staining, apoptotic cells were identified based on sub-G1 apoptotic peaks, which could be attributed to their lower DNA content [15]. As shown in Fig. 3A, apoptotic peaks and an increased percentage of apoptotic cells were observed after treatment with 40 μM AA-I for 24 h. After transfection with the AQP1-specific siRNA, the percentage of apoptotic cells decreased. The percentage of apoptotic cells following transfection with the AQP1-specific siRNA and subsequent treatment with AA-I (40 μM) for 24 h is summarized in Fig. 3B.

3.5. Detection of apoptosis and necrosis

By analyzing the appearance of the sub-G1 peaks via cell-cycle analysis, we further characterized the AA-I-induced apoptosis by Annexin V/PI dual staining. On the basis of their affinity for Annexin V (resulting from phosphatidylserine exposure at the outer leaflet of the plasma membrane), apoptotic cells can be distinguished from living cells by using microscopic and flow cytometric procedures [16]. Annexin V staining in combination with PI allows a further distinction between necrotic (Annexin V^+/PI^+) and apoptotic (Annexin V^+/PI^-) cells. This assay helps classify cells into early-stage apoptotic cells (Annexin V^+/PI^-) and late-stage apoptotic/necrotic (Annexin V^+/PI^+) cells [13,17]. HK-2 cells transfected with the AQP1-specific siRNA and then treated with AA-I at 40 μM for 24 h were assayed for the number of early-apoptotic and necrotic cells. The number of Annexin V^+/PI^- cells increased significantly and progressively, while the number of Annexin V^+/PI^+ cells remained low. Furthermore, AQP1 knockdown dramatically attenuated the AA-I-induced early apoptosis in HK-2 cells, but had no effect on the number of necrotic cells (Fig. 3C and D).

3.6. Activation of caspase 3 in AA-I-induced apoptosis

Caspase 3 is one of the key executioners of apoptosis [18]. To determine whether caspase 3 activity was involved in AA-I-induced apoptosis, we performed an assay using Ac-DEVD-pNA, which is a colorimetric substrate for caspase 3-like proteases. AA-I increased caspase 3-like activity in a concentration-dependent manner, while pretreatment of HK-2 cells with the AQP1-specific siRNA inhibited this activity, as shown in Fig. 4. These data suggested that inhibition of AQP1 expression was able to attenuate AA-I-induced apoptosis via a caspase 3-dependent pathway.

4. Discussion

Aristolochic acid nephropathy, a progressive tubulointerstitial renal disease, is primarily caused by AA-I intoxication [19].

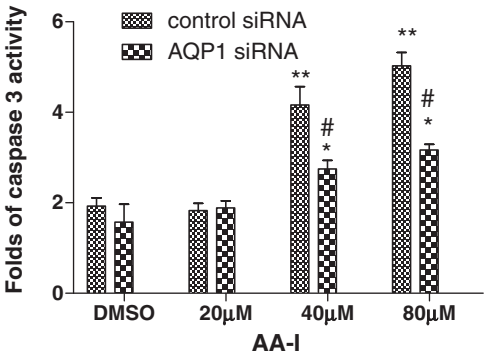

Fig. 4. Caspase 3 activity measurements using a colorimetric assay. AQP1 siRNA transfected HK-2 cells and control cells were induced with AA-I at 20, 40, and 80 μM for 24 h. ($^*P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. AA-I group).

Concerning the possible mechanism of AA-I-mediated tubular injury, pervious reports suggest that tubular epithelial cells could be the target of AA-I and that tubular cell apoptosis plays an important role in the development of kidney damage [6,20,21] . In this study, we showed that AA-I-induced (40 μM) apoptosis in HK-2 cells, and this was supported by changes in morphological characteristics, the presence of sub-G1 apoptotic peaks, and activation of caspase 3.

Apoptosis, programmed cell death, has been found in many multicellular organisms and occurs as a part of the normal physiological process in the body. However, excessive apoptosis will damage the structure of tissues and lead to loss of function. Unlike necrosis, apoptosis is mediated by the active participation of dying cells. Therefore, apoptosis appears to be much more amenable to pharmacological intervention compared with the irreversible progress of necrosis.

We used an AQP1-specific siRNA to selectively down regulate the expression of AQP1 in HK-2 cells. After AQP1 knockdown by siRNA, the cytotoxic effects of AA-I were investigated and compared. AA-I was found to exert an antiproliferation effect on HK-2 cells in a time- and dose-dependent manner, as determined by the results of the MTT assays. Knockdown of AQP1 resulted in a marked inhibition of AA-I-induced cytotoxicity at the concentration of 40 μM for 24 h. PI staining assays showed that the apoptotic cells were characterized by sub-G1 apoptotic peaks and that knockdown of AQP1 was able to prevent the increase of hypodiploid cells induced by AA-I. In the Annexin V/PI double-labeling assay, the results showed that HK-2 cell death occurred primarily in the early stage of apoptosis (Annexin V^+/PI^-) and that AQP1 knockdown dramatically attenuated AA-I-induced early apoptosis.

Caspase 3 is a central effector caspase that is implicated in several models of cell death [22]. Caspase 3-like enzyme activity in HK-2 cells was significantly enhanced in a concentration-dependent manner after treatment with AA-I in vitro. These data support the notion that a caspase 3-dependent mechanism is responsible for AA-I-induced apoptosis [1,21]. However, AQP1 knockdown may significantly inhibit the activity of caspase 3 in these cells, which are mediated through the inhibition of apoptosis in caspase 3-dependent pathway.

In conclusion, we demonstrated for the first time that AQP1 plays a crucial role in AA-I-induced apoptosis. Exposure to AA-I could activate caspase 3 and increase the rate of apoptosis in HK-2 cells. Knockdown of AQP1 via siRNA provided nearly complete protection against AA-I-induced apoptosis in vitro. As such, we suggest the possibility that AQP1 inhibition may be beneficial for preventing AA-I-induced apoptosis and could be a promising therapeutic strategy for treatment/prevention of aristolochic acid-induced kidney damage.

Conflict of interest statement

None declared.

Acknowledgments

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