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# Research Article

# Calcium-mediated activation of PI3K and p53 leads to apoptosis in thyroid carcinoma cells

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**Abstract.** The molecular mechanism responsible for cadmium-induced cell death in thyroid cancer cells (FRO) is unknown. We demonstrated that apoptosis of FRO cells induced by cadmium was concentration and time dependent. Cadmium caused the rapid elevation of intracellular calcium and induced phosphorylation of Akt, p53, JNK, ERK and p38. Inhibition of PI3K/Akt attenuated the cadmium-induced apoptosis, but the inhibition of JNK inhibitor, ERK or p38 aggravated it, indicating that activation of PI3K/Akt was a pro-apoptosis signal in response to cadmium treatment, whereas the activation of stress-activated protein kinase JNK, ERK and p38 functioned as survival signals to counteract the cadmium-induced apoptosis. Buffering of the calcium response attenuated mitochondrial impairment, recovered the cadmium-activated Akt, p53, JNK, ERK and p38, and subsequently blocked the apoptosis. These results suggested that apoptosis induced by cadmium in FRO cells was initiated by the rapid elevation of intracellular calcium, followed by calcium-mediated activation of PI3K/Akt and mitochondrial impairment.

**Keywords.** Apoptosis, cadmium, calcium, PI3K/Akt, p53, thyroid cancer.

## Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most aggressive malignancies in humans and carries a very poor prognosis. Surgery, radiotherapy and chemotherapy, based primarily on doxorubicin and cisplatin, do not meaningfully improve the survival of patient with ATC [1, 2]. Consequently, there is a need to find new therapeutic tools to treat these tumors. Studies have indicate that cadmium treatment can either abolish or substantially reduce chemically induced and spontaneous tumor formation in the mouse liver and lung [3–5]. Arsenic trioxide has recently been utilized as a mitochondria-targeting drug for acute promyelocytic leukemia, and beneficial effects have been reported [6, 7]. The main intracellular action of Cd and As seems to involve different cell death pathways.

Calcium ions are central to multiple signal transduction pathways to accomplish a variety of biological functions including differentiation and proliferation. The versatility of the $Ca^{2+}$-signaling mechanism in terms of speed, amplitude and spatio-temporal pat-

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terning enables elevations of $Ca^{2+}$ to regulate many processes of cell activity. $Ca^{2+}$ exhibits a cross-talk among a variety of signaling pathways [8, 9]. However, a cellular $Ca^{2+}$ overload or the perturbation of intracellular $Ca^{2+}$ compartmentalization can cause cytotoxicity and trigger apoptosis or necrosis [10]. Under such circumstances, various $Ca^{2+}$-dependent signaling cascades with kinases and phosphatases directly or indirectly influence cellular signaling, including activation of p53 [11], MAPKs [12–14], phosphoinositide 3-kinase (PI3K) and Akt signal pathway [15].

p53 is widely recognized as a protein functioning during the cell cycle (*i.e.*, an inducer of cell cycle arrest) and apoptosis [16]. Cells with activated p53 will arrest in the G1 phase of the cell cycle and will either institute DNA repair or undergo apoptosis. One mechanism whereby p53 inhibits cell cycle progression is by transcriptionally activating expression of the p21Waf1/Cip1 gene, a crucial downstream factor of p53, which inhibits proliferating cell nuclear antigen (PCNA) and cyclin-dependent kinases [17]. p21WAF1/PiC1 is important for p53 function and negatively regulates the cell cycle.

PI3K is a key signaling enzyme that responds to growth factors, cytokines, and environmental agents [18]. Activated PI3K can support various cell functions, including growth, migration, and survival. This latter function of PI3K has received a great deal of attention. Consequently, it has been established that PI3K can induce the phosphorylation/activation of Akt/protein kinase B, which in turn triggers cytoprotective events such as BAD phosphorylation and NF-κB activation [19]. However, evidence has been recently accumulating that indicates a role of PI3K contrary to its cytoprotective influence. For example, it was reported that the inhibition of PI3K using specific inhibitors or dominant negative mutants resulted in the suppression of cell death induced by arsenite [20], hypoxia [21], glucose deprivation [22], and serum withdrawal [23]. These observations suggest that PI3K promotes cell death under these specific experimental conditions. Therefore, it appears that PI3K does not always support cell survival, but rather it regulates the cellular viability in either of the two contrasting modes depending on the experimental conditions. In the present study we explored the intracellular mechanism of Cd-induced cell death in FRO cells, cells of an ATC cell line. Specifically, we investigated the roles and relationships of intracellular $Ca^{2+}$, the cell cycle, mitochondria, MAPKs, p53 and PI3K/Akt in the cells treated by Cd. Our results suggest that Cd-induced apoptotic cell death in FRO cells is mediated by rapid elevation of intracellular $Ca^{2+}$, followed by the activation of p53 and PI3K/Akt and G1 block, and a mitochondria-mediated apoptosis pathway.

## Materials and methods

**Cell culture.** FRO cells, cells of an ATC cell line bearing wild-type p53, were cultured in RPIM 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$/95% air.

**Assessment of cell death.** To assess the cytotoxicity and DNA fragmentation induced by Cd, cells were cultured to 80% confluence and then exposed to different concentrations of Cd for 24 h or to 100 μM Cd for 3, 6, 12, 24, 48 or 72 h. Cytotoxicity induced by Cd was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DNA fragmentation was determined with Apo-BrdU *in situ* DNA fragmentation assay kit (BioVision Research Products, Mountain View, CA) by flow cytometry. Various inhibitors were used to explore their effects on cell viability. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD; ALEXIS, Lausen, Switzerland) was added 2 h prior to the treatment with 100 μM Cd. SP600125 (Sigma, St Louis, MO), PD098059 and SB203580 (both from Promega, Madison, WI), LY294002 and Wortmannin (Calbiochem, Darmstadt, Germany), pifithrin-α (A.G. Scientific, San Diego, CA) and BAPTA (Sigma) were added 1 h prior to the treatment with 100 μM Cd for 72 h.

**Cell cycle analysis.** The cell phase distribution was assayed by determination of the DNA content. The cells were fixed overnight in 5 ml 70% ethanol at –20°C. They were then washed with PBS and incubated with propidium iodide (20 μg/ ml) and RNase A (200 μg/ ml) in the dark for 30 min. The stained cells were subjected to flow cytometry. A DNA content frequency histogram generated by deconvolution software was employed to analyze the data.

**Detection of intracellular $Ca^{2+}$ and mitochondrial transmembrane potential by flow cytometry.** To evaluate intracellular $Ca^{2+}$ levels, the cells were incubated with 5 μM Fluo-4 AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. To assess changes in mitochondrial transmembrane potential ($\Delta\psi m$), the cells were incubated with 40 nM $DiOC_6$ [3] (Aldrich Chem. Co.) for 15 min at 37°C. The cells were then harvested by trypsin and washed with PBS. Following re-suspension in PBS, the cells were subjected to flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

**Preparation of proteins in the cytosolic fractions.** The cells were washed twice in ice-cold PBS and resuspended in five volumes of ice-cold extract buffer (20 mM HEPES-KOH, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.5). The resuspended cells were homogenized with ten strokes of a Teflon homogenizer. The homogenates were centrifuged at 10 000 *g* for 15 min at 4°C. Cytosolic fractions were obtained after further centrifugation at 100 000 *g* for 1 h at 4°C. The resulting supernatant was stored at –70°C for further use.

**Western blotting analysis.** Protein (10 μg) from the cytosolic fraction and 50 μg total protein from treated cells were loaded onto l2% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the separated proteins transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). To detect the levels of cytochrome c (cyto c), p21, apoptosis-inducing factor (AIF), phospho-JNK1/2 (Thr183/Tyr185), phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), total JNK1/2, total p38, total p53 and cleaved PARP, mouse-anti-human monoclonal antibodies for cyto c and p21 (Zymed Laboratories, San Francisco, CA), mouse-anti-human monoclonal antibodies for AIF, total JNK1/2, total p38 and total p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse-anti-human monoclonal antibodies for phospho-JNK1/2, phospho-ERK1/2, phospho-p38 and cleaved PARP (Cell Signaling Technology, Beverly, MA) were employed with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology). To detect the levels of phospho-Akt (Thr308), phospho-p53 (Ser15), phospho-MDM2

(Ser166), total ERK, total Akt, and caspase-3, a rabbit monoclonal antibody for phospho-Akt (Thr308), rabbit polyclonal antibodies for phospho-p53 (Ser15), phosphor-MDM2 and total ERK (Cell Signaling Technology), for total Akt and caspase-3 (Santa Cruz Biotechnology) were used as the primary antibody and a polyclonal goat anti-rabbit IgG antibody as the secondary antibody (Santa Cruz Biotechnology). A goat anti-human actin antibody was used to detect the levels of actin, which was used as a control for equal loading. Detection of the target protein signal was achieved using an ECL system (Amersham Biosciences, Piscataway, NJ).

**Statistical analysis.** Data were expressed as a mean ± SD. Statistical difference between groups was examined using the ANOVA and Student's *t*-test. There was a statistically significant difference when $p<0.05$.

## Results

### Cell death induced by Cd was dose and time dependent.

To quantify cell death induced by Cd, cytotoxicity and DNA fragmentation were examined. The result showed that the cytotoxicity of Cd on FRO cells was elevated with increasing Cd concentrations and incubation periods ($p<0.01$), as evidenced by the cell viability assay (Fig. 1a, b). Cd was able to significantly induce DNA fragmentation ($p<0.01$), and the amount of DNA fragmentation peaked at 24 h after treatment with 100 μM Cd, and thereafter it decreased (Fig. 1c).

### Cell death induced by Cd is associated with the elevation of intracellular $Ca^{2+}$ level.

To explore whether the cell death induced by Cd is associated with the elevation of intracellular $Ca^{2+}$ level, we first determined the intracellular $Ca^{2+}$ level in the cells treated by Cd. The intracellular $Ca^{2+}$ level in Cd-treated FRO cells was analyzed by flow cytometry. The elevation of intracellular $Ca^{2+}$ level was observed in FRO cells as early as 0.5 h following treatment with Cd. The elevation of intracellular $Ca^{2+}$ level could be inhibited by BAPTA, a $Ca^{2+}$ buffer (Fig. 2a). To explore whether cell death induced by Cd was related to elevation of intracellular $Ca^{2+}$ level, FRO cells were treated with BAPTA 1 h prior to the treatment with Cd. Cell death was assessed by MTT. As shown in Figure 2b, cell death induced by Cd was inhibited by BAPTA to a level close to the control, confirming that the elevation of intracellular $Ca^{2+}$ level was a pivotal signal involved in the induction of cell death by Cd in FRO cells.

### PI3K/Akt signal pathway was pro-apoptotic, whereas three MAPKs functioned in an intrinsic survival pathway to counteract stress caused by Cd.

To determine whether the activation of MAPKs and PI3K/Akt is involved in the cell death induced by Cd in FRO cells, we treated FRO cells with Cd and analyzed the levels of JNK, ERK, p38 and Akt. The activation of

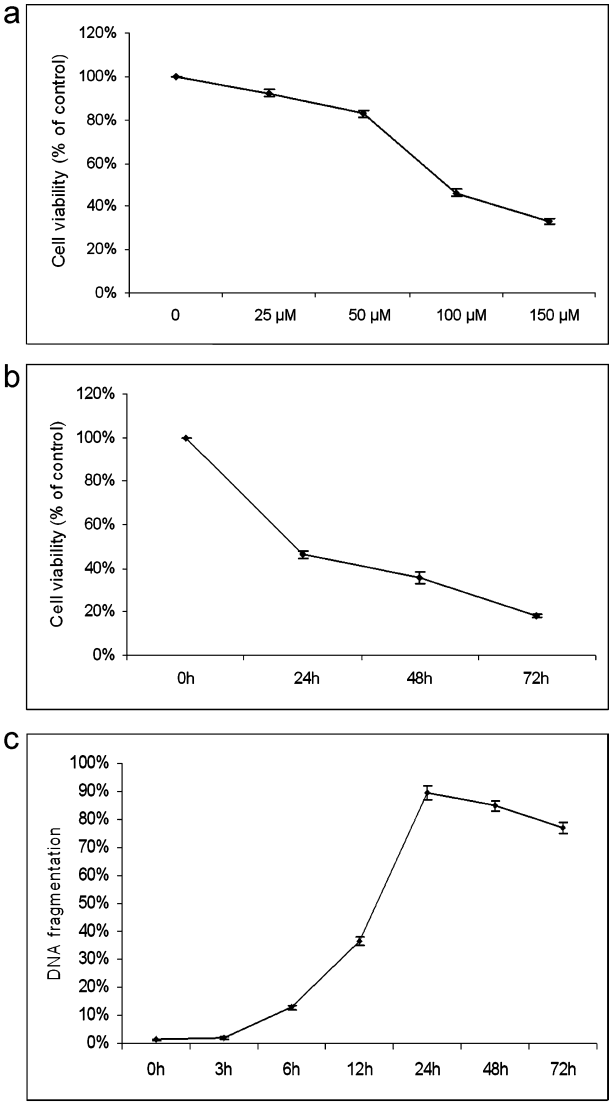


**Figure 1.** Cell death induced by Cd. FRO cells were treated with various concentrations of Cd (0–150 μM) for 24 h or with 100 μM Cd for the indicated periods up to 72 h. MTT assay was used to determine the viability of cells treated with various concentrations of Cd (*a*) and at different time points (*b*). The DNA fragmentation was examined by Apo-BrdU *in situ* DNA fragmentation assay kit (*c*). The data represent a mean of three independent experiments.

Akt, JNK1/2, ERK1/2, and p38 was assayed by Western blot analysis using antibodies that respectively recognized the phosphorylated forms of these kinases and total proteins of these kinases. The results showed that Cd induced activation of these four kinases and their activities peaked at around 12–24 h (Fig. 3a). The levels of activated Akt and JNK fell from the peak at 48 and 72 h, but were still higher than the control. The activated ERK1/2 and p38 maintained a high level until at least 72 h. The effects of activation of these four kinases on the induction of cell death were investigated using their specific inhibitors, PD098059 for ERK1/2, SB203580 for p38, SP600125 for JNK1/2, LY294002 and Wortmannin

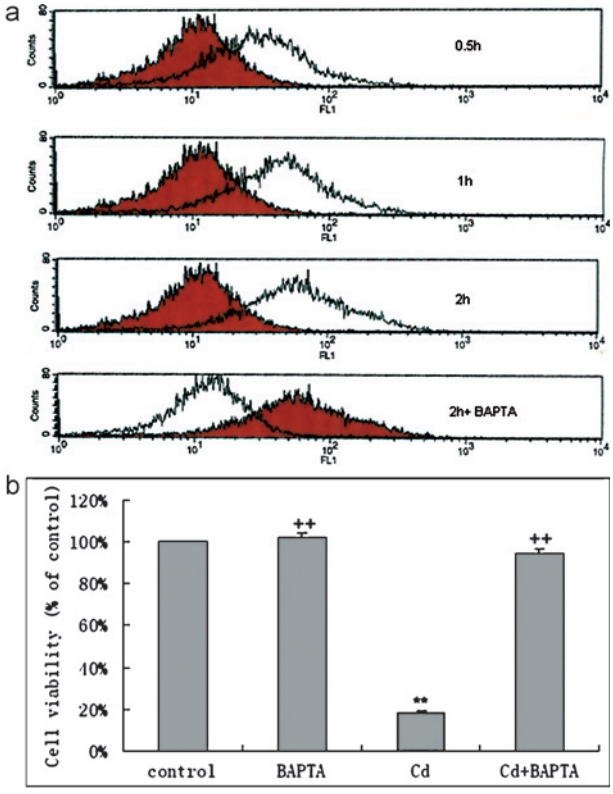


**Figure 2.** The intracellular $Ca^{2+}$ was elevated by Cd. (*a*) FRO cells were treated with 100 μM Cd for 0.5, 1 and 2 h. After treatment, cells were stained with 5 μM Fluo-4 AM for 30 min at 37°C and analyzed by flow cytometry. The horizontal axis shows the relative fluorescence intensity, and the vertical axis shows cell numbers. Shaded peaks are control cells without Cd treatment; open peaks are Cd-treated cells. In the experiment of inhibition by BAPTA on the elevation of intracellular $Ca^{2+}$, FRO cells were pretreated with 100 μM BAPTA 1 h prior to the treatment with 100 μM Cd for 2 h. The shaded peak is Cd-treated cells; the open peak is Cd plus BAPTA. The results shown are representative of three independent experiments. (*b*) FRO cells were treated with 100 μM Cd for 72 h or with 100 μM BAPTA 1 h prior to the treatment with 100 μM Cd for 72 h, and the percentage of cell viability of treated cells was assayed by MTT. The data represent a mean of three independent experiments. ** $p<0.01$, compared with control, $^{++}$ $p<0.01$, compared with Cd-treated cells.

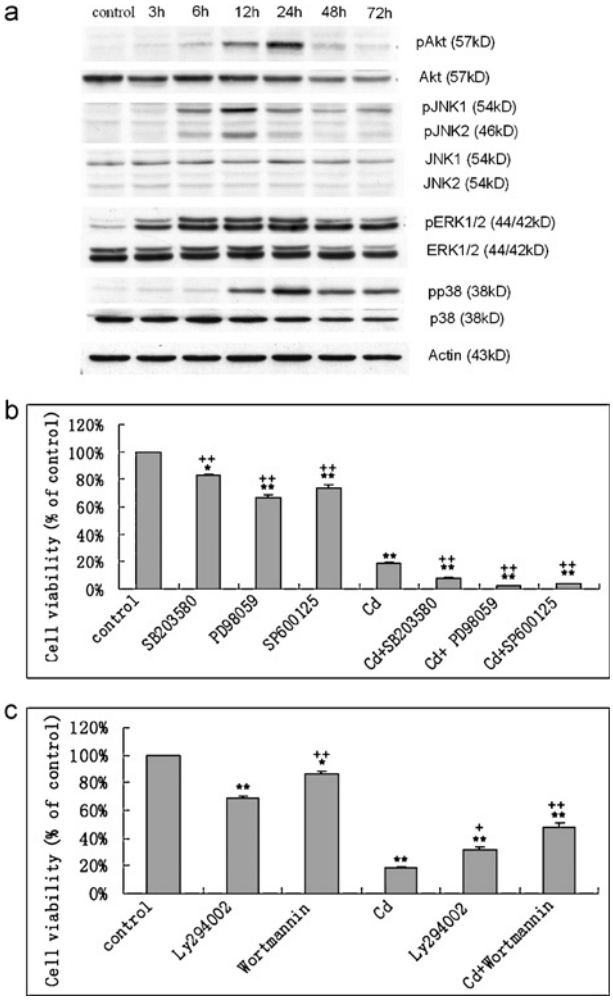


**Figure 3.** The effect of activation of Akt and MAPKs on cell death induced by Cd. (*a*) FRO cells were treated with 100 μM Cd for the indicated periods. The activation of Akt, JNK, ERK and p38 was determined by Western blot analysis using antibodies against phosphorylated and total molecules of these kinases. The level of actin in the total protein was used as a control. (*b*, *c*) FRO cells were treated with 100 μM Cd for 72 h, with 50 μM SP600125, with 40 μM PD098059, with 40 μM SB203580 or with 40 μM LY294002 and 100 nM Wortmannin, 1 h prior to the treatment with 100 μM Cd for 72 h, and the percentage of cell viability of treated cells then assayed by MTT. The data represent a mean of three independent experiments, ** $p<0.01$, compared with control, $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$, compared with Cd-treated cells.

for PI3K/Akt. Treatment with 40 μM PD098059, 40 μM SB203580 or 50 μM SP600125 accelerated Cd-induced cell death in FRO cells (Fig. 3b), suggesting that ERK1/2, p38 and JNK1/2 are activated and that the inhibition of these kinases has an additive effect with Cd in FRO cells. However, treatment with 40 μM LY294002 or 100 nM Wortmannin caused a significant reduction in Cd-induced cell death (Fig. 3c), indicating that activation of PI3K/Akt signaling pathway contributed to the induction of cell death in FRO cells treated with Cd.

**Activation of p53 is involved in the cell death induced by Cd.**

To examine the role of p53 in the cell death induced by Cd in FRO cells, we first determined whether p53 was up-regulated and activated by Cd. The result of Western blot analysis showed that total p53 protein was up-regulated by Cd (Fig. 4a). The phosphorylation of p53 at Ser15 was induced in a time-dependent manner, and it peaked at 12 h after the treatment. These results indicated that p53 was activated by Cd. The involvement of p53 activation in the induction of cell death was confirmed by a p53-specific inhibitor, pifithrin-α. Treatment with 50 μM pifithrin-α caused a significant reduction in Cd-induced cell death (Fig. 4b), reinforcing that activation of p53 contrib-

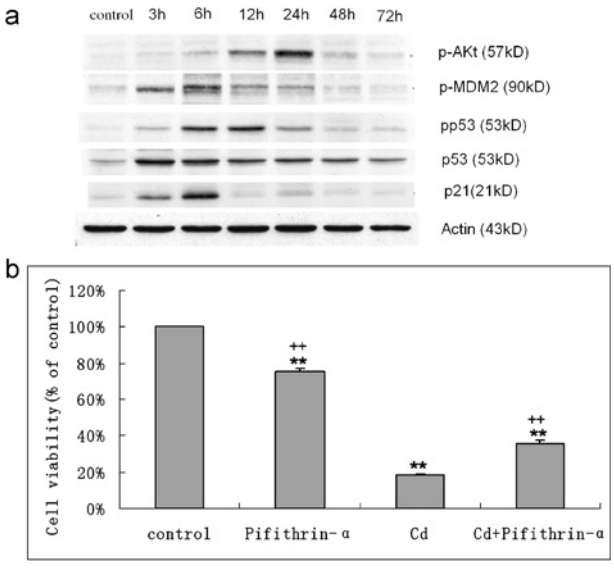


**Figure 4.** The effect of activation of p53 on cell death induced by Cd. (*a*) FRO cells were treated with 100 μM Cd for the indicated periods. The levels of phospho-Akt, phospho-MDM2, phospho-p53, total p53, and p21 were determined by Western blot analysis. The level of actin in the total protein was used as a control. (*b*) FRO cells were treated with 100 μM Cd for 72 h, with 50 μM of pifithrin-α 1 h prior to the treatment with 100 μM Cd for 72 h, and the percentage of cell viability of treated cells then assayed by MTT. The data represent a mean of three independent experiments, ** $p<0.01$, compared with control, $^{++}$ $p<0.01$, compared with Cd-treated cells.

uted to the induction of cell death in FRO cells treated with Cd.

### Elevation of intracellular $Ca^{2+}$ is involved in the activation of Akt, JNK, ERK, p38 and p53.

To examine whether the elevation of intracellular $Ca^{2+}$ is involved in the activation of Akt, JNK, ERK, p38 and p53, we explored whether the activation of these molecules were inhibited by BAPTA, a $Ca^{2+}$ buffer. The Western blot analysis showed that the phosphorylation levels of Akt, JNK, ERK, p38 and p53 could be inhibited to the control level when FRO cell were pretreated with BAPTA (Fig. 5). This result suggested that the elevation of intracellular $Ca^{2+}$ is the pivotal signal involved in the activation of Akt, JNK, ERK, p38 and p53; all of them are downstream events of the intracellular $Ca^{2+}$ signal.

### G1 block was involved in cell death induced by Cd.

To determine whether the change in cell phase distribution was related to Cd-induced cell death, the cell phase distribution after Cd treatment was analyzed by flow cytometry. When FRO cells were treated with 100 μM Cd, the percentage of cells at G1/G0 phase increased gradually in a time-dependent manner (Fig. 6). This result indicates that the G1 block was involved in cell death induced by Cd in FRO cells.

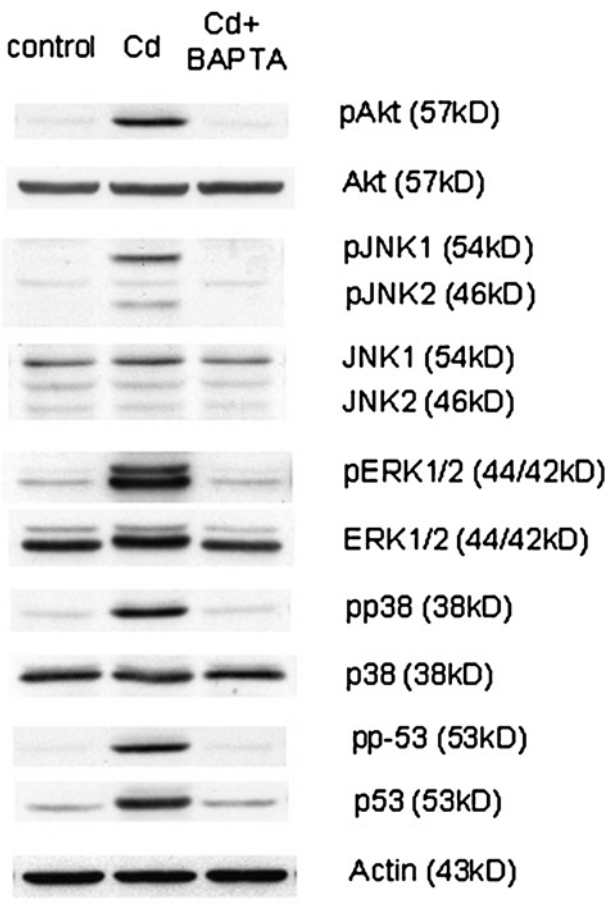


**Figure 5.** The elevation of intracellular $Ca^{2+}$ was related to the activation of Akt, MAPKs and p53. FRO cells were treated with 100 μM Cd for 12 h or with 100 μM of BAPTA 1 h prior to the treatment with 100 μM Cd for 12 h. The activation of Akt, JNK, ERK, p38 and p53 was determined by Western blot analysis using antibodies against phosphorylated and total molecules of these kinases or p53. The level of actin in the total protein was used as a control. The results shown are representative of three independent experiments.

### Mitochondrial pathway was involved in cell death induced by Cd.

Two approaches have been employed to determine whether a mitochondrial pathway was associated with cell death induced by Cd. In our first approach, we found that, following treatment with Cd, a reduction in $\Delta\psi m$ of FRO cells was observed. The $\Delta\psi m$ in FRO cells reduced gradually following treatment with Cd (Fig. 7). Furthermore, it was noted that the reduction in $\Delta\psi m$ could be attenuated by BAPTA, a $Ca^{2+}$ buffer. These results indicate that the reduction in $\Delta\psi m$ was involved in the cell death induced by Cd and that the reduction in $\Delta\psi m$ by Cd was associated with the elevation of intracellular Ca. In our second approach, FRO cells were treated with 100 μM Cd for various periods of time. The levels of cyto c and AIF in the cytosol were then analyzed by Western blot. The results showed that the levels of cyto c and AIF in the

cytosol of Cd-treated FRO cells increased in a time-dependent manner (Fig. 8). This implies that after Cd treatment, the release of cyto c and AIF from mitochondria into the cytosol was involved in cell death induced by Cd.

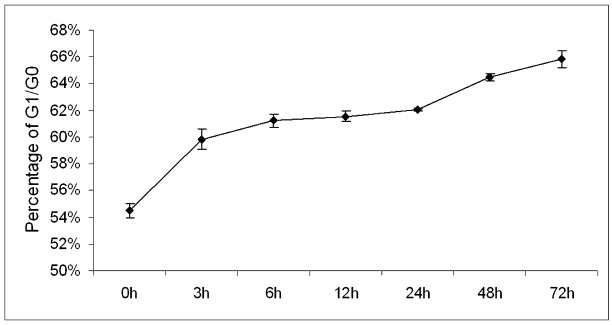


**Figure 6.** G1 block was induced by Cd. FRO cells were treated with 100 μM Cd for the indicated periods. Cell cycle was analyzed by flow cytometry and an increase in G1/G0 phase was recorded in the cells treated with Cd. The data represent a mean of three independent experiments.

### Activation of caspase-3 and cleavage of PARP was involved in cell death induced by Cd.

FRO cells were treated with 100 μM Cd for different periods of time. The activation of caspase-3 and cleavage of PARP (a substrate of caspase-3) were analyzed by Western blot. The result showed that the level of full-length procaspase-3 (32 kDa) decreased and the level of the active cleaved caspase-3 (20 kDa) increased in a time-dependent manner (Fig. 9a). Parallel to the activation of caspase-3, 89-kDa cleaved PARP was produced. To determine whether the activation of caspases was involved in the cell death induced by Cd, FRO cells were treated with zVAD, a general inhibitor for caspases, 2 h prior to the treatment with Cd. Cell death was assessed by MTT. As shown in Figure 9b, cell death induced by Cd was inhibited by zVAD, confirming that cell death induced by Cd was caspase-dependent in FRO cells.

## Discussion

Cd is known to induce toxic effects in human, and has shown to induce apoptosis in many tissues and cell types *in vivo* and *in vivo* [24–26]. However, the intracellular signaling mechanisms involved in Cd-induced apoptosis have only been investigated in a limited number of studies and not in thyroid cancer cells. Moreover, the mechanism of apoptosis seems to depend on the cell type. In the present study, we have provided convincing evidence of Cd-induced apoptotic cell death in anaplastic thyroid carcinoma cells, based on results from cell viability, TUNEL, caspase activity, cleavage of PARP, cyto c release and alteration of mitochondrial membrane potential.

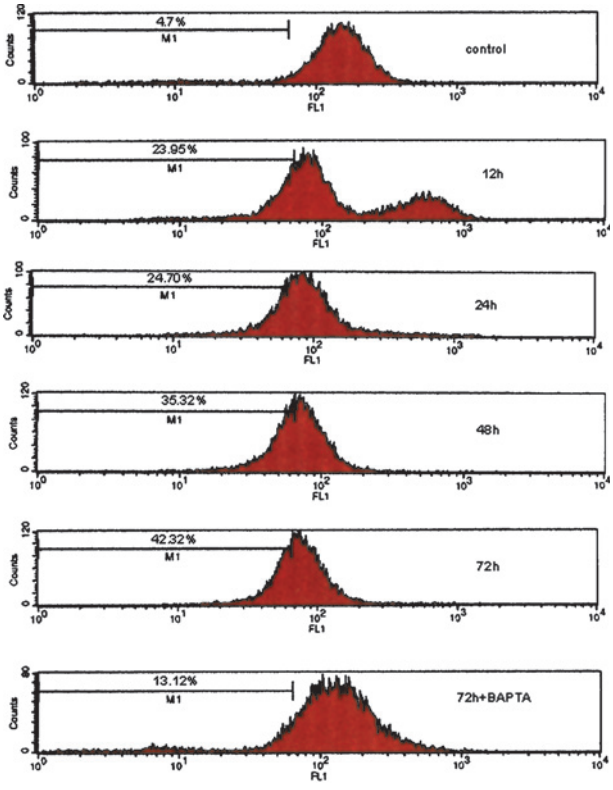


**Figure 7.** Reduction in mitochondrial transmembrane potential ($\Delta\psi m$) of FRO cells treated with Cd. FRO cells were treated with 100 μM Cd for 12, 24, 48 and 72 h. After the treatment, cells were stained with 40 nM $DiOC_6$ [3] for 15 min at 37°C and analyzed by flow cytometry. To determine the effect of BAPTA on Cd-induced alteration of $\Delta\psi m$, the FRO cells were pretreated with 100 μM BAPTA 1 h prior to treatment with 100 μM Cd for 72 h. The horizontal axis shows the relative fluorescence intensity and the vertical axis the cell number. The percentage of cells with a low $\Delta\psi m$ is indicated. The results shown are representative of three independent experiments.

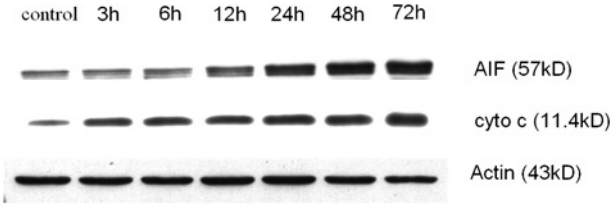


**Figure 8.** The release of cytochrome c (cyto c) and apoptosis-inducing factor (AIF) from mitochondria into the cytosol in FRO cells treated with Cd. FRO cells were treated with 100 μM Cd for indicated periods. After the treatment, the cytosolic fraction was extracted. The levels of cyto c and AIF in the cytosolic fraction were analyzed by Western blot. The level of actin in the total protein was used as a control. The results shown are representative of three independent experiments.

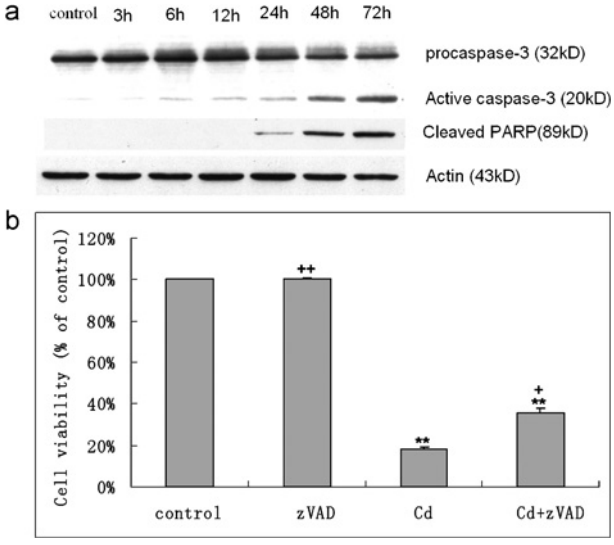


**Figure 9.** Activation of caspase-3, cleavage of PARP, and the effect of the caspase inhibitor on cell death in FRO cells treated with Cd. (*a*) FRO cells were treated with 100 μM Cd for the indicated periods. After the treatment, cells were collected and lysed. Activation of caspase-3 and cleavage of PARP were assayed by Western blot analysis with specific antibodies for pro- and active caspase-3, and for cleaved PARP. The level of actin in the total protein was used as a control. (*b*) FRO cells were treated with 100 μM Cd for 72 h or with 50 μM zVAD 2 h prior to treatment with 100 μM Cd for 72 h, and the percentage of cell viability of treated cells was assayed by MTT. The data represent a mean of three independent experiments. ** $p<0.01$, compared with control, $^{+}$ $p<0.05$, compared with Cd-treated cells.

In this study, we have demonstrated that exposure of FRO cells to Cd results in a rapid increase of cytosolic $Ca^{2+}$. Although $Ca^{2+}$ as a general mediator in apoptosis has been extensively studied, current knowledge about the exact role of $Ca^{2+}$ in Cd-induced apoptosis is very limited [27] and has not been studied in thyroid cancer. There is some indirect evidence showing the possible role of $Ca^{2+}$ in Cd-induced apoptosis. For instance, El Azzouzi *et al.* [28] found that a $Ca^{2+}$ channel blocker inhibited Cd-induced apoptosis in a human T cell line. Moreover, Cd has been found to activate $Ca^{2+}$-dependent endonuclease, protein kinase C, and many other proteins in $Ca^{2+}$-dependent signaling pathways that are closely related to the apoptotic process [29]. In the present study, we have provided strong evidence in support of the conclusion that the rapid elevation of intracellular $Ca^{2+}$ concentration is a key mediator in Cd-induced apoptotic cell death in FRO cells. First, the elevation of intracellular $Ca^{2+}$ level responds quickly as it occurs as early as 0.5 h following treatment with Cd (Fig. 2a). Second, BAPTA, an intracellular free $Ca^{2+}$ chelator, abolished Cd-induced $Ca^{2+}$ elevation and suppressed cell death induced by Cd almost to the control level (Fig. 2b). Finally, $Ca^{2+}$ chelator BAPTA abolished the phosphorylation of Akt, JNK, ERK, p38 and p53 induced by in Cd (Fig. 5), suggesting that $Ca^{2+}$ is required for the phosphorylation. The activation of p53, Akt, JNK, ERK and p38 has been demonstrated to be involved in the apoptotic cell death induced by Cd in FRO cells, with the first two as pro-apoptotic signals but the other three as anti-apoptotic signals (Figs 3 and 4).

Cd can directly damage isolated mitochondria, prevent respiration and cause mitochondrial swelling and loss of inner membrane potential [30]. On the other hand, some recent studies revealed that changes of intracellular $Ca^{2+}$ concentration are able to affect mitochondria and induce cyto c release, which is a well-proven upstream initiator for caspase-3 [31]. The release of cyto c from mitochondria usually results from mitochondrial membrane permeability transition (MPT) pore opening and/or mitochondrial membrane potential (MMP) disruption [32]. In our study, exposure of FRO to Cd caused disruption of MMP, the release of cyto c and AIF, and activation of caspase-3 and cleavage of PARP. Moreover, the disruption of MMP by Cd was attenuated by pretreatment of BAPTA, a $Ca^{2+}$ inhibitor. Therefore, a mitochondrial caspase-dependent pathway is involved in the apoptotic cell death induced by Cd in FRO cells. The elevation of intracellular $Ca^{2+}$ contributes to the disruption of MMP. Cd may disrupt MMP *via* its direct effect on mitochondria and/or indirectly through the elevated intracellular $Ca^{2+}$.

MAPK cascades include ERK, JNK and p38, and are all involved in the regulation of cell growth, differentiation and apoptosis. In general, the ERK signaling cascade is activated by growth factors and is associated with cell survival and proliferation. On the other hand, p38 and JNK are mainly activated by cellular stress and are often associated with inflammation and apoptosis [33]. The cellular functions regulated by ERK, p38 or JNK seem to depend on the cell type, the stimulus, the duration and strength of kinase activities. For instance, p38 MAPK can function to inhibit apoptosis by promoting the survival signal and NF-κB-targeted genes, stimulating c-IAP2 expression and stabilizing cyclooxygenase-2 transcript [34]. Inhibition of c-JNK induces apoptosis of human tumor cells in a p53-dependent manner [35, 36]. In our study, JNK, ERK and p38 were all activated by Cd prior to the occurrence of apoptosis in FRO cells. Pretreatment with their specific inhibitors, SP600125 for JNK1/2, PD098059 for ERK1/2, and SB203580 for p38, aggravated the cell death induced by Cd in FRO cells. Interestingly, PI3K/Akt was also activated by Cd, and pretreatment with LY294002 and Wortmannin, specific inhibitors for PI3K/Akt, caused significant reduction in Cd-induced cell death (Fig. 3c). Thus, ERK, p38, and JNK played a role in a survival pathway to counteract cell death induced by Cd in

FRO cells. Conversely, activation of PI3K/Akt signaling pathway contributed to the induction of cell death in FRO cells treated with Cd. Although it has been established that PI3K/Akt pathway functions as a survive signal to trigger cytoprotective events, such as BAD phosphorylation and NF-κB activation [19], evidence has been recently accumulating that PI3K may act against cytoprotection. For example, it was reported that the inhibition of PI3K by its specific inhibitors or dominant negative mutants resulted in the suppression of cell death induced by arsenite [20], hypoxia [21], glucose deprivation [22], and serum withdrawal [23]. These results suggest that the function of PI3K/Akt is to promote cell death under these specific experimental conditions. Therefore, it appears that PI3K does not always support cell survival, but rather it regulates the cellular viability in either of the two contrasting modes depending on the experimental conditions. It should be pointed out that factors other than PIK also participate the cell death pathway induced by Cd since the inhibition of PI3K results in at most 40 % abrogation of the cytotoxic effect of Cd.

Cytotoxic damage initiates a cellular response involving the activation of p53, which acts to stimulate apoptosis [37]. The phosphorylation of p53 disrupts the p53–MDM2 interaction, thus preventing p53 ubiquitination and degradation [38]. In our study, Cd induced phosphorylation of Akt, MDM2 and p53, with phosphorylation of MDM2 preceding that of Akt and p53. These results suggest that MDM2 is not primarily phosphorylated by Akt but probably by other stress-related kinases. The phosphorylated MDM2 was increased by Cd and such an increase occurred at about same time as the elevation of total p53 but predated the increase of the phosphorylated p53, indicating that the elevated level of p53 might result from not only its own phosphorylation but also the consequence of the phosphorylated MDM2, as the phosphorylated MDM2 has a reduced ability to interact with and inhibit p53 [38]. Cells with elevated levels of p53 will arrest in the G1 phase of the cell cycle and will either institute DNA repair or undergo apoptosis. One mechanism whereby p53 inhibits cell cycle progression is by transcriptionally activating expression of the p21Waf1/Cip1 gene, a crucial downstream factor of p53, which inhibits PCNA and cyclin-dependent kinases [17]. In our study, the increased expression of p21 was observed as early as 3 h following treatment with Cd (Fig. 4a). Parallel to the activation of p53 and up-expression of p21, the percentage of G1/G0 phase of cell cycle increased gradually from 3 h following Cd treatment (Fig. 6). Moreover, pifithrin-α, a specific inhibitor of p53, caused a significant reduction in Cd-induced cell death (Fig. 4b). However, similar to the inhibition of PI3K, the inhibition of p53 can only lead to at most 30 % reduction of Cd-induced cell dearth. The findings suggest that Cd-induce cell death is a complicated process that involves multiple factors. Nevertheless, our study provided evidence that the activation of p53 and increased expression of p21 contributed to G1 block and subsequent apoptotic cell death induced by Cd in FRO cells.

In conclusion, our study demonstrated the critical role of intracellular $Ca^{2+}$ overloading in Cd-induced apoptotic cell death in ATC cells. Upon Cd exposure, there was a rapid intracellular $Ca^{2+}$ elevation, followed by the activation of PI3K/Akt, p53 and MAPKs, G1 block, mitochondrial impairment, caspase-3 activation and cleavage of PARP. BAPTA, a specific intracellular $Ca^{2+}$ chelator, abolished Cd-induced $Ca^{2+}$ overloading and subsequently suppressed the activation PI3K/Akt, p53, MAPKs, mitochondrial impairment and apoptotic cell death. Interestingly, activation of PI3K/Akt is a pro-apoptosis signal in the process of induction of apoptotic cell death, whereas the activation of c-JNK, ERK and p38 MAPK functions as survival signals to counteract the cell death induced by Cd in ATC cells.

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