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Research Article

Mitochondria and calpains mediate caspase-dependent apoptosis induced by doxycycline in HeLa cells

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Abstract. Doxycycline (Dc) has been demonstrated to inhibit cell growth and induce apoptosis in tumor cells, although its mechanism of action is not fully understood. The present study demonstrates that apoptosis can be induced in HeLa cells. Western blot data demonstrated that cytochrome c (Cyt c), Smac (the second mitochondria-derived activator of caspase), calpain I, caspase-9, -3 and -8 were involved in the apoptotic process, while the pan caspase inhibitor zVAD-fmk almost completely inhibited Dc-induced apoptosis. We further demonstrated that the release of mitochondrial proteins and the activation of calpains occurred upstream of the caspase cascade, in which caspase-9 was activated in response to the release of Cyt c, that caspase-8 activation was caspase and calpain dependent, and that caspase-3 was activated mainly by caspase-8 and -9. Caspase-8 played important roles in the activation of caspase-3 and induction of apoptosis, whereas the role of the caspase-9 was limited.

Keywords. Doxycycline, apoptosis, mitochondria, calpain, caspase.

Introduction

Apoptosis is a highly regulated and energy-dependent process that is an intricate part of various physiological and pathological conditions. Caspase activation is a crucial step at which cells become committed to undergo apoptosis. Two pathways of caspase activation have been described during apoptosis. The first is mediated by death receptors, such as Fas and TNF receptors. Ligand binding to death receptors results in the recruitment of the adaptor molecule FADD/Mort-1 to the receptor [1–3], allowing binding and autoactivation of procaspase-8 [4–6]. Once caspase-8 is activated, it can process effector caspases (caspase-3, -6, and -7), to induce a caspase signaling cascade [7–9]. In the second pathway, diverse pro-apoptotic signals converge at the mitochondrial level, resulting in the translocation of cytochrome c (Cyt c) from the mitochondria to the cytoplasm [10], where it binds to Apaf-1, a mammalian CED-4 homologue, and mediates the recruitment of procaspase-9 [11, 12]. Oligomerization results in autoactivation of procaspase-9 [13]. Active caspase-9 then cleaves and activates procaspase-3. In the mitochondrial pathway, the 'apoptosome', a complex formed by Cyt c, Apaf-1 and caspase-9, is a critical activator of effector caspases.

The tetracycline antibiotic family provides broad antibacterial protection by inhibiting bacterial protein synthesis. Doxycycline (Dc) is a semi-synthetic tetracycline made by modifying the chemical structure of a naturally occurring tetracycline, known as oxytetracycline, so as to enhance its antibiotic activity. Recent research has focused on possible non-anti-microbial effects of Dc, especially anti-tumor functions, such as inhibition of solid malignant tumor proliferation, invasion and metastasis as well as the induction of apoptosis in cultured tumor

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cells [14–17]. Although it has been demonstrated that tetracycline could interfere with mitochondrial protein synthesis, inhibit matrix metalloproteinases and activate caspase-3 [18–20], the apoptotic mechanism of action is not fully understood. In the current study, we demonstrate that Dc induced caspase-dependent apoptosis in HeLa cells. During the apoptosis process, the caspase cascade was mediated by mitochondria and calpains.

Materials and methods

Materials. Anti-Cyt c antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA); anti-calpain I large subunit (procalpain I and autolyzed calpain I), caspase-3, -8, -9 and Smac antibodies from Cell Signaling Technology, Inc. (MA, USA); and monoclonal antibody to GAPDH, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG from Kangcheng (Shanghai, P.R. China). Dc, rhodamine 123 and digitonin were from Sigma (MO, USA). Calpeptin, zVAD-fmk, zIETD-fmk and zLEHD-fmk were from Calbiochem (CA, USA). Arsenic trioxide was a kind gift from Professor Chen Zhu.

Cell culture and Dc treatment. The human cervix carcinoma cell line, HeLa, was purchased from Shanghai Type Culture Collection of Chinese Academy of Sciences [21]. HeLa cells were sub-cultured twice, at 2–3 days intervals (37 °C, 5% CO_2), in RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded (7×10^4 cells/cm^2) 24 h prior to treatment. Dc stock solution was freshly prepared in double-distilled water (5 mg/ml). HeLa cells were treated with 10–25 μg/ml Dc for indicated treatment times.

Inhibition of caspase and calpain activity. Calpeptin, zVAD-fmk, zIETD-fmk and zLEHD-fmk were dissolved in DMSO (50 mM stock solution). Cells were pretreated with caspase inhibitors (100 μM) or calpeptin (25 μM) for 2 h prior to addition of Dc (25 μg/ml) to culture medium. Cells treated with DMSO (0.2%) and Dc (25 μg/ml) plus DMSO (0.2%) were used as the negative and positive controls, respectively.

Hoechst 33258 staining and quantification of apoptotic cells. Adherent and suspended cells were collected, after treatment, with 0.25% trypsin-EDTA, centrifuged (500 *g*, 5 min), fixed in 70% ethanol (8 h, 4 °C) and washed twice with phosphate-buffered saline (PBS, pH 7.4). Cells were then incubated in PBS (containing Hoechst 33258, 50 ng/ml, 30 min, room temperature), washed twice with PBS, mounted onto glass slides and observed by fluorescence microscopy. Apoptotic cells were identified by nuclei condensation and fragmentation. The percentage of apoptotic cells was calculated based on the ratio of apoptotic cells to total cells counted. A minimum of 500 cells were counted for each treatment (n = 3/treatment).

Mitochondrial membrane potential assay. Changes in mitochondrial membrane potential were measured utilizing potential-sensitive rhodamine 123 as previously described [22]. Briefly, cells were collected and incubated (15 min, 37 °C, in the dark) in RPMI 1640 containing 10% FBS and 100 nM rhodamine 123. Data were analyzed by FACSCalibur (B&D, USA) and CellQuest software.

Preparation of cytosolic proteins. Cells were scraped, washed twice with PBS, then suspended in 200 μl digitonin lysis buffer (0.025% digitonin in 250 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM Tris, pH 7.4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 10 min followed by a brief centrifugation (2 min, 15 000 *g*). The supernatant was then collected for analysis of cytosolic proteins.

Genomic DNA ladder analysis. Genomic DNA ladder was assayed as previously described [23]. In brief, after treatment with Dc, floating and adherent cells were collected and pooled. Fragmented DNA from an aliquot of 2×10^6 cells was extracted with 100 μl lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) for 10 s. Clear extracts were brought to 1% SDS, treated with RNase A (1.5 mg/ml, 2 h, 56 °C), and digested with proteinase K (1 mg/ml, at least 2 h, 37 °C). DNA fragments were precipitated with 2.5 volume of 100% cold ethanol after addition of 1/10 volume of 3 M sodium acetate, then dissolved and separated on a 1.8% TBE-agarose gel.

Western blots analysis. Cytoplasmic and total cell extracts from HeLa cells were prepared as previously described [24]. The protein concentration of each extract was determined by the bicinchoninic acid (BCA) assay using BSA as the standard. Cell extracts were separated by electrophoresis on 10% or 15% standard SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with 5% nonfat milk or BSA (following the manufacturer's instructions for antibodies) in Tris-buffered saline Tween solution (TBST; 20 mM Tris, 137 mM NaCl, pH 7.6, with 0.1% Tween 20, 60 min, room temperature). Membranes were incubated with primary antibody in TBST containing 5% nonfat milk or BSA (12 h, 4 °C), washed three times at room temperature then incubated in HRP-conjugated second antibody (1:5000) for 1 h at room temperature. Membranes were then incubated with SuperSignal

Western blotting detecting reagents (Perkin Elmer Life Sciences, USA) for 1 min and exposed to x-ray film for detection of bands.

Results

Dc induces typical apoptosis in HeLa cells. HeLa cells treated with Dc displayed multiple features of apoptosis, including morphological changes and DNA fragmentation (Fig. 1a, b). A DNA ladder was detected at higher doses or longer time of Dc treatment, *i.e.*, 12 h at 20 µg/ml or 30 h at 10 µg/ml (Fig. 1b). Nuclear staining with Hoechst 33258 demonstrated morphological changes characteristic of apoptotic cells, such as nuclear fragmentation and condensation (Fig. 1a). Quantification of apoptotic nuclei indicated that Dc-induced apoptosis was time and concentration dependent (Fig. 1c, d).

Inhibition analysis of Dc-induced apoptosis in HeLa cells. To further elucidate the mechanism of action of Dc-induced apoptosis, we investigated the effects of protease inhibitors on apoptosis, including zVAD-fmk (pan caspase inhibitor), zLEHD-fmk (caspase-9 inhibitor), zIETD-fmk (caspase-8 inhibitor) and calpeptin (calpain I and calpain II inhibitor). Inhibitors were added 2 h prior to induction of apoptosis. After treatment with Dc (25 µg/ml, 22 h), cells were collected for nuclear staining and DNA extraction for ladder experiments. The data demonstrated that morphological changes and DNA fragmentation of HeLa cells treated with Dc were almost completely blocked by zVAD-fmk (Fig. 2a, b). These results suggested that caspases played important roles during Dc-induced apoptosis in HeLa cells. Quantification of apoptotic nuclei showed that apoptosis ratios decreased by 55.0% (zVAD-fmk), 45.2% (zIETD-fmk and zLEHD-fmk), 28.6% (zIETD-fmk), 27.6% (calpeptin and zLEHD-fmk), 14.9% (calpeptin) and 8.3% (zLEHD-fmk), respectively, compared with 56.7% in the non-inhibited sample (Fig. 2c). These results indicated that in addition to caspase-8 and -9, calpains were also involved in Dc-induced apoptosis, similar to what has been previously reported in G418-induced apoptosis [25].

Decrease of mitochondrial membrane potential during Dc-induced apoptosis. To confirm whether mitochon-

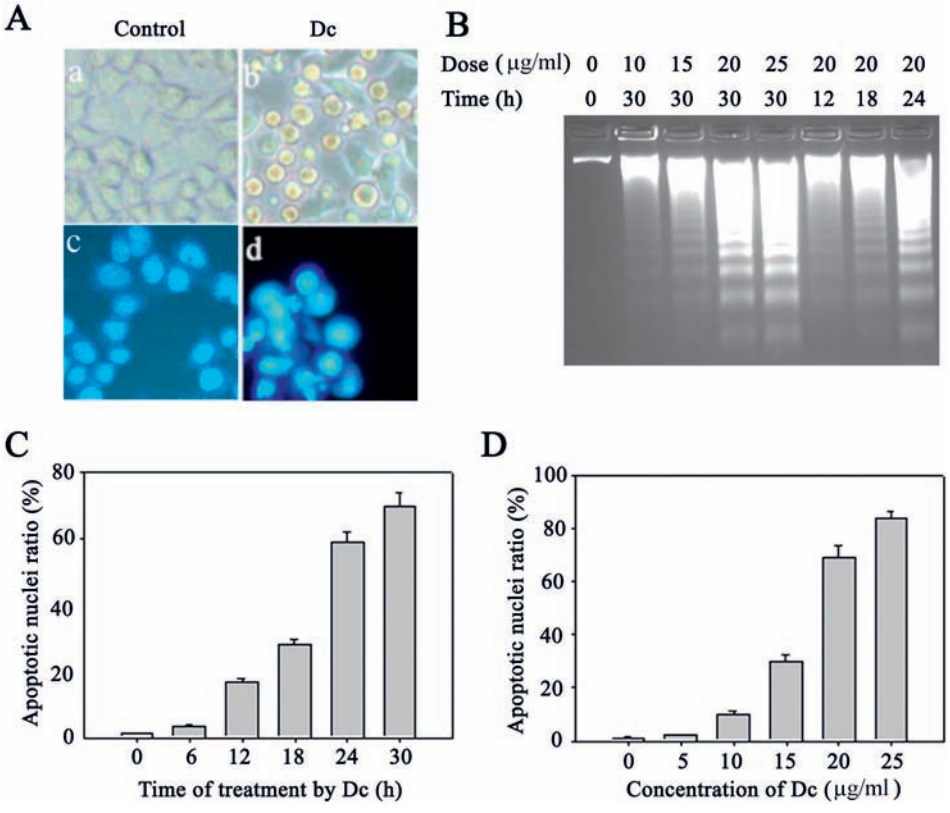

Figure 1. Induction of typical apoptosis in HeLa cells by Dc. (*a*) HeLa cells were treated with Dc (25 µg/ml, 22 h) and analyzed as follows: non-treated and treated cells were imaged by phase-contrast microscopy (*a* and *b*) and by fluorescence microscopy with Hoechst 33258 staining (*c* and *d*). Treated cells show shrinkage and nuclear fragmentation. (*b*) Dose range and time course of generation of DNA ladders. (*c*) Quantification of the rate of apoptosis in Dc-treated cells (25 µg/ml), from 0 to 30 h, by counting apoptotic nuclei. (*d*) Quantification of apoptosis rates in cells treated with 10–25 µg/ml Dc for 30 h. The error bars represent SD (n = 3 independent experiments per time point).

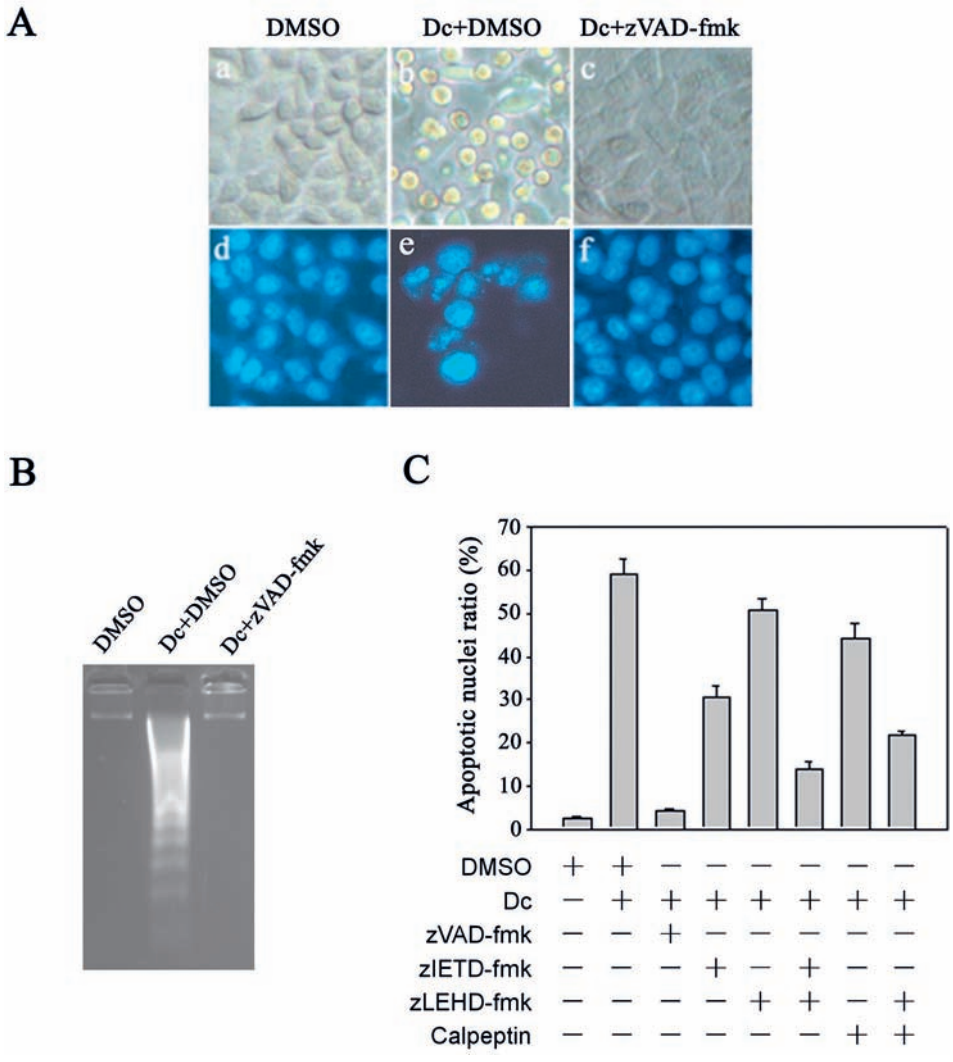

Figure 2. (*a*) Cells treated with DMSO, Dc+DMSO or Dc+zVAD-fmk were imaged by phase-contrast microscopy (*a–c*), and by fluorescence microscopy after Hoechst 33258 staining (*d–f*). (*b*) Genomic DNA ladder extracted from cells treated with DMSO, Dc+ DMSO and Dc+zVAD-fmk, respectively. (*c*) Quantification of apoptosis rates of cells treated with Dc, with concomitant inhibition of caspases or calpains. Graph data given as mean ± SD (n = 3). The concentrations of DMSO and inhibitors are according to the Materials and methods.

dria were involved in Dc-induced apoptosis, changes in mitochondrial membrane potential were detected. Cells treated with arsenic trioxide (3 μM, 36 h) were used as apoptosis control [26]. The mitochondrial membrane potential index was calculated as the product of the mean fluorescence intensity of positive cells and the percent of positive cells. The membrane potential indexes of apoptosis control and Dc-treated cells decreased about 53.2% and 31.6%, respectively, compared with that of non-treated cells (Fig. 3), suggesting that mitochondria were involved in Dc-induced apoptosis.

Caspase-independent release of Cyt c and Smac. To further explore the roles of mitochondria during Dc-induced apoptosis, the release of Smac and Cyt c was investigated. Cytosolic fractions extracted at 0, 6, 9, 12, 17 and 22 h after Dc treatment (25 μg/ml) were analyzed by Western blot methods using anti-Smac and anti-Cyt c antibodies, respectively. Although very little Smac and Cyt c were detected in the cytosol after treatment for 6 h, large amounts of Cyt c and Smac were released at 9 h post treatment (Fig. 4a). In addition, Cyt c and Smac release continuously increased as the treatment time extended.

To further examine the relationship between caspase activation and the release of Smac and Cyt c, cells were treated with DMSO, zVAD-fmk or zIETD-fmk prior to Dc treatment (25 μg/ml). Smac and Cyt c in cytosol were detected 12 h after Dc treatment. Although the two inhibitors delayed or inhibited apoptosis, they had no obvious effects on the release of Smac and Cyt c (Fig. 4b), suggesting that the release of Smac and Cyt c was independent of caspase activity during Dc-induced apoptosis.

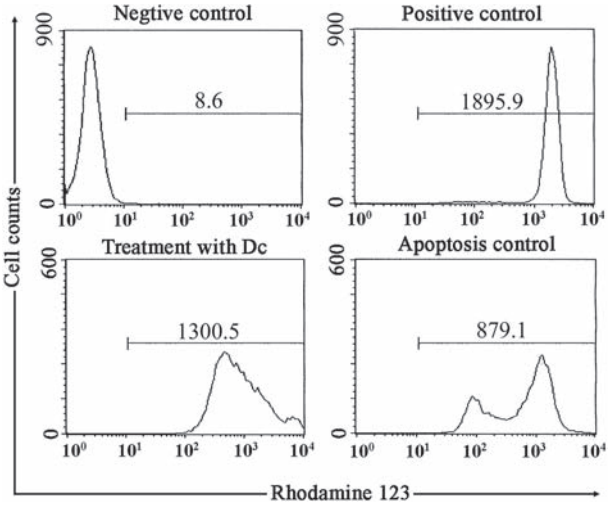

Figure 3. Decrease of mitochondrial membrane potential during Dc-induced apoptosis. Unstained HeLa cells were used as negative control; stained untreated cells (positive control), cells treated with arsenic trioxide (3 µM, 36 h, apoptosis control) and Dc-treated cells (25 µg/ml, 22 h) were stained with rhodamine 123, and the fluorescence intensity was detected. The results show that the mitochondrial membrane potential of cells treated with arsenic trioxide and Dc decreased by approximately 53.2% and 31.6%, respectively, compared with positive control. These results are representative for three independent experiments.

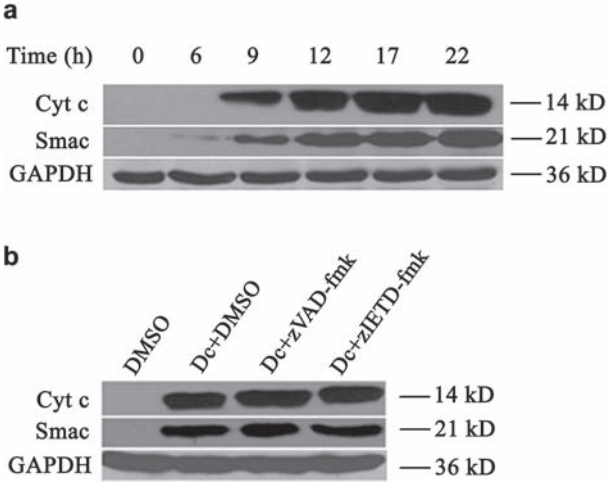

Figure 4. The effects of caspase inhibitors on Dc-induced release of Smac and Cyt c. (*a*) Time course of Smac and Cyt c release during Dc-induced apoptosis. (*b*) Effects of zVAD-fmk and zIETD-fmk on the release of Smac and Cyt c at 12 h after Dc treatment. These results are representative for three independent experiments.

Western blot analysis of calpain I, caspase-9, -8 and -3. Activation of caspases is a crucial point at which cells become committed to apoptosis. To better understand the Dc-induced caspase cascade, we investigated the activation of initiator caspases-8 and -9 and the executioner caspase-3. Antibodies specific to caspase-3, -8, and -9

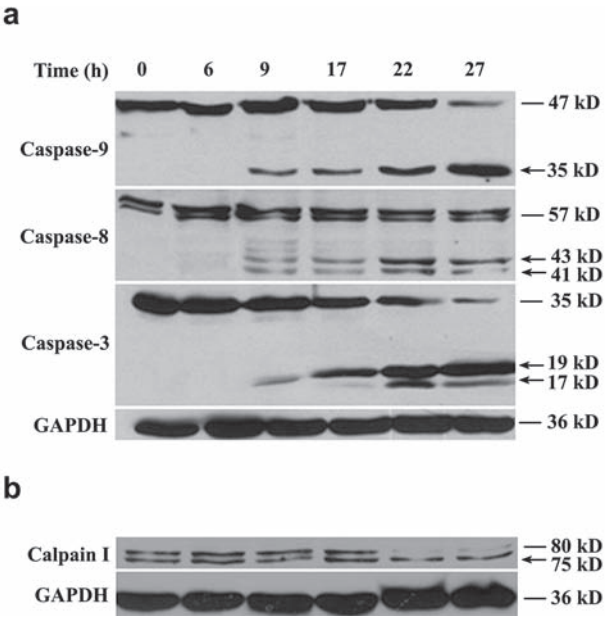

Figure 5. Calpain I, caspase-3, -8 and -9 are activated during Dc-induced apoptosis. (*a*) Caspase-3, -8 and -9 are activated in a time-dependent manner, after Dc treatment (25 µg/ml). (*b*) Calpain I is autolyzed in untreated HeLa cells, and procalpain I is undetected at 22 h after Dc treatment. Solid lines indicate intact forms of substrates; arrows indicate cleavage products. These experiments were repeated three times with similar results.

were used to probe total protein content of extracts at 6, 9, 17, 22 and 27 h after treatment with Dc (25 µg/ml). The results demonstrated that caspase-9, -8 and -3 were activated within 9 h of Dc treatment, and that the time course of activation was similar for all three. Additionally, the ratio of cleaved caspases to procaspases exhibited a time-dependent increase in Dc-treated cells (Fig. 5a). However, these results did not confirm the order in which the caspases were activated. This may have been due to the different sensitivity of varying antibody. Activation of calpain I, a Ca^{2+}-activated cysteine protease, was also detected by Western blot methods. The results showed that about half of procalpain I was autolyzed in non-treated HeLa cells, and that the cleavage ratio had no obvious change during 17 h of treatment. By 22 h post treatment, the procalpain I could no longer be detected (Fig. 5b). These results suggested that procalpain I was cleaved into activated fragments by 22 h after Dc treatment. The activation of caspase-2 could not be detected in Dc-treated cells (data not shown).

Caspase-8 activation through caspase-9 and calpains pathways. Activation of caspase-8 was detected in Dc-induced apoptosis. To elucidate the activation mechanism, HeLa cells were pretreated with DMSO, zLEHD-fmk, calpeptin or zLEHD-fmk+calpeptin prior to Dc treatment. Total protein was then extracted at 22 h after Dc treatment. Western blot analysis results indicated

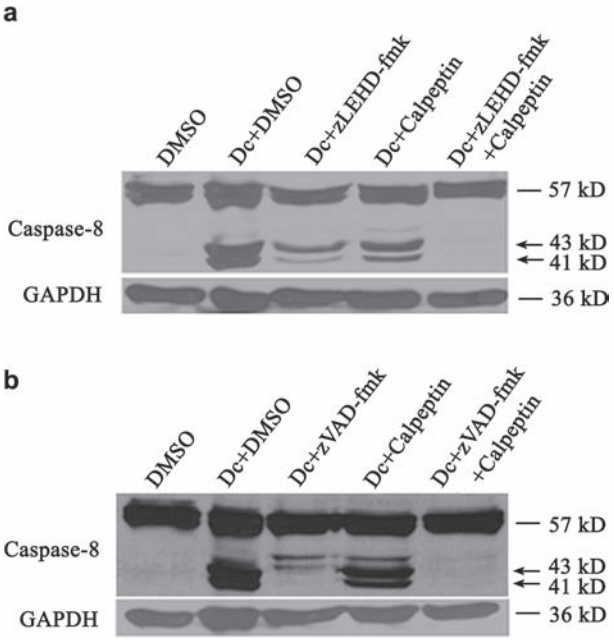

Figure 6. Caspase inhibitors and calpeptin have inhibitory effects on the activation of caspase-8 during Dc-induced apoptosis in HeLa cells. (*a*) Activation of caspase-8 is inhibited when both caspase-9 and calpains are inhibited. (*b*) Activation of caspase-8 is inhibited by zVAD-fmk and calpeptin. Solid lines indicate intact forms of substrates, arrows indicate cleavage products. These experiments were repeated three times with similar results.

that both zLEHD-fmk and calpeptin had an inhibitory effect on the activation of caspase-8, and the cleaved fragments could not be detected when caspase-9 and calpains were inhibited simultaneously (Fig. 6a). These data suggested that activation of caspase-8 was located downstream of caspase-9 and calpains during Dc-induced apoptosis.

To further elucidate the signaling pathway of caspase-8 activation, zVAD-fmk and/or calpeptin were used to inhibit Dc-induced activation of caspase-8. The results showed that the activation of caspase-8 was inhibited when both zVAD-fmk and Calpeptin were used (Fig. 6b), and that, compared with caspase-9-specific inhibitor, the pan-caspase inhibitor had a greater inhibitory effect on the activation of caspase-8 (Fig. 6b). This may have been due to the fact that zVAD-fmk inhibited the feedback activation of caspase-8 by effector caspases, and that zVAD-fmk had inhibitory effects on calpains [27]. It has been reported that caspase-3, -9 and -6 were required to activate caspase-8, and caspase-6 directly activated caspase-8 during mitochondria-triggered apoptosis [28]. It has also been demonstrated that caspase-8 was the direct substrate of calpains in an *in vivo* system [29]. Taken together, these data suggested that in addition to the caspase-9 pathway, the calpains pathway could activate caspase-8 during Dc-induced apoptosis.

Caspase-9 activation in response to the release of Cyt c. We have demonstrated that the release of Cyt c was independent of caspase activity and that caspase-9 was located upstream of caspase-8. We consequently hypothesized that the activation of caspase-9 might occur in response to Cyt c release, independent of other caspases. To confirm this hypothesis, cells were pretreated with DMSO, zVAD-fmk, Calpeptin and zVAD-fmk+calpeptin, respectively, and the activation of caspase-9 was detected at 12 h after Dc treatment. The results showed that the activation of caspase-9 was not inhibited when pan-caspases and/or calpains were inhibited (Fig. 7a). This confirmed that caspase-9 was located upstream of other caspases, and that it was activated in response to the release of Cyt c during Dc-induced apoptosis.

Caspase-9 and -8 were involved in the activation of caspase-3. Activated caspase-3 is one of the most important effector caspases in cell apoptosis. It is involved in the cleavage of death substrates, such as poly(ADP-ribose) polymerase (PARP), caspase-activated DNase (CAD) and endonuclease G [30]. Western blot analysis showed both zLEHD-fmk and, in particular, zIETD-fmk had inhibitory effects on the activation of caspase-3 during Dc-induced apoptosis. The activated 17- and 19- kDa fragments were almost completely inhibited when caspase-8 and -9 were both blocked. However, an abnormal cleaved caspase-3 fragment of approximately 24 kDa was detected when the inhibitors were added. Although these

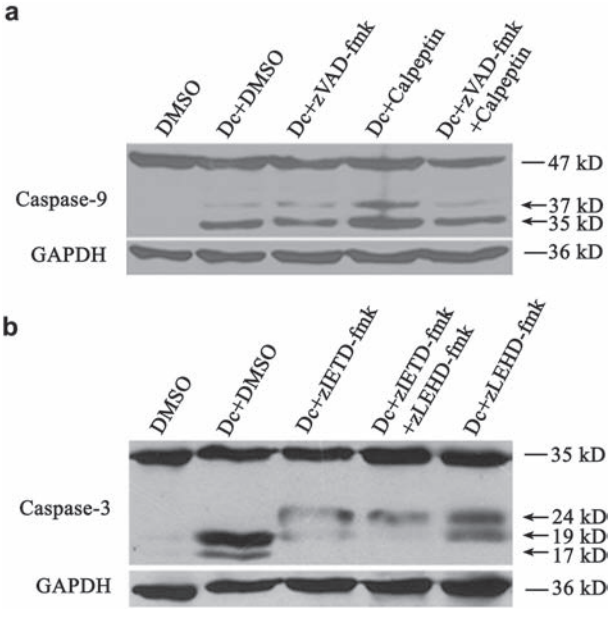

Figure 7. (*a*) Activation of caspase-9 is not inhibited by zVAD-fmk, calpeptin or zVAD-fmk+calpeptin at 12 h after Dc treatment (25 μg/ml). (*b*) zIETD-fmk and/or zLEHD-fmk has inhibitory effects on the activation of caspase-3, while an abnormal cleaved fragment of about 24 kDa is detected when caspase-9 or/and -8 are inhibited. These results are representative for three independent experiments.

results implied that caspase-9 and -8 mediated the activation of caspase-3, it cannot be ruled out that other substitute factors may have become active when caspase-8 and -9 were inhibited.

Discussion

The mitochondrion, which was once thought simply to generate energy for a cell is, in fact, a pivotal decision center; as it controls cell life and death by releasing death-promoting factors into the cytosol. One of these factors is Cyt c, a protein that normally shuttles electrons between protein complexes in the inner mitochondrial membrane [10]. The release of Cyt c triggers the activation of caspase-9 through the formation of apoptosomes, which then results in the activation of executioner caspases, including caspase-3, -6, and -7 [11, 31]. In this study, we detected the release of Cyt c and the activation of caspase-9 9 h after treatment with Dc. We also found that the activation of caspase-9 was independent of caspases and calpains. These data lead us to conclude that the activation of caspase-9 could occur through a mitochondrial pathway.

During apoptosis, two additional proteins are also released from the mitochondria and facilitate the activation of caspases by neutralizing the anti-apoptotic activity of two protein inhibitors of apoptosis (IAPs), Smac and HtrA (the mammalian homologue of the *Escherichia coli* heat shock–inducible protein) [32–35]. Both proteins bind IAPs by direct interaction with specific domains. It has been reported that the release of both Cyt c and Smac was a prerequisite for apoptotic cell death in several model systems, such as nerve growth factor deprivation-induced cell death of sympathetic neurons and anticancer drug-induced tumor cell death [36, 37]. In our research, release of Cyt c and Smac was detected about 9 h after treatment with Dc. We also demonstrated that zVAD-fmk-sensitive caspases and caspase-8 were not required for the release of Smac and Cyt c from mitochondria during Dc-induced apoptosis. Our data suggested that release of Cyt c and Smac did not occur through a caspase-dependent pathway.

Dc treatment resulted in activation of caspase-9 as an initiator caspase, while activation of caspase-8 was dependent on the activity of caspase-9 and calpains. The activation of caspase-8 was partially inhibited by zVAD-fmk, zLEHD-fmk or calpeptin and it was almost completely inhibited by calpeptin+zVAD-fmk or calpeptin+zLEHD-fmk.

Previous research demonstrated that immunodepletion of caspase-9 from the cytosol resulted in the subsequent inhibition of Cyt c-triggered caspase-8 activation in a Jurkat cell extract system [31]. It was also demonstrated that caspase-3, -9 and -6 were required to activate caspase-8 and that purified caspase-6 could directly cleave caspase-8 in Cyt c-activated extracts [28]. These reports support our conclusion that caspase-9 is involved in the activation of caspase-8 during Dc-induced apoptosis, and that this process may be mediated by the effector caspases.

Calpains, Ca^{2+}-dependent intracellular cysteine proteases, are activated by endoplasmic reticulum (ER) stress and release of Ca^{2+} [38]. Calpain activity has been shown to be crucial for a diverse spectrum of cellular responses, including apoptosis, cell proliferation, adhesion and motility, most of which can be accomplished by the two ubiquitous isoforms, calpain I and II. Given that calpains, present in multiple subcellular locations, have many target molecules *in vivo*, the spatial and temporal nature of calpain activation is probably crucial for their function [39]. It has been demonstrated *in vitro* that caspase-8 is a substrate of calpains [29]. In our study, calpeptin had inhibitory effects on the activation of caspase-8 induced by Dc. However, the precise mechanisms of this process are still unknown. The inhibitory effects of calpeptin on the activation of caspase-3, and -9 and on the release of mitochondrial proteins were not detected by Western blot methods (data not shown).

Caspase-3, an important effector caspase, was activated mainly by caspase-8 and -9 during Dc-induced apoptosis. However, when these caspases were inhibited, an abnormal cleaved fragment of caspase-3 was detected. As apoptosis induced by drugs is a complex process, we cannot exclude the possibility of the existence of substitute factors in this apoptosis model. Apoptosis analysis showed that the inhibitory effect of zIETD-fmk was much

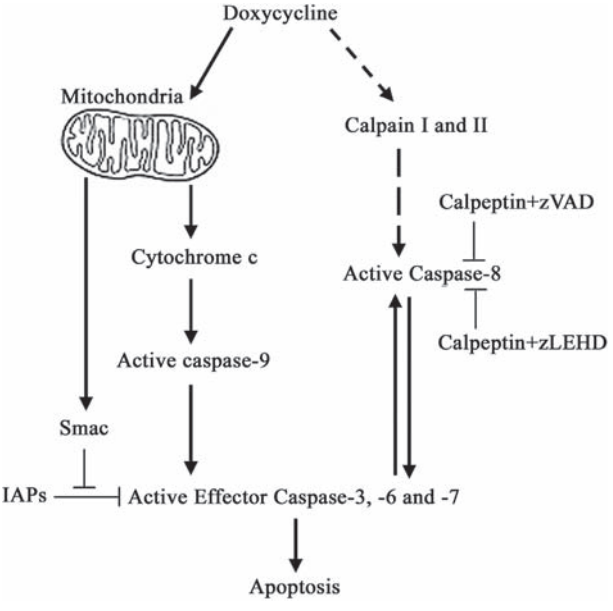

Figure 8. Mitochondria and calpains pathways mediate the activation of the caspase cascade in Dc-induced apoptosis in HeLa cells. Cyt c released from mitochondria initiates the activation of caspase-9. Activation of caspase-8 occurs through a caspase- and calpain-dependent pathway.

stronger than that of zLEHD-fmk, while zLEHD-fmk could enhance the inhibitory effect of zIETD-fmk. These data indicated that caspase-8 is a pivotal caspase in Dc-induced apoptosis.

In Dc-induced apoptosis of HeLa cells, the caspase cascade, including caspase-9 -8 and -3, was activated. Apoptosis was largely inhibited when caspase-9 and -8 were both inhibited, and it was completely inhibited by pan-caspases inhibitor. These data suggested that Dc-induced apoptosis of HeLa cells was caspase dependent, although zVAD-fmk had some inhibitory effects on the non-caspase proteases.

In conclusion, release of pro-apoptotic factors from mitochondria and activation of calpains mediate the caspase-dependent apoptosis of Dc-treated HeLa cells. Caspase-9, as an initiator caspase, was activated through the mitochondrial pathway, and was involved in the activation of caspase-3 and -8. Caspase-8 was activated through the caspase-9 and calpains pathways, playing important roles in amplifying the activation of effector caspases and the induction of apoptosis (Fig. 8).

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