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Caspase-3 mediated feedback activation of apical caspases in doxorubicin and TNF-α induced apoptosis

Shihe Yang · Ann D. Thor · Susan Edgerton · XiaoHe Yang

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Abstract Aberrant apoptosis has been associated with the development and therapeutic resistance of cancer. Recent studies suggest that caspase deficiency/downregulation is frequently detected in different cancers. We have previously shown that caspase-3 reconstitution significantly sensitized MCF-7 cells to doxorubicin and etoposide. In contrast to the well established role of caspase-3 as an effector caspase, the focus of this study is to delineate caspase-3 induced feedback activation of the apical caspases-2, -8, -9 and -10A in doxorubicin and TNF-α induced apoptosis. Using cell-free systems we show that caspases-9 and 2 are the most sensitive, caspase-8 is less sensitive and caspase-10A is the least sensitive to caspase-3 mediated-cleavage. When apoptosis is induced by doxorubicin or TNF-α in an intact cell model, cleavage of caspases-8 and -9, but not caspase-2, was markedly enhanced by caspase-3. Caspase-3 mediated-feedback and activation of caspase-8 and -9 in MCF-7/C3 cells is further supported by an increase in the cleavage of caspase-8 and 9 substrates and cytochrome *c* release. These data indicate that, in addition to its function as an effector caspase, caspase-3 plays an important role in maximizing the activation of apical caspases and crosstalk between the two major apoptotic pathways. The significant impact of caspase-3 on both effector and apical caspases suggests that modulation of caspase-3 activity would be a useful approach to overcome drug resistance in clinical oncology.

Keywords Caspase-3 · Apoptosis · Apical caspase · Doxorubicin · TNF-α

Abbreviations

DISC	death-inducing signaling complex
CHX	cycloheximide
TnT	*in vitro* transcription and translation
DEVD-CHO	Ac-Asp-Glu-Val-Asp-CHO
YVAD-CHO	Ac-Tyr-Val-Ala-Asp-CHO
Z-IETD-AMC	Z-Ile-Glu-Thr-Asp-AMC
Ac-LEHD-AMC	Ac-Leu-Glu-His-Asp-AMC
PARP	poly(ADP-ribose)polymerases

Introduction

Caspases are a group of cysteine proteases that cleave after an aspartic acid residue of a specific recognition site. Activation of these enzymes is a biochemical hallmark of apoptosis [1]. Among the 14 mammalian caspases that have been cloned to date, caspases-2, -3, -6, -7, -8, -9, -10 and -12, are the most closely related to apoptotic execution, whereas others are involved in the regulation of inflammatory processes [2]. It has been well established that activation of caspases requires a signal transduction mediated by proteolysis, a process known as the caspase cascade [3]. Depending on the point of entry to the cascade, apoptotic caspases may be classified as apical/initiator or effector caspases. Apical caspases, such as caspases-2, -8, -9 and -10, are characterized by a longer prodomain at their N terminus and are involved in the initiation of apoptosis.

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S. Yang · X. Yang (✉)
Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 USA
e-mail: xiaohe-yang@ouhsc.edu

A. D. Thor · S. Edgerton
Department of Pathology, University of Colorado at Denver & Health Sciences Center, Denver, CO 80220, USA

Effector caspases, such as caspases 3, 6 and 7, having a shorter prodomain, are activated by apical caspases and mediate the cleavage of cellular death substrates [4, 5].

Numerous stimuli induce apoptosis through two major pathways: the external death receptor pathway and the internal mitochondrial pathway [6]. In the death receptor pathway, caspases 8 and/or 10 are recruited to the death-inducing signaling complex (DISC) [7] and activated by autocleavage [8]. In the mitochondrial pathway, DNA damage and other cellular stresses induce changes in mitochondrial membrane permeability and the release of cytochrome *c* into the cytosol [9, 10]. This in turn activates caspase-9 during the formation of apoptosome, which is composed of cytochrome *c*, caspase-9 and Apaf-1 (apoptotic protease activating factor-1) [11, 12]. Activated apical caspases from either pathway are able to activate the effector caspases (caspases-3, -6 and -7) [13]. Since the substrates of the effector caspases are critical cellular proteins involved in cell survival, proliferation and structural maintenance [14], activation of caspase cascade leads to cellular dysfunction and dissociation.

Caspase-3 is a major effector caspase that plays a critical role in the apoptotic cascade [15]. Caspase-3 knockout results in defects in cerebral and thymic development [16]. Caspase-3 deficient cells, such as MCF-7 breast cancer cells, fail to show a typical apoptotic phenotype with apoptotic stimulation [17]. Using caspase-3 deficient and reconstituted cells we have studied the role of this important protein in apoptosis induced by different stimuli. We have demonstrated that caspase-3 reconstitution significantly sensitizes MCF-7 cells to granzyme B, chemo- and radiation therapy [18–20]. These data suggest that caspase-3 deficiency contributes to a more aggressive carcinogenic phenotype and therapeutic resistance. Caspase-3 downregulation/deficiency has been frequently detected in breast and other cancers [21]. Hence, these findings have clear clinical relevance.

Recent studies have also suggested that caspase-3 functions beyond a typical effector caspase. Studies of a mouse cell line model indicate that caspase-3 has feedback action on caspase-9 [22]. Caspase-3 mediated activation of caspase-9 was also involved in cisplatin induced apoptosis [23]. We also observe enhanced activation of caspase-9 by caspase-3 in granzyme-B and TNF-α induced apoptosis [20, 24]. Although caspase-3 mediated feedback on certain apical caspases has been reported before, the relative susceptibility of different apical caspases and the underlying mechanisms remain unclear. In particular, the role of caspase-3 as a rate control factor in apoptotic initiation has not been fully appreciated. In this study, we performed a systematic analysis of caspase-3 mediated-feedback activation of the apical caspases-2, -8, -9 and -10A in both cell-free and intact cell systems. Our results provide deeper insight into caspase-3 mediated feedback on multiple caspases. In particular, we show that the caspase-3 status has profound impact on the activation of these apical caspases, suggesting that caspase-3 also plays an important role in the initiation of apoptosis induced by both death receptor and mitochondrial pathways.

Materials and methods

Cell culture and drug treatment

MCF-7/PV and MCF-7/C3 cells were derived from the MCF-7 breast cancer cell line by transfecting control and caspase-3 encoding pBabepuro vector, as described previously [18]. The cells were maintained in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and penicillin/streptomycin. For drug treatments, 1×10^6 cells were seeded into 60-mm dishes 24 h before drug treatment. The cells were then treated with doxorubicin (Bedford Laboratories, Bedford, OH) or TNF-α (Bristol-Myers Squibb Co., Princeton, NJ) and cycloheximide (CHX) (Sigma, St. Louis, MO) at indicated conditions. In all cases, medium from individual dishes, which might contain floating dead cells, was collected and mixed with the cell pellet from the same dish.

Cleavage of ^{35}S-labeled apical caspases prepared by in vitro transcription and translation

In vitro transcription and translation (TnT) of the caspases were performed as described previously [25]. In brief, plasmids encoding caspase-2, -8, -9 or -10A cDNA (a generous gift from Dr. Christopher Froelich, Northwestern University), under the control of a T7 RNA polymerase promoter, were used for TnT [20, 25]. ^{35}S-Methionine-labeled proteins were prepared from these vectors using a T7-coupled reticulocyte lysate transcription translation (TnT) system (Promega, Madison, WI). The raw TnT products were purified with DEAE-cellulose column (Promega) and recovered by collecting fractions containing labeled proteins. For cleavage assays using recombinant caspase-3, the reaction system consisted of 20 μl of adjusted TnT product ^{35}S labeled caspase-2, -8, -9 or -10A and 20 μl of reaction buffer (100 mM Hepes, pH 7.5, 20% glycerol, 0.5 mM EDTA, 5 mM DTT) containing recombinant full length caspase-3 (Calbiochem, San Diego, CA) at a final concentration of 100 nM. At the times indicated, the reaction was stopped by adding SDS containing sample loading buffer and immediately heating at 95°C for 6 min. The samples were then separated by 12% SDS-PAGE gels. Dried gels were imaged and scanned.

For the cleavage assay using apoptotic lysate, MCF-7/PV and MCF-7/C3 cells were treated with 10 μM of doxorubicin for 20 hrs or 80 ng/ml of TNF-α plus 5 μg/ml of CHX for 4 hrs, respectively. The cell lysate was prepared with NP-40 containing lysis buffer as previously described [20]. Twenty μl of TnT product was incubated with 20 μg of cell lysate, in the presence or absence of 50 μM of caspase inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) or Ac-Tyr-Val-Ala-Asp-CHO (YVAD-CHO) (BD Biosciences, San Diego, CA), for the times indicated. The samples were then processed as described above.

Western blotting

For regular cell lysate, PBS-washed cells were treated with lysis buffer [26] on ice for 30 min. Lysed cells were centrifuged at 14,000 rpm for 10 min to remove cellular debris. Protein concentrations of the supernatant were determined using BCA Protein Assay (Pierce, Rockford, IL). Fifty μg of cell lysate were loaded onto each lane of a gel. Protein was separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBS-T (5% milk in Tris-buffered saline-Tween 20) washing buffer and probed with specific primary antibodies. Concentrations of the primary antibodies used ranged from 1:500 to 1:2,000 dilutions. Antibodies against PARP, cytochrome *c*, caspases 2, 7, 8 and 9 were purchased from BD Biosciences (San Diego, CA). Antibodies against caspase-3 and 6 and β-actin were from Santa Cruz Biotechnology Inc. Washed membranes were then probed with horseradish peroxide-labeled anti-mouse, anti-rabbit, or anti-goat secondary antibodies (Amersham Pharmacia, Arlington Heights, IL), respectively. The membranes were washed again and treated with enhanced chemiluminescence reagents (Amersham Pharmacia). The specific protein bands were visualized by autoradiography [18].

Detection of cytochrome *c* release by subcellular fraction and Western blot

The cytosolic extract for cytochrome *c* release experiments was prepared according to published protocol [27]. In brief, treated cells were washed with PBS and resuspended in sucrose containing buffer. After 30 min incubation, the cells were disrupted by 30 strokes with a glass Dounce (Type B). The large debris in the cell homogenates was cleaned by centrifugation at 880*g* for 10 min. Mitochondria in the supernatant were separated by further centrifugation at 22,000*g* for 15 min. The resulting supernatants were saved as cytosolic extract. Cytochrome *c* in this extract was detected using Western blotting described above.

Cleavage of fluorogenic substrates of caspases-8 and 9

Drug-treated cells were washed with PBS and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 130 mM KCl, 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin] at 320 μl/60-mm dish. After incubation at 37°C for 10 min, the samples were spun for 3 min (5000 rpm), and the supernatant was collected. After adding 100 μl of lysate to each well of a fluorometer plate, 100 μl of substrate solution containing 2 μM of Z-Ile-Glu-Thr-Asp-AMC (Z-IETD-AMC, caspase-8 substrate) or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC, caspase-9 substrate) (Calbiochem, San Diego, CA) in lysis buffer was added right before reading. Fluorescence was measured in a microplate fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Results are reported as the fluorogenic activity over 1 h (T_{60} to T_0). Samples were prepared in triplicate. The differences between MCF-7/PV and MCF-7/C3 cells were statistically analyzed using Student's *t*-test.

Results

Processing of caspases-2, -8, -9 and -10 by recombinant caspase 3 *in vitro*

The cleavage of a caspase substrate can be affected by many factors, including the enzymatic kinetics, accessibility of the substrate to enzyme and co-factors that may modulate the enzymatic activity. To exclude interference from the indirect factors presented in intact cells, we compared the susceptibility of the four initiator caspases-2, -8, -9 and -10A, to recombinant caspase-3, in a simple cell free system. We first prepared ^{35}S labeled caspase-2, -8, -9 and -10A using *in vitro* transcription and translation (TnT) and then treated the resulting caspases with purified recombinant caspase-3. Among the four apical caspases, caspase-9 and caspase-2 were the most sensitive to caspase-3 treatment (Fig. 1), as indicated by markedly decreased pro-caspases and the appearance of cleaved subunits. In contrast, caspase-3 treatment resulted in little cleavage of caspases-8 and -10A. Caspase-3 induced cleavage of caspase-9 yielded three products, p37, p20 and p10 (Fig. 1C). This pattern of cleavage suggests that the major cleavage site is D330, which yields a majority of p37 and p10 bands [28]. A minor p20 band suggests that caspase-3 may also cleave caspase-9 between the prodomain and the pLarge subunit (p20) [29]. The caspase-3 cleavage products of pro-caspase-2 appeared to be p33 and p12 (Fig. 1(A)). This pattern suggests that the cleavage site of procaspase-2 is between the pSmall and pLarge subunits [30]. Caspase-3 treatment of procaspase-8

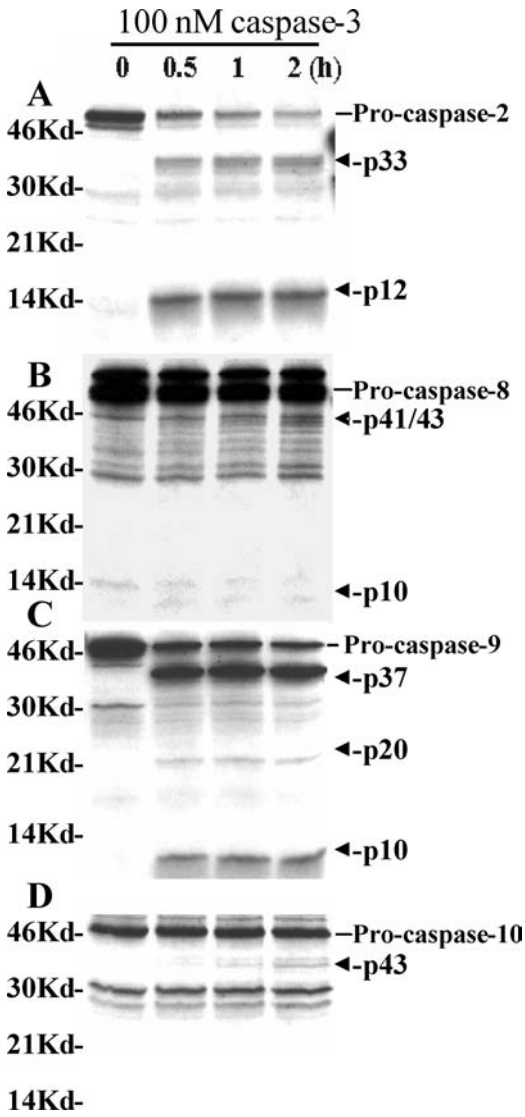

Fig. 1 Processing of caspases 2, 8, 9, and 10A by recombinant caspase 3 *in vitro*. ^{35}S labeled caspases-2 (A), 8 (B), 9 (C) or 10A (D) was prepared using *in vitro* transcription and translation (TnT) system, respectively. Radio-labeled TnT products were purified with DEAE-cellulose column. Approximately 100 ng of TnT product was treated with 100 nM of recombinant caspase-3 (full length) at the times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes

results in modest cleavage, yielding p41/p43 and p10 subunits (Fig. 1(B)). A trace of the p43 subunit of caspase-10A was detected only after a 2-hour treatment of the pro-cases with caspase-3 (Fig. 1(D)). In these experiments, we have adjusted enzyme/substrate ratio among different caspases. However, pro-caspase-8 signals were still higher than the other caspases. This could be due to more Met residues in caspase-8 (16 total) and/or other subtle factors. Since caspase-3 mediated cleavage of caspases-2, 9 and 10 was not correlated with pro-caspase amount, the quantity of pro-caspase-8 appears not to be a major factor that affects its sensitivity to caspase-3 under the given conditions. In aggregate, these *in vitro* data indicate that pro-caspases 9 and 2 are the preferred substrates for caspase-3.

Cleavage of apical caspases by cytosolic extract from apoptotic MCF-7 cells with different caspase-3 status

We examined the cleavage of the four apical caspases by endogenous caspase-3 using apoptotic cell lysates *in vitro*. This reaction system tests cellular caspase-3 activity and avoids interference by other factors in intact cells, such as the cellular compartment. To demonstrate the specificity of caspase-3, we prepared the lysate from isogenic MCF-7 cells, MCF-7/PV and MCF-7/C3 (caspase-3 deficient or reconstituted, respectively). ^{35}S-labeled caspase-2, -8, -9 and -10A were treated with the lysate from doxorubicin or TNF-α/CHX treated cells (Figs. 2 and 3). In general, the processing of the four apical caspases by the MCF-7/C3 cell lysate was greatly enhanced as compared to the MCF-7/PV cell lysate. Caspase-3 reconstitution particularly enhanced caspase-9 processing, followed by caspases-2 and 8 (Figs. 2A–C and 3A–C). The effect of caspase-3 on caspase-10A processing was minimal (Figs. 2D and 3D). The processing patterns of each apical caspase in response to doxorubicin or TNF-α treated samples were similar, suggesting that caspase-3 mediated cleavage of the apical caspases-9, -2 and -8 occurs in both the mitochondrial and death receptor pathways. Of note, caspase-3 mediated cleavage of caspases-2, 8 and 9 was blocked by DEVD-CHO but not YVAD-CHO (Figs. 2(A)–(C) and 3(A)–(C)). Since DEVD-CHO and YVAD-CHO are inhibitors for caspase-3-like or caspase-1-like caspases respectively, enhanced cleavage of the apical caspases in drug treated MCF-7/C3 lysate was most likely mediated-by a caspase-3-like activity. Insignificant differences between caspase-10A processing in MCF-7/C3 cell lysate, with and without DEVD-CHO, also suggest that caspase-10A is not a sensitive substrate of caspase-3. Although DEVD-CHO may also affect caspases-6 and 7, its effect was only detected in MCF-7/C3 cells but not MCF-7/PV cells. Therefore, DEVD-CHO-mediated blockage of caspases-2 and 9 cleavage in these cells is the antagonist effect of caspase-3. In addition, we noticed a difference in the cleaved products of caspase-8, between recombinant and cellular caspase-3 treatments. Instead of the p10 subunit detected in the reactions using recombinant caspase-3 (Fig. 1), a p18 subunit was detected in the reactions that used MCF-7/C3 lysate (Figs. 2(B) and 3(B)). This discrepancy suggests that endogenous caspase-3 might cleave caspase-8 at a site different from recombinant caspase-3, or that p18 was derived indirectly by active caspase-3. Taken together, the cell-free system data indicate that caspase-9 and 2 are

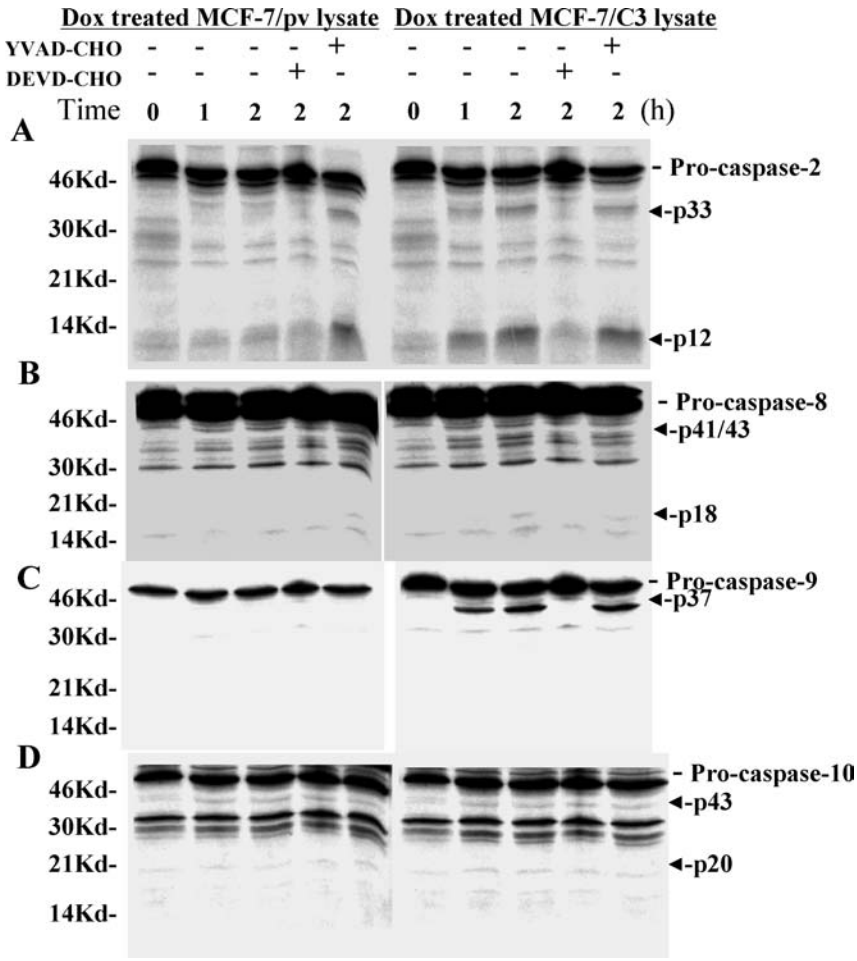

Fig. 2 Processing of 2, 8, 9, and 10A by endogenous caspase-3 in apoptotic lysate induced with doxorubicin. Apoptotic cytosolic extract was prepared from MCF-7/PV and MCF-7/C3 cells treated with 10 μM of doxorubicin for 20 h. Approximately 100 ng of ^{35}S labeled caspases- 2 (A), 8 (B), 9 (C) or 10A (D) was incubated with 20 μg of cytosolic extract, in the presence or absence of 50 μM of DEVD-CHO or YVAD-CHO, at the times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes

the most sensitive to endogenous caspase-3, followed by caspase-8.

Processing of apical caspases in doxorubicin and TNF-α treated control and caspase-3 reconstituted MCF-7 cells

To examine the effects of caspase-3 on the cleavage of the apical caspases-2, -8 and -9 in intact cells, we used the caspase-3 deficient MCF-7/PV and caspase-3 containing MCF-7/C3 cell lines to study the cleavage of these apical caspases in response to doxorubicin or TNF-α treatment. Results from this system reflect the influence of factors presented in intact cells, including the subcellular compartment, physiological enzyme/substrate ratios and the factors that may affect caspase-3's activity. As shown in Figs. 4 and 5, the cleavage patterns of the selected caspases were markedly different between drug-treated MCF-7/PV and MCF-7/C3 cells. Caspase-3 expressed by MCF-7/C3 cells was functional, as indicated by a drug induced decrease (cleavage) of pro-caspase-3 (Figs. 4(C) and 5(C)). Cleavage of PARP, a general indicator of apoptotic proteolysis, in drug treated MCF-7/C3 cells was markedly enhanced by drug treatment, as shown by an earlier appearance of the cleaved subunit (p85) and a decrease, or disappearance of full length PARP (p116) (Figs. 4(A) and 5(A)). Consistent with our previous reports, cleavage of caspases-6 and -7 was largely caspase-3 dependent (Figs. 4(D) and (E), 5(D) and (E)). Our results demonstrate that caspase-3 primarily cleaves caspase-6 and -7 between the N-terminal prodomain and the pLarge (p20) subunits, generating Δ-caspase-6 and Δ-caspase-7 (pro-caspase-6 and 7 minus the N terminal propeptide, respectively). An increase of the p10 subunit of caspase-7 in drug treated MCF-7/C3 cells indicates that caspase-3 is also able to cleave caspase-7 between the pLarge and pSmall (p10) subunits. Caspase-3 is therefore critical for effective cleavage of cellular death substrates, such as PARP, as well as for the full activation of other effector caspases.

With the above defined profiles of apoptotic activities in drug treated MCF-7/PV and MCF-7/C3 cells, we then analyzed the activation of apical caspases-2, -8 and -9 in these cells. Interestingly, caspase-2 cleavage was not detected in any of the cell lines treated with either stimulus

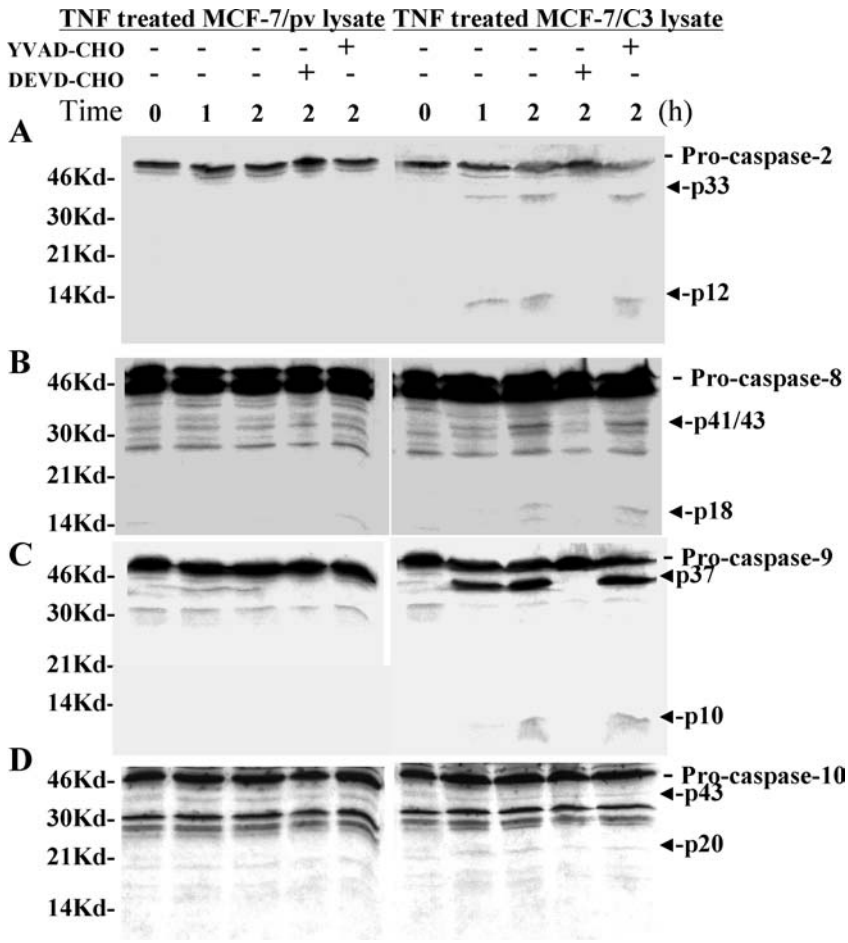

Fig. 3 Processing of 2, 8, 9, and 10A by endogenous caspase-3 in apoptotic lysate induced with TNF-α. Apoptotic cytosolic extract was prepared from MCF-7/PV and MCF-7/C3 cells treated with 80 ng of TNF-α plus 5 μg of CHX for 4 h, respectively. Approximately 100 ng of ^{35}S labeled caspases-2 (A), 8 (B), 9 (C) or 10A (D) was incubated with 20 μg of cytosolic extract, in the presence or absence of 50 μM of DEVD-CHO or YVAD-CHO, at the times indicated. The processing was analyzed by SDS-PAGE and autoradiography. Arrows indicate the cleaved products of pro-enzymes

under the given conditions (Figs. 4B and 5B). In contrast to a sensitive response to caspase-3 in cell-free systems, caspase-3 overexpression in MCF-7/C3 cells did not enhance caspase-2 cleavage/activation in intact cells. This suggests that the cellular compartment and/or caspase-2 binding protein may interfere with caspase-3 mediated-cleavage of caspase-2.

Although caspase-8 was much less sensitive to caspase-3 than caspase-2 *in vitro*, caspase-3 mediated cleavage of caspase-8 was more evident in intact cells. When MCF-7/PV cells were treated with 10 μM doxorubicin for up to 20 h only a weak band was detected, which appeared to be the p41 subunit of caspase-8 (Fig. 4(F)). In contrast, the p41/p43 subunits were readily detected as early as 8 h post treatment in MCF7/c3 cells (Fig. 4F). These results clearly demonstrate that caspase-3-mediated feedback to caspase-8 is involved in apoptosis initiated through the mitochondrial pathway. In apoptosis induced by TNF-α, the difference of caspase-8 processing between the two cell lines was not as definitive as in the doxorubicin treated cells. As a major apical caspase in the death receptor pathway, the processing of caspase-8 was evident in the absence of caspase-3 (MCF-7/PV cells) (Fig. 5(F)). Nevertheless, on the basis of the decrease in pro-caspase-8, the processing of caspase-8 in TNF-α treated MCF-7/C3 was also modestly increased (Fig. 5(F)). These results indicate that caspase-3 can enhance the activation of caspase-8, and that the magnitude of the amplification is pathway dependent. It is more evident in apoptosis initiated via mitochondrial pathway, as compared to the death receptor pathway. Since caspase-8 is not a preferred substrate of caspase-3 *in vitro*, enhanced processing of caspase-8 in MCF-7/C3 cells suggests a feedback effect involving indirect interactions.

Consistent with our *in vitro* results, caspase-9 cleavage was enhanced by caspase-3 in intact cells. Although caspase-9 is the primary apical caspase in DNA damage induced apoptosis, only weak signals of the p35 subunit of caspase-9 were detectable in doxorubicin treated MCF-7/PV cells, which appears to be the result of caspase-9 autocleavage (Fig. 4(G)). In contrast, caspase-9 processing in drug treated MCF-7/C3 cells was significantly increased, as demonstrated by the appearance of p37 and increased signals of p35. These results suggest that caspase-3 cleaves caspase-9 at the same site (D330) as in our *in vitro* experiments, and that caspase-3-mediated activation of caspase-9 also enhances caspase-9 auto-cleavage/activation, further amplifying the feedback

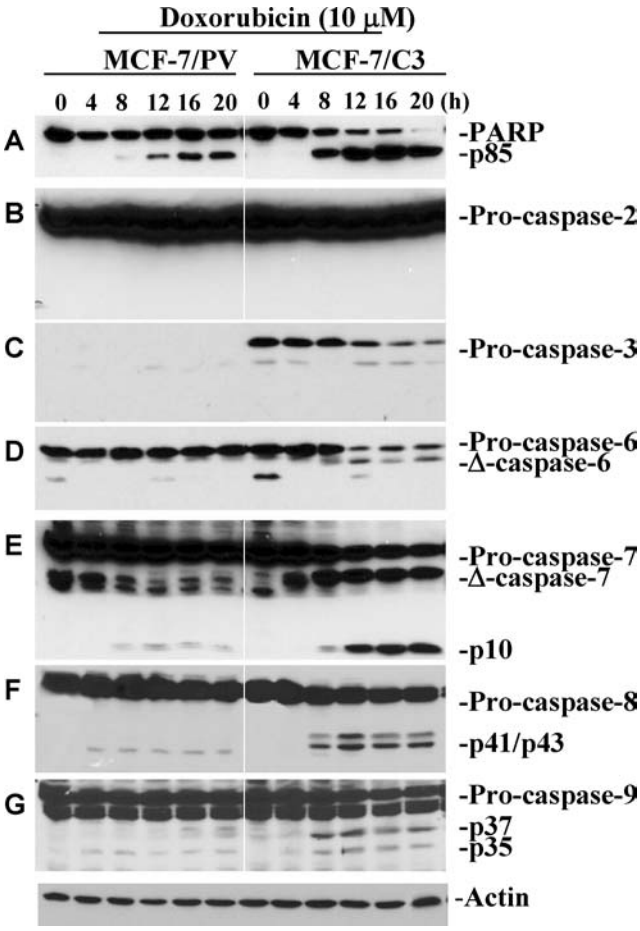

Fig. 4 Cleavage of PARP, caspases-2, 3, 6, 7, 8 and 9 in doxorubicin treated MCF-7/C3 cells, as compared to MCF-7/PV cells. MCF-7/PV and MCF-7/C3 cells were treated with 10 μM of doxorubicin for indicated periods. PARP (A), caspases-2 (B), 3 (C), 6 (D), 7 (E), 8(F), 9 (G) and actin were detected using Western blot

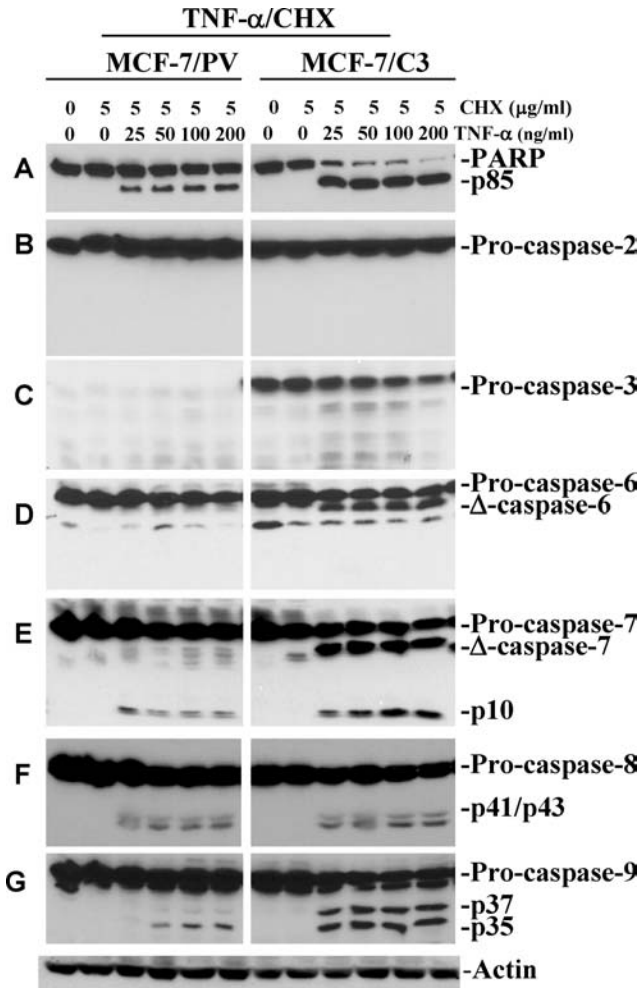

Fig. 5 Cleavage of PARP, caspases-2, 3, 6, 7, 8 and 9 in TNF-α and CHX treated MCF-7/C3 cells, as compared to MCF-7/PV cells. MCF-7/PV and MCF-7/C3 cells were treated with 80 ng/ml TNF-α plus 5 μg/ml CHX at indicated concentrations for 4 h. PARP (A), caspases-2 (B), 3 (C), 6 (D), 7 (E), 8(F), 9 (G) and actin were detected using Western blot

loop. Caspase-3 mediated activation of caspase-9 in TNF-α treated cells was more evident than doxorubicin treated cells (Fig. 5(G)). These data support a role for caspase-3 in communicating between these two pathways to enhance apoptotic execution.

Caspase-3 enhances the cleavage of synthetic substrates for caspase-8 and -9 in doxorubicin and TNF-α induced apoptosis

To demonstrate that caspase-3 mediated-cleavage of caspases-8 and -9 generates functional enzymes, we analyzed the cleavage of fluorescent-conjugated substrates of caspase-8 and -9 using apoptotic lysates derived from MCF-7/PV and MCF-7/C3 cells, treated with either doxorubicin or TNF-α/CHX. As shown in Fig. 6, the apoptotic lysate from MCF-7/C3 cells displayed significantly stronger enzymatic activity with both substrates. In doxorubicin induced apoptosis, a difference in the cleavage of caspase-9 substrate was more evident than cleavage of caspase-8 substrate. Conversely, the enzymatic activity on caspase-8 substrate in TNF-α/CHX induced apoptosis was significantly higher (more than 15-fold) in the MCF-7/C3 cell lysate as compared to the MCF-7/PVcell lysate. Although the substrates for caspases-8 and 9 are not terribly specific, based on their preferential sensitivity to caspases-8 and 9, these quantitative data provide additional evidence that supports caspase-3-mediated activation of procaspases-8 and -9.

Enhanced cytochrome *c* release from mitochondria to the cytosol in MCF-7 cells reconstituted with caspase-3

The release of cytochrome *c* from mitochondria to the cytosol is a critical event involved in both apoptosis initiation pathways. This event is tightly controlled by Bcl-2 family members. One of the mechanisms that triggers cytochrome

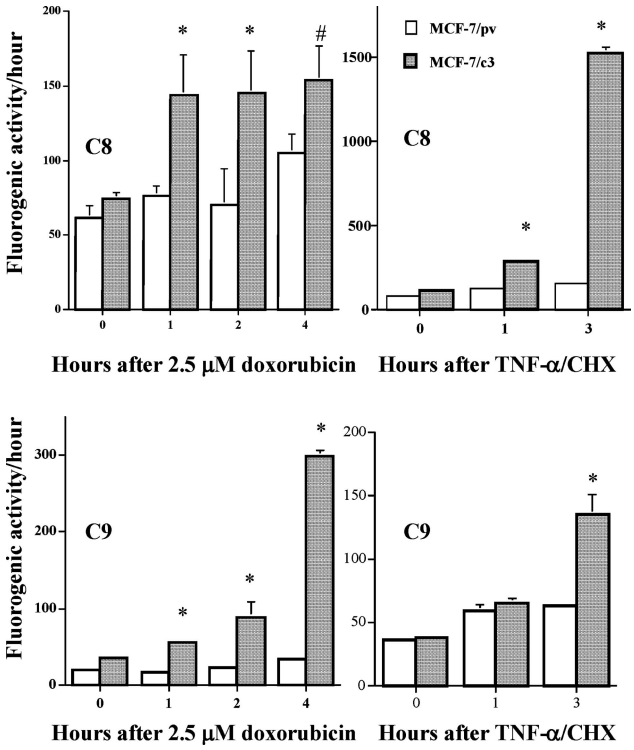

Fig. 6 Cleavage of caspases-8 and 9 substrates by apoptotic lysate from MCF-7/PV and MCF-7/C3 cells. Apoptotic cytosolic extract was prepared from MCF-7/PV and MCF-7/C3 cells treated with 10 μM of doxorubicin or 80 ng of TNF-α plus 5 μg of CHX for 0, 1 and 3 h. Z-IETD-AMC (caspase-8 substrate) and Ac-LEHD-AMC (caspase-9 substrate) cleavage was determined by their fluorogenic activity in response to different apoptotic lysate as detailed in the Methods. A. Cleavage of caspase-8 (C8) substrate by doxorubicin induced apoptotic lysate. B. Cleavage of caspase-9 (C9) substrate by doxorubicin induced apoptotic lysate. C. Cleavage of caspase-8 (C8) substrate by TNF-α/CHX induced apoptotic lysate. D. Cleavage of caspase-9 (C9) substrate by TNF-α/CHX induced apoptotic lysate. $^*p < 0.01$; $\#p < 0.05$; in the comparison between MCF-7/PV and MCF-7/C3 cells under the same conditions

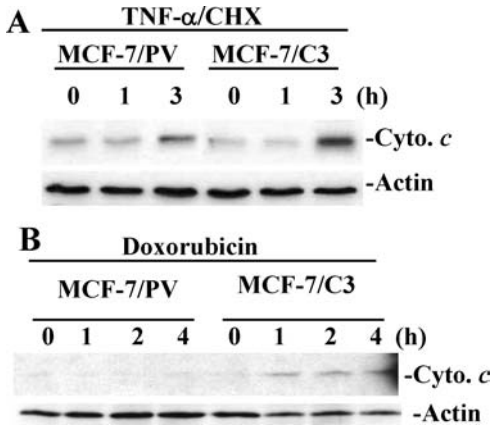

Fig. 7 Feedback action of caspase-3 on cytochrome *c* release in doxorubicin or TNF-α/CHX treated MCF-7/PV and MCF-7/C3 cells. MCF-7/PV and MCF-7/C3 cells were treated with 2 μM of doxorubicin (A) or 80 ng/ml TNF-α plus 5 μM/ml CHX (B) at the times indicated. Cytosolic extract was prepared from the treated cells using a glass Dounce and fractioning centrifugation as detailed in the Methods. Fifty μg of the extract of each sample was separated by SDS-PAGE. Cytochrome *c* released to the cytosol was detected using western blot

c release is the translocation of tBid (truncated Bid) to the mitochondrial membrane, as a result of Bid cleavage by caspase-8 [31]. Since enhanced cleavage of caspase-8 was observed in MCF-7/C3 cells treated with either doxorubicin or TNF-α, we examined whether the release of cytochrome *c* was enhanced in these cells. As shown in Fig. 7, relative cytochrome *c* levels in the cytosolic fraction of MCF-7/C3 cells treated with TNF-α for 3 h, or doxorubicin for 1 to 4 h were much higher than we observed in the cytosolic factions of MCF-7/PV cells treated similarly. These data are consistent with those reported by Slee et al [32], and confirm a role of caspase-3 in cytochrome *c* release.

Discussion

Although activation of the caspase cascade has been extensively studied, most of the relevant literature has focused on signaling from the apical to effector caspases. In this report, we studied the action of the effector caspase-3 on four apical caspases. Our results demonstrate that caspase-3 cleaves and activates apical caspases, particularly caspase-9 and -8, and to a less degree caspase-2. Integrating our results from cell-free and in intact cell systems suggest that the underlying mechanisms of caspase-3-mediated activation of individual apical caspases are likely different.

Caspase-9 is an apical caspase mainly induced by intracellular signals. It is tightly regulated by multiple mechanisms and its activation is initiated in the apoptosome via autoprocessing [11]. Its activity can be negatively regulated by the caspase inhibitor XIAP and Bcl-2 family proteins, such as Bcl-xL [33, 34]. Phosphorylation of pro-caspase-9 at Ser-196 by Akt and/or at Thr-125 by Erk also inhibits its protease activity [35, 36]. Caspase-3 mediated-feedback activation of caspase-9 appears to be an additional regulatory mechanism that enhances its activity. Previous *in vitro* studies have demonstrated that caspase-9 can be autocleaved at D315, resulting in p35 and p12 subunits [28]. Caspase-3 cleaves caspase-9 primarily at D330, resulting in p37 and p10 subunits [28]. Caspase-3 may also cleave caspase-9 at D130, which separates the prodomain from the rest of the molecule [29]. Similar cleavage patterns have also been observed with mouse caspase-9 [22]. In our experiments, treatment of caspase-9 with recombinant caspase-3 results in two major subunits, p37 and p10, and a minor subunit p20. These data suggest that although caspase-3 can cleave caspase-9 at both sites, the two sites have different sensitivities to caspase-3, with D330 as the major site and D130 as a minor one. Treatment of caspase-9 with an apoptotic lysate of MCF-7/C3 cells yielded two major subunits, p37 and p10.

This difference may result from different enzyme/substrate ratios between the two reaction systems and/or factors in the apoptotic lysate that may interfere with caspase-3's cleavage at the D130 site. Detection of both p37 and p35 in TNF-α treated MCF-7/C3 cells, but not in MCF-7/PV cells, suggests that caspase-3 mediated-activation of caspase-9 leads to more autocleavage of caspase-9. This mechanism further enhances the feedback loop. Although it has been reported that cleavage of caspase-9 *in vitro* does not necessarily mean caspase-9 activation [37], our data show that enhanced cleavage of caspase-9 in MCF-7/C3 cells is accompanied by a drastic increase of caspase-9 activity, as indicated by LEHD-AMC cleavage data (Fig. 6). Mechanistically, cleavage of caspase-9 might stabilize its dimerization, leading to enhanced activity [38]. Consistent cleavage/activation of caspase-9 by caspase-3 *in vitro* and in intact cells suggests that caspase-3's feedback on caspase-9 occurs via direct mechanisms. In aggregate our results indicate that caspase-3 activity is required for maximal activation of caspase-9 in both the mitochondrial and death receptor pathways.

Caspase-8 activation is generally involved in death receptor induced apoptosis. Activated in DISC, caspase-8 initiates the caspase cascade by activation of caspase-3 [39]. It also triggers the mitochondrial pathway by cleaving Bid. Translocation of cleaved Bid (tBid) to the mitochondrial membrane leads to Bax/Bak oligomerization, cytochrome *c* release and caspase activation [31, 40]. In this study, we found that caspase-3 status has a significant impact on caspase-8 cleavage and activation. Although caspase-8 cleavage/activation is modestly enhanced by caspase-3 in TNF-α induced apoptosis (Fig. 5(F)), doxorubicin induced apoptosis data show that caspase-3 plays a critical role in the activation of caspase-8 in apoptosis via the mitochondrial pathway (Fig. 4(F)). These results underscore the significance of caspase-3 in the cross talk between the death receptor and mitochondrial pathways. However, with limited cleavage of caspase-8 by caspase-3 *in vitro*, it is hard to attribute the significant increase of caspase-8 cleavage/activation in MCF-7/C3 cells totally to the direct action by caspase-3. Caspase-3 may enhance caspase-8 cleavage/activation indirectly as well. Recently, it was reported that caspase-6 is a direct activator of caspase-8 in cytochrome *c*-induced apoptosis. Removal of the caspase-6 prodomain is believed to be absolutely required for its activation [41]. Since it has been shown that caspase-9 cannot process caspase-6 [28], and we found that caspase-3 reconstitution significantly enhances caspase-6 activation by removing its prodomain [18], it appears that the indirect activation of caspase-8 by caspase-3 might be through signaling from caspase-9 to caspase-3 to caspase-6 to caspase-8. This is consistent with previous reports indicating that all caspases -9, -6 and -3 are required for caspase-8 activation in cytochrome *c* induced apoptosis [42].

Responses of caspase-2 to caspase-3 were also different *in vitro* and in intact cells. We found that caspase-2 was sensitive to caspase-3 *in vitro* but it was not cleaved in intact cells under the given conditions. The discrepancy between these two systems suggests that the access of caspase-3 to caspase-2 in intact cells could be blocked by certain inhibitory factors. We observed a similar discrepancy in the analysis of granzyme B mediated-cleavage of caspase-7, as caspase-7 was very sensitive to granzyme B *in vitro* but insensitive in an intact cell system [20]. Given the sensitive response of caspase-2 to caspase-3 in cell-free models, caspase-3 mediated-cleavage/activation of caspase-2 in a different cell line or under different conditions cannot be ruled out. For example, it has been reported that caspase-3 is required for caspase-2 activation in UV and TNF-α induced apoptosis [43]. The factors that affect caspase-3-mediated cleavage of caspase-2 needs to be addressed by future studies. In the case of caspase-10A, results from both *in vitro* and intact cell analysis suggest that it is not a sensitive substrate of caspase-3. Caspase-3-mediated activation of apical caspases is therefore likely to be selective. Interactions between caspase-3 and caspases-2, -10A and other apical caspases will require further investigation, although we have shown an essential role of caspase-3 in the full activation of caspase-8 and -9. It is worth noting that our study emphasizes the relative sensitivities of the four apical caspases to caspase-3. Prolonged or intensified treatment may lead to more or differential activation of the apical caspases.

Although caspase-3 is best known as an effector caspase, accumulating evidence now suggests an "apical nature" of caspase-3. Our finding that the activation of caspases- 6 and -7 is largely dependent on caspase-3 [18] (Figs. 4 and 5) indicates that caspase-3 acts as an upstream factor in a mini-cascade composed of these three effector caspases. Furthermore, several reports have demonstrated that caspase-3-mediated cleavage of death substrates is not the final event [14]. Many cleavage products of caspase-3, such as Bcl-2 [44], become active pro-apoptotic molecules that feedback to the apoptotic pathways. Caspase-3 mediated-feedback cleavage/activation of apical caspase-8 and -9 highlights its "initiator role." By integrating our data with previous reports [24], we propose a model for caspase-3 mediated-feedback activation on caspase-8 and -9 (Fig. 8). This model indicates that once the caspase cascade is initiated, caspase-3 activity is required for effective communication between the death receptor and mitochondrial pathways. Caspase-3, therefore, plays a central role in apoptosis regulation and execution. Given that caspase-3 deficiency or downregulation has been detected in numerous human cancers [21], defects in caspase-3 might be a critical factor facilitating carcinogenesis and/or therapeutic resistance. Targeting caspase-3, therefore, may be a useful novel strategy to treat many of the diseases associated with aberrant apoptosis.

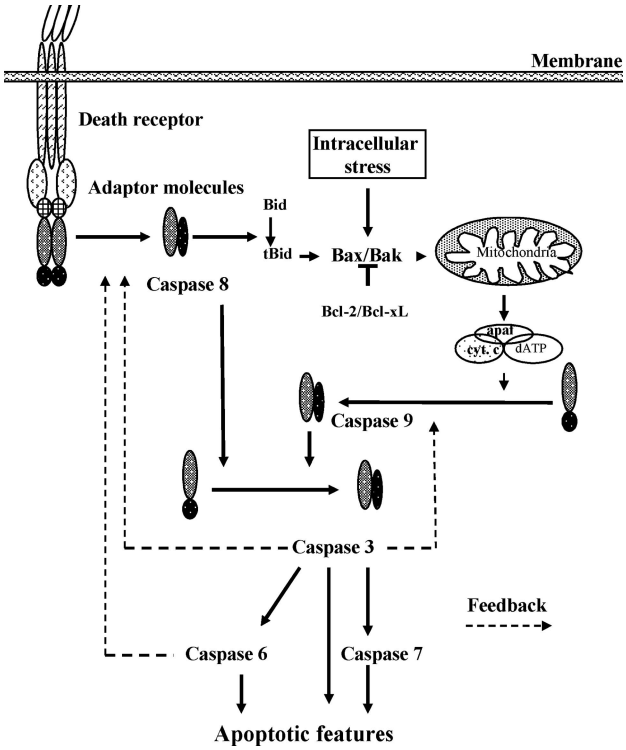

Fig. 8 Caspase-3-mediated feedback activation on caspase-8 and -9. In this model, caspases-8 and 9 are primarily activated by death receptor and mitochondrial signals, respectively. Caspase-3, which is downstream of activated caspase-8 and -9, enhances the activation of caspase-6 and -7 and cleaves cellular death substrates directly. In feedback reactions, caspase-3 activates caspase-9 by direct cleavage. It also activates caspase-8 by direct cleavage (minor) and by enhancing caspase-6 mediated-cleavage of caspase-8 (major)

Conclusion

We have systematically examined the effect of caspase-3 on apical caspases-2, 8, 9 and 10A in doxorubicin and TNF-α induced apoptosis using cell free and intact cell systems. Although caspase-3 mediated feedback activation has been reported before, we found that caspases-9 and 2 are sensitive to caspase-3 *in vitro*, whereas caspases- 9 and 8 are sensitive to caspase-3 in intact cells, suggesting that caspase-3 mediated feedback on different apical caspases could be mechanistically different. In particular, caspase-3 mediated cleavage/activation of caspase-8 is more evident in doxorubicin induced apoptosis, as is caspase-3 mediated cleavage/activation of caspase-9 in TNF-α induced apoptosis. This suggests that caspase-3 mediated activation not only amplifies the apoptotic signal in general, but also plays a pivotal role in the communication between death receptor and mitochondrial pathways. The striking differences in the cleavage/activation of the apical caspases between caspase-3 deficient and reconstituted cells strongly suggest that caspase-3 is a rate limiting factor in apoptosis initiation. During the preparation of this manuscript, a recent report in *Science* [45], which was based on knockout animal model, demonstrated that caspases- 3 and 7, known to be involved in the later stage of apoptotic execution, also act in the early stage of apoptosis. Although our experiments are based on biochemical and cell biology systems, this report delivers a similar message.

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