CMLS, Cell. Mol. Life Sci. 61 (2004) 1816–1825
1420-682X/04/141816-10
DOI 10.1007/s00018-004-4143-7

CMLS Cellular and Molecular Life Sciences

Research Article

Cytochrome c release and endoplasmic reticulum stress are involved in caspase-dependent apoptosis induced by G418

Q. H. Jin, B. Zhao and X. J. Zhang*

Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, 320 YueYang Road, Shanghai 200031 (China), Fax: +86 21 54921403, e-mail: xjzhang@sibs.ac.cn

Received 1 April 2004; received after revision 21 April 2004; accepted 26 May 2004

Abstract. G418 is used extensively in transfection experiments to select eukaryotic cells that have acquired neomycin resistance genes, but the mechanism is still elusive. To investigate this, we treated normal rat kidney cells with G418 for 3 days and found that the cells presented typical apoptotic features such as cell shrinkage, nuclear fragmentation, and caspase-3 activation. However, there was no low-molecular DNA ladder. The pan caspase inhibitor z-VAD-fmk completely inhibited this type of apoptosis, suggesting a caspase-dependent mechanism. Caspase cascades in apoptosis induced by G418 were initiated by at least two pathways: the release of cytochrome c from mitochondria, which was observed under confocal microscopy, and endoplasmic reticulum stress, demonstrated by the increase in Ca^{2+} concentration and the cleavage of m-calpain and procaspase-12. Both pathways activated caspase-9. Inhibition of caspase-9 activity by z-LEHD-fmk prevented most of the cells from apoptosis, and E-64d, an inhibitor of calpain accentuated this block. The cleavage of casapse-9 and caspase-12 was blocked only by simultaneous application of z-VAD-fmk and E-64d, but not by either alone. E-64d did not prevent the release of cytochrome c. These results indicated that these two pathways were independent of each other.

Key words. G418; apoptosis; cytochrome c; endoplasmic reticulum stress.

Apoptosis is an active form of cell death that plays an essential role in physiological and pathological conditions [1, 2] and can be initiated by three distinct pathways involving caspase activation. One pathway involves signal transduction through cellular death receptors to activate caspase-8, -2, and -10, and in turn activates downstream effector caspases, including caspase-3 and -7 [3]. This pathway is also called the extrinsic pathway. The second pathway, also called the intrinsic pathway, is mediated by the release of cytochrome c from mitochondria. Once released, cytochrome c forms an oligomeric complex with dATP and Apaf-1 [4, 5], followed by recruitment of procaspase-9 and its activation. The active caspase-9 then activates effector caspases, such as caspase-3, -6, and -7 [3, 5, 6]. The third pathway is initiated by activation of caspase-12 in response to endoplasmic reticulum (ER) stress, such as apoptosis induced by tunicamycin, thapsigargin or calcium ionophores [7]. Activated caspase-12 directly activates procaspase-9 independently of cytochrome c and Apaf-1, and finally activates effector caspases, caspase-3 and 7 [8, 9]. Although caspases play a central role in the implementation of apoptosis, cells can still undergo apoptosis without caspase activation. One caspase-independent mechanism involves the release of apoptosis-inducing factor (AIF) from mitochondria,

* Corresponding author.

which is then translocated to the nucleus to trigger nuclear condensation [10, 11].

G418 is a neomycin analog widely used in conjunction with eukaryotic expression vectors encoding genes whose products inactivate G418. But the molecular mechanism by which G418 exerts its selection effect is still unclear. G418 has various roles in cells. It is involved in inhibition of protein synthesis by binding to 80S ribosomes [12], activation of phosphatidylinositol phospholipase C (PI-PLC) [13] that leads to release of GPI-anchored proteins [14], increase of dihydroxyacetone phosphate acyltransferase and peroxismal β-oxidation activity [15]. G418 has also been reported to induce apoptosis [16]. In this study, we showed that G418 induced caspase-dependent apoptosis. The caspase cascades were initiated by at least two pathways: cytochrome c release and ER stress.

Materials and method

Materials

G418 and RPMI 1640 were purchased from Invitrogen; normal rat kidney (NRK) cells were from the cell bank of the Chinese Academic of Science (Shanghai, China). Antibody to cleaved caspase-3 was from Cell Signaling Technology. Antibody to cytochrome c, and the pan-caspase inhibitor z-VAD-fmk [benzoyloxycarbonyl-Val-Ala-Asp(OMe)-CH_2F] were from Promega Life Science. Antibody to caspase-9, E-64d (epoxysuccinyl-L-leucyl-amido-3-butane ethyl ester), fluo-3 acetoxymethyl ester (fluo-3/AM), and the caspase-9 inhibitor z-LEHD-fmk [(benzoyloxycarbonyl-Leu-Glu (OMe)-His-Asp(OMe)-CH_2F)] were from Calbiochem. The TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling) kit was from Roche Molecular Biochemicals. MitoTracker Red CMXRos was purchased from Molecular Probes. Horseradish peroxidase-coupled goat anti-mouse or anti-rabbit IgG, fluorescein isothiocyanate-coupled goat anti-mouse IgG, rhodamine-coupled goat anti-rabbit IgG secondary antibodies and the ECL (kit) were from Santa Cruz Biotechnology. The caspase-8 polyclonal antibody was from NeoMarkers and caspase-12 polyclonal antibody from Biovision. Monoclonal antibodies to m-calpain and to β-actin were from Sigma.

Cell culture and treatment with G418

NRK cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and 4 mM glutamine. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. When they had reached 60–80% confluency, the medium was changed with fresh culture medium to which G418 was added. The cells were cultured for 1 to 4 days before harvested for various measurements.

Isolation of DNA fragment

DNA isolated from apoptotic NRK cells was extracted as previously described [17] with minor modifications. Briefly, the living cells or cells (3×10^6) treated with 400 μg/ml G418 for 1 to 4 days were harvested and washed in PBS (10 mM K_2HPO_4, 1 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). Cells were then pelleted and resuspended in 40 μl 0.2 M Na_2HPO_4/0.1 M citric acid (192:8) at room temperature for at least 60 min. After centrifugation at 1500 g for 15 min, supernatants were transferred to new 0.5-ml Eppendorf tubes, and 3 μl 0.25% NP-40 in distilled water and 3 μl RNase A (10 mg/ml) were added. After incubation at 37 °C for 60 min, 3 μl proteinase K (10 mg/ml) was added and samples were further incubated for 30 min at 50 °C. After suspension in 5 μl loading buffer, the DNA was loaded onto a 1% agarose gel containing 5 μg/ml ethidium bromide. Electrophoresis was performed at 10 V/cm for 2 h. Fragmented DNA was visualized under UV light.

Flow cytometric analysis of cell apoptosis

The cells treated with various drugs were collected by centrifugation after treatment with trypsin and then fixed with 70% ethanol. The cells were pelleted and resuspended in 400 μl PBS, and treated with 10 μg/ml RNase A for 1 h at 37 °C, followed by staining with 50 μg/ml propidium iodide (PI) for at least 30 min, and analyzed with a flow cytometer (Becton-Dickinson FACSorter) using CELLQuest software.

Double staining of immunoflurescence labeled cleaved caspase-3 fragment and TUNEL

The cells were grown on glass slides and treated with 400 μg/ml G418 or 400 μg/ml G418 plus 100 μM z-VAD-fmk. After one washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 45 min at 4 °C and then permeabilized with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 5 min on ice. After block in 3% BSA in PBS for 1 h at 4°C, the slides were incubated with anti-cleaved caspase-3 antibody diluted 1:100 for 24 h at 4 °C. After washing three times with PBS, the slides were incubated with secondary antibody conjugated with rhodamine anti-rabbit IgG diluted 1:100 for 1 h. Afterwards the slides were washed three times with PBS and stained with 10 μM Hoechst 33258, or were further incubated with 50 μl TUNEL reaction mixture for 60 min at 37 °C in a dark and humidified atmosphere and stained with Hoechst 33258 before the slides were observed under a Nikon fluorescence microscope.

Visualization of cytochrome c, nuclei, and mitochondria by confocal microscopy

Cells grown on chambered coverslips were treated with 400 μg/ml G418 or with 400 μg/ml G418 plus 100 μM

zVAD-fmk for 3 days. The cells still remaining on the coverslips were washed once with PBS and fixed with 4% formaldehyde for 1 h at 4 °C and then permeabilized with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 5 min on ice. After gently washing three times, the cells were blocked in 3% BSA in PBS. The cells were then stained with anti-cytochrome c antibody (diluted 1:100 in 3% BSA in PBS) for 1 h at 37 °C in humidified atmosphere, followed by washing three times with PBS. The secondary antibody, FITC-conjugated goat anti-mouse IgG, diluted 1:100 in 3% BSA in PBS, was added and the cells were incubated for 30 min at 37 °C in a humidified atmosphere, and then washed three times with PBS. To double-label nuclei, cells were further stained with 10 µM Hoechst 33258 for 5 min at room temperature. To double-label mitochondria, cells were first incubated with 2 µM MitoTracker Red in culture medium for 30 min in a 5% CO_2 humidified incubator and then immunostained for cytochrome c. The coverslips were observed under a laser scanning confocal microscope (Bio-Rad radiance 2100).

Transmission electron microscopy

NRK cells treated with G418 were collected by centrifugation after trypsin treatment. The cells were then fixed with 2.5% glutaraldehyde in PBS, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections were stained with uranylacetate and lead acetate and examined on a Zeiss EM 902 transmission electron microscope.

Calcium imaging

NRK cells were grown on glass slides and treated with G418. The relative concentration of calcium was measured as described elsewhere [18], the cells were washed twice with normal salt buffer (NS buffer) containing 124 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, and 10 mM dextrose, pH 7.4, and then loaded with 2 µM fluo-3/AM in NS buffer for 30 min at 37 °C in a humidified 5% CO_2 incubator. After washing with NS buffer, the cells were imaged using the Leica TCS SP2 confocal system and a 100 UV objective lens. Calcium fluorescence was analyzed with Leica confocal software (version: 2.00 Build 0770).

Western blot analysis of caspase-3, -8, -9, -12 and m-calpain

The cells either treated with 400 µg/ml G418 or untreated were collected and lysed in 1×SDS PAGE gel loading buffer [50 mM Tris pH 6.8, 100 mM DTT, 2% (w/v) SDS, 10% (v/v) glycerol, freshly added 1 µg/ml aprotinin and 10 mM PMSF] and boiled for 10 min. Protein concentration was determined by the bicinchoninic acid (BCA) method. Bromophenol blue (final concentration 0.01%) was added to the samples before an equal amounts of proteins were loaded in each lane for electrophoresis and then electrotransferred onto nitrocellulose membranes. After blocked with TBST (10 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.05% Tween-20) containing 5% skimmed milk for 1 h, the membranes were incubated with a primary antibody (capsase-12 diluted to 1/500, caspase-9 to 1/1000, caspase-8 to 1/500, caspase-3 to 1/1000, or m-calpain to 1/500, or β-actin to 1/4000) in 5% skimmed milk in TBST for 2 h at 37 °C. After washing with TBST three times, the membranes were incubated with anti-rabbit or anti-mouse IgG antibody conjugated with HRP diluted 1:2000 in 5% skimmed milk in TBST for 1 h at 37 °C. The results were visualized by the ECL method.

Results

Induction of apoptosis in NRK cells by G418

Apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation. NRK cells treated with 400 µg/ml G418 for 3 days presented obvious apoptotic features: cell rounding and shrinkage and nuclear condensation and fragmentation (fig. 1Ad and e). Since activation of caspases is a hallmark of apoptosis, we used an antibody specific to the cleaved caspase-3 fragment to examine the presence of active caspase-3 by immunocytofluorescence. As shown in figure 1Af, the activated caspase-3 fragment was present in cells with condensed and fragmented nuclei, but not in normal cells (fig. 1Ab and c). In G418-treated NRK cells, no low-molecular DNA ladder was observed, and only a high-molecular DNA smear was seen, even after 4 days (fig. 1B). However, in some apoptosis processes, a low-molecular DNA ladder is absent, and ultrastructural features and the morphology of the nucleus after staining with Hoechst 33258 are reliable criteria of apoptosis [19]. Thus, although no low-molecular DNA ladder was observed, the alterations in cell and nuclear morphology, and the activation of caspase-3 proved that G418 exerted cell toxicity by induction of cell apoptosis.

Transmission electron microscopy observation revealed the alteration of ultrastructural features

To further investigate the apoptosis induced by G418, we observed the ultrastructural features of apoptotic NRK cells by transmission electron microscopy. As in figure 2C, shown, G418-treated cells revealed prominent chromatin condensation, nuclear pyknosis and fragmentation, in agreement with the results of Hoechst 33258 staining. The cytoplasm of apoptotic cells was full of vacuoles (fig. 2E). Some of the vacuoles may have been derived from ER, which was destroyed before nuclear condensation (fig. 2B), and part was from swollen mitochondria (fig. 2E). Mitochondria play an important role in apopto-

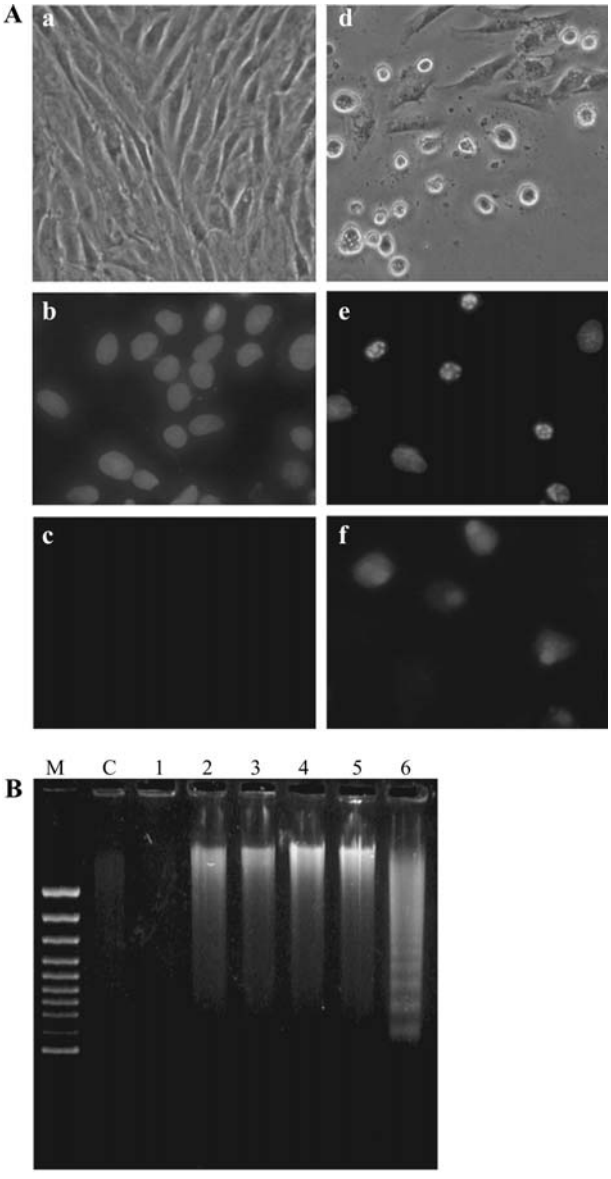

Figure 1. G418 induced NRK cell apoptosis. (*A*) NRK cells treated with 400 μg/ml G418 for 3 days showed rounding up and shrinkage (*d*), nuclear condensation and fragmentation (*e*), and positive immunofluorescence staining for the cleaved caspase-3 fragment (*f*), compared with the cell morphology (*a*), nuclei (*b*), and staining for cleaved caspase-3 fragment (*c*) in untreated NRK cells (*b* and *c*, *e* and *f* are in the same observation field). (*B*) A DNA smear but no DNA ladder appeared in G418-treated NRK cells. M, marker; C, control; lanes 1–5, G418 treatment for 1–5 days; lane 6, staurosporine treatment for 2 days, as a positive control for DNA ladder.

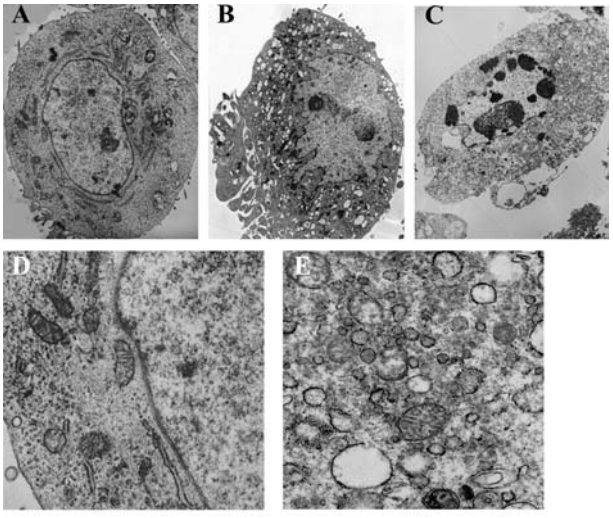

Figure 2. Transmission electron microscopy revealed the alteration of ultrasturctral features. untreated control NRK cell (*A*, *D*). NRK cells treated with 400 μg/ml G418 for 3 days (*B, C, E*). The ER is destroyed before nuclear condensation (*B*). Typical apoptotic features include chromatin condensation, nuclear pyknosis, and fragmentation (*C*). Apoptotic cells were full of vacuoles, and mitochondria were swollen (arrows (*E*)). (*A*–*C*) ×7000; ((*D, E*) ×30,000)

sis. The swollen mitochondria could release apoptotic factors including cytochrome c and AIF to participate in G418-induced apoptosis.

Caspases played an important role in G418 induced apoptosis

Because a cleaved caspase-3 fragment was produced in apoptotic NRK cells induced by G418, 100 μM of the pan caspase inhibitor z-VAD-fmk was used to block caspase activity and to investigate the role of caspases. NRK cells treated with 400 μg/ml G418 plus 100 μM z-VAD-fmk for 3 days still rounded up, and blebbed (fig. 3E). However, by flow cytometry examination, only 2 ± 0.4% cells were apoptotic, even lower than the control (6.3 ± 1.6%) z-VAD-fmk alone (100 μM) had no toxic effect on NRK cells (data not shown). To further investigate whether capsase activity was blocked, the cleaved caspase-3 fragment was detected by immunofluorescence. It was totally absent from NRK cells induced by G418 plus z-VAD-fmk, even in the TUNEL-positive cells (fig. 3F–H). These results suggested that caspases played a critical role in G418 induced apoptosis. Interestingly, the nuclei of TUNEL-positive cells induced by G418-alone showed condensation and fragmentation, whereas the nuclei only showed condensation after z-VAD-fmk was added. This indicated that caspase cascades were the chief pathway leading to apoptosis induced by G418, but not the unique pathway.

Caspase-9 was partially activated by the release of cytochrome c from mitochondria independent of caspase activity

Because caspase cascades played an important role, we further investigated the signal pathways that activate caspases. One pathway triggering caspase cascades is initiated through cellular death receptors to activate caspase-8. But caspase-8 was not cleaved in G418-induced apoptosis in NRK cells within 3 days. Another initiator, caspase-9, was cleaved at 2 days, and the increase in

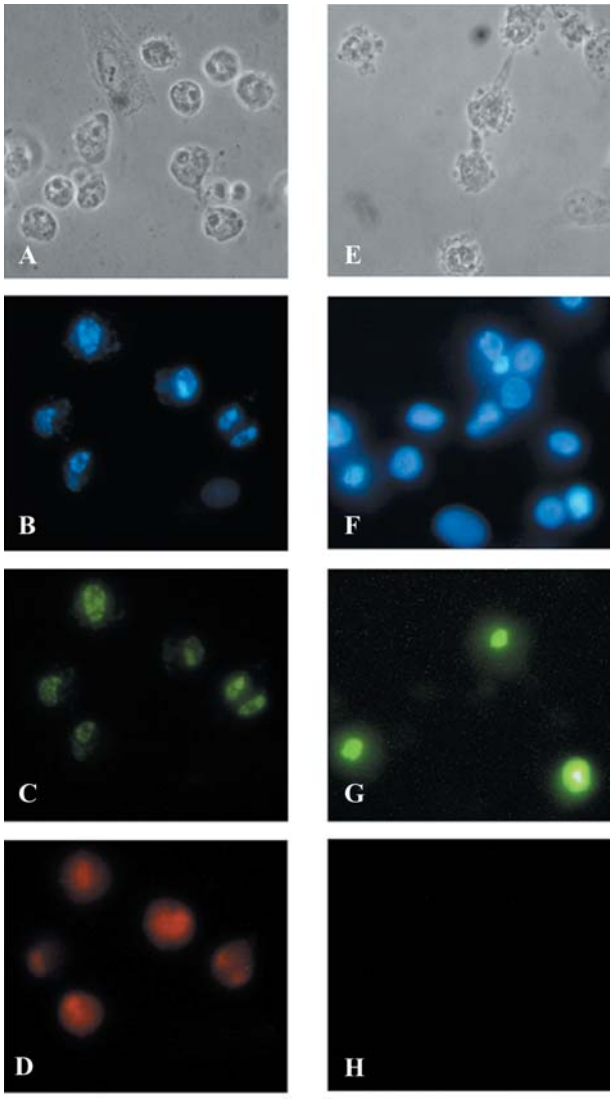

Figure 3. Different apoptotic features appeared with complete inhibition of caspase activity by 100 μM z-VAD-fmk. NRK cells treated with 400 μg/ml G418 plus 100 μM z-VAD-fmk for 3 days rounded up and blebbed (*E*) more seriously than cells treated only with 400 μg/ml G418 for 3 days (*A*). In cells treated with G418 alone, cells with nuclear condensation and fragmentation (*B*) were positively labeled by TUNEL (*C*) and immunfluorescence labeled for cleaved caspase-3 (*D*). In cells treated simultaneously with G418 and z-VAD-fmk, cells were negative for the activated caspase-3 fragment (*H*), and nuclei of apoptotic cells positively labeled by TUNEL (*G*) only showed condensation, but no fragmentation (*F*). (*B*–*D* are in the same observation field, and *F*–*H* are in the same field).

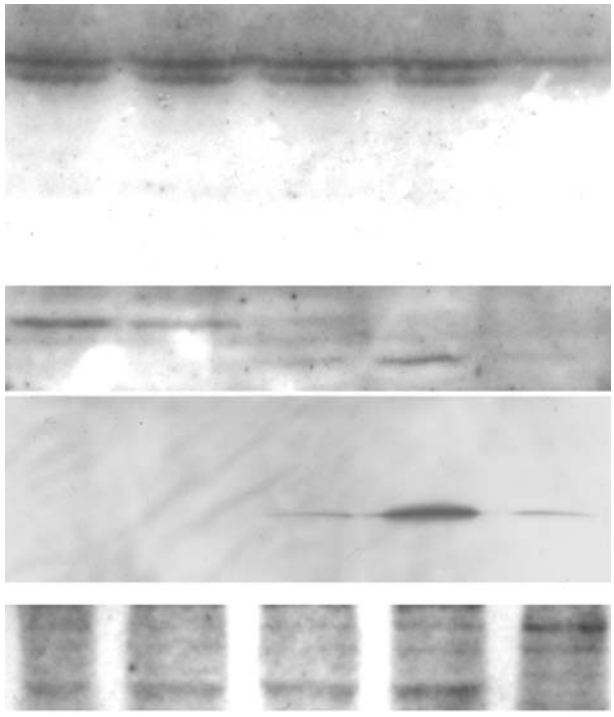

Figure 4. Caspase-3 and caspase-9 were activated, but not caspase-8 within 3 days. (*A*) Caspase-9 (*Ab*) and caspase-3 (*Ac*), but not caspase-8 (*Aa*) were cleaved in G418 induced apoptosis within 3 days. (*B*) The cleavage of caspase-9 was prevented by simultaneous use of E-64d and z-VAD-fmk, but neither alone. (lane 1: G418; lane 2: G418+DMSO; lane 3: G418+E-64d; lane 4: G418+z-VAD-fmk; lane 5: G418+E-64d+z-VAD-fmk. G418, 400 μg/ml; DMSO, 0.5%; E-64d, 25 μM; Z-VAD-fmk, 100 μM)

cleaved caspase-9 was simultaneous with the increase in cleaved caspase-3 fragment (fig. 4A). At day 4, the degeneration of procaspase-8 might be caused by nonspecific cleavage by apoptosis proteases, as activated caspase-3 and caspase-9 also degenerated at the same time.

The main pathway to activate caspase-9 is initiated by the release of cytochrome c from mitochondria. In addition, swollen mitochondria were observed by transmission electron microscopy, so we investigated whether cytochrome c was released from mitochondria. In living NRK cells, cytochrome c staining was punctated and located in mitochondria stained by MitoTracker (fig. 5B). Treated with either G418 or G418 plus 100 μM z-VAD-fmk for 3 days, the cells exhibited diffusive cytosolic cytochrome c staining. In the cells treated with G418 plus z-VAD-fmk, cytochrome c gathered in the nuclei, and in the cells treated with only G418, partial cytochrome c staining was still punctuate, although the nuclei had been fragmented (fig. 5A). The results suggested that cytochrome c was partially released from mitochondria of NRK cells treated with G418, and its release was independent of caspase activity. In fact, cytochrome c release was more complete after caspase activity had been blocked.

The activation of caspase-9 through the cytochrome c pathway needs caspase activity. But caspase-9 was still cleaved after caspase activity had been blocked by 100 μM z-VAD-fmk. 25 μM, E-64d an irreversible inhibitor of cysteine proteases, had no effect on the cleavage of caspase-9 alone, but the cleavage of caspase-9 was completely blocked when E-64d was combined with 100 μM z-VAD-fmk (fig. 4B). The caspase-9-specific inhibitor z-LEHD-fmk (50 μM) or E-64d (25 μM) alone only partially inhibited apoptosis induced by G418: the apoptosis

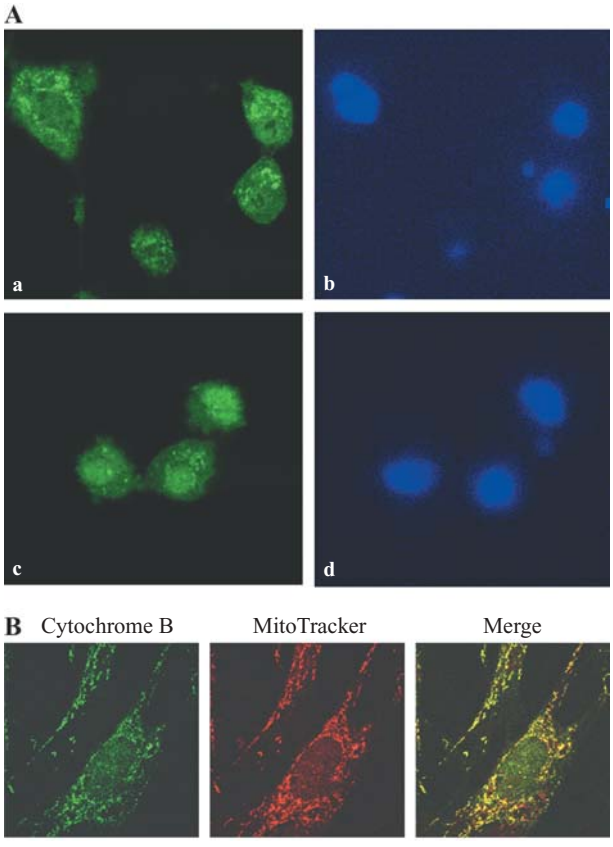

Figure 5. Cytochrome c was released from G418-treated NRK cells independently of caspase activity. Partial cytochrome c staining was still punctated (*Aa*) when the nucleus was contracted and fragmented (*Ab*). Suggesting that not all cytochrome c was released from the mitochondria. z-VAD-fmk (100 μM) did not prevent cytochrome c release (*Ac*). (*B*) In untreated NRK cells, cytochrome c was located in mitochondria stained by MitoTracker.

rate was 26.4 ± 4.1% and 45.6 ± 9.8%, respectively, compared with 74.7 ± 15% by G418 or 60.7 ± 12.8% by G418 with the drug vehicle DMSO. The inhibition of apoptosis was more effective after z-LEHD-fmk plus E-64d treatment, indicated by the decrease in the apoptosis rate to 13.9 ±1 .6% (fig. 6). Thus, there were at least two pathways leading to caspase-dependent apoptosis induced by G418: one via cytochrome c released from mitochondria to activate caspase-9, the other blockable by E-64d.

ER stress involved in G418 induced apoptosis

Another important pathway to trigger caspase cascades is initiated by ER stress. Transmission electron microscopy observation showed that the ER was destroyed before nuclear condensation. So we conjectured that ER stress was involved in the apoptosis process induced by G418. ER stress can be triggered by perturbation of Ca^{2+} homeostasis [20]. Hence we used fluo-3/AM to measure the relative Ca^{2+} concentration. As shown in figure 7A, the fluoresecence density gradually increased in NRK cells treated with G418 from 1 day to 3 days, and at day 3, it was nearly six fold higher than in untreated cells. An increase in Ca^{2+} can initiate activation of m-calpain, and activated m-calpain will completely autocleave at Ala^{9}-Lys^{10} within 1 min [21] and also cleave caspase-12 to activate it [22]. Caspase-12 is regarded as a representative molecule implicated in the apoptosis mechanisms relevant to ER stress [7, 22]. As figure 7B shows, the molecular weight of m-calpain decreased slightly after 3 days treatment with G418, indicating that m-calpain was activated and autocleaved. Meanwhile, caspase-12 was also cleaved, and completely cleaved at the third day. These results demonstrate that ER stress was involved in G418-induced apoptosis.

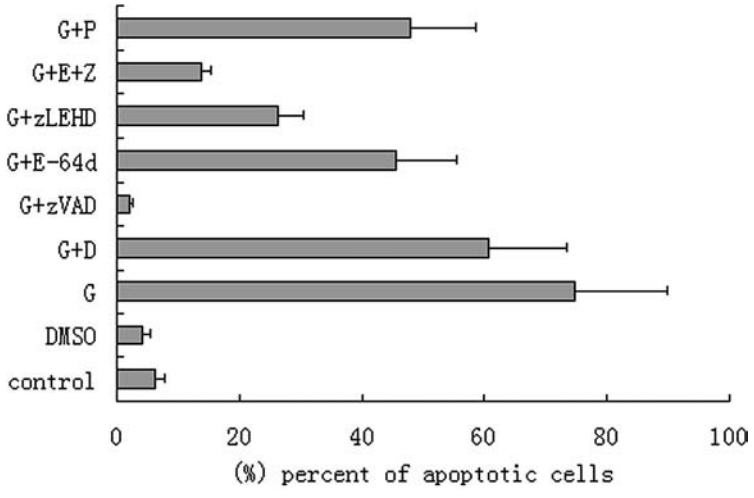

Figure 6. The inhibitory effects of various drugs on apoptosis induced by G418 in NRK cells (G418 (G), 400 μg/ml; DMSO (D), 0.5% (v/v); z-VAD-fmk (zVAD), 100 μM; E-64d (E), 25 μM; z-LEHD-fmk (zLEHD), 50 μM). z-VAD-fmk, E-64d, and z-LEHD were dissolved in DMSO, and all cells treated with these three drugs contained 0.5% DMSO.

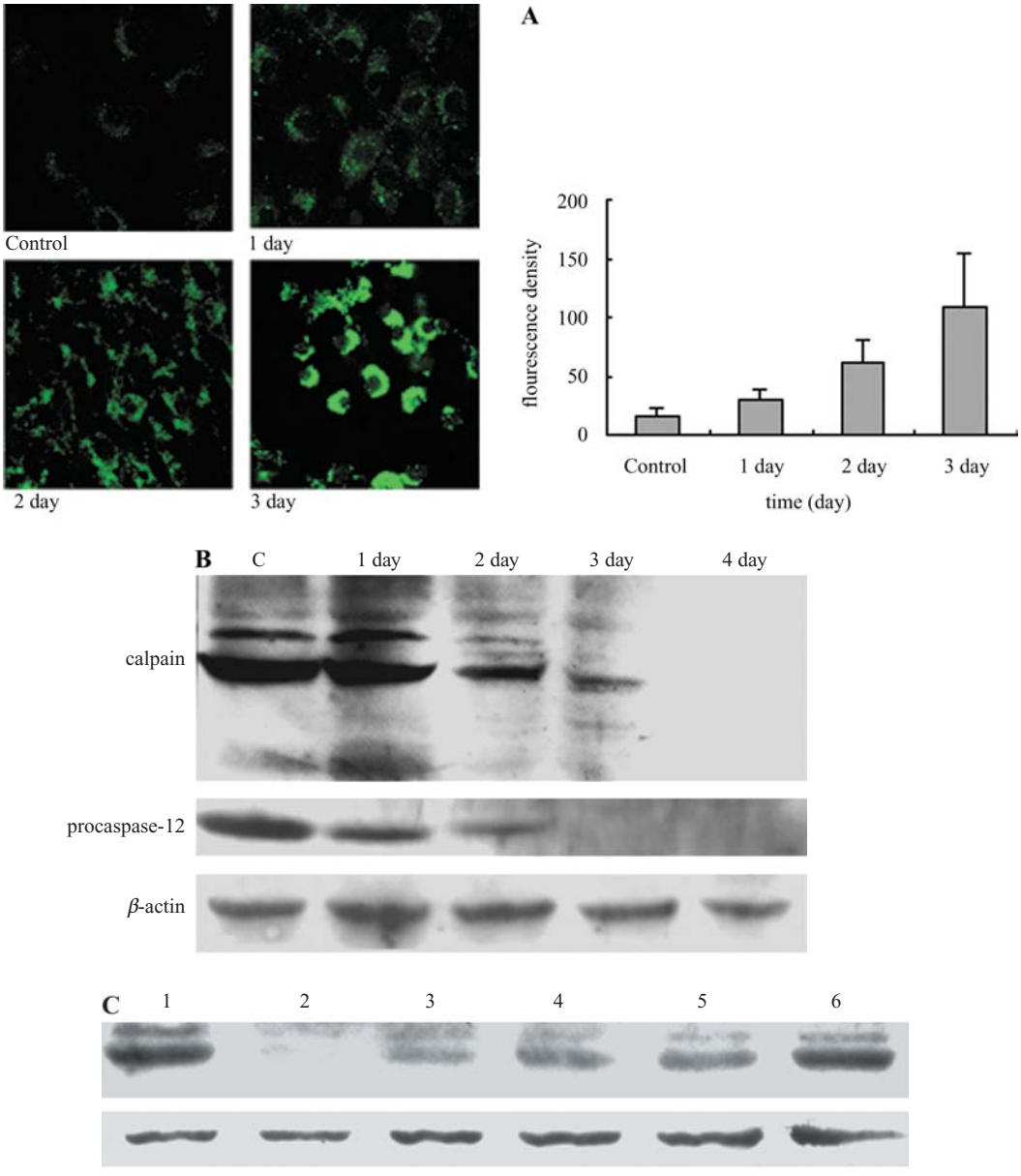

Figure 7. Endoplasmic reticulum (ER) stress was involved in G418 induced apoptosis. (A) Concentration of Ca^{2+} was gradually increased in NRK cells treated with 400 μg/ml G418. (B) m-calpain and caspase-12 were cleaved. (C) The cleavage of caspase-12 was prevented by simultaneously E-64d and z-VAD-fmk, but neither alone. β-actin acts as inner reference. (lane 1: control; lane 2: G418; lane 3: G418+DMSO; lane 4: G418+E-64d; lane 5: G418+z-VAD-fmk; lane 6: G418+E-64d+z-VAD-fmk. G418, 400 μg/ml; DMSO, 0.5%; E-64d, 25 μM; Z-VAD-fmk, 100 μM)

However, caspase-12 was cleaved at 2 days, before m-calpain was activated, and moreover, 25 μM E-64d alone, an inhibitor of m-calpain, only partially prevented the cleavage of caspase-12 (fig. 7C, lane 4). This suggested that other proteases, in addition to m-calpain, were involved in cleavage of caspase-12. Caspase-7 has been reported to be translocated to the ER surface and cleaves caspase-12 [23]. So, 100 μM z-VAD-fmk, which cannot inhibit m-calpain activity, was used to abolish caspase activity. Z-VAD-fmk alone also partially inhibited the cleavage of casapse-12 (fig. 7C, lane 5), but the cleavage of caspase-12 was completely prevented by simultaneous treatment with z-VAD-fmk and E-64d (fig. 7C, lane 6). These results indicated that both m-calpain and caspase-7 were involved in the cleavage of caspase-12.

Discusssion

Although the aminoglycoside G418 is used extensively in transfection experiments to select for eukaryotic cells that have acquired neomycin resistance genes, the molecular

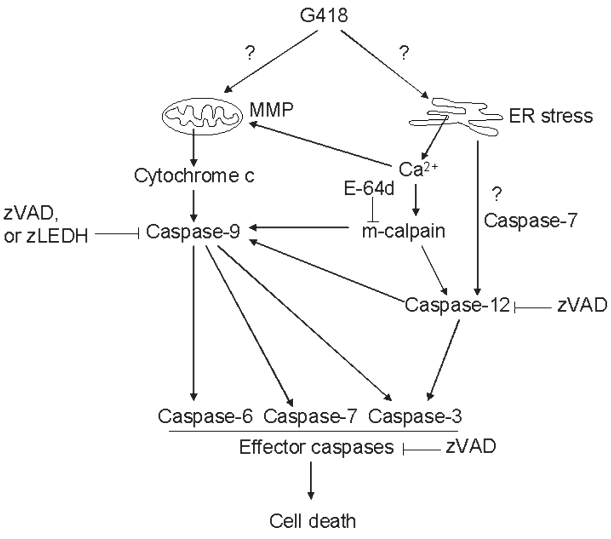

Figure 8. G418 initiated two relatively independent pathways to apoptosis. One pathway was initiated by ER stress that resulted in an increase in intracellular Ca^{2+} concentration and the cleavage of m-calpain, and then caspase-12 was activated, and active caspase-12 continued to cleave effector caspases, such as caspase-3, or to cleave and activate caspase-9, which activated effector caspases. In addition of m-calpain, caspases were involved in the cleavage of caspase-12, the possible caspase involved being caspase-7. The other pathway was initiated by the release of cytochrome c from mitochondria that might be caused by an increase in intracellular Ca^{2+} concentration or the direct effect of G418. The release of cytochrome c resulted in the activation of caspase-9, and then active caspase-9 activated the effector caspases. Caspase-9 could be cleaved by caspase-9 itself, or caspase-12, and m-calpain. MMP; mitochondrial membrane permeabilization.

mechanism is elusive. In the present study, we reported that G418 exerted its function by inducing cellular apoptosis through investigation of the morphological changes, DNA fragmentation, and caspase-3 activation.

Hygromycin B, which is an aminoglycoside antibiotic and also widely used to establish stable mammalian cell lines, induces apoptosis in CHO LR73 cells in 18 h and exhibits a DNA ladder [24]. G418 required 6–7 days to induce apoptosis in CHO LR73 cells, which was not accompanied by a DNA ladder [24]. This indicates that there are different apoptotic mechanisms involved in apoptosis induced by hygromycin B and G418. In this study, we elucidated the apoptotic pathway in G418-induced apoptosis.

One effect of G418 on NRK cells was to prevent cell proliferation. Neomycin, another aminoglycoside antibiotic, blocks thrombin initiation of cell proliferation by inhibition of thrombin-stimulated phosphoinositide turnover [25]. There is an important second messenger molecule, diacylglycerol (DAG), involved in this turnover, and DAG can activate protein kinase C (PKC). Several reports show that PKC inhibits apoptosis by decreasing caspase-8 activation [26, 27]. We conjectured that G418 induced apoptosis by inhibition of PKC activation. But phorbol-12-myristate-13-Acetae (PMA), an agonist of PKC, failed to block apoptosis induced by G418 (data not shown). In addition, caspase-8 was not activated in NRK cells treated with G418 within 3 days. So this indicated that the G418-induced NRK cell apoptosis had no relationship to inhibition of phospoinositide turnover.

Mitochondria play a pivotal role in apoptosis regulation. In the process of apoptosis, mitochondria matrix swelling and outer membrane rupture [28] are caused by Bcl-2 family members via formation of pores in the outer membrane [29] or through binding to channels in either the outer or the inner mitochondrial membrane, thereby inducing hyperpolarization or a permeability transition [30–32]. Some apoptotic factors, such as cytochrome c [33, 34], AIF [10], and endonuclease G [35], are released from mitochondria to the cytosol. Cytochrome c, together with Apaf-1, dATP and cytosolic procaspase-9, forms the apoptosome, and then activates caspase-9 to initiate caspase cascade. AIF can induce cell apoptosis by a caspase-independent pathway [36], and endonuclease G induces DNA degradation independently of caspase activation [35, 37]. These two factors might function after caspase activity was blocked in G418-induced apoptosis. In this study, swollen mitochondria were present in apoptotic NRK cells treated with G418 for 3 days, and at the same time, cytochrome c, but not all of it, was released from mitochondria. According to another report, once cytochrome c release is initiated, it will continue until it has been completely released from all mitochondria in individual cells within a 5-min time range [38]. Moreover, the release of cytochrome c occurs at an early stage in apoptosis [38]. In apoptotic NRK cells exhibiting nuclear fragmentation induced by G418, cytochrome c was still partially held in mitochondria. This suggested that cytochrome c was released in the process of other apoptosis pathways, or that G418 affected mitochondria later. Although caspase-9 was cleaved in G418-induced apoptosis, it can also be cleaved by calpain [39, 40] and caspase-12 [8]. The cleavage of caspase-9 was blocked only by simultaneous use of E-64d (a calpain inhibitor) and z-VAD-fmk. This suggested that calpain was involved in the cleavage of caspase-9.

The ER participates in the initiation of apoptosis by at least two different mechanisms, namely the unfold protein response (UPR) and Ca^{2+} signaling [20]. Caspase-12, specifically localized on the cytoplasmic side of ER, is thought to play a role in ER stress-mediated apoptosis [7]. G418 can induce misreading of mRNA by reducing the fidelity of aminoacyl-tRNA selection [41], and leads to accumulation of misread, abnormal proteins [42]. It may trigger the UPR and result in ER stress. An increase of $[Ca^{2+}]$ and cleavage of caspase-12 testified that ER stress was involved in apoptosis induced by G418 in NRK cells. An ER stress-specific caspase cascade is one in which active caspase-12 cleaves caspase-9, and then acti-

vates effector caspases [8, 9]. Obviously, caspase-9 has an essential role in both the ER stress-specific caspase cascade and the caspase cascade initiated by the release of cytochrome c. In this study, the caspase-9 inhibitor z-LEHD-fmk blocked apoptosis in a large proportion of the cells, but not as efficiently as z-VAD-fmk, indicating that caspase-9 was not responsible for the activation of all effector caspases. One possible pathway was cleavage by activated caspase-12 of effector casapases, such as caspase-3 [43], because E-64d could further block apoptosis inhibited by z-LEHD-fmk.

Besides cleaving caspase-12, calpain also cleaves Bax [44] and Bid [45], and it cleaves Bcl-X_L to convert it to a pro-apoptotic form [22]. These cleaved molecules then act on mitochondria to result in the release of cytochrome c. But in this study, E-64d did not change the release model of cytocrome c in cells treated with either G418 or G418 plus z-VAD-fmk (data not shown), suggesting that other factors might result in the release of cytochrome c, such as Ca^{2+} [46]. Because G418 inhibits protein synthesis, it can prevent the turnover of some proteins, such as MCL-1, which is an antiapoptotic member of the Bcl-2 family with a rapid turnover [47], and this might lead to cytochrome c release. ER stress can also induce caspase-8 activation, which subsequently cleaves Bid and results in cytochrome c release. The release of cytochrome c in this pathway is blocked by the caspase inhibitor z-VAD-fmk [48]. But in this study, cytochrome c release was not blocked by z-VAD-fmk. Moreover, caspase-8 was not activated in G418-induced apoptosis within 3 days. G418 might also act directly on the mitochondria because the viability of the cells decreased after treatment with over 800 μg/ml G418 for 1 day, but few apoptotic cells were detected (data not shown). Thus, these two apoptotic pathways triggered by cytochrome c release and ER stress were relatively independent events. These two pathways are shown in figure 8.

Caspase-12 has an important role in apoptosis triggered by ER stress. However, Fischer et al. [49] reported that the human caspase-12 gene has acquired deleterious mutations that prevent the expression of a functional protein. However, there are no functional data confirming this conclusion, and it remains highly hypothetical. Thus, in human cells, ER stress induced by G418 may trigger different apoptotic signaling pathways with those in rat cells, such as activation of caspase-8 [48].

One other possible pathway to apoptosis is triggered by lysosome. Lysosome is the primary reservoir of nonspecific proteases in mammalian cells. Accumulating evidence shows that lysosome has an important role in apoptosis [50]. Caspase-3 can be activated by the elevation of lysosomal pH [51]. Aminoglycosides can accumulate in lysosomes and raise lysosomal pH [52]. G418 also raised lysosomal pH independent of caspase activity (data not shown). But 2.5 μM pepastatin A, a lysosomal protease inhibitor, did not prevent cell apoptosis. E-64d, another lysosomal protease inhibitor, might inhibit cell apoptosis by blocking the ER stress apoptosis pathway, but not lysosomal protease. So, although G418 raised lysosomal pH, we had no evidence for lysosomal involvement in G418-induced apoptosis.

In summary, G418 exerted its toxicity by induction of caspase-dependent apoptosis. The caspase cascades were activated by at least two independent pathways: the release of cytochrome c and ER stress.

Acknowledgements. We thank Dr. Y.-f. Shi (Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School) for critical review of the manuscript. This work was supported by the National High Technology '863' Programs of China (Grant No. 2002BA711A07), the National Natural Science Foundation of China (Grant No. 30240028), and Grant No. KSCX2-SW-211 from the Chinese Academy of Sciences.

1 Wyllie A. H., Kerr J. F. and Currie A. R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol. **68:** 251–306
2 Cohen G. M. (1997) Caspases: the executioners of apoptosis. Biochem. J. **326 (Pt 1):** 1–16
3 Earnshaw W. C., Martins L. M. and Kaufmann S. H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. **68:** 383–424
4 Li P., Nijhawan D., Budihardjo I., Srinivasula S. M., Ahmad M., Alnemri E. S. et al. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91:** 479–489
5 Zou H., Li Y., Liu X. and Wang X. (1999) An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. **274:** 11549–11556
6 Saleh A., Srinivasula S. M., Acharya S., Fishel R. and Alnemri E. S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. J. Biol. Chem. **274:** 17941–17945
7 Nakagawa T., Zhu H., Morishima N., Li E., Xu J., Yankner B. A. et al. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature **403:** 98–103
8 Morishima N., Nakanishi K., Takenouchi H., Shibata T. and Yasuhiko Y. (2002) An endoplasmic reticulum stress-specific caspase cascade in apoptosis: cytochrome c-independent activation of caspase-9 by caspase-12. J. Biol. Chem. **277:** 34287–34294
9 Rao R. V., Castro-Obregon S., Frankowski H., Schuler M., Stoka V., del Rio G. et al. (2002) Coupling endoplasmic reticulum stress to the cell death program: an Apaf-1-independent intrinsic pathway. J. Biol. Chem. **277:** 21836–21842
10 Susin S. A., Lorenzo H. K., Zamzami N., Marzo I., Snow B. E., Brothers G. M. et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature **397:** 441–446
11 Kroemer G. and Reed J. C. (2000) Mitochondrial control of cell death. Nat. Med. **6:** 513–519
12 Bar-Nun S., Shneyour Y. and Beckmann J. S. (1983) G-418, an elongation inhibitor of 80 S ribosomes. Biochim. Biophys. Acta **741:** 123–127
13 Morris J. C., Ping-Sheng L., Zhai H. X., Shen T. Y. and Mensa-Wilmot K. (1996) Phosphatidylinositol phospholipase C is activated allosterically by the aminoglycoside G418: 2-deoxy-2-fluoro-scyllo-inositol-1-O-dodecylphosphonate and its analogs inhibit glycosylphosphatidylinositol phospholipase C. J. Biol. Chem. **271:** 15468–15477
14 Kung M., Stadelmann B., Brodbeck U. and Butikofer P. (1997) Addition of G418 and other aminoglycoside antibiotics to

mammalian cells results in the release of GPI-anchored proteins. FEBS Lett. **409:** 333–338
15 Allen L. A. and Raetz C. R. (1992) Partial phenotypic suppression of a peroxisome-deficient animal cell mutant treated with aminoglycoside G418. J. Biol. Chem. **267:** 13191–13199
16 Billaut-Mulot O., Fernandez-Gomez R., Loyens M. and Ouaissi A. (1996) *Trypanosoma cruzi* elongation factor 1-alpha: nuclear localization in parasites undergoing apoptosis. Gene **174:** 19–26
17 Gong J., Traganos F. and Darzynkiewicz Z. (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal. Biochem. **218:** 314–319
18 Sberna G., Saez-Valero J., Beyreuther K., Masters C. L. and Small D. H. (1997) The amyloid beta-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. J. Neurochem. **69:** 1177–1184
19 Fournel S., Genestier L., Rouault J. P., Lizard G., Flacher M., Assossou O. et al. (1995) Apoptosis without decrease of cell DNA content. FEBS Lett. **367:** 188–192
20 Ferri K. F. and Kroemer G. (2001) Organelle-specific initiation of cell death pathways. Nat. Cell Biol. **3:** E255–E263
21 Elce J. S., Hegadorn C. and Arthur J. S. (1997) Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. J. Biol. Chem. **272:** 11268–11275
22 Nakagawa T. and Yuan J. (2000) Cross-talk between two cysteine protease families: activation of caspase-12 by calpain in apoptosis. J. Cell Biol. **150:** 887–894
23 Rao R. V., Hermel E., Castro-Obregon S., Rio G. del, Ellerby L. M., Ellerby H. M. et al. (2001) Coupling endoplasmic reticulum stress to the cell death program: mechanism of caspase activation. J. Biol. Chem. **276:** 33869–33874
24 Chen G., Branton P. E. and Shore G. C. (1995) Induction of p53-independent apoptosis by hygromycin B: suppression by Bcl-2 and adenovirus E1B 19-kDa protein. Exp. Cell Res. **221:** 55–59
25 Carney D. H., Scott D. L., Gordon E. A. and LaBelle E. F. (1985) Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell **42:** 479–488
26 Zhuang S., Demirs J. T. and Kochevar I. E. (2001) Protein kinase C inhibits singlet oxygen-induced apoptosis by decreasing caspase-8 activation. Oncogene **20:** 6764–6776
27 Gomez-Angelats M., Bortner C. D. and Cidlowski J. A. (2000) Protein kinase C (PKC) inhibits fas receptor-induced apoptosis through modulation of the loss of K^+ and cell shrinkage: a role for PKC upstream of caspases. J. Biol. Chem. **275:** 19609–19619
28 Vander Heiden M. G., Chandel N. S., Williamson E. K., Schumacker P. T. and Thompson C. B. (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell **91:** 627–637
29 Basanez G., Nechushtan A., Drozhinin O., Chanturiya A., Choe E., Tutt S. et al. (1999) Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. USA **96:** 5492–5497
30 Heiskanen K. M., Bhat M. B., Wang H. W., Ma J. and Nieminen A. L. (1999) Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. J. Biol. Chem. **274:** 5654–5658
31 Marchetti P., Castedo M., Susin S. A., Zamzami N., Hirsch T., Macho A. et al. (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. J. Exp. Med. **184:** 1155–1160
32 Vander Heiden M. G., Chandel N. S., Schumacker P. T. and Thompson C. B. (1999) Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell **3:** 159–167
33 Liu X., Kim C. N., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:** 147–157
34 Kluck R. M., Martin S. J., Hoffman B. M., Zhou J. S., Green D. R. and Newmeyer D. D. (1997) Cytochrome c activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. EMBO J. **16:** 4639–4649
35 Li L. Y., Luo X. and Wang X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature **412:** 95–99
36 Lorenzo H. K., Susin S. A., Penninger J. and Kroemer G. (1999) Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ. **6:** 516–524
37 Loo G. van, Schotte P., Gurp M. van, Demol H., Hoorelbeke B., Gevaert K. et al. (2001) Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ. **8:** 1136–1142
38 Goldstein J. C., Waterhouse N. J., Juin P., Evan G. I. and Green D. R. (2000) The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat. Cell Biol. **2:** 156–162
39 Wolf B. B. and Green D. R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. J. Biol. Chem. **274:** 20049–20052
40 Ruiz-Vela A., Gonzalez de Buitrago G. and Martinez A. C. (1999) Implication of calpain in caspase activation during B cell clonal deletion. EMBO J. **18:** 4988–4998
41 Eustice D. C. and Wilhelm J. M. (1984) Fidelity of the eukaryotic codon-anticodon interaction: interference by aminoglycoside antibodies. Biochemistry **23:** 1462–1467
42 Buchanan J. H., Stevens A. and Sidhu J. (1987) Aminoglycoside antibiotic treatment of human fibroblasts: intracellular accumulation, molecular changes and the loss of ribosomal accuracy. Eur. J. Cell Biol. **43:** 141–147
43 Hetz C., Russelakis-Carneiro M., Maundrell K., Castilla J. and Soto C. (2003) Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. EMBO J. **22:** 5435–5445
44 Wood D. E. and Newcomb E. W. (2000) Cleavage of Bax enhances its cell death function. Exp. Cell Res. **256:** 375–382
45 Chen M., He H., Zhan S., Krajewski S., Reed J. C. and Gottlieb R. A. (2001) Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. J. Biol. Chem. **276:** 30724–30728
46 Orrenius S., Zhivotovsky B. and Nicotera P. (2003) Regulation of cell death: the calcium-apoptosis link. Nat. Rev. Mol. Cell Biol. **4:** 552–565
47 Yang T., Kozopas K. M. and Craig R. W. (1995) The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. J. Cell Biol. **128:** 1173-1184
48 Jimbo A., Fujita E., Kouroku Y., Ohnishi J., Inohara N., Kuida K. et al. (2003) ER stress induces caspase-8 activation, stimulating cytochrome c release and caspase-9 activation. Exp. Cell Res. **283:** 156–166
49. Fischer H., Koenig U., Eckhart L. and Tschachler E. (2002) Human caspase 12 has acquired deleterious mutations. Biochem. Biophys. Res. Commun. **293:** 722–726
50 Bursch W. (2001) The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ. **8:** 569–581
51 Hishita T., Tada-Oikawa S., Tohyama K., Miura Y., Nishihara T., Tohyama Y. et al. (2001) Caspase-3 activation by lysosomal enzymes in cytochrome c-independent apoptosis in myelodysplastic syndrome-derived cell line P39. Cancer Res. **61:** 2878–2884
52 Mingeot-Leclercq M. P., Laurent G. and Tulkens P. M. (1988) Biochemical mechanism of aminoglycoside-induced inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases. Biochem. Pharmacol. **37:** 591–599