p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2

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Downstream mediators of p53 in apoptosis induction remain to be elucidated. We report that p53-induced apoptosis occurred in the absence of cytochrome c release into the cytosol. Although Bax was upregulated, it remained largely in the cytosol and there was no detectable translocation to the mitochondria. Bid was not activated as no cleavage could be detected. Thus, the absence of cytochrome c release may be due to the lack of Bax translocation to mitochondria and/or Bid inactivation. Nevertheless, p53-induced apoptosis is still caspase dependent because it could be abolished by z-V-AD-fmk. To search for alternative downstream targets of p53, we detected production of reactive oxygen species (ROS) as well as mitochondrial membrane potential (Δψ). p53 induced ROS generation, which then caused a transient increase of Δψ followed by a decrease. Antioxidants could inhibit the alterations of Δψ, thereby preventing apoptosis. z-VAD-fmk was unable to abrogate Δψ elevation but inhibited Δψ decrease, indicating that Δψ elevation and its decrease are two independent events. Bcl-2 may abolish elevation as well as decrease of Δψ without interfering with ROS levels. Thus, the ROS-mediated disruption of Δψ constitutes a pivotal step in the apoptotic pathway of p53, and this pathway does not involve cytochrome c release.

Keywords: apoptosis/cytochrome c/mitochondrial membrane potential/p53/reactive oxygen species

Introduction

p53 is a transcription factor that can not only induce growth arrest through transactivation of the p21Cip1 gene, but can also trigger cell death via apoptosis in certain cell types (Levine, 1997; Agarwal et al., 1998). There are several lines of evidence demonstrating the mechanisms by which p53 induces apoptosis. p53 may use transcriptional activation to regulate the gene products of the pro-apoptotic protein Bax (Miyashita and Reed, 1995) or insulin-like growth factor-binding protein-3 (Buckbinder et al., 1995). However, p53-induced apoptosis does not necessarily require transcriptional activation, because p53-mediated apoptosis initiated by DNA damage occurs in the presence of actinomycin D or cycloheximide, which block RNA or protein synthesis (Caelles et al., 1994).

Inhibition of translation by cycloheximide could not block apoptosis induced by a temperature-sensitive p53 (Wagner et al., 1994). Furthermore, inhibitors of protein phosphatases induce p53-dependent apoptosis in the absence of transactivation (Yan et al., 1997). In addition, the introduction of a p53 cDNA fragment that encodes amino acid residues 1–214, and that does not bind to DNA or act as a transcription factor, is able to induce apoptosis (Haupt et al., 1995). In general, how p53 implements the apoptotic programme remains to be explored in further studies.

Recent studies show that cytochrome c participates in activating the programme of cell death. Addition of exogenous cytochrome c to cytosol can trigger apoptotic programmes in a cell-free apoptosis system (Liu et al., 1996; Kluck et al., 1997a), and microinjection of cytochrome c to cytosol also results in induction of apoptosis (F.Li et al., 1997). Cytochrome c may bind to apoptotic protease activating factor-1 (Apa-f-1), which then activates caspase-9 (P.Li et al., 1997). The cytochrome c–required caspase activation pathway is found to be necessary for diverse stimuli to trigger apoptosis and is unlikely to be cell type dependent. Stauroporine and etoposide led to cytochrome c release and caspase-3 activation during induction of apoptosis in HL-60 cells (Yang et al., 1997). Irradiation induces release of cytochrome c in human U-937 cells and in human multiple myeloma cells (Chauhan et al., 1997; Kharbanda et al., 1997). Tumour necrosis factor α induces cytochrome c redistribution in 293 cells (Duckett et al., 1998). It is of note that Bax is able to induce cytochrome c release (Jurgensmeier et al., 1998; Rosse et al., 1998). Since Bax has been shown to be a downstream mediator of p53, it is reasonable to hypothesize that cytochrome c may be involved in p53-induced apoptosis. However, it is not yet clear whether cytochrome c release is a prerequisite for the induction of apoptosis by p53.

Besides releasing cytochrome c, mitochondria may participate in apoptosis by opening their membrane permeability transition pores (MPT), an event that is an early step in the signalling cascade of apoptosis (Green and Reed, 1998). Little information exists about whether p53 needs to trigger MPT in order to induce apoptosis.

Bcl-2 and Bcl-XL may regulate apoptosis by controlling cytochrome c release. For example, release of cytochrome c in cells undergoing apoptosis could be prevented by overexpression of Bcl-2 or Bcl-XL, thereby abrogating the activation of caspase-3 (Kharbanda et al., 1997; Kluck et al., 1997b; Yang et al., 1997). It has been proposed that Bcl-2 functions as an antioxidant to prevent apoptosis. It may decrease lipid peroxidation and increase the cell resistance to reactive oxygen species (ROS) (Hockenbery et al., 1993; Kane et al., 1993). On the other hand, Bcl-2 may block ROS production by regulating the opening of MPT (Marzo et al., 1998; Zamzami et al., 1998). However,
an opposing proposal indicates that Bel-2 can protect cells from death independently of ROS, because cell death can be rescued by Bel-2 effectively even in the absence of ROS (Shimizu et al., 1995). It remains enigmatic whether Bel-2 prevents p53-induced apoptosis by controlling cytochrome c release or by interfering with ROS production.

The aim of our present study was to investigate whether p53-induced apoptosis requires cytochrome c release and to assess how Bel-2 can interfere with p53-induced apoptosis.

Results

p53 induces apoptosis independently of cytochrome c release

p53 was overexpressed in HeLa cells, which have been found to contain very low levels of wild-type p53 (Matlashewski et al., 1986). As shown in Figure 1A, p53 was nearly undetectable in adenovirus β-galactosidase (Adβ-gal)-infected HeLa cells, whereas a time-dependent increase of p53 protein levels could be detected in HeLa cells infected with adenovirus p53 (Adp53). Less than 10% of cells were dead in cultures infected with Adβ-gal. However, a time-dependent increase of cell death could be observed in cells infected with Adp53 (Figure 1B). A cell death ELISA was used to assess whether p53-induced cell death occurred by apoptosis in our present study. As depicted in Figure 1C, DNA fragments were noticeably increased in cells infected with Adp53, but not in those infected with Adβ-gal. In order to estimate the significance of p53-induced apoptosis in our model, we used 1.0 mM hydrogen peroxide, which is known to be a potent pro-apoptotic stimulus (Hampton et al., 1998). Treatment with 1.0 mM hydrogen peroxide resulted in a significant increase of DNA fragmentation (Figure 1C). The amount of DNA fragments is in accordance with the percentage of dead cells. Annexin V was next used to assess apoptosis. p53 induces a time-dependent increase in Annexin V-positive cells (Figure 1D). In addition, Adp53-infected cells demonstrated shrinkage and membrane blebbing (Figure 1E). Taken together, these data suggest that overexpression of p53 induces apoptosis in HeLa cells.

In order to detect whether cytochrome c release is involved in p53-induced apoptosis, we examined the distribution of cytochrome c after Adp53 infection. Subcellular fractions including either cytosol or mitochondria-enriched heavy membranes (HM) were prepared, and cytochrome c protein levels were measured by immunoblotting (Figure 2). The majority of cytochrome c remained in the mitochondria-enriched HM and there was no increase of cytochrome c levels in the cytosolic fractions after Adp53 infection.

p53-induced apoptosis does not involve the subcellular translocation of Bax and cleavage of Bid

To explore the reasons for the lack of cytochrome c release in p53-induced apoptosis, we studied factors known to induce cytochrome c release in HeLa cells overexpressing p53. p53 is known to transactivate Bax and therefore it is believed that Bax plays an important role in p53-induced apoptosis. Immunoblot analysis demonstrates that there was a time-dependent increase of Bax protein levels after Adp53 infection (Figure 3A). However, upregulated Bax in Adp53-infected cells remained largely in the cytosolic fraction and there was no detectable translocation to the mitochondria-enriched HM (Figure 3B).

In order to exclude a lack of pro-apoptotic function of Bax in HeLa cells, we transiently overexpressed Bax in these cells. Overexpression of Bax induced cell death in HeLa cells, which was accompanied by a time-dependent release of cytochrome c (data not shown). In contrast to cells overexpressing p53, overexpression of Bax itself resulted in an increase of Bax protein not only in the cytosol but also in the mitochondria-enriched HM (Figure 3C).

Bid, a pro-apoptotic factor causing cytochrome c release, is known to be activated by its cleavage. As shown by immunoblotting (Figure 3D), Bid was not cleaved in p53-overexpressing cells. In conclusion, these results suggest that the absence of cytochrome c release is probably due to the lack of subcellular translocation of p53-upregulated Bax and/or the inactivation of Bid.

p53-induced apoptosis depends on caspases

Released cytochrome c has been characterized as binding to Apaf-1 and then activating caspase-9 (P.Li et al., 1997). It would be of interest to detect whether caspases are still required in p53-induced apoptosis that is devoid of cytochrome c release (Figure 2). z-VAD-fmk (benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone) was used to treat the cells after Adp53 infection. z-VAD-fmk could prevent cell death in a dose-dependent manner with 100 μM z-VAD-fmk almost completely blocking cell death (Figure 4A). To assess the potential involvement of caspase-3, activation of caspase-3 was evaluated by detecting its cleavage by immunoblotting. There was no apparent cleavage of caspase-3 proenzyme and no detectable active p17 in Adp53-infected cells (Figure 4B). However, we did observe the existence of caspase-3-like activity in p53-infected cells by assaying DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) cleavage (Figure 4C).

The activation of downstream caspases can also be initiated through activation of caspase-8. We therefore detected caspase-8 activity by assessing its ability to cleave IETD-pNA (Ile-Glu-Thr-Asp-p-nitroanilide). There was no significant caspase-8 activity in p53-infected cells (data not shown). We detected the expression of Fas after Adp53 infection because Fas is able to activate caspase-8 (Li et al., 1998). Fas expression levels remained unaltered in Adp53-infected cells as detected by immunoblotting (data not shown). In addition, the binding of Fas to FADD (Fas-associated death domain protein), as determined by immunoprecipitation, was found to be unchanged (data not shown). Taken together, these data indicate that caspases other than caspase-8 participate in p53-induced apoptosis.

p53 regulates Δψ through reactive oxygen species

The lack of a role for cytochrome c implied the search for alternative downstream targets that are causally involved in p53-induced apoptosis. In the following experiments we investigated the role of ROS and Δψ in this context. We detected intracellular ROS production in HeLa cells overexpressing p53 by staining these cells with the ROS-
p53 regulates mitochondrial membrane potential

sensitive fluorescent dyes 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (HE), respectively. p53 overexpression led to a sharp and transient increase in the intracellular concentration of superoxide peaking at 8 h, as assessed by HE, which was followed by a decrease in the intracellular concentration. Adβ-gal did not induce significant alterations of superoxide concentrations (Figure 5A). In p53-overexpressing cells, hydrogen peroxide, as detected by DCFH-DA, was noticeably elevated at 16 h and reached a peak at 24 h. Adβ-gal led to no significant alterations of hydrogen peroxide concentrations (Figure 5B). Thus, it seems that the peak in superoxide generation precedes that of hydrogen peroxide generation, although the specificity of DCFH-DA for hydrogen peroxide and that of HE for superoxide is limited. To test whether augmented ROS levels play a role in mediating the death signal of p53, N-acetylcysteine (NAC) and 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), two antioxidants, were used to treat the cells immediately after Adp53 infection. Both NAC and tiron showed a protective
Fig. 2. Overexpression of p53 does not induce cytochrome c release. 
Cytochrome c distribution was detected by immunoblotting. Cells 
were harvested at the time indicated and then subcellular fractions 
including either cytosol or mitochondria-enriched HM were prepared. 
A representative result of six independent experiments is shown.

Fig. 3. p53 induces upregulation of Bax. 
(A) Upregulation of Bax by p53. Cells were infected with Adp53 and harvested 
at the time indicated for the detection of Bax protein levels by immunoblotting. 
(B) Determination of Bax distribution. Subcellular fractions including 
either cytosol or mitochondria-enriched HM were prepared at the 
times indicated. Bax protein levels were determined by immunoblotting. 
Representative results of three independent experiments are shown. 
(C) Overexpression of Bax induces Bax translocation. HeLa cells were transfected with 
pADBax and subcellular fractions including either cytosol or mitochondria-enriched 
HM were prepared at the times indicated. Bax was detected by immunoblotting. 
Representative results of three independent experiments are shown. 
(D) Determination of Bid by immunoblotting.

effect against p53-induced apoptosis in a dose-dependent 
manner (Figure 5C). These data suggest that the generation 
of ROS is essential for the induction of apoptosis by p53.

We next detected whether p53-induced apoptosis involves 
alterations of \( \Delta \psi \). As shown in Figure 6A, Ad\( \beta \)-
gal infection led to no significant change in \( \Delta \psi \) detected 
by 3,3'-dihexyloxacarbocyanine iodide [DiOC\( _{6}(3) \)]. How-
ever, \( \Delta \psi \) began to decrease 24 h after Adp53 infection. 
Interestingly, there was a transient increase of \( \Delta \psi \) 12–
24 h after Adp53 infection, preceding the reduction of \( \Delta \psi \). 
Similar results were obtained using tetramethylrhodamine 
ethyl ester (TMRE) to assess \( \Delta \psi \) (data not shown). 
Administration of carbonyl cyanide \( m \)-chlorophenylhydra-
zone (CCCP), a protonophore that dissipates \( \Delta \psi \), led to 
a significant decrease of \( \Delta \psi \) either in control cells without 
adenvirus infection or in cells 16 h after Adp53 infection 
(Figure 6B), indicating that DiOC\( _{6}(3) \) is predominantly 
located in mitochondria and that the uptake of DiOC\( _{6}(3) \) really 
reflects \( \Delta \psi \). To determine whether increased or 
decreased uptake of DiOC\( _{6}(3) \) represents proliferation 
of mitochondria or originates from a difference in the amount 
of mitochondria within cells, the cellular content of 
cytochrome c was detected by immunoblotting using
p53 regulates mitochondrial membrane potential

**Fig. 5.** p53 induces the generation of ROS. ROS were detected with HE (A) or DCFH-DA (B). The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. (C) Effects of antioxidants on cell death induced by p53. Cell death was determined by Trypan Blue exclusion 48 h after Adp53 infection. Adβ-gal infection served as a control. Cells were treated with tiron or NAC immediately after Adp53 infection. Data are expressed as the mean ± SEM of four independent experiments.

whole-cell lysates. As shown in Figure 6C, cytochrome c levels remained constant after Adp53 infection, indicating that the increased or decreased uptake of DiOC₆(3) is due neither to mitochondrial proliferation nor to an unequal amount of mitochondria.

To determine the relationship between ROS and Δψ in the apoptotic cascade of p53, we used antioxidants to test whether they could influence p53-induced alterations of Δψ. The results show that 1 mM tiron or 50 mM NAC, respectively, could inhibit the increase as well as decrease of Δψ (Figure 6A), suggesting that ROS are responsible for the alterations of Δψ.

**Fig. 6.** p53 induces alterations of Δψ that can be abrogated by antioxidants. (A) Analysis of Δψ by DiOC₆(3) at the time indicated after Adβ-gal or Adp53 infection; 1 mM tiron or 50 mM NAC were added immediately after Adp53 infection. The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. Data are expressed as the mean ± SEM of three independent experiments. (B) Effect of CCCP on Δψ. Cells were harvested 16 h after Adp53 infection, then Δψ was detected with DiOC₆(3) in the absence or presence of CCCP. The control cells were treated under the same conditions without Adp53 infection. (C) Immunoblotting detection of cytochrome c levels using whole-cell lysates of cells infected with Adp53. A representative result of three independent experiments is shown.

Caspase activation is downstream of ROS production and Δψ elevation but upstream of Δψ decrease

The protective effect of z-VAD-fmk against p53-induced apoptosis led us to test the relationship between caspase activation and ROS production as well as Δψ alterations. z-VAD-fmk at 100 μM, a dose at which cell death could be abolished almost completely (Figure 4A), could prevent neither the production of ROS as detected by HE or DCFH-DA (Figure 7A) nor the increase of Δψ (Figure 7B). However, z-VAD-fmk at 100 μM could abolish the decrease of Δψ (Figure 7C). Also, DNA fragmentation is abrogated completely by 50–100 μM z-VAD-fmk.
P.-F. Li, R. Dietz and R. von Harsdorf

Fig. 7. Effect of z-VAD-fmk on ROS generation and Δψ alterations. (A) z-VAD-fmk does not prevent ROS production. ROS were assessed 8 h (HE) or 20 h (DCFH-DA) after Adβ-gal and Adp53 infection. z-VAD-fmk was at 100 μM. (B) z-VAD-fmk does not influence Δψ elevation detected by DiOC₆(3) 20 h after Adp53 infection. z-VAD-fmk was at 100 μM. (C) z-VAD-fmk abolishes the decreases in Δψ detected by DiOC₆(3) 48 h after Adp53 infection. z-VAD-fmk was at 100 μM. (D) z-VAD-fmk inhibits DNA fragmentation detected by cell death ELISA. Data are expressed as the mean ± SEM of two independent experiments. Each figure in (A), (B) and (C) is a representative result of three independent experiments.

(Figure 7D). These data suggest that caspases play a role downstream of ROS production and Δψ elevations, and are involved in the decrease of Δψ.

Bcl-2 maintains Δψ after ROS generation

To elucidate the effect of Bcl-2 on p53-induced generation of ROS and alterations of Δψ, we used HeLa-Bcl-2 cells infected with Adp53. As shown in Figure 8A, Adp53-infected HeLa-Bcl-2 cells underwent apoptosis significantly less than Adp53-infected HeLa-neo cells, suggesting that Bcl-2 could attenuate p53-induced apoptosis. Adβ-gal did not induce significant alterations of Δψ in HeLa-neo cells. Overexpression of p53 in HeLa-neo cells resulted in an increase of Δψ 12–24 h after Adp53 infection and a subsequent decrease of Δψ. However, in HeLa-Bcl-2 cells, p53 was unable to cause the elevation and subsequent decrease of Δψ (Figure 8B), suggesting that Bcl-2 may maintain Δψ. In addition, in Adp53-infected HeLa-Bcl-2 cells, CCCP could decrease the fluorescence levels of DiOC₆(3) significantly, whereas the cellular content of cytochrome c remained unchanged (data not shown). It is thus suggested that the uptake of DiOC₆(3) represents Δψ and that the stability of Δψ in HeLa-Bcl-2 cells is not due to the unequal amount of mitochondria.

Finally, we determined whether Bcl-2 regulates Δψ by influencing ROS levels. ROS levels were detected in Adp53-infected and Adβ-gal-infected HeLa-Bcl-2 cells. Adβ-gal infection could not change ethidium bromide (EB) and DCF fluorescence significantly in HeLa-Bcl-2 cells. In contrast, both EB and DCF signals were increased in Adp53-infected HeLa-Bcl-2 cells (Figure 8C and D), and the fluorescence signal was comparable to that obtained in Adp53-infected HeLa cells without Bcl-2 overexpression (Figure 5A and B). These data reveal that Bcl-2 prevents p53-induced alterations of Δψ downstream of ROS.

Discussion

Our data provide evidence that the regulation of Δψ through the generation of ROS constitutes an important pathway of p53-induced apoptosis. Importantly, this pathway involves neither the subcellular translocation of Bax, which is upregulated by p53, nor the activation of Bid or the release of cytochrome c. Therefore, these results provide a model for cytochrome c-independent induction of apoptosis mediated by p53. Despite the absence of cytochrome c release, caspases are still activated in p53-induced apoptosis and they function downstream of ROS generation and Δψ elevation. Moreover, our observation that Bcl-2 is able to antagonize p53-induced disruption of Δψ downstream of ROS provides a new insight into the anti-apoptotic action of Bcl-2.

Although p53 could upregulate Bax expression, the release of cytochrome c as a consequence of Bax activation could not be detected. In contrast, overexpression of Bax itself resulted in the translocation of cytochrome c from mitochondria to the cytosol. Thus, p53 and Bax may initiate apoptosis using distinct pathways in which cytochrome c is released. However, our observations suggest that Bax may initiate apoptosis through an intrinsic pore-forming activity because it forms channels in synthetic membranes (Antonsson et al., 1997). Such a property of Bax has been shown to regulate the release of cytochrome c. Direct evidence is obtained from an in vitro study where addition...
p53 regulates mitochondrial membrane potential

Fig. 8. Effects of Bcl-2 on p53-induced alterations of Δψ and generation of ROS. (A) Bcl-2 prevents p53-induced apoptosis. HeLa-neo cells or HeLa-Bcl-2 cells were infected with Adp53 and cell death was assessed by Trypan Blue exclusion at the indicated time. (B) Bcl-2 attenuates p53-induced alterations of Δψ. Δψ was detected by DiOC₆(3) at the time indicated after Adβ-gal or Adp53 infection. (C and D) Bcl-2 does not prevent p53-induced generation of ROS detected by HE (C) or DCFH-DA (D). The results are expressed as a percentage of the fluorescence intensity compared with uninfected control cells. Data are expressed as the mean ± SEM of three independent experiments.

of recombinant Bax protein to isolated mitochondria can induce cytochrome c release (Jurgensmeier et al., 1998). An in vivo study also indicated that overexpression of Bax results in cytochrome c release (Rosse et al., 1998). In addition, Bax has been shown to be located in the cytosol rather than at mitochondrial membranes in cells under physiological conditions. Induction of apoptosis results in the translocation of Bax from the cytosol to mitochondrial membranes, which is a necessary step to trigger apoptosis (Hsu et al., 1997). Thus, the discrepancy between the effects of p53-dependent and -independent expression of Bax may reside in the failure of p53-induced Bax to translocate to the mitochondria, the reason for which remains to be determined. Nevertheless, our data provide an explanation for previous studies reporting Bax-independent apoptosis induced by p53 (Knudson et al., 1995; Brady et al., 1996).

In our study, p53-induced apoptosis involves the activation of caspases. However, the immunoblot analysis demonstrated that caspase-3 was not cleaved, although caspase-3-like activity could be detected. One explanation for such a discrepancy could be that other caspases carrying a catalytic domain similar to caspase-3 may contribute predominantly to the DEVD-pNA cleavage. Downstream caspases are activated not only by the cytochrome c–Apaf-1–caspase-9 pathway, but also by the Fas–caspase-8 pathway (Li et al., 1998; Luo et al., 1998). It appears that the Fas–caspase-8 pathway is absent from our model. Although it has been shown that p53 is able to induce the translocation of Fas from the cytosol to the cell surface, resulting in increases in Fas–FADD binding in vascular smooth muscle cells (Bennett et al., 1998), this is unlikely to occur in our case, since an increase of Fas–FADD binding could not be detected. Furthermore, p53 was unable to upregulate Fas expression in our hands. The lack of activation of caspase-8 and Bid further suggests the absence of a functional Fas pathway, since it would include both the activation of caspase-8 and cleavage of Bid (Li et al., 1998; Luo et al., 1998). It is not surprising that caspase-3-like activity is detectable in the absence of caspase-8 activation and cytochrome c release, because caspase-3 could be activated by other caspases such as caspase-6 (Xanthoudakis et al., 1999). Active caspase-3 or caspase-7 create an amplification loop activating upstream caspases (Sun et al., 1999; Xanthoudakis et al., 1999). Future studies should reveal which caspases are activated by p53.

To date, there is no direct evidence indicating that caspase activation requires ROS. Instead, caspases may regulate ROS generation. For example, inhibition of caspases can block ROS production (Krumn et al., 1998; Tan et al., 1998). On the other hand, caspases may function as negative regulators of ROS production because caspase activation can counteract ROS generation (Vercammen et al., 1998). Notably, ROS may even lead to the inactivation of caspases (Hampton and Orrenius, 1997). Our observation that z-VAD-fmk could not prevent ROS production indicates that ROS production is upstream of caspase activation in p53-induced apoptosis. This is consistent with previous work indicating that ROS production
is an early event, whereas the cleavage of poly(ADP-ribose) polymerase is a late event in p53-induced apoptosis (Polyak et al., 1997). Our recent work also reveals that ROS-induced apoptosis requires the activation of caspases and that the spectrum of activated caspases is ROS-type dependent (von Harsdorf et al., 1999).

Our data suggest the involvement of mitochondria in p53-induced apoptosis with Δψ being the target of p53. p53 induces a transient increase of Δψ followed by a decrease. Δψ is a component of the overall proton motive force that drives ATP production in mitochondria. Its elevation has been shown to have implications for a variety of pathophysiological conditions, in particular for apoptosis. Induction of apoptosis by alkali results in an elevated level of Δψ and overexpression of manganese superoxide dismutase restores Δψ, thereby preventing apoptosis (Majima et al., 1998). The elevation of Δψ has also been observed in apoptosis triggered by growth factor withdrawal and Bcl-XL was shown to prevent the increase of Δψ, thereby inhibiting apoptosis (Vander Heiden et al., 1997). Our results, which indicate that antioxidants prevent the increase of Δψ, suggest that ROS may be responsible for the elevation of Δψ. This is consistent with a recent report showing that superoxide is able to augment Δψ (Majima et al., 1998). The increase of Δψ is an early event in the apoptotic cascade and may be a sign of mitochondrial swelling and disruptions of the outer mitochondrial membrane (Vander Heiden et al., 1997). Most recently, the biochemical basis of the increase of Δψ in apoptosis has been demonstrated to be due to a failure to exchange ADP for ATP. Cells with a high Δψ are committed to undergoing apoptosis, whereas those with a low Δψ are capable of exiting the apoptotic pathway (Heiden et al., 1999).

Interestingly, z-VAD-fmk is unable to block the increase of Δψ, but it can abrogate the decrease of Δψ (Figure 7B and C). These data indicate that Δψ elevation and Δψ decrease are two independent events, or that they are taking place at two different stages of the apoptotic programme in which caspase activation is an intermediate step. Such a phenomenon is not restricted to p53-induced apoptosis, since most recently apoptotic induction by Fas was shown to involve a similar pattern of alterations of the mitochondrial membrane potential (Banki et al., 1999). Here, z-VAD-fmk only prevents the decrease of Δψ and not its elevation. Thus, p53-induced Δψ hyperpolarization is upstream of Δψ decrease. Our present work and that of others (Majima et al., 1998; Banki et al., 1999) shows that ROS are responsible for Δψ elevation. Superoxide generation reached a peak 8 h after Adp53 infection (Figure 5A), a time at which Δψ started to increase (Figure 6A). The protective effect of tiron against Δψ elevation suggests that superoxide triggers Δψ elevation. In the subsequent cascade, hydrogen peroxide served as a substitute for superoxide to keep Δψ at high levels (Figures 5B and 6A). The ROS production pattern is probably related to the biochemical property of each species with superoxide having a short lifespan but hydrogen peroxide having a long one.

Our results demonstrate for the first time that Bcl-2 may counteract p53-induced apoptosis by maintaining Δψ. Bcl-2 has been shown to elevate Δψ from low levels either by enhancing H+ efflux (Shimizu et al., 1998) or by preventing the opening of MPT (Zamzami et al., 1998). Here we found that Bcl-2 stabilizes Δψ by preventing both the increase and decrease of Δψ induced by p53. Since p53 is still able to induce the generation of ROS in Bcl-2-overexpressing cells, Bcl-2 appears to exert its stabilizing effect on Δψ downstream of ROS. This is consistent with previous observations indicating that the anti-apoptotic action of Bcl-2 does not involve the reduction of ROS levels (Satoh et al., 1996, 1997). The inability of Bcl-2 to block ROS production may be related to the sources of ROS production. Bcl-2 can prevent ROS production when it is induced by cytochrome c release (Cai and Jones, 1998), or by the opening of MPT (Zamzami et al., 1998), because Bcl-2 blocks cytochrome c release and MPT opening. Thus, Bcl-2 may function at multiple levels and it elicits its anti-apoptotic effect in a manner largely dependent on the stimuli and apoptotic pathways.

Our present work provides evidence that mitochondria participate in p53-induced apoptosis by the alteration of Δψ. However, the biochemical mechanisms behind the disruption of Δψ remain to be identified. In this regard, it would be of great interest to understand how ROS lead to the alteration of Δψ and by which means Bcl-2 interacts with this process. Furthermore, the interpretation of our results is limited to the specific model used, which is the overexpression of p53. Therefore, there is a great need for future studies employing different models of p53-dependent apoptosis in order to understand the signalling in p53-induced apoptosis fully.

Materials and methods

Materials

Cell death detection ELISA kit and G418 were purchased from Boehringer Mannheim. Polycyclonal anti-Bcl-2 antibody, polyclonal anti-Bax antibody, anti-caspase-3 polyclonal antibody and anti-cytochrome c monoclonal antibody were from Pharmingen. Anti-p53 monoclonal antibody and z-VAD-fmk were from Calbiochem. Anti-Fas and FADD antibodies were from Transduction Laboratories. Anti-Bid antibody was from Santa Cruz. Caspase-8 assay kit and Annexin V kit were from R&D System. Caspase-3 assay kit was from BioMol. HE and TMRE were from Molecular Probes Inc. CCCP, DiOC$_6$(3), DCFH-DA, NAC and tiron were from Sigma (St Louis, MO). The pADBax plasmid harbouring the human full-length Bax cDNA driven by the cytomegalovirus (CMV) promoter, the pEBBS7-425-Bcl-2 plasmid containing the human full-length Bcl-2 cDNA driven by the CMV promoter and the pCMVneo plasmid were kindly provided by Dr Karsten Brand, Max-Delbrück-Center, Berlin.

Cell culture

HeLa cells (HeLa 229, No. CCL-2.1, American Type Culture Collection, Rockville, MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cell death was determined by Trypan Blue exclusion. Cells were harvested by trypsinization and washed in phosphate-buffered saline (PBS). The numbers of Trypan Blue-positive and -negative cells were counted on a haemocytometer.

Adp53 infection

The adenoviral vector contains deletions of the E1A and E1B region, is dependent (von Harsdorf et al., 1999). The identical vector was used to create the adenoviral construct (Ad5-gal) (Sandig et al., 1997). Viruses were amplified in 293 cells and purified on a CsCl gradient. HeLa cells were infected at a multiplicity of infection (m.o.i.) of 50 for 90 min. After washing with PBS, culture medium was added and cells were cultured until the indicated time. For the administration of z-VAD-fmk, cells were treated.
immediately after Adp53 infection. After 24 h the inhibitor was added once more. For the treatments with antioxidants, NAC and tiron were added immediately after Adp53 infection.

Transfection with pADBax
Cells that had been passaged the previous day were transfected by the calcium phosphate precipitation method (Sambrook et al., 1989). Plasmid DNA (20 μg) was used for the transfection of 1 × 10⁶ cells. The calcium phosphate–DNA precipitates were left on the cells for 24 h. The control cells were transfected with a plasmid containing no Bax cDNA using the same method.

Establishment of HeLa cells stably overexpressing Bcl-2
Cells were co-transfected with pEBST-425-Bcl-2 and pCMVneo at a ratio of 10:1 by the method of calcium phosphate precipitation (Sambrook et al., 1989). Transfected cells were selected in medium containing 1 mg/ml G418 for 30 days and Bcl-2 expression was determined using immunoblotting analysis as described below. The cells stably expressing Bcl-2 are referred to as HeLa-Bcl-2 cells. The cells only transfected with pCMVneo served as a control and are referred to as HeLa-neo cells.

Analysis of DNA fragmentation
DNA fragmentation was detected by cell death detection ELISA, which was performed according to the manufacturer’s instructions and as described previously (P.F.Li et al., 1997).

Immunoblotting analysis
Cells were lysed for 1 h at 4°C in a lysis buffer [20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 10 μg/ml each of leupeptin, aprotinin and pepstatin A]. Samples containing 30 μg of protein were subjected to 12% SDS–PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Equal protein loading was controlled by Ponceau Red (Sigma) staining of membranes. Blots were probed using primary antibodies, followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG. Antigen–antibody complexes were visualized by enhanced chemiluminescence.

Preparation of subcellular fractions
Cells were washed twice with PBS and the pellet was suspended in 0.5 ml of buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml each of leupeptin, aprotinin and pepstatin A) containing 250 mM sucrose. The cells were homogenized by 10 strokes in a Dounce homogenizer. The homogenates were centrifuged twice at 750 g for 5 min at 4°C to collect nuclei and debris. The supernatants were centrifuged at 10 000 g for 15 min at 4°C to collect the HM pellet. The resulting supernatants were centrifuged at 100 000 × g for 1 h at 4°C to yield light membrane pellets (LM). The final supernatants are referred to as cytosolic fractions.

Flow cytometry analysis of cell death
Cells were labelled with Annexin V according to the manufacturer’s instructions. Samples (10⁵ events) were analysed with a flow cytometer (Coulter Epics) and the distribution of cells was determined using Multicycle Software (Coulter EPICS XL/XL-MCL System II).

Detection of caspase-3 and caspase-8 activity
Caspase-3 and caspase-8 activity were detected using assay kits. The detection of caspase-3 and caspase-8 activity in the absence of transcriptional activation of p53-target genes. The cells stably expressing Bcl-2 showed restored DNA damage-induced cell death in the absence of p53. EMBO J., 15, 1221–1230.


Analysis of DNA fragmentation
DNA fragmentation was detected by cell death detection ELISA, which was performed according to the manufacturer’s instructions and as described previously (P.F.Li et al., 1997).

References


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