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Apoptosis induced by microinjection of cytochrome *c* is caspase-dependent and is inhibited by Bcl-2

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Abstract

Microinjection of cytochrome *c* induced apoptosis in all the cell types we tested (IPC-81, Swiss 3T3, Clone 8 fibroblasts, NRK, H295, Y1, HEK 293). The apoptotic phenotype induced by injected cytochrome *c* was characterized by externalization of phosphatidyl serine, cell detachment from substratum and from neighbor cells, and had the classic ultrastructural features of membrane budding, chromatin condensation and cell shrinkage. Depending on the cell type and concentration of cytochrome *c*, the induction of apoptosis was remarkably rapid. The development of apoptosis was prevented by the caspase inhibitor Z-VAD.fmk. Four of the cell types (Clone 8, Swiss 3T3, NRK, Y1) were transfected with *bcl-2* and these all showed a markedly decreased sensitivity towards injected cytochrome *c*. Our data suggest that extramitochondrial cytochrome *c* is a general apoptogen in cells with a functioning caspase system. They also indicate that, in preventing apoptosis, Bcl-2 acts not only at the level of regulation of cytochrome *c* release from mitochondria, but can also interfere with caspase activation induced by cytochrome *c* microinjected directly into the cytoplasm.

Keywords: apoptosis; microinjection; cytochrome *c*; caspase; Bcl-2

Abbreviations: Apaf, apoptotic protease activating factor; TRITC, tetramethylrhodamine isothiocyanate; Z-VAD.fmk, Z-Val-Ala-DL-Asp-fluoromethylketone

Introduction

Apoptosis is a distinct form of cell suicide, controlled by an internally encoded death program. Apoptotic cells are characterized by specific biochemical and morphological changes, including the activation of a proteolytic cascade leading to the cleavage of selective cytoplasmic and nuclear proteins, chromatin and RNA degradation, cell shrinkage and blebbing of cell membranes accompanied by the appearance of phosphatidyl serine on the outer surface of the plasma membrane.

The biochemical machinery involved in the killing and degradation of the cell is constitutively expressed. During the last few years the 'main players' in the induction and execution cascade of reactions in apoptotic cells were identified as a family of aspartic acid-specific cysteine proteases, the caspases (Alnemri *et al*, 1996). Over-expression of any of these proteases leads to apoptotic cell death. Moreover, mice deficient in caspase-3 have a striking defect in the extensive apoptosis that occurs during brain development. Peptide inhibitors of caspases can prevent apoptosis both in isolated cells and in *in vivo* models of development. Caspases are synthesized in a precursor form, and an apoptotic signal then converts the pro-caspase into the mature enzyme (for review see Zhivotovsky *et al*, 1997). At least two pro-caspases, pro-caspase-8 and -1, are activated via oligomerization-induced autoproteolysis (Yang *et al*, 1998). In contrast, pro-caspase-3, the best characterized executioner caspase, does not initiate activation in an autocatalytic manner. Activation of this enzyme requires an initiating first step cleavage, probably by other pre-existing cellular caspases, followed by a second autoproteolytic step.

In a search for biochemical components involved in caspase cascade activation, three apoptotic protease activating factors (Apaf 1–3) were recently purified from a cell-free system based on cytosol from normal cells (Liu *et al*, 1996; Zou *et al*, 1997; Li P *et al*, 1997). One of these proteins (Apaf-2) was recognized as cytochrome *c*. Apaf-1 has a shared similarity with pro-domain of CED-3 and CED-4, and Apaf-3 was identified as a member of the caspase family, pro-caspase-9. The binding of Apaf-3 to Apaf-1 is dependent on the presence of cytochrome *c* and dATP or ATP. This event leads to pro-caspase-9 activation. Activated caspase-9 in turn cleaves and activates pro-caspase-3. Thus, cytochrome *c* plays a cofactor role in the activation of the proteolytic cascade. Although holocytochrome *c* is a mitochondrial protein, it has been demonstrated that following exposure of cells to apoptotic stimuli, cytochrome *c* is rapidly released from mitochondria into the cytosol (Yang *et al*, 1997; Kluck *et al*, 1997a; Kharbanda *et al*, 1997). Additional experiments showed that cytochrome *c* release in apoptotic cells can precede changes in mitochondrial membrane potential (Yang *et al*, 1997; Kluck *et al*, 1997a). It was further reported that anti-apoptotic proteins of the Bcl-2 family prevent this translocation of cytochrome *c* from mitochondria to cytosol and thereby interfere with the subsequent activation of cytosolic caspases and apoptosis.

It has recently been demonstrated that addition of exogenous cytochrome *c* to cytosolic extracts from normal

cells induces caspase activation (Liu *et al*, 1996). Cytosolic caspase activation was only observed with intact holocytochrome *c*. Neither heat-denatured cytochrome *c*, nor enzyme-degraded cytochrome *c* (microperoxidase-11, the heme group of cytochrome *c* with amino acids 11–21) were able to substitute for holocytochrome *c* (Kluck *et al*, 1997b; Hampton *et al*, 1998). Cytochrome P-450, cytochrome b_5, hemoglobin, biotinylated cytochrome *c* and apo-cytochrome *c* were also ineffective in this respect, showing the importance of the presence of unmodified cytochrome *c* in this reaction (Hampton *et al*., 1998).

Based on these observations, several important questions were addressed in the present study. First, could high cytosolic concentrations of exogenous cytochrome *c* induce apoptosis in most cells, making cytochrome *c* a plausible general apoptosis inducer? Second, is the time course of the apoptotic process in this case compatible with the development of apoptosis induced by diverse stimuli? Finally, what effect might overexpression of Bcl-2 cause in this situation? To address these questions we decided to microinject cytochrome *c* into different types of cells in an attempt to mimic the release of endogenous cytochrome *c* into the cytosol. Seven types of cells were injected: adrenocortical Y-1 tumor cells; human embryonal kidney HEK 293 cells; human adrenocortical tumor cell line H295; normal rat kidney (NRK) epithelial cells; mouse embryonal Swiss 3T3 fibroblasts; Clone 8 mouse embryonal fibroblasts; and rat promyelocytic IPC-81 leukemia cells. The type of cell death induced by microinjection was verified by co-staining with FITC-conjugated Annexin V and propidium iodide, as well as by electron microscopy. Four of the cell types were transfected with *bcl-2* and the effect of the Bcl-2 overexpression on the response to injected cytochrome *c* was investigated. Our results indicate that injected cytochrome *c* is able to induce apoptosis in various cell types and that this effect is caspase-dependent. Furthermore, in our experiments Bcl-2-overexpressing cells were protected from apoptosis induced by injected cytochrome *c*. A brief report of this study has recently appeared (Zhivotovsky *et al*, 1998).

Results and Discussion

Microinjected cytochrome *c* induces apoptosis in a number of cell types

To investigate if microinjection of cytochrome *c* is sufficient to kill cells and, if so, whether this is a general phenomenon, different types of cells were analyzed (various fibroblasts, leukemia cells, and epitheloid cells of normal and tumor origin).

Injected cytochrome *c* appeared to be able to kill all cell types tested (Figures 1–5, Table 1). The cytochrome *c* effect was specific since microinjection of the vehicle failed to induce cell death or any apparent changes in cellular morphology (shown for IPC-81 cells in Figure 1). Further, injection of biotinylated cytochrome *c* (shown for Clone 8 fibroblasts in Figure 2) or apo-cytochrome *c* (data not shown) induced no morphological signs of apoptosis. The inability to induce apoptosis by injection of modified cytochrome *c* into intact cells is in accordance with the recent observation that biotinylated cytochrome *c*, in contrast to native cytochrome *c*, does not activate caspases in cytosolic extract (Hampton *et al*, 1998).

The first indications of cytochrome *c*-induced cell death were budding and partial or complete detachment of cells from plastic. Cells were considered apoptotic when they showed clear budding (Figures 1–5C). The kinetics of apoptosis induction were characterized in all cell types by a lag period followed by a first order accumulation of apoptotic cells (shown for Y1 cells, Swiss 3T3 cells and NRK cells in Figures 3–5). The estimated intracellular concentration of cytochrome *c* in these experiments was 20 μM (Mellgren *et al*, 1993). A difference in lag phase (5–40 min) was related to the sensitivity of different cells to undergo apoptosis. The relatively short lag period between cytochrome *c* injection and morphological signs of apoptosis suggests that mitochondrial leakage of cytochrome *c* into the cytoplasm might also rapidly activate apoptosis in the intact cell. Thus, it appears likely that the control of cytochrome *c* release from mitochondria represents a critical step in the apoptotic process.

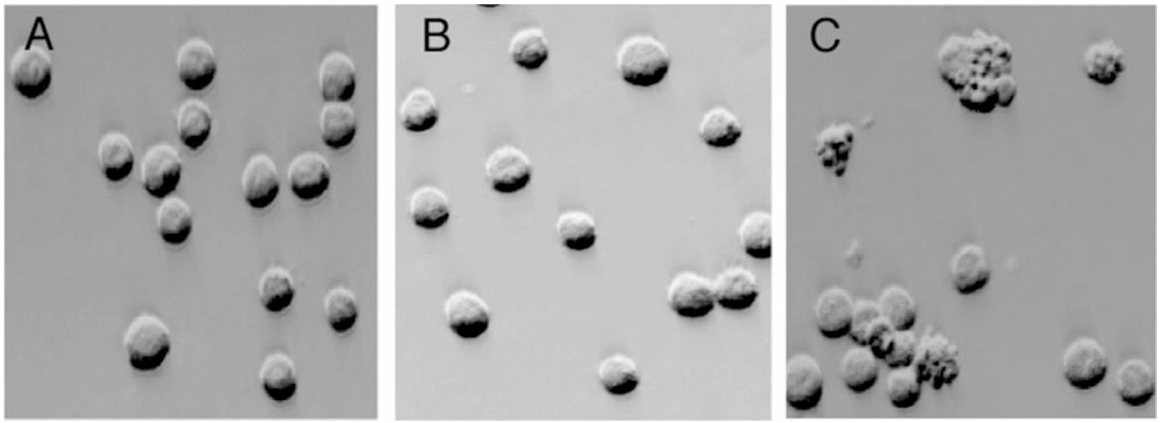

Figure 1 The appearance of IPC-81 cells 2 h after microinjection of cytochrome *c*. (**A**) Control (non-injected) cells. (**B**) Cells injected with water. (**C**) Cells injected with 20 μM cytochrome *c* dissolved in water. Magnification 300 ×

The first order rate constant ($K_{apop}=\ln 2/t_{1/2}$) of apoptosis induction in these experiments was 2.8/h for Y1, 1.4/h for Swiss 3T3 fibroblasts, and 0.54/h for NRK cells. One hour after injection, apoptotic morphology was seen in $79\pm4.0\%$ of Y1 cells, $70\pm8.3\%$ of Swiss 3T3 fibroblasts, $31\pm5.1\%$ of NRK cells, $22\pm1.3\%$ of IPC-81 cells, $36\pm3.0\%$ of H295 cells, $46\pm6\%$ of HEK 293 cells, and more than 99% of the Clone 8 cells. Thus, microinjection of cytochrome *c* was sufficient to kill all cell types tested.

To confirm the morphological characteristics of apoptosis, the injected cells were analyzed by transmission electron microscopy. The ultrastructure of cells injected with cytochrome *c* (Figure 6) was characterized by surface protrusions, some of which became pinched off to form apoptotic bodies while the plasmalemma remained intact. The cytoskeleton was dramatically altered, and in addition to gross morphological changes, clustering of actin filaments was observed (Figure 6B). The mitochondria

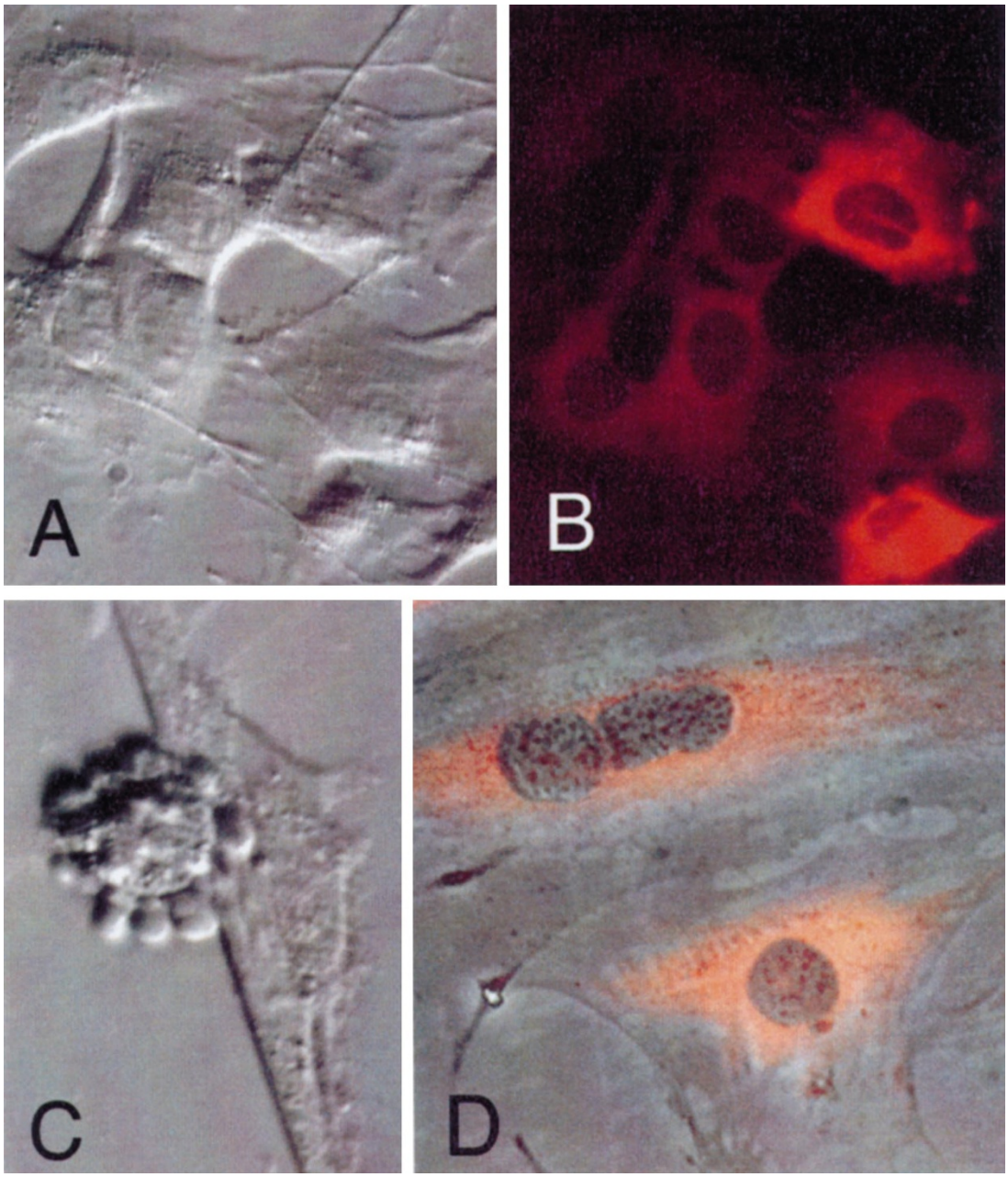

Figure 2 Comparison of Clone 8 cells injected with biotinylated and non-biotinylated cytochrome *c*, and of wild-type and *bcl-2* transfected Clone 8 cells injected with cytochrome *c*. (**A**) Wild-type cells injected with TRITC-dextran and 25 μM biotinylated cytochrome *c*. Magnification: 300 $\times$. (**B**) Fluorescent micrograph of the same field as in panel **A**. Magnification 300 $\times$. (**C**) Wild-type cells injected with TRITC-dextran and 2 μM cytochrome *c*. Magnification 500 $\times$. (**D**) *bcl-2* transfected Clone 8 cells injected with TRITC-dextran and 2 μM cytochrome *c* viewed under combined phase and fluorescent microscopy. Magnification 400 $\times$

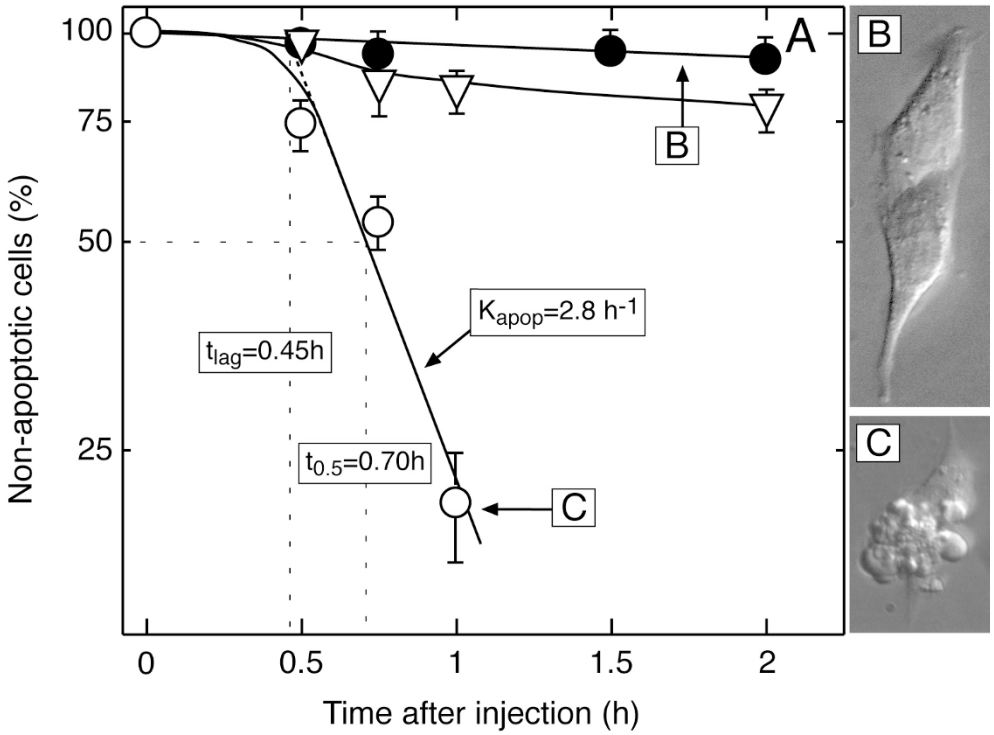

Figure 3 Time course of apoptosis development in wild-type and *bcl-2* transfected Y1 adrenocortical cells injected with cytochrome *c*. (**A**) Wild-type cells injected with 20 μM cytochrome *c* without (○) and with (●) preincubation with caspase inhibitor (20 μM Z-VAD.fmk), and *bcl-2* transfected cells injected with 20 μM cytochrome *c* (▽). Note the semilogarithmic nature of the graph and the insets showing the lag time (0.45 h) before the wild-type cell population started to undergo apoptosis after injection (see the Materials and Methods section for further details). (**B**) Wild-type Y1 cells preincubated with Z-VAD.fmk 105 min after injection of cytochrome *c*. Magnification: 400×. (**C**) Wild-type Y1 cells 1 h after injection with cytochrome *c*. Magnification 400×

were redistributed within the cytoplasm, presumably secondary to cytoskeletal alterations. Otherwise, the mitochondrial ultrastructure appeared normal. These structural changes were similar to those described in cells undergoing apoptosis in response to diverse stimuli (Gjertsen *et al*, 1994). In addition to electron microscopy, cells microinjected with cytochrome *c* were studied by co-staining of cells with FITC-conjugated Annexin V and propidium iodide. Cells dying in response to microinjection of cytochrome *c* became Annexin V-positive, demonstrating that microinjection of cytochrome *c* is also associated with phosphatidyl serine exposure on the cell surface. The plasma membrane remained intact, since the cells excluded propidium iodide and trypan blue (data not shown). In cells left for hours after injection, or in cells deliberately injected under supra-optimal pressure, necrosis was observed as evidenced by internalization of propidium iodide in addition to positive staining with Annexin V (data not shown).

Z-VAD.fmk inhibits cytochrome *c*-induced apoptosis

It has previously been shown that cytochrome *c* in cell-free extracts triggers caspase activation (Liu *et al*, 1996). To determine if apoptosis induced by microinjection of cytochrome *c* also involves the caspase-mediated pathway, cells were preincubated for 30–60 min with 20 μM of the caspase inhibitor Z-VAD.fmk. For all cell types except Clone 8, less than 13% of the pretreated cells became apoptotic and remained at the same level of apoptosis for more than 3 h after injection of 20 μM cytochrome *c* (Figures 3–5; Table 1). The Clone 8 cells were particularly sensitive to cytochrome *c* and were injected with significantly lower concentrations (2–10 μM) of this protein. Z-VAD.fmk was also effective in Clone 8 cells injected with these low concentrations of cytochrome *c* (Table 1). Thus, our results indicate that injected cytochrome *c* is able to induce caspase-dependent apoptosis. This is compatible with the proposed mechanism (Li P *et al*, 1997) that cytochrome *c* released from mitochondria can promote pro-caspase-9 processing and thereby the subsequent activation of the caspase cascade involved in the cellular execution machinery.

Bcl-2 overexpression prevents cell death after cytochrome *c* injection

In many experimental models overexpression of Bcl-2, or its close family member Bcl-x_L, protects cells from apoptosis (for review see Reed, 1997). It has been suggested that the role of Bcl-2 protection is linked to its mitochondrial location and the prevention of release of cytochrome *c* into the cytoplasm (Yang *et al*, 1997; Kluck

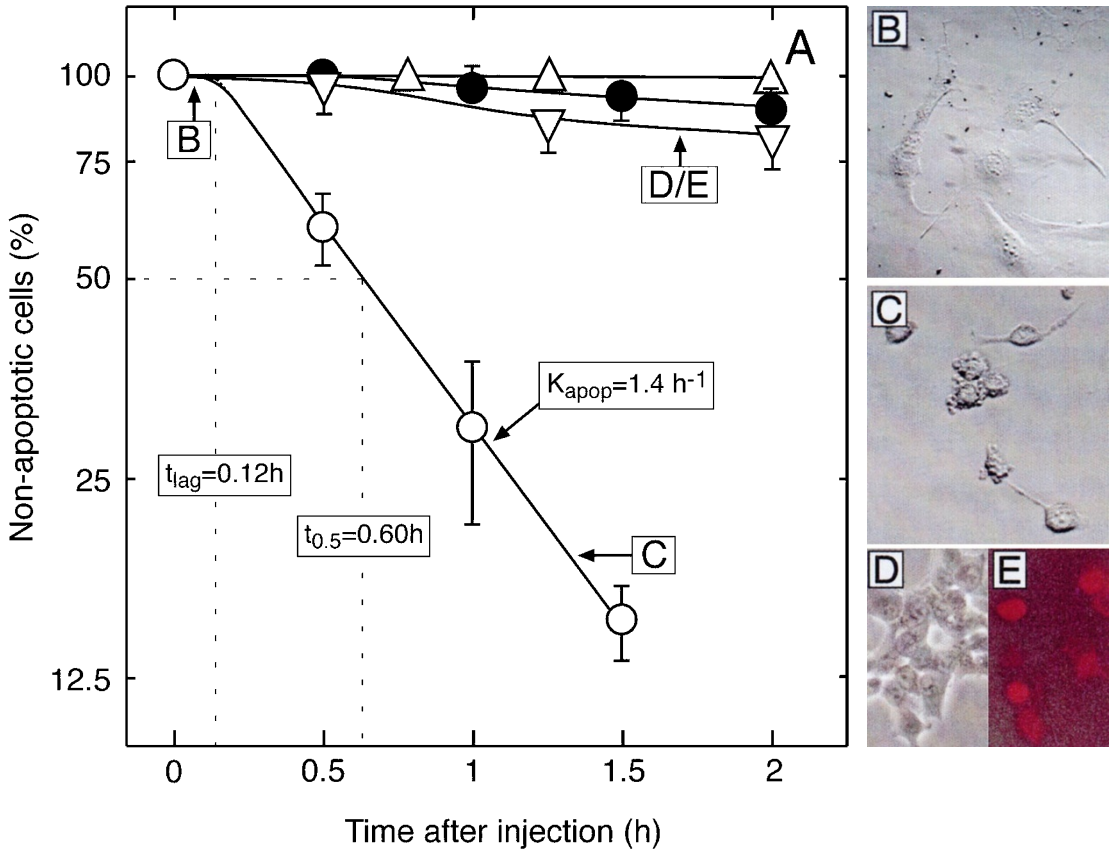

Figure 4 Time course of apoptosis development in wild-type and *bcl-2* transfected Swiss 3T3 cells injected with 20 μM cytochrome *c*. (**A**) Corresponds to that for Y1 cells in Figure 3, except that an additional experiment is shown in which the 3T3 cells were injected with 20 μM biotinylated cytochrome *c* (△). (**B**) Wild-type 3T3 cells about 5 min after injection of cytochrome *c*, i.e. before onset of apoptosis. (**C**) Wild-type 3T3 cells 90 min after injection of cytochrome *c*. (**D**) *bcl-2* transfected cells 2 h after injection of 20 μM cytochrome *c*. (**E**) Fluorescent micrograph of the same field as in **D** to demonstrate that all cells were successfully injected. Magnification of all photos: 200 ×

et al, 1997a). This idea was supported by experimental data obtained from a mammalian cell-free system and in whole cells treated with apoptosis-inducing agents (Yang *et al*, 1997). In these systems, Bcl-2 was not able to prevent the effect of cytochrome *c*, once it had been released from the mitochondria. We had therefore expected that microinjected cytochrome *c* should act equally well in triggering apoptosis whether cells over-expressed Bcl-2 or not. Surprisingly, in our experiments overexpression of Bcl-2 significantly delayed cell death even when cytochrome *c* was injected into the cytosol (Figures 3–5). Moreover, the cells did not undergo an incomplete or partial apoptosis as judged by their normal morphology after injection with cytochrome *c* (Figures 2D, 4D and E, 5B). To ascertain that the *bcl-2* transfected cells were adequately injected, cytochrome *c* was co-injected with TRITC-dextran and successfully injected cells were scored by fluorescence microscopy. The same percentage (60–99%) of successfully injected cells was achieved in wild-type and *bcl-2* transfected cells. Figures 2D and 4E show non-apoptotic *bcl-2* transfected cells co-injected with fluorescent TRITC-dextran and cytochrome *c*. Cells were fluorescent-positive and morphologically normal for more than 24 h after injection. It is important to note that the protective effect of Bcl-2 was dependent on the level of free cytoplasmic cytochrome *c* (Table 2). By increasing the concentration of cytochrome *c* progressively, the protection from death by overexpression of Bcl-2 could be overcome (data shown for Clone 8 cells in Table 2).

Several explanations can be offered for our unexpected observation. First, Bcl-2 and other Bcl-2-like proteins are located not only in the mitochondrial outer membrane but also in other organelles (Hockenbery *et al*, 1990) - hence the anti-apoptotic property might be due to an action outside the mitochondria. In fact, it has been shown that Bcl-2/Bcl-x_L and pro-caspase-8 are cooperatively associated with the Bap31 complex in the endoplasmic reticulum (Ng and Shore, 1998). Based on this observation, the authors suggested that 'this complex in the ER might cooperate with events in the mitochondria to control proximal and distal steps in a Bcl-2-regulated caspase cascade'. Second, Bcl-2 may also be a member of the 'apoptosome' complex and prevent cell death by directly binding to Apaf-1/Apaf-2/Apaf-3, or it may prevent the formation of an active Apaf 1-3 complex. This would be

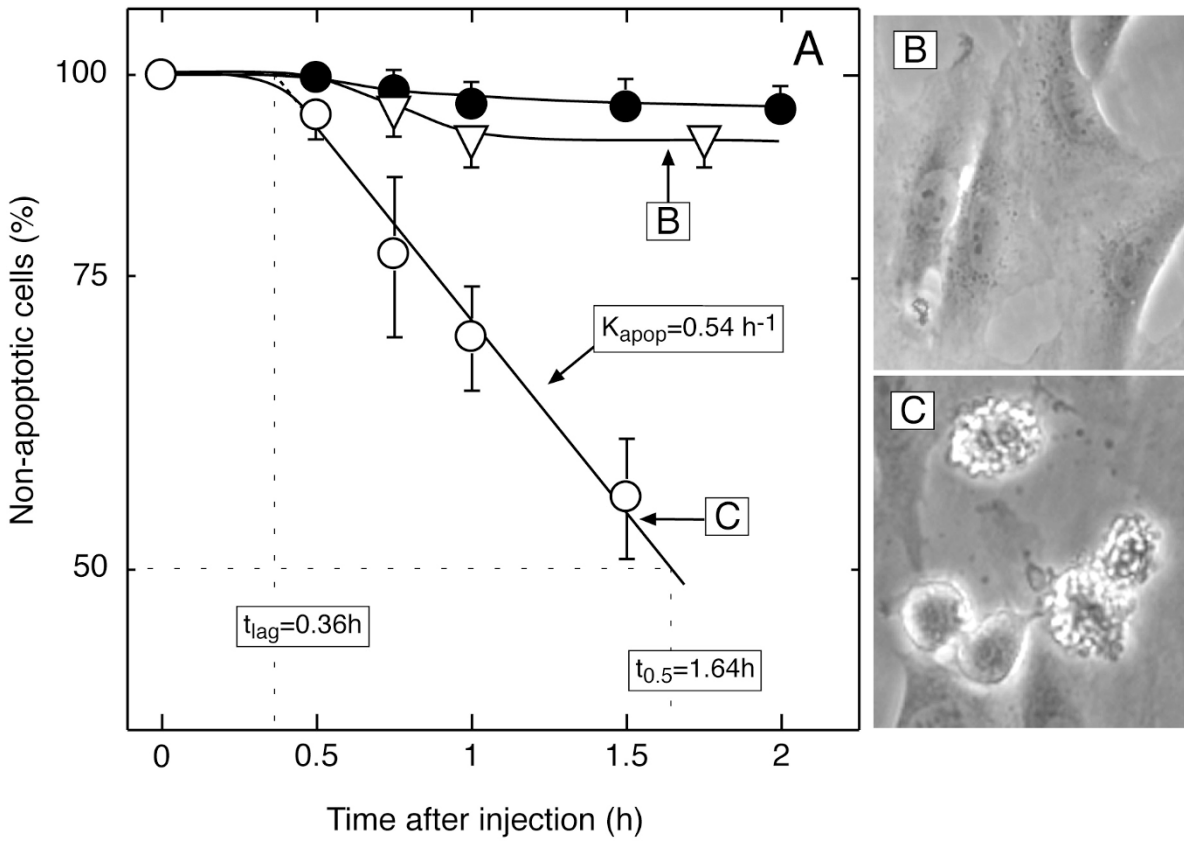

Figure 5 Time course of apoptosis development in wild-type and *bcl-2* transfected NRK cells injected with 20 μM cytochrome *c*. (**A**) Corresponds to that for Y1 cells in Figure 3. (**B**) *bcl-2* transfected NRK cells 105 min after injection of 20 μM cytochrome *c*. (**C**) Wild-type NRK cells 90 min after injection of 20 μM cytochrome *c*. Magnification 400 $\times$

analogous to the nematode system, where CED-9 binds to the CED-3/CED-4 complex to prevent CED-3 activation (Hengartner, 1997, 1998). Third, it has been reported that cytochrome *c* binds to Bcl-x_L (Kharbanda *et al*, 1997), and it seems reasonable that Bcl-2 may exert a similar quenching effect outside the mitochondria. Fourth, Bcl-2 targets several proteins, including the protein kinase Raf-1, to mitochondria (Wang *et al*, 1996a). It has been shown that a Bcl-2 interacting protein, Bag-1, binds to and activates the kinase Raf-1 (Wang *et al*, 1996b). Moreover, Bag-1 modulates the chaperone activity of Hsp70 (Takayama *et al*, 1997). Thus, it seems possible that when Bcl-2 is overexpressed, it may act, perhaps together with other anti-apoptotic proteins (Bag-1?), as a transporter for cytochrome *c* back to mitochondria (Scaife, 1966). The capacity of the Bcl-2 to facilitate this putative re-entry of cytochrome *c* into mitochondria could simply be overloaded. This might explain the fact, that high concentrations of injected cytochrome *c* overcome the Bcl-2 defense (Table 2).

In addition to serving as a source for cytochrome *c* release, the importance of mitochondria for apoptosis is underscored by the co-localization of pro-apoptotic and anti-apoptotic proteins to the outer mitochondrial membrane (Hockenbery *et al*, 1990); the requirement of ATP for several critical steps in the apoptotic process (Nicotera and Leist, 1997; Tsujimoto, 1997); and the disruption of mitochondrial physiology, including swelling and opening of mitochondrial megachannels (so-called permeability transitions, PT) during apoptosis (Kroemer, 1997; Vander Heiden *et al*, 1997). The mechanism by which cytochrome *c* is released from mitochondria is still unknown. In a recent publication (Vander Heiden *et al*, 1997), the alterations in mitochondrial physiology that follow apoptotic stimuli were investigated to determine which, if any, of them could be affected by Bcl-x_L. The authors concluded that Bcl-x_L promoted cell survival by regulating the membrane potential and volume homeostasis of mitochondria. Consistent with their observation, Bcl-x_L was not able to inhibit apoptosis induced by microinjection of cytochrome *c* in 293 cells (Duckett *et al*, 1998). On the other hand, it has recently been shown that microinjection of cytochrome *c* into MCF-7 cells did not overcome the anti-apoptotic effect of Bcl-x_L, suggesting that the ability of Bcl-x_L to inhibit cell death cannot be due solely to the inhibition of cytochrome *c* release from mitochondria (Li F *et al*, 1997). Moreover, it is quite possible that Bcl-2 and Bcl-x_L do not use exactly the same pathway for their anti-apoptotic effect. In fact, ectopic expression of Bcl-2, but not Bcl-x_L rescues B

Table 1 The effect of caspase inhibitor on cytochrome *c*-induced apoptosis

Cell type	Cytochrome c (μM in cell)	Apoptotic cells (%±SEM) No Z-VAD	20 μM Z-VAD	n
Clone 8	2	23±6.6	4.6±1.5	3
Clone 8	4	40±8.9	7.2±0.3	3
Clone 8	10	96±2.9	76±7.7	3
Clone 8	20	99±0.3	n.d.	3
H295	20	36±3.0	12±3.0	3
HEK 293	20	46	6.8	2

Clone 8 fibroblasts, H295 adrenocortical cells and HEK 293 kidney cells were injected with TRITC-dextran and cytochrome *c* to give the intracellular concentrations as indicated. 20 μM Z-VAD.fmk was added 30–60 min before injections. Cells were scored for apoptotic morphology 90 min after injections. The standard error of the mean (SEM) and the number of separate experiments (n) are indicated

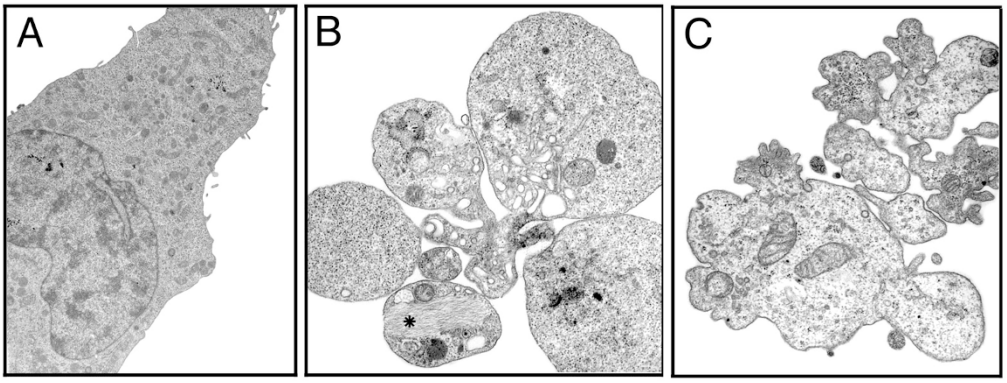

Figure 6 Electron micrographs of NRK cells fixed 2 h after injection with water (**A**) or 20 μM cytochrome *c*. (**B, C**). Panel **B** shows a cell in a moderately advanced stage of apoptosis and panel **C** shows a cell in a later stage of apoptosis. Note the condensed assembly of microfilaments in panel **B** (indicated by asterix). Magnification: **A**: ×3.200, **B**: ×10.250, **C**: ×12.500

Table 2 The concentration dependence of cytochrome *c* action in wild-type and *bcl-2* transfected cells

Cell type	Cytochrome c (μM in cell)	Apoptotic cells (%±SEM)	n
Clone 8	2	23±6.6	3
Clone 8-bcl2	2	0±0	3
Clone 8	4	40±8.9	3
Clone 8-bcl2	4	4.7±0.8	5
Clone 8	10	96±2.9	3
Clone 8-bcl2	10	78±9.1	3
Clone 8	20	99±0.3	3
Clone 8-bcl2	20	96±1.9	3

The experiments were conducted in parallel with those shown in Table 1 (data for wild-type cells are the same). For experimental details, see the legend to Table 1

cells from Fas-mediated apoptosis (Alam *et al*, 1997). However, further experimentation is required to resolve these apparent discrepancies.

There is an additional, fifth, possibility to explain the Bcl-2 protective effect. The injection of cytochrome *c* could initiate the 'circle of death' proposed by Hengartner (1998), in which initially activated caspases could promote further mitochondrial cytochrome *c* release, leading to a second loop of caspase activation and apoptosis. This mechanism would also be compatible with the finding that Bcl-2 protects only against moderate amounts of injected cytochrome *c*. In fact, it has been shown that in apoptotic cells cleavage of Bcl-2 by caspases leads to conversion of Bcl-2 to a Bax-like death effector protein (Cheng *et al*., 1997). It is likely that cleavage of mitochondrial Bcl-2 can also promote the release of cytochrome *c*. In this case the overexpression of Bcl-2 might act not only by blocking the cytochrome *c* release, but also downstream via (an) unknown mechanism(s), possibly by enhancing cellular anti-oxidant defence or by regulating the flow of different ions, including Ca^{2+}, across the endoplasmic reticulum membrane (He *et al*., 1997). In fact, in cells transiently transfected with *bax* (pro-apoptotic homologue of *bcl-2*), Bax was localized to mitochondria and promoted the release of cytochrome *c*. In this case the overexpression of Bcl-2 failed to prevent Bax-induced cytochrome *c* release, however, it did increase the survival of cells with significant amounts of cytochrome *c* in the cytoplasm (Rosse *et al*, 1998). These data, as well as ours, suggest that Bcl-2 might have other functions than the prevention of mitochondrial cytochrome *c* release.

Thus, it appears that cytochrome *c*-induced apoptosis can be prevented by Bcl-2 not only at the level of its release from mitochondria, but also downstream of this release, possibly by one of the mechanisms described above. Work is in progress in our and other laboratories to investigate the precise mechanism of the protective effect of the Bcl-2 family of proteins in apoptosis.

Materials and Methods

Materials

Bovine cytochrome *c*, hexa-biotinylated cytochrome *c*, geneticin and TRITC-labeled dextrane were obtained from Sigma (St Louis, MO, USA). Z-VAD.fmk was from Bachem (Bubendorf, Switzerland). SuperFect transfection agent was obtained from Qiagen GmbH (Hilden, Germany). Expression vector for bcl-2 (p442) was a kind gift from Dr Timothy McDonnell (MD Anderson Cancer Center, Houston, TX, USA). Bi-biotinylated cytochrome *c* was prepared in our laboratory by David H Burgess.

Cell culture and microinjection

Normal rat kidney NRK cells, Swiss 3T3 fibroblasts, mouse Y1 adrenocortical tumor cells, human embryonal kidney HEK 293 cells, Clone 8 embryonal mouse fibroblasts and IPC-81 promyelocytic leukemia cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The human adrenocortical tumor cell line H295 was cultured in a 1 : 1 mixture of DMEM and Ham's F-12 medium (Gibco–BRL, Gaitherburg, MD, USA) supplemented with insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium (6.25 ng/ml), linoleic acid (5.35 μg/ml; ITS plus, Collaborative Research, Bedford, MA, USA), 2% Nu-Serum (Collaborative Research, Bedford, MA, USA) and antibiotics. All cell types were cultured in 9.8 cm^2 dishes with grid patterns forming 4 mm^2 squares. Microcapillaries (type BF100-10, 1.00/0.78) and puller (Model P-87) were from Sutter Instrument Co. (Novato, CA, USA). The micromanipulator (Eppendorf 5171), microinjector (Eppendorf 5246), and an incubator providing 5% CO_2 and 37°C were mounted on an inverted Nikon Diaphot 300 microscope. NRK, Y1, Swiss 3T3, Clone 8, HEK 293, H295 and the corresponding bcl-2-transfected cells were seeded at 10 000 cells/cm^2 and were injected 24 h after seeding. IPC-81 cells to be injected were seeded at $3 \times 10^4/cm^2$ in dishes pre-coated with fibronectin (1 $\mu g/cm^2$). After seeding, the cells were treated with 100 nM of the phorbol ester TPA to further improve cell attachment. Cells were injected 4–8 h after seeding.

Cytochrome *c* was routinely co-injected with TRITC-dextran (to 0.1 μg/μl intracellular concentration) to detect positively-injected cells with a fluorescence microscope, and was delivered in H_2O. Control injections were done with TRITC-dextran in H_2O alone. There was no enhancement or attenuation of the effect of injected cytochrome whether TRITC-dextran was present or not and whether the cytochrome was dissolved in physiological buffer or in water. Optimal injections were obtained with capillary pipettes with a tip diameter of less than 0.3 μm, which were made shortly before the experiment. Pipettes were loaded by retrograde filling. The injected volume was estimated as previously shown (Mellgren *et al*, 1993) to be close to 2% of the cellular volume, corresponding to a 50-fold dilution of injected material. The inhibitor of caspase activity, Z-VAD.fmk, was dissolved in dimethylsulfoxide (DMSO) and added to the culture medium 30–60 min before the injections. The final concentration of DMSO was less than 0.5% and 0.5% DMSO by itself did not affect the cells.

Injections were performed in 50–150 cells for each experimental condition in each experiment, and data is presented as the average from at least four experiments, unless otherwise indicated.

Transfections

Y1 cells were transfected using the calcium phosphate protocol. Swiss 3T3 and NRK were transfected using the SuperFect agent from Qiagen, as described in the protocol from the manufacturer. The transfected cells were selected with neomycin 1 mg/ml or geneticin for several weeks. Bcl-2 expressing cells were almost completely resistant against anti-Fas- and daunorubicin-induced apoptosis, while non-transfected or mock-transfected cells treated with anti-Fas or daunorubicin died within 30–36 h (not shown). Some cultures were co-transfected with *lacZ*-containing plasmid for evaluation of transfection efficiency. Cells were microinjected 24 h following transfection with the fluorogenic β-galactosidase substrate CMFDG (Brustugun *et al*, 1995). The *bcl-2* transfected Clone 8 cells were a kind gift from R. Ahlgren and T. Sandal at the Department of Anatomy and Cell Biology, University of Bergen.

Evaluation of apoptotic morphology and determination of rate constants for apoptosis development

For routine assessment of apoptosis, cell morphology was evaluated by inverted phase microscopy using phase and Nomarski optics. Apoptotic cells of all cell types were easily discriminated from non-apoptotic (both normal and necrotic) cells by the appearance of surface protrusions, and in some experiments cells were treated with FITC-conjugated Annexin V to verify apoptotic cells. In brief, cells were exposed to 5 μg/ml FITC-conjugated Annexin V and counter-stained with 5 μg/ml propidium iodide. Cells were scored under UV light using filter sets as for FITC-staining, containing a long pass filter for simultaneous detection of propidium iodide.

When the decrease of normal cells after cytochrome *c* injection was plotted semilogarithmically against time (Figures 3–5), a near rectilinear curve resulted. This allowed the determination of the rate constant for the transition from normal to apoptotic phenotype (K_{apop}), which is the slope of the rectilinear part of the curve.

For ultrastructural analysis, both injected and control NRK-cells were fixed in 2% glutaraldehyde and buffered with Na-cacodylate (pH 7.4). Cell culture dishes were post-fixed in 2% OsO_4 and dehydrated in graded alcohol solutions. Embedding was performed by filling Agar 100 resin into a gelatin cyclinder that was placed over the injected area. Ultra-thin sections were stained in uranyl acetate and lead citrate and viewed with a Phillips 300 electron microscope.

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