The protonophore CCCP induces mitochondrial permeability transition without cytochrome *c* release in human osteosarcoma cells

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Abstract Mitochondrial permeability transition (MPT) and cytochrome *c* redistribution from mitochondria are two events associated with apoptosis. We investigated whether an MPT event obligatorily leads to cytochrome *c* release in vivo. We have previously shown that treatment of human osteosarcoma cells with the protonophore *m*-chlorophenylhydrazone (CCCP) for 6 h induces MPT and mitochondrial swelling without significant cell death. Here we demonstrate that release of cytochrome *c* does not occur and the cells remain viable even after 72 h of treatment with CCCP. Bax is not mobilized to mitochondria under these conditions. However, subsequent exposure of CCCP-treated cells to etoposide or staurosporine for 48 h results in rapid cell death and cytochrome *c* release that is accompanied by Bax association with mitochondria, demonstrating competency of these mitochondria to release cytochrome *c* with additional triggers. Our findings suggest that MPT is not a sufficient condition, in itself, to effect cytochrome *c* release.

Key words: Mitochondrial permeability transition; Cytochrome *c*; Bax; Cell death; Mitochondrion

1. Introduction

Mitochondria play a pivotal role in apoptosis [1,2]. Two mitochondrial events that are proposed to mediate this control of apoptosis are the mitochondrial permeability transition (MPT) and release of pro-apoptotic proteins from the intermembrane space of mitochondria.

MPT is characterized by an abrupt increase in the permeability of the inner mitochondrial membrane to solutes with molecular mass below 1.5 kDa. This has been attributed to the opening of a mitochondrial megachannel, the MPT pore, proposed to be located at contact sites between the inner and outer mitochondrial membranes [3]. Pore opening inevitably leads to the dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$), chemical equilibration of solutes across the membranes and, as studied in isolated mitochondria, large amplitude swelling of the mitochondrial matrix with consequent loss of outer membrane integrity [4,5]. The role of MPT in cell death is supported by pharmacological interventions in which the use of MPT inducers (e.g. atractyloside) promote apoptotic changes [6,7] whilst MPT inhibitors (e.g. bongkrekic acid) attenuate onset of apoptosis [6,8].

The translocation of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytosol is another key event, which leads to the activation of caspases. The best studied of these apoptogenic proteins is cytochrome *c*, which upon translocation into the cytosol, forms a multimeric complex with Apaf-1, the mammalian homologue of the death initiating protein CED-4 of *Caenorhabditis elegans* [9], in an ATP-requiring reaction. This complex is able to recruit procaspase-9 and activate it. In turn, caspase-9 can proteolytically cleave downstream caspases, such as caspase-3, to execute the biochemical and morphological features of apoptosis [10,11]. The role of cytochrome *c* as a mediator of mitochondria-dependent caspase activation and cell death is supported by in vivo studies involving microinjection of cytochrome *c* into intact cells [12,13], and in vitro studies using cell-free systems [14].

How cytochrome *c* is released from the mitochondria is not fully understood [15]. One hypothesis is that MPT may precipitate this outcome. This is based, first, on findings that isolated mitochondria undergoing an MPT-dependent swelling release intermembrane space proteins including cytochrome *c* [16,17]. Second, in apoptotic cells, cytochrome *c* redistribution from mitochondria is preceded by, or occurs concurrently with, depolarization of $\Delta\Psi_m$ [8,18–20]. However, not all the data available support this model. In particular, there has been inconsistency in the results obtained for $\Delta\Psi_m$ changes associated with cytochrome *c* release. In some apoptotic cell systems, $\Delta\Psi_m$ loss was observed only after cytochrome *c* efflux [21–23] whilst in others, an absence of change in $\Delta\Psi_m$ was observed [24,25]. In addition, some studies on the pro-apoptotic Bcl-2 family member, Bax, have demonstrated that cytochrome *c* could be released independently of an MPT event [26,27]. This has led to proposed alternative models involving a specific release mechanism via a Bax-regulated channel [2,15].

In this study, we have investigated whether MPT and cytochrome *c* release are obligatorily connected events, particularly testing the hypothesis that MPT in vivo leads to cytochrome *c* release. We have previously reported that treatment of human osteosarcoma cells (an immortal adherent cell line) with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) resulted in MPT events. The mitochondria in such CCCP-treated cells showed loss of $\Delta\Psi_m$ and morphological swelling [28]. Significantly, mitochondria preloaded in vivo with calcein (M_r 623) released this marker dye upon treatment of cells with CCCP; this release was inhibited by

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cyclosporin A [28], thus confirming the response as an authentic MPT [4] in intact cells. Here, we report that MPT induced by CCCP-mediated mitochondrial depolarization in osteosarcoma cells does not lead to cytochrome *c* release or cell death, even after extended periods of treatment (up to 72 h), indicating that MPT, as such, is not a sufficient condition for cytochrome *c* release in vivo.

2. Materials and methods

2.1. Reagents

CCCP, staurosporine, and etoposide were purchased from Sigma (St. Louis, MO, USA). Monoclonal mouse anti-cytochrome *c* and anti-Bax IgGs were from Pharmingen (San Diego, CA, USA), goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) was from Biosource International (Camarillo, CA, USA), and goat anti-mouse IgG conjugated to Alexa 568 and rhodamine-123 (Rh123) were from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

Human osteosarcoma cells 143B TK$^-$ were cultured as previously described [28]. A derivative of this cell line stably expressing mitochondrially targeted green fluorescent protein (mtGFP) was also generated. A recombinant plasmid pCZ34 [29], expressing GFP5 fused at its N-terminus to the 16 amino acid mitochondrial targeting sequence of human 3-oxoacyl-CoA thiolase (hOACTL), was transfected into osteosarcoma cells using Lipofectamine Plus (Gibco BRL). Briefly, 1 μg plasmid was pre-complexed with the Plus and Lipofectamine reagents, and then incubated with the cells in serum-free and antibiotic-free culture medium for 3 h at 37°C. At 48 h post transfection, cells containing the expressed plasmid were selected by adding G418 (A.G. Scientific, CA, USA) at a final concentration of 600 μg/ml. The cells were monitored for 1 week with medium change every 2–3 days, maintaining selection. The surviving cells were harvested and seeded at 100 cells per 100 mm culture dish to allow colony formation. The colonies were analysed for GFP expression and one colony (denoted B4) was selected for use in this study.

2.3. Treatment of cells

Cells were seeded at a density of 1×10^5 into six-well plates the day before treatment. One group of cells was treated with either 10 μM CCCP, 100 μM etoposide or 100 nM staurosporine alone whilst another group of cells was initially treated with 10 μM CCCP for 6 h and subsequently treated with either etoposide or staurosporine.

2.3. Immunocytochemical staining of cytochrome c and Bax

For each treatment group, a portion of cell suspension containing viable (adherent) and non-viable (detached) cells was placed onto glass slides coated with poly-L-lysine (0.01%) by centrifugation at 1000 rpm for 5 min using a Cytospin (Shandon Scientific, UK). Cells were fixed with 3.5% paraformaldehyde (PFA) for 10–15 min, permeabilized with 0.1% Triton X-100 for 10 min, and incubated for 1.5–2 h with either monoclonal anti-cytochrome *c* or anti-Bax primary antibody (diluted 1/50) and then for 1 h with secondary antibody (diluted 1/100) conjugated either with FITC or Alexa 568. Coverslips were mounted onto glass slides with Fluoroguard anti-fade reagent (Bio-Rad Laboratories, CA, USA) and sealed with clear nail polish. Cells were either scored for release/non-release of cytochrome *c* or cytosolic/mitochondrial localization of Bax in situ following visualization with a Leica TCS-NT upright confocal microscope using a 63× oil immersion objective.

2.5. Cell viability

Trypan blue exclusion was performed in order to assess viability of cells. Following incubation with the appropriate reagent(s) for 24, 48 and 72 h, detached and adherent fractions were collected separately, and each fraction was stained with 0.2% trypan blue. Viable (trypan blue-negative) and non-viable (trypan blue-positive) cells were counted using a haemocytometer under a light microscope.

2.6. Monitoring $\Delta\Psi_m$

Loss of $\Delta\Psi_m$ as a result of CCCP treatment was monitored by Rh123 staining. Cells were incubated with 10 μM Rh123 for 15 min at 37°C and then washed three times with culture medium. Images of Rh123 stained cells incubated with and without CCCP were obtained with a Leica TCS-NT inverted confocal microscope. For each group, images of at least 10 fields of cells (with each field containing ≥100 cells) were taken using a 16× oil/water immersion objective lens. Both fluorescence and phase contrast images were obtained and subsequently merged for quantification of fluorescence intensity using an MCID image analysis software (Imaging Research Inc., Canada). The total number of pixels per unit area of cell was quantified and adjusted for background. The mean pixel value was then calculated per image and the mean of the mean was obtained for each group.

2.7. Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test to compare the results in experimental and control groups of cells.

3. Results

3.1. MPT and loss of $\Delta\Psi_m$ induced by CCCP do not lead to cytochrome c release or cell death

Intact osteosarcoma cells treated with CCCP were previously observed to undergo MPT and mitochondrial swelling in the absence of cell death. We further investigated whether CCCP-induced MPT under these conditions led to the release of cytochrome *c* from mitochondria. In brief, cells were treated with CCCP for a period of 72 h and total cell population (viable+non-viable) was collected every 24 h and immunostained for cytochrome *c*. Visualization of FITC fluorescence revealed two types of population of cells. One typically showed a mitochondrially compartmentalized pattern of fluorescence (Fig. 1A), which was categorized as negative for cytochrome *c* release; the other showed clearly diffused fluorescence including coverage of the nuclear region (Fig. 1B), which was designated positive for cytochrome *c* release.

Examination of cells after CCCP treatment revealed that at all time points tested (24, 48, and 72 h) about 1–3% of cells had released cytochrome *c*, comparable to that of untreated cells (0.4–2%) (data not shown in detail here, but see also Fig. 3A below). The small percentage of cells positive for cytochrome *c* release in the untreated group is attributed to the spontaneous release of cytochrome *c* associated with a stochastic (and as yet uncharacterized) activation of the death mechanism in these cells. The lack of release in the CCCP-treated cells is paralleled by the minimal loss in cell viability such that greater than 95% of cells remained viable even after 72 h of treatment, as also observed in untreated controls (data

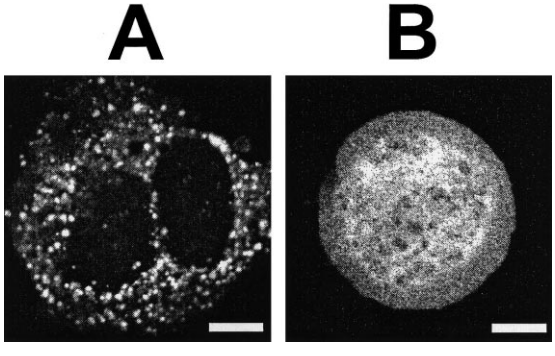

Fig. 1. Distribution of cytochrome *c* in situ. Immunocytochemical staining for cytochrome *c* revealed two types of cells: those showing no release of cytochrome *c* (A) or those showing release of cytochrome *c* (B). Scale bars indicate 10 μm.

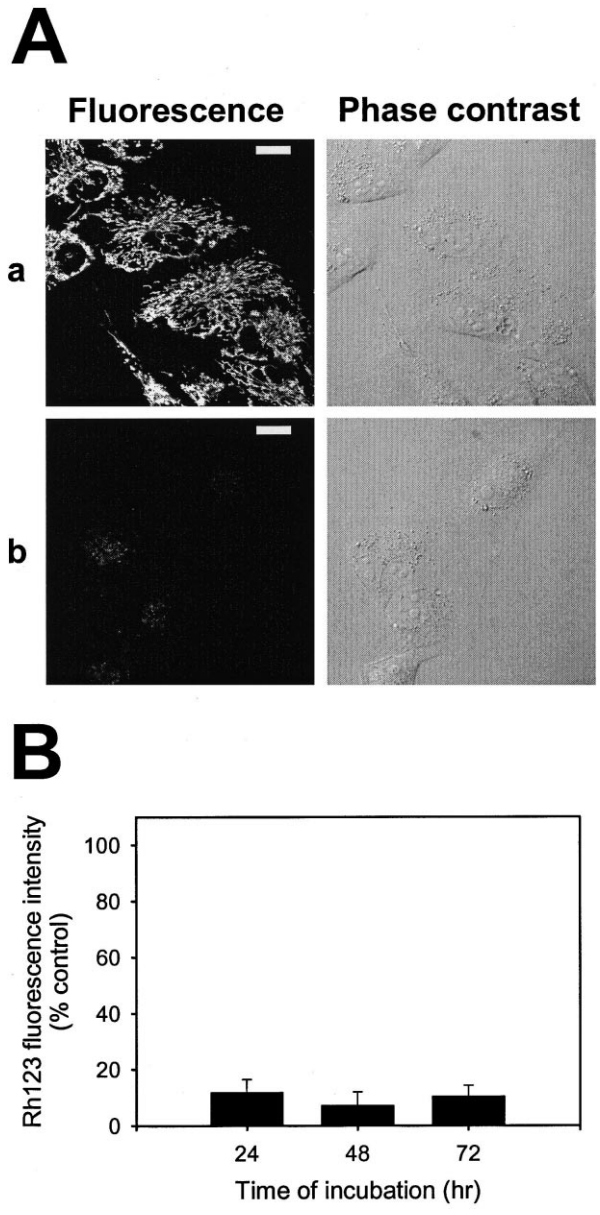

Fig. 2. Cells treated with CCCP for extended periods maintain loss of $\Delta\Psi_m$. A: Representative images of cells stained with Rh123, without (a) or with (b) CCCP treatment for 48 h. For comparison of fluorescence intensity, the fluorescence gain level was first calibrated for untreated control where there is intense mitochondrial Rh123 fluorescence (indicative of high $\Delta\psi_m$) and CCCP-treated cells were imaged using the same photomultiplier gain level. Scale bars indicate 20 μm. B: Quantitative analysis of Rh123 fluorescence in CCCP-treated cells relative to untreated control. Rh123 fluorescence in CCCP-treated cells was expressed as a percentage of that in untreated control, which was designated to have 100% fluorescence.

not shown in detail here, but see also Fig. 3B below). These cells also did not stain with either annexin V or propidium iodide, indicating that they are not apoptotic.

To confirm that $\Delta\Psi_m$ remained dissipated under these extended conditions of CCCP treatment, cells were stained with Rh123, a reporter dye which accumulates in a Nernstian manner in mitochondria and is thus sensitive to changes in inner membrane potential. Rh123 fluorescence was monitored every 24 h for 72 h by confocal microscopy. For both untreated and CCCP-treated groups, the mean fluorescence intensity was determined and loss of Rh123 fluorescence in CCCP-treated cells was expressed as percentage of untreated control. As shown in Fig. 2, the Rh123 fluorescence was reduced by approx. 80% at all time points, indicating that the mitochondria in CCCP-treated cells were continuously depolarized during the course of treatment.

3.2. *Additional treatment of CCCP-treated cells with etoposide or staurosporine causes release of cytochrome c with concomitant cell death*

In order to determine whether cytochrome *c* could be released from mitochondria of CCCP-treated cells, having undergone MPT, the cells were further treated with the apoptotic inducers etoposide or staurosporine. Cells were pretreated with CCCP for 6 h during which time MPT has occurred and were then subsequently treated with either

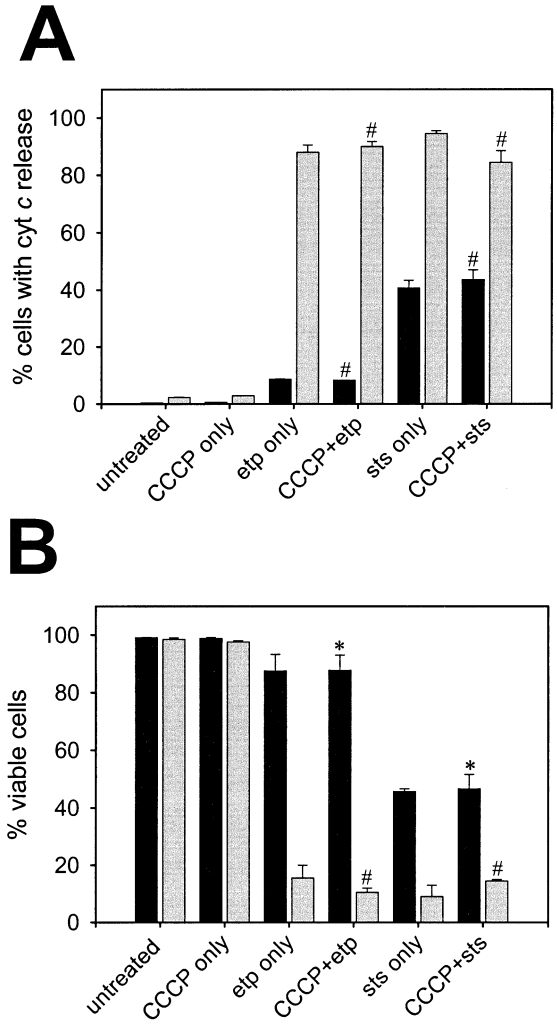

Fig. 3. Effects of etoposide and staurosporine treatment on cytochrome *c* release and cell viability. A: Percentage of cells that have released cytochrome *c* out of the total cells counted in untreated, CCCP, etoposide (etp), CCCP plus etoposide, staurosporine (sts), and CCCP plus staurosporine groups. B: Cell viability of the various treatment groups, determined by trypan blue exclusion. Treatment times were 24 h (black bars) and 48 h (shaded bars). Symbols in A and B indicate significant differences between the CCCP-treated reference group and either CCCP plus etoposide-treated cells, or CCCP plus staurosporine-treated cells, for both time points ($^*P < 0.1$; $^\#P < 0.05$).

etoposide or staurosporine for 24, 48 and 72 h whilst maintaining CCCP in the medium. A cell population analysis was carried out after the appropriate time of incubation, involving cytochrome *c* immunocytochemistry and determination of cell viability.

Etoposide or staurosporine treatment alone did not cause release of cytochrome *c* in a significant proportion of cells ($< 50\%$) after 24 h (Fig. 3A). By 48 h, however, the proportion of cells showing cytochrome *c* release had increased to near maximal levels (about 90%), associated with an increase in the percentage of non-viable cells to greater than 85% by that time (Fig. 3B). Prior treatment of cells with CCCP did not significantly affect this outcome, suggesting that the mechanism enabling cytochrome *c* release is functional in CCCP-treated cells upon exogenous induction of apoptosis.

3.3. The release of cytochrome c is accompanied by Bax localization into mitochondria

Movement of Bax from the cytosol into mitochondria has been reported to facilitate the release of cytochrome *c* following apoptotic induction [30,31]. We have therefore monitored localization of endogenous Bax in cells subjected to the different treatment regimes. Mitochondrial fluorescence was visualized in osteosarcoma cells expressing mitochondrially targeted GFP (mtGFP) and Bax was detected by indirect immunostaining using a secondary antibody conjugated to Alexa 568, a red fluorophore. Confocal microscopy of untreated B4 cells showed diffused Bax immunoreactivity encompassing most cell regions, including the cytosol, but excluding the nucleoli (Fig. $4A_1$). This was also the case in CCCP-treated cells, consistent with their failure to release cytochrome *c* (Fig. $4A_2$). However, cells treated with etoposide (Fig. $4A_3$) or staurosporine (Fig. $4A_4$) showed a more punctate distribution of Bax, the vast majority of which co-localizes well with mtGFP. These data clearly demonstrate translocation of Bax from the cytosol to mitochondria under these conditions of apoptotic induction.

The proportion of cells showing mitochondrial localization of Bax was quantified in the same manner as for cytochrome *c* and the results are depicted in Fig. 4B. CCCP treatment alone did not cause any significant change in Bax distribution such that 99% of cells showed cytosolic Bax staining after 48 h of treatment. In contrast, approx. 40% and 50% of cells treated with etoposide or staurosporine, respectively, showed mitochondrial Bax distribution at that time. CCCP pretreatment did not have any significant effect on the values obtained for both these conditions of apoptotic induction. Thus, the results correlate with the cytochrome *c* release data, although the

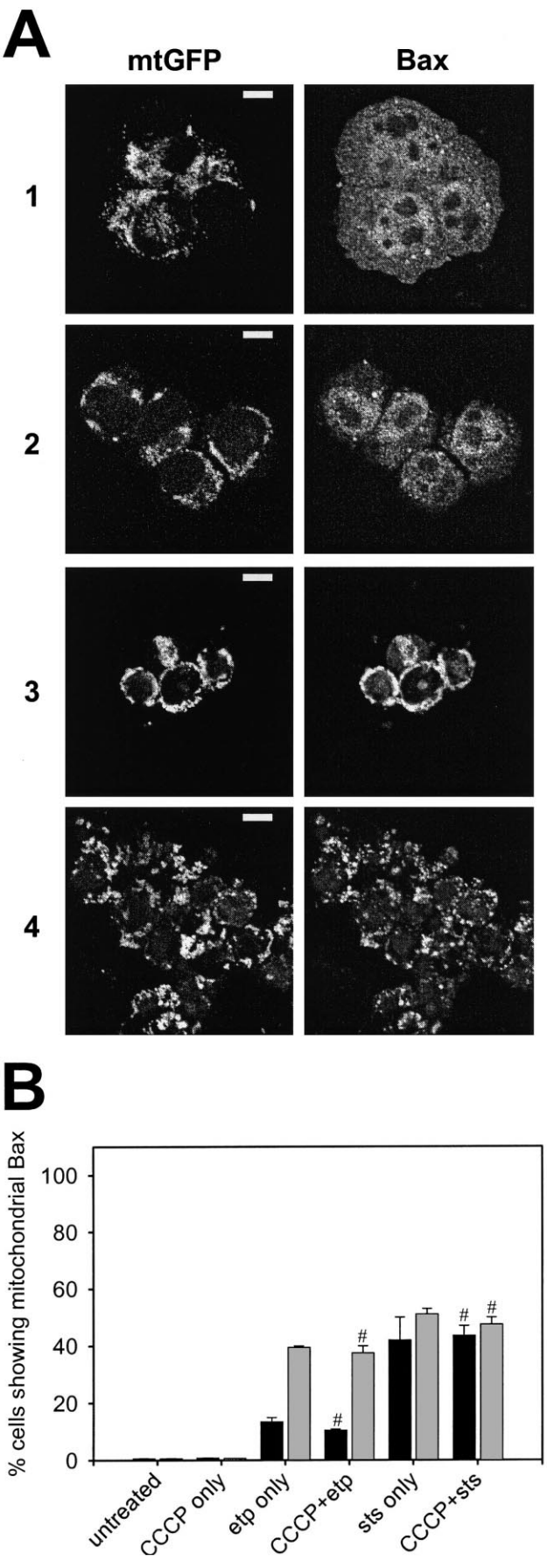

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Fig. 4. Bax distribution following CCCP, etoposide or staurosporine treatment. A: Representative images of cells showing (a) mtGFP-labelled mitochondria (green emission channel) and (b) Bax immunostaining (red emission channel). Panel 1, untreated cells. Panels 2, 3 and 4, cells treated for 48 h with CCCP, etoposide and staurosporine, respectively. Scale bars indicate 10 μm. Note that in untreated cells, the mitochondria no longer appear filamentous (cf. Fig. 2A) due to trypsinization and centrifugation in this experiment. B: Percentage of cells showing mitochondrial localization of Bax out of the total cells counted for the various treatment groups. Treatment times were 24 h (black bars) and 48 h (shaded bars). Symbols indicate significant differences between the reference group of cells treated with CCCP only and those treated with CCCP plus another agent ($^{\#}P < 0.05$).

absolute proportions quantified were generally lower for Bax compared with cytochrome *c*. In particular, the proportion of cells showing mitochondrial Bax following etoposide or staurosporine treatment for 48 h did not exceed 50% whilst greater than 90% of these cells showed release of cytochrome *c*. We therefore cannot exclude that cytochrome *c* may be released via a Bax-independent pathway in these cells.

4. Discussion

4.1. Occurrence of MPT without cytochrome c release in vivo

It has been proposed that MPT can induce apoptosis via the release of cytochrome *c* and other pro-apoptotic proteins from the intermembrane space due to MPT-mediated swelling of mitochondria and subsequent permeabilization of the outer membrane. This proposal is based primarily on studies in vitro whereby induction of MPT in isolated mitochondria directly causes swelling with concomitant non-specific release of intermembrane space proteins [17,18]. In this study, we tested whether this situation actually applies in vivo, in intact cells.

Our approach to monitoring MPT in intact cells entails use of the fluorescent dye calcein, whose fluorescence in mitochondria is specifically revealed when cytosolic calcein fluorescence is quenched with cobalt (Co^{2+}) [28,32]. We have observed that treatment of human osteosarcoma cells with CCCP not only causes dissipation of $\Delta\Psi_m$ as its primary action but also induces pore opening within 30 min, as visualized by the release of mitochondrially entrapped calcein [28]. This calcein release was significantly inhibited by the addition of cyclosporin A (a known MPT inhibitor [4]) validating our inference that a genuine MPT has taken place. This MPT was maintained for a period of up to 6 h during which time no apparent cellular damage was observed and was also accompanied by mitochondrial swelling judged by confocal microscopy [28].

We have extended this line of investigation to determine whether CCCP-induced MPT eventually leads to cytochrome *c* efflux from mitochondria in these osteosarcoma cells. Analysis of cytochrome *c* distribution by immunofluorescence in conjunction with confocal microscopy showed that there was no significant difference in the CCCP-treated cells compared to the untreated control even after extended periods of treatment. This demonstrates that an MPT event coupled with prolonged disruption of the proton gradient by CCCP does not facilitate release of cytochrome *c*. Thus, MPT and cytochrome *c* release are not mutually exclusive events in this in vivo system.

4.2. Response of mitochondria to additional triggers: possible role of Bax

Cytochrome *c* redistribution in CCCP-treated cells did occur, however, following additional treatment with either etoposide or staurosporine for 48 h, and this was concomitant with cell death. This suggests that the mitochondria are still responsive to signals conveyed by extramitochondrial changes brought about by etoposide (DNA topoisomerase II inhibitor) or staurosporine (protein kinase inhibitor). CCCP-treated depolarized mitochondria thus remain as integral components of the apoptotic machinery despite disruption in their bioenergetic integrity. The lack of effect of CCCP pretreatment on the rate and degree of cytochrome *c* release in etoposide-treated or staurosporine-treated cells further suggests that MPT is not a prerequisite for cytochrome *c* redistribution. Instead, it is likely that additional effector molecules are recruited into mitochondria during chemical-induced apoptosis to promote cytochrome *c* efflux. Such molecules may include the pro-apoptotic member of the Bcl-2 family, Bax, which is known to translocate from the cytosol into mitochondria where it oligomerizes and forms large conductance channels in the outer membrane [33,34].

Previous studies in vivo have shown that treatment of various mammalian cells with etoposide or staurosporine causes mobilization of Bax to mitochondria, which in turn effects release of cytochrome *c* [35,36]. The residence of Bax in cells not induced apoptotically includes various cytoplasmic locations as well as the nucleus [36,37]. CCCP by itself had no effect on the distribution of Bax, suggesting that cytochrome *c* release in osteosarcoma cells may require stimulus such as Bax localization to mitochondria. Indeed, additional incubation with either etoposide or staurosporine for 48 h revealed Bax to localize in the mitochondria in a significant proportion of cells, and this, in general, paralleled cytochrome *c* release. This suggests that Bax translocation into mitochondria was involved in the release of cytochrome *c*. However, it may not be the sole mechanism governing this event. In only about half of the cells in which release of cytochrome *c* occurred, was there observed mitochondrially localized Bax, and this was obtained for both etoposide and staurosporine treatments. Thus, there may be other signalling factors, such as other pro-apoptotic members of the Bcl-2 family and caspases [38,39], that may effect release of cytochrome *c* in this system. Nevertheless, our data suggest that MPT pore opening and loss of $\Delta\Psi_m$ are not sufficient for the translocation of cytochrome *c* from mitochondria in vivo. The additional participation of Bax and possibly of other signalling molecules, either in conjunction with or independently of MPT, is required to activate cytochrome *c* release.

4.3. Differences in mitochondrial behaviour in vivo and in vitro

The absence of cytochrome *c* release following MPT detected here contrasts with that observed in vitro, and suggests that a different mechanism of release may occur in intact cells. In particular, one may consider that a more specific mode of release via an outer membrane channel could be favoured in vivo rather than a non-specific mode of release due to rupture of the outer membrane. Some authors [2,15] have reported that mitochondria do not swell but become condensed before cytochrome *c* release. However, MPT with consequent mitochondrial swelling cannot be altogether excluded as a possible mechanism for the release of cytochrome *c* in certain individual cell types subjected to particular treatments. In rat thymocytes for instance, induction of MPT has been shown to lead to cytochrome *c* redistribution and cell death [18]. Similarly, mitochondrial swelling detected by electron microscopy in Jurkat T cells undergoing Fas-induced or staurosporine-induced apoptosis preceded release of cytochrome *c* [40,41].

Although we have observed swelling as a result of CCCP-induced MPT in osteosarcoma cells by confocal microscopy, it is possible that the degree of swelling achieved was insufficient to cause breakage of the outer membrane. We obtained similar observations to those reported here, following treatment of osteosarcoma cells with valinomycin, a K^+ ionophore that also collapses $\Delta\psi_m$ and induces MPT. Under these con-

ditions, there was marked swelling of mitochondria that was more profound than that observed in CCCP-treated cells but, again, cytochrome *c* was not released (M.L.R. Lim and P. Nagley, unpublished data). In these situations of MPT and swelling in vivo, the absence of cytochrome *c* release following MPT is likely to be due to the outer membrane remaining intact. It can be postulated that the process of mitochondrial swelling in vivo differs from that occurring in isolated mitochondria such that the degree of swelling may be subjected to a more tight regulation. Bcl-X_L, an anti-apoptotic Bcl-2 protein localized in the mitochondrial outer membrane, has been shown to play a role in maintaining osmotic homeostasis of mitochondria [40]. The presence of this protein may therefore restrict the extent of swelling of mitochondria in intact cells, which may also vary amongst different cell types.

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