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Cytochrome c Is Dispensable for Fas-Induced Caspase Activation and Apoptosis

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Cytochrome c is thought to play an important role in the initiation of apoptosis following its release from mitochondria. It is controversial whether such release is also involved in caspase activation and apoptotic cell death after ligation of the cell surface molecule Fas. We addressed this issue by investigating cells from the human cell lines Jurkat and SKW6 which had been treated with the inhibitor of the mitochondrial F0/F1-ATPase, oligomycin. Oligomycin-treatment led, over a wide range of concentrations, to ATP-depletion and, at similar concentrations, abrogated the appearance of caspase-3-like activity caused by stauroporine. Electroporation of cytochrome c protein into intact cells induced caspase activation in both cell lines and significant nuclear apoptosis in Jurkat cells. In ATP-depleted cells, electroporation of cytochrome c induced neither caspase activation nor nuclear fragmentation. Fas-induced caspase activation and nuclear apoptosis, however, were unaffected by the depletion of ATP. Thus, cytochrome c is unlikely to be an important factor in Fas-induced cell death. © 1999 Academic Press

Apoptotic cell death is the result of the activation of a specialized intracellular signaling system. The components of the apoptosis machinery which are required for the implementation of cell death are probably present in all cells and are activated upon a specific signal. Over the last few years, it has become clear that members of the caspase family of cysteine proteases are critical for at least most forms of apoptosis, and the question of how caspases are activated has, accordingly, attracted much attention (for review see (1)). Several points of evidence support the concept that cytochrome c plays a role in the induction of caspase activity: In a cell-free system, cytochrome c can activate caspase-3 via a complex with Apaf-1 and caspase-9 (2–4). When microinjected or electroporated into normal cells, cytochrome c can trigger apoptosis (5, 6). Furthermore, cytochrome c-release from its normal location in the mitochondria into the cytosol can be detected as an early event in several forms of apoptosis such as cell death initiated by treatment with high concentrations of the kinase-inhibitor staurosporine (7). It appears therefore likely that cytochrome c can act as an initiator of the apoptotic system.

Whether the induction of apoptosis by the cell surface receptor Fas/APO-1/CD95 is mediated by cytochrome c is controversial. Fas-ligation leads to the recruitment of a signaling complex and subsequently the activation of caspase-8 (8, 9). Caspase-8 can by itself process other caspases such as caspase-3 which is known to be involved in central events of apoptosis (1); it is therefore plausible that this could be a direct way of bypassing the caspase-activating capacity of cytochrome c. Mice deficient for caspase-9, the only caspase known to be initially activated via cytochrome c, have severe developmental defects and a disturbed apoptotic response to many death-inducing stimuli; cell death induced by Fas-signaling, however, appears to be normal (10, 11).

Recent data, on the other hand, contradict this model. Several groups of authors have reported that cytochrome c is released from mitochondria following ligation of Fas (12, 13) although other publications dispute this finding (14–16). Moreover, a mechanism has been described which provides a molecular explanation for such release as a consequence of caspase-8-activation: The protein Bid can be cleaved by active caspase-8 and one of the cleavage products is capable of associating with mitochondria and triggering the release of cytochrome c (17–19). Along the same lines, both a naturally occuring and an engineered dominant negative form of caspase-9 has been found to inhibit fas-induced cell death (20–22).

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In this study, we addressed the question of the respective contribution of cytochrome c to the activation of caspases and apoptosis induced by either Fas-ligation or treatment with staurosporine. Cells from the two human cell lines Jurkat and SKW6 were depleted of ATP by treatment with the inhibitor of the mitochondrial F0/F1 ATPase, oligomycin, during culture in glucose-free medium. Under these conditions, caspase-activation by staurosporine was precluded but caspase-activity initiated by anti-Fas-treatment was unaltered. To characterise this further, the dependency of cytochrome c on ATP for the induction of apoptosis was tested by electroporation of the protein into intact cells.

It has been reported recently that different cell lines react in a different fashion to a Fas-signal and a model has been proposed which allocates cells to either of two groups (12). We therefore used for these studies two cell lines in parallel, the human T cell line Jurkat (a "type II" cell line) and the human SKW6 lymphoblastoid line (a "type I" cell line).

MATERIALS AND METHODS

Cell lines and treatment of cells. Jurkat cells were from the American Type Culture Collection. SKW6 cells were obtained from Dr. Andreas Strasser, Walter and Eliza Hall Institute, Melbourne, Australia. Both cell lines were grown in Click's RPMI supplemented with 10% FCS, glutamine and antibiotics. For ATP-depletion, cells were collected by centrifugation, washed once in glucose-free RPMI medium (Gibco BRL) containing 0.5% FCS and 2 mM pyruvate (here referred to as depletion medium) and cultured in depletion medium at a density of 10^6 cells/ml in 12-well plates. Oligomycin (Sigma) was added to the concentrations indicated in the figure legends and cells were cultured at 37°C/7% CO_2 for the time periods specified in the figure legends. To induce apoptosis, to some samples anti-Fas mAb (Upstate Biotechnologies; 100 ng/ml for Jurkat cells, 50 ng/ml for SKW6 cells) or staurosporine (Sigma, 1 μM for Jurkat cells, 2 μM for SKW6 cells) was added, and culture was continued for two hours before cells were extracted and caspase-activity was determined.

Measurement of ATP levels in oligomycin-treated cells. Jurkat or SKW6 cells were cultured at 10^6/ml in depletion medium in the presence of various concentrations of oligomycin as indicated in the figure legends. After one or two hours, 0.5 ml-samples were taken, washed once in PBS and boiled in 50 μl of 40 mM Tris-borate buffer (pH 9.2) at 100°C for five minutes (23). Samples were centrifuged at 15 000 rpm for five minutes to pellet debris and supernatants were frozen at −70° for up to two weeks. Measurement of ATP content was done using a luciferin-luciferase kit (Sigma) in a Berthold luminometer in duplicates (23). Data are expressed as percent of ATP in control cells (cells cultured for the same length of time in the absence of oligomycin).

Electroporation of cells. For electroporation with cytochrome c, cells were collected by centrifugation, washed once in depletion medium (without oligomycin) and resuspended in depletion medium containing oligomycin as indicated in the figure legends (3×10^6 per 3 ml-sample) and cultured in 6-well plates for one hour. Cells were then centrifuged again and resuspended in 400 μl PBS supplemented with 100 μg/ml BSA and cytochrome c (400 μg per sample) as indicated. Mock transfections were carried out by exactly the same procedure in the absence of cytochrome c. Cells were kept in 4 mm electroporation cuvettes for 10 minutes on ice, electroporated in a BioRad Gene Pulser (960 μF and 220 V for SKW6 cells, 230 V for Jurkat cells) and kept on ice for an additional 20–30 minutes. Cell suspensions were then placed back into the original 3 ml of depletion medium and culture was continued at 37°. Cells were extracted for measurement of caspase-activity after one hour or stained for FACS analysis (see below for procedures) after 4 hours.

Measurement of caspase activity. Extracts were prepared from cells stimulated as indicated. Cells were collected by centrifugation and washed once in PBS. Lysis was performed by incubating cells at a density of 2×10^7/ml in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40) for ten minutes on ice followed by vigorous vortexing. Extracts were then cleared by centrifugation for 5 minutes at 10 000 × g at 4°. For assay of DEVD-cleaving activity, extracts were diluted 1:10 in reaction buffer (mitotic dilution buffer (24) [10 mM HEPES-KOH pH 7.0, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT] supplemented with 0.1% CHAPS and 100 μg/ml BSA) containing the caspase substrate Ac-DEVD-AMC (Bachem) at a final concentration of 10 μM. Reactions were performed in triplicates in flat bottomed 96-well plates at 37° for one hour. Free AMC was then measured by determining fluorescence at 390 nm (excitation) and 460 nm (emission) in a Millipore Cytofluor 96-reader. Values were calculated by subtracting background fluorescence (buffer/substrate alone) and are presented as mean/three times standard error of the mean.

Assay for nuclear apoptosis. Analysis of nuclear apoptosis was carried out as described (25). Cells were washed in depletion medium and cultured at 2×10^5/200 μl depletion medium in triplicate cultures in flat bottomed 96-well plates. Various concentrations of oligomycin were added (in the experiment shown here concentration was 1.25 μM). To some samples, anti-Fas mAb was added one hour after the addition of oligomycin (100 ng/ml for Jurkat cells, 50 ng/ml for SKW6 cells), and culture was continued for five hours. Cells were then washed once in PBS, resuspended in 200 μl of staining buffer (0.1 M sodium citrate, 0.1% Triton-X-100, 50 μg/ml propidium iodide) and kept at 4°C overnight. Samples were then analyzed in a FACS-Calibur flow cytometer (Becton Dickinson).

RESULTS

Effect of oligomycin treatment on the appearance of caspase activity induced by staurosporine or anti-Fas signaling. In the absence of glucose in the culture medium, treatment with oligomycin leads to the depletion of cells for ATP (26, 27). To assess the effect of this treatment on the appearance of caspase-activity, Jurkat or SKW6 cells were cultured in the absence of glucose with 2.5 μM oligomycin for one hour. Then stauroporine was added and culture was continued for two more hours. Cells were then extracted and caspase-3-like activity was measured as the activity able to cleave the substrate DEVD-AMC in the extracts. As shown in Fig. 1, staurosporine induced DEVD-cleaving activity in both cell lines. Addition of oligomycin did not induce caspase-activity but showed the tendency to lower the basal level of this activity. When cells where pre-cultured with oligomycin, the capacity of staurosporine to induce caspase-activity was greatly reduced, in some experiments completely abrogated in both cell lines (Fig. 1 and data not shown).

In a similar setup, cells were pre-treated with oligomycin and the capacity of Fas-signaling to induce caspase-activity was investigated. As shown in Fig. 2

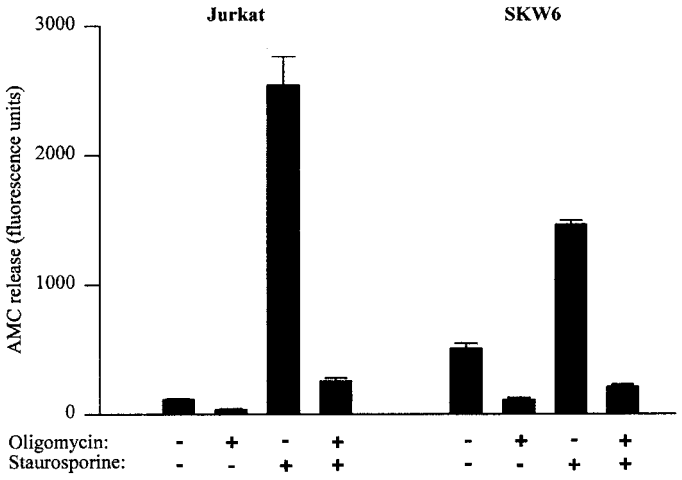

FIG. 1. Treatment with oligomycin prevents the appearance of staurosporine-induced DEVD-cleaving activity. Jurkat or SKW6 cells were cultured (10^6 cell in 1 ml) either in depletion medium alone or in the presence of 2.5 μM oligomycin as shown. After 1 h, staurosporine (1 μM for Jurkat cells; 2 μM for SKW6 cells) was added to some samples, and culture was continued for another 2 h. Cells were then collected by centrifugation and washed in PBS. Cell pellets were extracted and activity was determined as described under Materials and Methods. Columns show means, error bars 3× SEM of triplicate assays. Similar results were obtained in five independent experiments.

(left panel), oligomycin-treatment did not reduce the Fas-induced DEVD-cleaving activity in either cell line. In some experiments, there was even a tendency towards enhancement of this proteolytic activity in Jurkat cells (for example in the experiment shown in Fig. 2). To obtain additional evidence that cells still underwent apoptosis, cell nuclei were stained with propidium iodide and analyzed by flow cytometry. In accordance with the observed DEVD-cleaving activity, nuclei of oligomycin treated Jurkat cells did exhibit this sign of apoptosis and there was even a consistent increase in the percentage of cells with sub-G1-DNA staining (Fig. 2, right panel). For SKW6 cells, there was either no difference or a slight decrease in this percentage (Fig. 2).

In order to establish a correlation between ATP-depletion and reduction in staurosporine-induced caspase-activation, experiments were performed in which the concentration of oligomycin was titrated over a wide range. We had chosen the original micromolar ranges of oligomycin because this concentration had proved effective in inhibiting nuclear apoptosis in recent studies (26, 27). The concentration of oligomycin required to induce an almost complete depletion of cellular ATP-levels was much lower and lay between about 10 and 40 nM (Fig. 3). The effective concentration was similar for Jurkat and SKW6 cells. After one hour, depletion had reached a level which was stable over the next hour of incubation (Fig. 3).

The oligomycin concentration was then titrated over a similar range and the effect on the induction of caspase activity was investigated. As detailed in Fig. 4, the concentration required to reduce the staurosporine-induced DEVD-cleaving activity by half was between 10 nM and 50 nM (there was some variation within that range probably depending on the

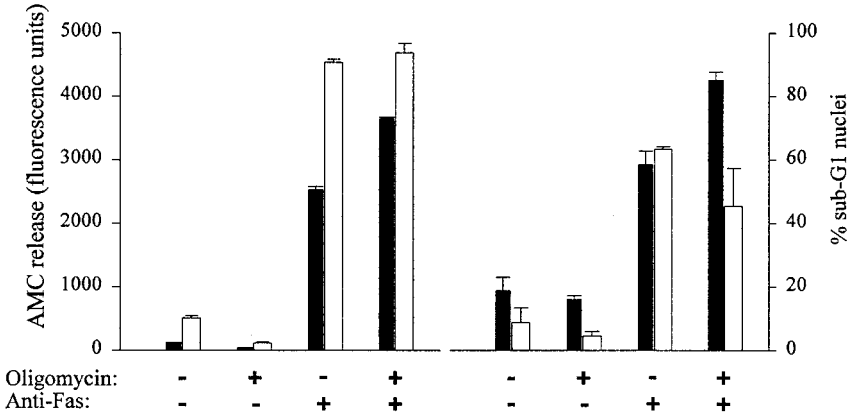

FIG. 2. Fas-induced caspase activation and appearance of sub-G1 nuclei are not inhibited by oligomycin. (Left) 10^6 Jurkat cells (filled bars) or SKW6 cells (open bars) were cultured in 1 ml depletion medium for 1 h either alone or in the presence of 2.5 μM oligomycin as indicated. After 1 h, anti-Fas mAb was added as indicated (100 ng/ml for Jurkat cells; 50 ng/ml for SKW6 cells), and culture was continued for another 2 h. Cells were harvested and DEVD-cleaving activity was measured as described under Materials and Methods. Data are given as mean (error bars are 3× SEM) of triplicate assays. Similar results were obtained in five independent experiments. (Right) 2×10^5 Jurkat cells (filled bars) or SKW6 cells (open bars) were cultured in triplicates in flat-bottom, 96-well plates in depletion medium either alone or in the presence of 1.25 μM oligomycin for 1 h. Anti-Fas mAb was added to some samples as indicated (the final concentrations were 100 ng/ml for Jurkat cells and 50 ng/ml for SKW6 cells), and culture was continued for an additional 5 h. Cells were permeabilized, stained and analyzed as described under Materials and Methods. Data are presented as mean and 3× SEM of the percentages of events with PI staining less than G1 staining (see also Fig. 6a). Similar results were obtained in three independent experiments.

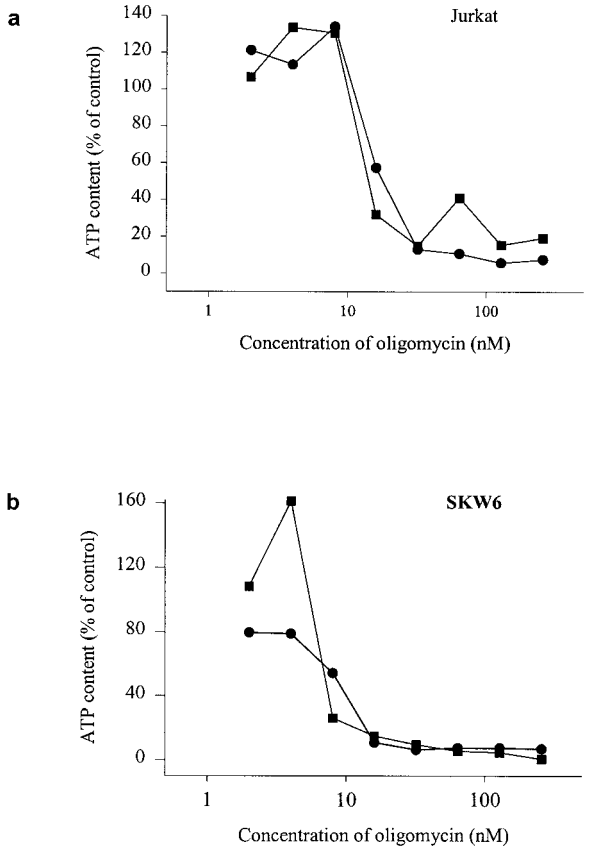

FIG. 3. Correlation of ATP content and oligomycin concentration. 10^6 Jurkat (a) or SKW6 (b) cells were cultured in 1 ml of depletion medium either alone or in the presence of various concentrations of oligomycin. After 1 (circles) or 2 (squares) h, 5×10^5 cells were collected and processed as described under Materials and Methods. ATP levels were determined in a luciferin–luciferase assay, and results are presented as percentage of untreated control [values are means of two measurements (single measurements in the SKW6 2-h series)]. Similar results were obtained in two independent experiments for each cell line.

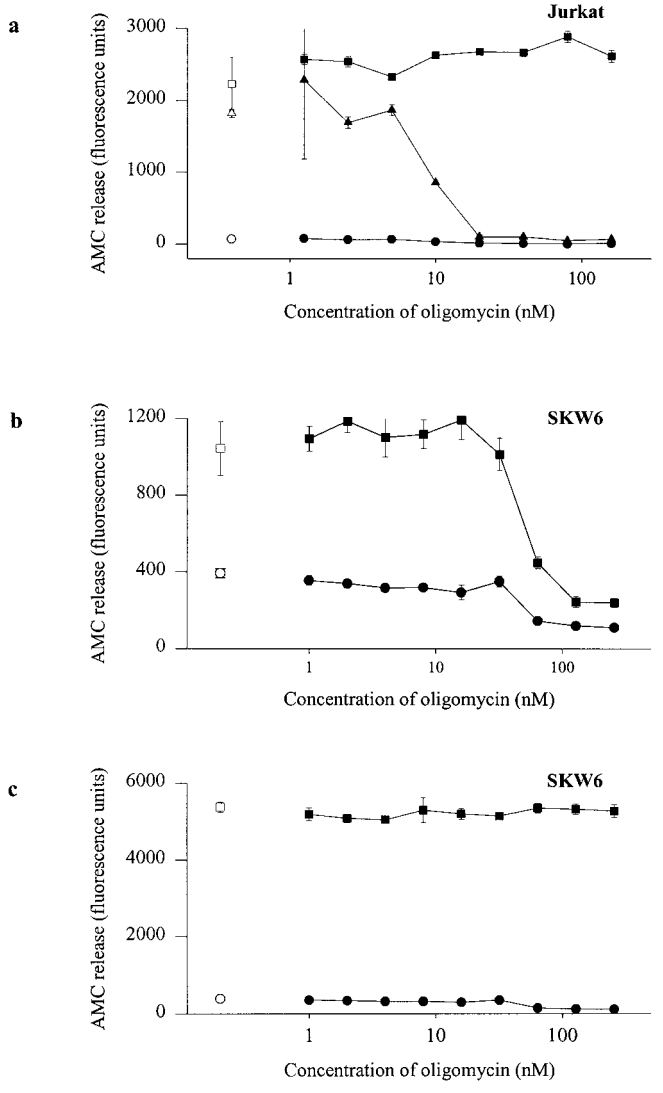

FIG. 4. Effect of various concentrations of oligomycin on the induction of DEVD-cleaving activity by staurosporine and Fas ligation. (a) Jurkat cells (10^6 cells/sample in 1 ml of depletion medium) were cultured either without (open symbols) or with various concentrations of oligomycin (filled symbols). After 1 h, one set of cells was left untreated (circles); to the other cultures, either anti-Fas mAb (100 ng/ml, squares) or staurosporine (1 μM, triangles) was added. Culture was continued for another 2 h, and cells were extracted and enzyme activity was measured as described under Materials and Methods. The data are presented as mean and 3× SEM of triplicate reactions. Similar results have been obtained in three independent experiments. (b and c) SKW6 cells were cultured for 1 h as described (a) with (filled symbols) or without (open symbols) oligomycin in various concentrations as indicated. To some samples, anti-Fas mAb (50 ng/ml) or staurosporine (2 μM) was added, and enzyme activity was measured 2 h later. (b) Circles, no apoptosis stimulus; squares, staurosporine. (c) Circles, no stimulus; squares, anti-Fas mAb. The data shown in (b) and (c) are taken from the same experiment and are presented in two diagrams because of the difference in activity induced by staurosporine and anti-Fas treatment. The data are presented as mean and 3× SEM of triplicate reactions. Similar results were obtained in three independent experiments.

condition of the cells) for both Jurkat (Fig. 4a) and SKW6 (Fig. 4b and c) cells. The capacity of a Fas-signal to induce such activity was unaffected over the entire concentration range (Fig. 4).

It is well established that staurosporine causes apoptosis in a wide variety of cell lines and there is compelling evidence that this process involves the activation of caspases (1). Recent work further suggests that this caspase-activation is initiated by cytochrome c after it has been liberated from its normal localization in the mitochondria into the cytosol. The following experiments were devised to determine at which step the inhibition of caspase-activation by oligomycin takes place.

Electroporation of cytochrome c into cells leads to caspase activation and apoptosis in normal but not in oligomycin-treated cells. A recent report demonstrates that electroporation of cytochrome c protein

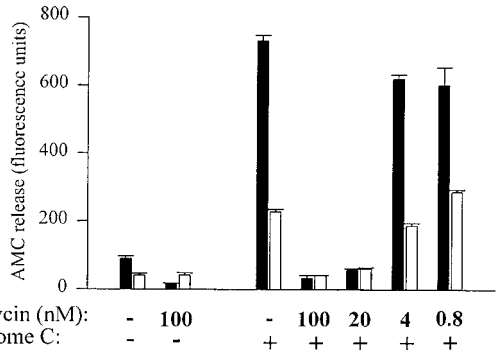

FIG. 5. Oligomycin treatment prevents the induction of DEVD-cleaving activity induced by electroporation with cytochrome c. Jurkat (filled bars) or SKW6 (open bars) cells were cultured in depletion medium either without or with various concentrations of oligomycin as indicated. After 1 h, cells were collected and electroporated in the absence or presence of cytochrome c as described under Materials and Methods. Cells were then taken back into culture and incubated for 1 h before they were extracted and DEVD-cleaving activity was determined as above. Similar results have been obtained in three independent experiments with each cell line.

into intact cells leads to apoptosis as measured by the appearance of nuclei with sub-diploid DNA-staining (6). It appears plausible to assume that the mechanism of this is the Apaf-1-dependent caspase-9/caspase-3 activation and subsequent apoptosis as it had been demonstrated earlier in cell extracts (4). We used this experimental setup to activate the apoptosis machinery in cells that had been pre-treated with oligomycin.

Jurkat and SKW6 cells were cultured in depletion medium in the presence of various concentrations of oligomycin for one hour. Cells were then electroporated in the absence or presence of cytochrome c protein and taken back into culture. Two events were further analyzed in these cells: After one hour, protein extracts were prepared to measure caspase- activity as above. After four hours, cells were permeabilised, DNA was stained with propidium iodide (PI) and apoptosis was quantified by flow cytometric analysis. As shown in Fig. 5, mock transfection of cells (i.e. electroporation under the same conditions but in absence of cytochrome c) induced little caspase-activity in either Jurkat or SKW6 cells. Electroporation in the presence of cytochrome c, however, led to significant caspase-activity in both Jurkat and SKW6 cells. When cells had been pre-treated with oligomycin, caspase-activation was suppressed in a dose-dependent manner (Fig. 5). The concentration of oligomycin required to render cells unresponsive to cytochrome c-electroporation was in a similar range as the concentration needed for ATP-depletion and to abolish staurosporine-induced caspase-activation (see Figs. 3 and 4). This is in agreement with the interpretation that both mechanisms of caspase-activation rely on the same molecular events, i.e. that staurosporine treatment leads to cytochrome c-release which acts in the same way as cytochrome c introduced by electroporation.

When nuclear apoptosis was measured in electroporated Jurkat cells, a similar picture was seen. Cells electroporated in the presence of cytochrome c displayed an increased percentage of apoptotic nuclei compared to control transfected cells (Fig. 6a). When the cells had been pre-treated with oligomycin, cytochrome c did not induce nuclear fragmentation (Fig. 6b). Similar experiments were performed in SKW6 cells but in five experiments like the one shown for Jurkat cells (Fig. 6), only in one experiment significant cytochrome c-induced nuclear fragmentation was observed (data not shown). We think it most likely that a lower percentage of SKW6 cells was transfected and that therefore not enough cells reacted with fragmentation of their DNA. Overall, the induction of caspase-activity was lower when cells were cultured and electroporated in depletion medium than when experiments were performed in complete culture medium (data not shown). This is probably explained by the less than optimal conditions that had to be used such as low protein concentrations.

Taken together, these data demonstrate that by ATP-depletion conditions can be generated under which cytochrome c is incapable of inducing caspase-activation and apoptosis. Activation of caspases by a Fas-signal, however, is not noticeably altered under these conditions. Thus, cytochrome c appears to be dispensable for Fas-induced apoptosis.

DISCUSSION

In this study evidence is presented that cytochrome c is dispensable for Fas-triggered caspase-activation and apoptosis. Treatment of cells with oligomycin efficiently blocked cytochrome c-mediated caspase-activation in two cell lines. Fas-induced caspase-activation, however, was unaffected under these conditions.

Over the past few years it has become clear that a conserved cell death pathway exists which is responsible for the implementation of both developmental cell death and physiological cell death in an adult organism (28). Although under experimental conditions exceptions can be demonstrated, members of the caspase family of cysteine proteases are likely to participate in at least the vast majority of cases of naturally occurring cell death (1). Based on this concept, much attention has been focused on the study of the initiation of caspase activity following an apoptotic stimulus. An apparently almost unlimited number of chemical substances and stimuli can induce caspase activity and apoptosis in a responsive cell.

A more recent line of research implicates cytochrome c in the activation of caspases and proposes this protein for the role of common mediator of caspase-activation.

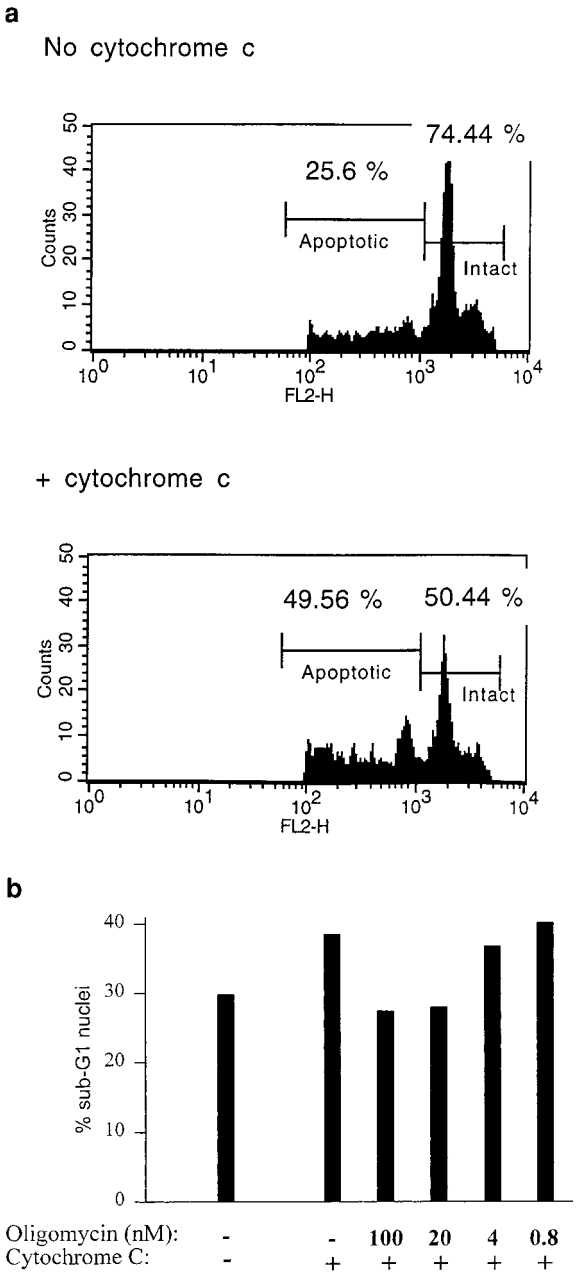

FIG. 6. Oligomycin treatment prevents the appearance of sub-G1 nuclei induced by electroporation with cytochrome c. (a) Flow cytometric profile of Jurkat cells after electroporation in the absence (upper panel) or the presence (lower panel) of cytochrome c. Cells were electroporated and taken back into culture. After 4 h, cells were permeabilized, stained and analyzed by flow cytometry. See Materials and Methods for details of electroporation and analysis procedures. Debris was gated out. The percentages of events with normal DNA content or reduced staining are given. (b) Percentage of cells with sub-G1 staining following electroporation. Jurkat cells were cultured in depletion medium either in the absence of oligomycin or in the presence of various concentrations of oligomycin as indicated for 1 h and then electroporated in the absence or presence of cytochrome c as indicated. After 4 h, cells were analyzed in (a). Data are percentages of cells in the sub-G1 gate as in (a). Similar results were obtained in three independent experiments.

Cytochrome c can, in concert with Apaf-1, activate caspase-9 which subsequently can process and activate caspase-3 (4). Additionally, cytochrome c has been demonstrated to be released from mitochondria during some forms of apoptosis (7, 14).

Earlier studies have found that ATP was required for apoptosis and depletion of cells for ATP has been proposed to convert apoptosis into necrosis (26, 27, 29) but the molecular events responsible were unclear. Recent work suggests that at least one step which requires ATP is the Apaf-1-mediated caspase activation.

In cell extracts, both dATP and ATP can in concert with cytochrome c trigger caspase activation (4). Since Apaf-1 contains an ATP-binding site which has been reported to be important for its function (4, 30), it is likely that an ATP-dependent function of Apaf-1 such as a conformational change is necessary for its function and that caspase-activation is blocked at this stage in the absence of ATP. In extracts, dATP appears to exert the same role as ATP, as the addition of an excess of an uncleavable ATP-analogue also inhibits the dATP-induced caspase activation and both dATP and ATP are hydrolysed by Apaf-1 (reference 31 and our unpublished results). Therefore, it is at present reasonable to assume that the ATP-dependent step in the pathway to caspase activation lies with Apaf-1.

In one recent study it was reported that in the presence of oligomycin the ability of Fas to activate DEVD-cleaving caspases was much reduced (26). These observations are in obvious conflict with our results and we are not certain what the reason for this discrepancy is. It is possible that different cell lines—or even subclones of the same line—have acquired defects which might cause such a phenotype. It is also possible that preparations of oligomycin might have side effects other than the inhibition of the F0/F1-ATPase which again might vary according to the cell type used. Additionally, the very high concentration of 10 μM used in that study (26) may have contributed to such side effects; in our hands, the oligomycin concentrations required for both ATP-depletion and inhibition of staurosporine-induced caspase-activation were about 1000-fold lower (see Results section).

The Fas-triggered pathway to cell death has been extensively studied, and a model has been established which is able to cover most of the distance between ligand binding and apoptotic phenotype. Oligomerisation and conformational changes recruit a complex consisting of the molecules FADD/MORT-1, FLASH and caspase-8 to the receptor. Individual caspase-8-molecules activate each other and, subsequently, activate "effector" caspases such as caspase-3 (8, 9, 32). However, recent work has suggested that cytochrome c is involved in fas-triggered cell death.

A number of studies have found that cytochrome c is released from mitochondria during Fas-induced apoptosis (12, 13), and a molecular mechanism has been proposed by which this could occur: Caspase-8 can cleave the protein Bid and one of the cleavage products is capable of inducing release of cytochrome c from mitochondria (17, 18) suggesting that caspase-8 does not directly activate caspase-3 but does so via cytochrome c-mediated activation of caspase-9. We and others have found that cytochrome c-release is at least not an early event during Fas-induced cell death (15, 16) and investigations of activation rates have suggested that caspase-8 is well capable of directly activating caspase-3 (33). Furthermore, Bid-induced cytochrome c-release from mitochondria could be inhibited by Bcl-2 (17) which indicates that, at least in cases where Bcl-2 does not inhibited Fas-induced cell death, Bid does not contribute to apoptosis.

In this study, we found that the Fas-induced caspase-activity was unaffected by pre-incubation with oligomycin in both cell lines used and over a wide range of oligomycin-concentrations, indicating that caspases themselves do not require ATP for their proteolytic activity. Moreover, a recent report demonstrated that in the absence of ATP Fas-ligation still led to the processing of pro-caspase-3 (13). We therefore believe that the evidence is very strongly favoring a model in which Fas-signaling can induce normal caspase-activation and -activity in the absence of ATP.

Downstream of caspases, some "post-mortem" events of apoptosis undoubtedly do require ATP. Changes in nuclear morphology and "ladder-like" DNA-fragmentation was greatly reduced in Fas-induced cell death by ATP-depletion (26, 27) and our own unpublished observations). The reduction in PI-uptake, however, which leads to the appearance of the "sub-G1-peak" on a FACS-profile characteristic of apoptosis, is not or only slightly affected (13) and see above). All of these events of nuclear apoptosis occur in parallel during normal apoptosis (25) and sub-G1 cells have been thought to represent nuclei with less than normal DNA, a consequence of internucleosomal DNA-cleavage. A recent study, however, shows that such sub-G1 cells can appear in the absence of apoptotic DNA-"laddering" (34), and the events of DNA-fragmentation and appearance of sub-G1-nuclei may therefore, although normally linked, result from different molecular mechanisms.

It has been found that different cell lines react in a different fashion to a Fas death-signal; while so-called "type I" (such as SKW6 cells) cells immediately activate caspases following Fas-engagement, this process is slower in "type II" cells (such as Jurkat cells). We used these two cell lines which have been reported to cover both known phenotypes of Fas-responders (12) and obtained the same results in both lines, i.e. normal appearance of caspase-3-like activity after ATP-depletion.

In "type I" cells such as Jurkat Bcl-2 does inhibit Fas-induced apoptosis (12). This could mean that in Jurkat cells other factors are required to pass on a Fas-signal. We found that in both cell types as exemplified with Jurkat and SKW6 cells, Fas can activate caspases under circumstances when cytochrome c does not have this capacity, i.e. in the absence of ATP. This indicates that cytochrome c is not this mediator which distinguishes Jurkat from SKW6 cells.

A wealth of data has accumulated to propose that cytochrome c participates during drug-induced apoptosis. There is, however, no evidence for such a role for cytochrome c during programmed cell death (PCD) during development; a recent study was even able to demonstrate that cytochrome c was not released during PCD in Drosophila and that visible changes to mitochondria in that model occur downstream of caspases (35). There is evidence that PCD was the evolutionary benefit which drove the evolution of a cell death pathway (28). It might therefore been argued that activation of this system by cytochrome c is a more recent adaptation of higher organisms and that, possibly, death receptors trigger a shortcut to activation of the ancient system.

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