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Inhibition of Actin Polymerization Enhances Commitment to and Execution of Apoptosis Induced by Withdrawal of Trophic Support

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Abstract We have previously shown, using jasplakinolide, that stabilization of the actin cytoskeleton enhanced apoptosis induced upon cytokine withdrawal (Posey and Bierer [1999] J. Biol. Chem. 274:4259–4265). It remained possible, however, that a disruption in the regulation of actin dynamics, and not simply F-actin stabilization, was required to affect the transduction of an apoptotic signal. We have now tested the effects of cytochalasin D, a well-characterized agent that promoted actin depolymerization. Actin depolymerization did not affect CD95 (Fas)-induced death of Jurkat T cells in the time course studied but did enhance the commitment to cytokine withdrawal-induced apoptosis of factor-dependent cell lines. The induction of cell death was not the result of direct cytoskeletal collapse, since treatment of the cells with cytochalasin D in the presence of IL-2 did not promote death. As with jasplakinolide, the enhancement of commitment to apoptosis could be delayed by overexpression of the anti-apoptotic protein Bcl-x_L, but, unlike jasplakinolide, cytochalasin D modestly affected the ''execution'' stage of apoptosis as well. Taken together, these results suggest that changes in actin dynamics, i.e., the rate of actin polymerization and depolymerization, modulate the transduction of the apoptotic signal committing lymphocytes, withdrawn from required growth factors, to the death pathway. J. Cell. Biochem. 88: 1066–1076, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: apoptosis; Cytochalasin D; actin; cytokine-withdrawal

The process of apoptosis, a form of programmed cell death, is characterized by cell morphological changes, membrane blebbing, and internucleosomal DNA cleavage [Wyllie et al., 1980]. Apoptosis is commonly divided into two phases, the initial "commitment" phase and the downstream "effector" phase [Cohen, 1997]. The molecular mechanisms and signaling pathways by which a cell commits to apoptotic death is specific to the death-inducing stimulus. For instance, CD95 (Fas)-induction of apoptosis [Chinnaiyan and Dixit, 1997] proceeds and is regulated by molecular mechanisms quite different from that of cytokine deprivation [Sarin et al., 1996; Deshmukh and Johnson, 1997]. Commitment results in activation of member(s) of a family of cysteinyl proteinases termed caspases. Once a cell has committed to apoptosis and caspases are activated, a relatively uniform program of apoptosis ensues, resulting in characteristic morphological changes of apoptosis and ultimately cell death [Cohen, 1997].

The actin cytoskeleton has been thought to be important for progression of the execution phase of apoptosis [Cotter et al., 1992; Mashima et al., 1999]. Actin has been shown to be involved in membrane blebbing, in which a fine F-actin ring delineates the bleb [Huot et al., 1998].

Abbreviations used: cRPMI-10%, complete RPMI media plus 10% FBS; PI, propidium iodide; TUNEL, terminal deoxynucleotide transferase-mediated nick end labeling; ICE, interleukin 1β-converting enzyme.

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Drug-induced inhibition of bleb formation, however, did not inhibit stress-induced apoptotic death, demonstrating that membrane blebs are not critical to the cell death pathway [Huot et al., 1998]. One substrate of caspase 3, a caspase central to the effector phase of apoptosis, is actin [Kayalar et al., 1996; Brown et al., 1997; Guenal et al., 1997; Mashima et al., 1997]; cleavage of endogenous actin may herald cytoskeletal collapse, nuclear condensation, and DNA fragmentation. Further, the subcellular localization of certain death effector molecules, including caspase-3 itself, appears to be dependent upon actin integrity [Suria et al., 1999].

In addition, the actin cytoskeleton plays a role in the initiation of apoptosis of adherent cells that have been forcibly detached. The actin cytoskeleton has been shown to be involved in maintaining the adhesion of anchorage-dependent cells to the extracellular matrix. Disruption of adhesion leads to a form of cell death, termed anoikis, which proceeds by apoptosis [Frisch and Ruoslahti, 1997]. While anchorage-dependent cells have been well analyzed, few studies have examined the involvement of the actin cytoskeleton in the induction of apoptosis of non-adherent cells.

We previously used jasplakinolide, a cell-permeant agent that stabilizes the actin cytoskeleton, to demonstrate that F-actin stabilization regulated the cell death-signaling pathway in suspension cells [Posey and Bierer, 1999]. Actin stabilization enhanced the commitment to cell death upon cytokine withdrawal from CTLL-20 cells, a murine IL-2-dependent cell line. These studies left open the possibility that any modulation of actin homeostasis (dynamics), and not just F-actin stabilization, could affect the cell death program. Here we have examined the effect on apoptosis of cytochalasin D, used to promote actin depolymerization. The addition of the actin-depolymerizing compound cytochalasin D to CTLL-20 cells, upon IL-2 deprivation, enhanced the rate at which the cells underwent apoptosis in a time- and concentration-dependent manner. No effect of cytochalasin D was observed on CD95 (Fas)-induced Jurkat cell death, suggesting that not all apoptotic signaling pathways are sensitive to actin modulation. Overexpression of the anti-apoptotic protein Bcl-x_L did not fully abrogate the effect of cytochalasin D, as it did the effect of jasplakinolide. Taken together, these data suggest that modification of the actin cytoskeleton affects both the execution phase and the early signal transduction pathways leading to apoptosis. We suggest that redistribution of the actin cytoskeleton dynamically regulates apoptosis, such that an alteration in the distribution of F- to G-actin, achieved either by actin stabilization or depolymerization, affects the sensitivity to the cell death signal.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Reagents

The human leukemia T cell line Jurkat was grown in RPMI-1640 (MediaTech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (Sigma, Chemical Company, St. Louis, MO), 10 mM HEPES (Sigma), 2 mM L-glutamine (MediaTech), 100 U/ml penicillin, and 100 μg/ml streptomycin (MediaTech) and 50 μM 2-mercaptoethanol (Sigma) (termed complete RPMI-10%, or cRPMI-10%). The IL-2-dependent murine T cell line CTLL-20 [Gillis and Smith, 1977] and the IL-3-dependent murine pro-B cell line BaF/3 were grown in complete RPMI-10% supplemented with 1–2% IL-2-containing supernatant derived from concanavalin A-stimulated rat splenocytes or 5% IL-3-containing Wehi-conditioned media. For CTLL-20 experiments, cells were expanded in cRPMI-10% supplemented with 100 U/ml rhIL-2 for 48 h prior to an experiment. A CTLL-2 clone transfected with pSFFVNeo-*bcl-*x_L and consequently overexpressing the Bcl-X_L protein (Bcl-x_L-CTLL-2) (a kind gift from Dr. Craig Thompson, University of Pennsylvania Cancer Center, Philadelphia, PA) [Boise et al., 1996] was grown in cRPMI-10% supplemented with 100 U/ml rhIL-2. Western blot analysis confirmed overexpression of the Bcl-X_L protein (data not shown). All cell lines were free of mycoplasma as determined by PCR analysis (Mycoplasma PCR Primers; Stratagene, La Jolla, CA).

For apoptosis assays, CTLL-20 or Ba/F3 cells were washed three times in cRPMI-10% and resuspended at a final density of 2×10^5 cells/ml in cRPMI-10% without growth factor supplementation to which either cytochalasin D (Sigma) or vehicle (DMSO, final concentration 0.02–0.1%, as indicated) had been added. Washed CTLL-20 cells resuspended in cRPMI-10% with growth factor rhIL-2 (100 U/ml) served as controls. Cytochalasin D was stored in DMSO at −20°C and diluted into media

immediately prior to use. Recombinant human IL-2 (rhIL-2) was kindly provided by Hoffman-LaRoche (Nutley, NJ). CD95 (Fas)-induced cell death was induced by treatment of washed Jurkat cells with anti-CD95 (Fas) mAb 7C11 (Upstate Biotechnologies, Lake Placid, NY) for the indicated periods of time.

Immunofluorescent Staining and Microscopy

Cells (1×10^6/sample) were adhered to glass coverslips coated with poly-L-lysine, incubated with either cytochalasin D or vehicle as indicated, then fixed in 3.7% paraformaldehyde. Cells were permeabilized with 0.1% Triton-X-100 in PBS for 2.5 min, and stained with phalloidin conjugated to Texas Red (Molecular Probes, Eugene, OR). Samples were mounted in ProLong Antifade (Molecular Probes) and visualized with an Axioskop MC80 DX (Carl Zeiss, Inc., Thornwood, NY) equipped with a halogen light. Images were captured using a SPOT32 CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and imported into Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).

Quantitation of F- and G-Actin

The method was performed essentially as described [Watts and Howard, 1992; Watts et al., 1995]. Cells (1×10^6/sample) incubated at RT with 1 μM cytochalasin D or 0.1% DMSO were either (1) fixed and then permeabilized for the determination of either total F-actin or G-actin or (2) permeabilized and then fixed for the determination of Triton-insoluble F-actin. Permeabilization buffer contained 10 mM imidazole (pH 7.2), 40 mM KCl, 10 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Fixation buffer contained 3.7% formaldehyde in 1× PBS. For determination of either total or insoluble F-actin, samples were incubated with 1 U Oregon-Green conjugated phalloidin (Molecular Probes) for 2 h at RT in the dark. For determination of G-actin, samples were incubated with 5 μg DNase I-FITC (Molecular Probes) for 2 h at RT in the dark. Phalloidin was extracted by overnight incubation in methanol. Fluorescence of each sample was determined by reading in a fMax fluorimetric plate reader (Molecular Devices, Sunnyvale, CA). Samples prepared as above with the exclusion of the addition of fluorescent stain were used as autofluorescence controls.

Assessment of Apoptosis

A number of different methods were used to assess induction of apoptosis. *Hoechst staining* was performed following the procedures described in Posey and Bierer [1999]. The 95% confidence interval for each sample within each experiment was determined according to the statistical definition of the variance of a proportion in a binomial experiment. The variance was computed as pq/n, where $p =$ the proportion apoptotic, $q = 1-p$, and $n =$ the number of cells counted. *DNA content analysis by flow cytometry* was determined as described [Cifone et al., 1993] with slight modifications. Briefly, cells (2×10^5/sample) were pelleted and resuspended in 100 μl of a hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100 μg/ml PI) with RNase added (2.5 μg/sample) and incubated for 30 min at 37°C. Fluorescence of each sample was analyzed by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA). The cytometer was equipped with an argon laser that emitted at a wavelength of 488 nM; fluorescence of PI was measured at 585 nM. Data were gated to exclude debris and cell aggregates and analyzed using the software packages Lysys, CellQuest (Becton Dickinson) or WinMDI Version 2.1.4 (J. Trotter, Scripps Institute, San Diego, CA). Events that fell below the G_0/G_1 peak were considered apoptotic and are enumerated. Terminal deoxynucleotide transferase-mediated nick end labeling (TUNEL) staining was performed with the Oncor (Gaithersburg, MD) ApopTag Plus kit according to the manufacturer's instructions with modifications [Posey and Bierer, 1999]. Fluorescence of each cell sample stained with fluorescein (FITC)-conjugated anti-digoxigenin antibody was measured by flow cytometry (FACScan; Becton-Dickinson) at a wavelength of 535 nM and the data analyzed using CellQuest. Data were gated based on forward and side scatter to exclude cell debris; M1 regions of FITC-bright cells (and therefore considered apoptotic) were selected for each histogram based on the background levels of staining within each sample. *Analysis of DNA fragmentation* was demonstrated as described [Bierer et al., 1991] with minor modifications. Cells (1×10^6/sample) were washed once with phosphate-buffered saline (PBS), resuspended in 20 μl lysis buffer (0.5% (w/v) sodium lauroyl sarkosinate, 10 mM EDTA pH 8, 50 mM Tris

pH 8, 0.5 μg/ml proteinase K), and incubated at 56°C for 1–3 h. RNase (5 μg/sample) was added for an additional hour of incubation. An equal volume of bromophenol blue loading dye was added to each lysate, and samples were heated to 65°C for 10 min. Lysates were electrophoresed on a 2% TAE agarose gel; DNA was visualized by ethidium bromide staining and photographed using the Eagle Eye system (Stratagene).

RESULTS

Cytochalasin D Promoted Disruption and Depolymerization of Actin in CTLL-20 and Jurkat T Cells

Although commonly assumed to promote actin depolymerization, cytochalasin D has been reported to nucleate actin filments and thereby promote actin polymerization in some cell types under certain conditions [Goddette and Frieden, 1986; Wilder and Ashman, 1991; Franki et al., 1992]. To confirm that cytochalasin D functioned to disrupt and depolymerize actin filaments in CTLL-20 and Jurkat T cells under conditions used in the experiments presented here, CTLL-20 and Jurkat T cells were incubated with either 1 μM cytochalasin D or the vehicle, 0.1% DMSO, stained with phalloidin conjugated to Texas Red, and visualized with fluorescence microscopy (Fig. 1 and data not shown). In both Jurkat (Fig. 1) and CTLL-20 (data not shown) T cells, treatment with cytochalasin D resulted in the disruption of cortical actin filaments. To confirm that the disruption of actin filaments visualized by microscopy correlated with actin depolymerization, Jurkat T cell lysates treated with either cytochalasin D or DMSO were incubated with either Oregon Green-conjugated phalloidin or FITC-conjugated DNase I. Incubation with cytochalasin D increased the relative amount of DNase I staining and decreased the relative amount of phalloidin binding (data not shown), indicating an increase in the amount of G-actin and a decrease in the amount of F-actin. Thus, in Jurkat and CTLL-20 T cells, the concentration and conditions of cytochalasin D used in the following experiments promoted actin filament disruption and depolymerization.

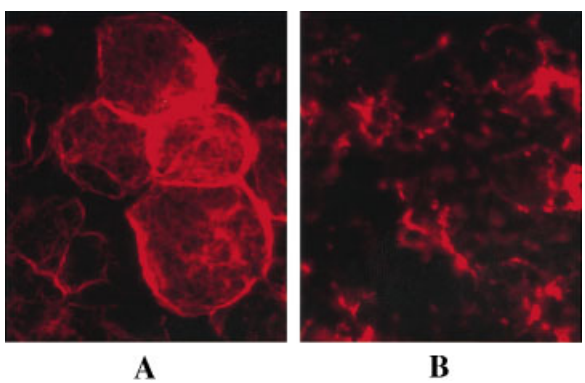

Fig. 1. Treatment with cytochalasin D disrupted actin filaments in Jurkat T cells. Jurkat T cells were incubated in RPMI with either 0.1% DMSO (**A**) or cytochalasin D (**B**) for 1 h at room temperature. Cells were fixed in paraformaldehyde, permeabilized, stained with phalloidin conjugated to Texas Red, and visualized using fluorescence microscopy as described. Images are representative of at least three independent experiments.

Cytochalasin D Enhanced Apoptosis Induced Upon IL-2 Deprivation of CTLL-20 Cells

We have previously observed that actin stabilization by treatment with jasplakinolide enhanced the commitment to cell death upon IL-2-withdrawal of cytokine-dependent cells. We wished to examine whether depolymerization of the actin cytoskeleton would affect apoptosis of cells deprived of required growth factor. CTLL-20 cells were cultured in the presence or absence of rhIL-2 with either cytochalasin D or vehicle (DMSO) control. After 15 h, cells were harvested and the percentage of apoptotic cells was quantitated using fluorocytometric analysis of DNA content (Fig. 2). The addition of cytochalasin D increased the percentage of sub G0/G1 events, interpreted as apoptotic cells [Darzynkiewicz et al., 1992; Ormerod et al., 1993] in the cells deprived of IL-2, but did not cause the death of cells maintained in IL-2. CTLL-20 cells withdrawn from IL-2 in the presence of cytochalasin D or DMSO exhibited the characteristic fragmentation of DNA into 180-bp multimeric ladders, while cells maintained in rhIL-2 in the presence of drug or vehicle did not (data not shown). These data demonstrate that actin depolymerization by cytochalasin D increased apoptosis induced by cytokine deprivation, but does not induce cell death in cells treated with cytochalasin D and maintained in growth factor.

Data obtained in additional independent experiments confirmed that the percent of apoptosis in cytochalasin-treated, IL-2 deprived CTLL-20 cells was greater than that of DMSO-treated, IL-2-deprived cells at multiple timepoints (Table I). There was some variability in

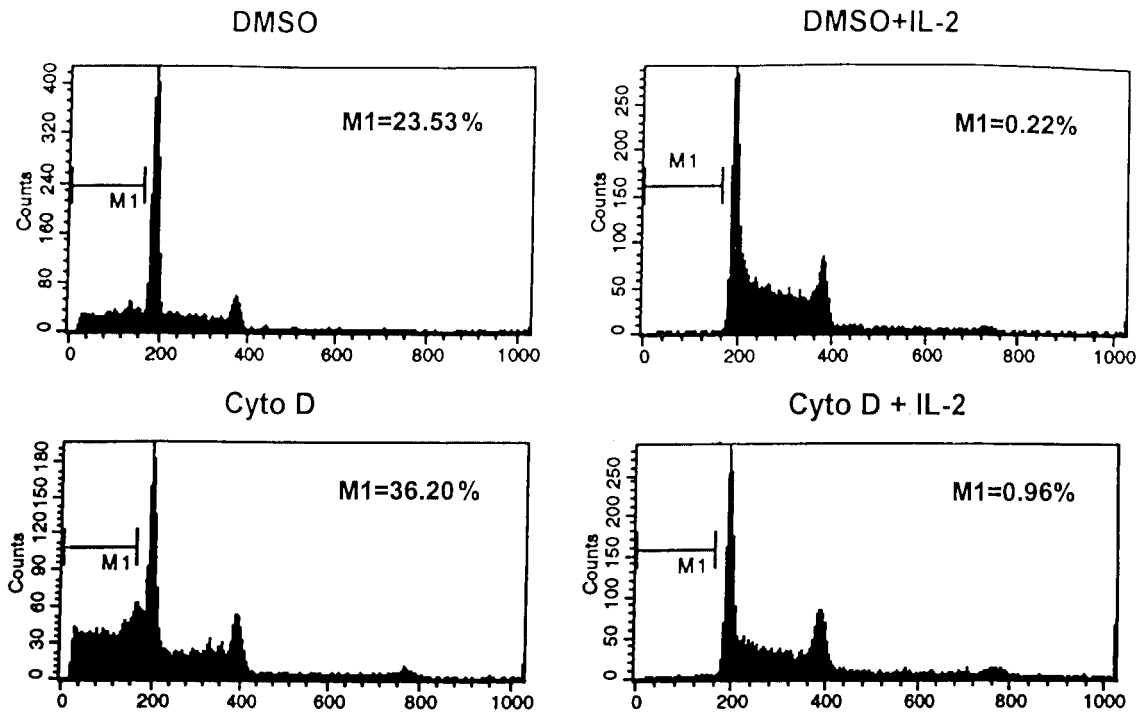

Fig. 2. Treatment with cytochalasin D increased the percentage of CTLL-20 cells that have undergone apoptosis following cytokine withdrawal. CTLL-20 cells were treated with 0.1% DMSO or 1 μM cytochalasin D (Cyto D) upon withdrawal from IL-2 (Left). Cell populations similarly treated with drug or vehicle were maintained in 100 U/ml rhIL-2 (Right). All cells were harvested after incubation for 15 h. Percent apoptotic cells was quantitated by analysis of DNA content, as analyzed by PI staining and flow cytometry, described in Materials and Methods. The marker M1 indicates the position of the "sub-G_0/G_1" peak; the percent apoptotic cells (M1) is given above the marker. Data are representative of three independent experiments.

the degree to which actin depolymerization enhanced apoptosis. That variability depended in part upon the assay method and duration of cytokine deprivation; importantly, although the absolute numbers varied, the direction of change was consistent. Concentrations of cytochalasin D of 0.5 μM or greater were required to increase the percentage of apoptotic cells observed above control levels (data not shown). In all assays, cell death was prevented by the addition of sufficient concentrations of rhIL-2 (see, e.g., Fig. 2; Table I).

Actin Depolymerization Modified Growth Factor Requirement for Survival

To determine whether modulation of the actin cytoskeleton increased the threshold of IL-2 required to maintain viability, CTLL-20 cells were incubated with varying concentrations of cytochalasin D (1, 3, or 5 μM) or DMSO (0.02%) for 24 h in the presence of varying concentrations of rhIL-2 (Fig. 3). Actin depolymerization increased the sensitivity of CTLL-20 cells to deprivation of IL-2; at insufficient concentrations (1 U/ml or less) of rhIL-2, addition of cytochalasin D enhanced the apoptotic response. These results imply either that the modulation of the actin cytoskeleton increased the IL-2 requirement for survival or that it accelerated an apoptotic program once such a program had been initiated. Additionally, these data indicated that the enhancement by cytochalasin D of apoptosis induced by cytokine withdrawal was concentration dependent, as the percentage of apoptotic cells in the sample treated with 5 μM cytochalasin D was greater than that of those treated with either 1 or 3 μM cytochalasin D (Fig. 3).

Cytochalasin D Affected Cell Death Induced by Cytokine Deprivation but not by CD95 (Fas)-Ligation

To determine whether the effect of cytochalasin D was specific to CTLL-20 cells or to IL-2 dependence, we treated IL-3-dependent Ba/F3 cells with cytochalasin D or vehicle control, in the absence or presence of IL-3. Cytochalasin D, in a concentration-dependent fashion, enhanced apoptosis of Ba/F3 cells following IL-3 deprivation (Fig. 4). The concentration dependence

TABLE I. Enhancement of apoptosis by treatment of CTLL-20 cells with cytochalasin D

Experiment	Method of quantitation of apoptosis	Duration of IL-2 withdrawal (h)	Treatment	Apoptosis (%)	
				No IL-2	IL-2 added
1	Hypotonic PI	12.5	DMSO	7.1	0.44
			Cyto D	20.8	0.61
2	Hypotonic PI	15	DMSO	10.0	1.6
			Cyto D	22.1	ND
3	Hypotonic PI	16	DMSO	46.2	0.27
			Cyto D	70.5	0.54
4	TUNEL	15.5	DMSO	13	4.0
			Cyto D	25.8	3.9
5	TUNEL	15	DMSO	42.4	0.49
			Cyto D	58.14	0.51

of the enhancement of apoptosis by cytochalasin D was similar between CTLL-20 and Ba/F3 cells (Fig. 3,4 and data not shown). However, cytochalasin D treatment did not alter CD95 (Fas)-induced cell death in a CD95-sensitive Jurkat T cell line at any time point (Fig. 5). These data suggest that the enhancement of apoptosis induced by actin modulation with cytochalasin D may be specific to the apoptotic stimulus; that is, cytochalasin D can accelerate commitment to or sensitize cells to apoptosis induced by cytokine deprivation, though not by Fas ligation.

Actin Depolymerization Shortened the Time of Commitment to Apoptosis

We have previously shown that actin stabilization affected the time of commitment of cells to apoptosis [Posey and Bierer, 1999], while others have shown that actin depolymerization affected not the induction, but the execution phase of apoptosis [Ghibelli et al., 1995; Huot et al., 1998; Rao et al., 1999]. Commitment to apoptosis, that is, the time at which cells could no longer be rescued from the cell death program, is readily assessed in this system as cytokine can be added back to the cultures at varying times after deprivation. Therefore, CTLL-20 cells were washed and deprived of IL-2 in the presence or absence of cytochalasin D; at varying times following deprivation, 100 U ml^{-1} rhIL-2 was added back to cultures. The percent of cells that could not be rescued from apoptosis by the readdition of rhIL-2 at a given timepoint was quantified at 25 h (Fig. 6). If rhIL-2 was added back at time 0, no apoptosis was observed. Cells could be rescued by readdition of rhIL-2 within 4–6 h of deprivation, although

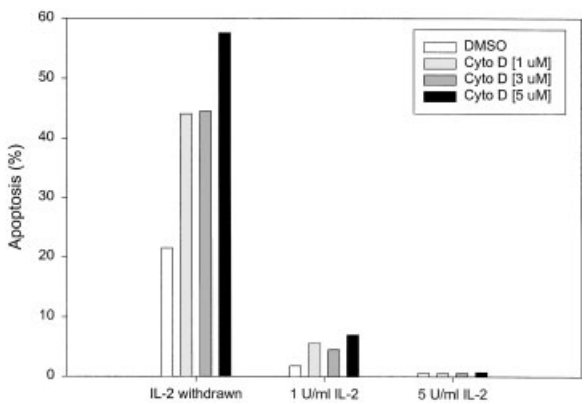

Fig. 3. Cytochalasin D sensitizes CTLL-20 cells to apoptosis at suboptimal concentrations of rhIL-2. CTLL-20 cells were incubated with 0.02% DMSO (open bars) or 1 μM (light gray), 3 μM (dark gray), or 5 μM (black) cytochalasin D, in media alone or rhIL-2 at the indicated concentration for 19 h. Data shown are percent apoptotic cells determined by Hoechst staining and are representative of two independent experiments.

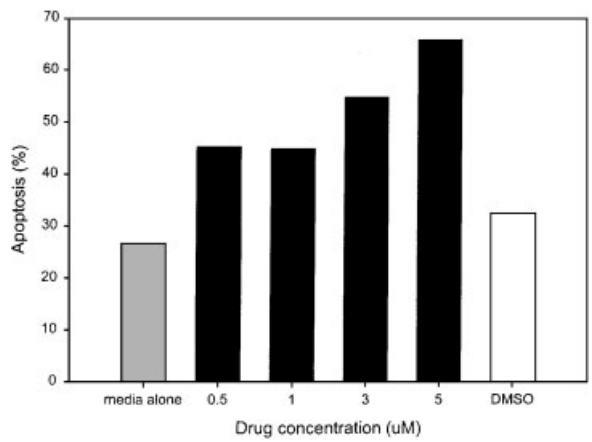

Fig. 4. The percentage of apoptotic Ba/F3 cells, deprived of IL-3, is increased by treatment with cytochalasin D. The IL-3-dependent cell line Ba/F3 were washed, resuspended in media, 0.1% DMSO, or cytochalasin D at the indicated concentrations. After 16 h, Ba/F3 cells were fixed and stained with Hoechst for quantification of apoptosis by nuclear morphology. The experiment shown is representative of three independent experiments.

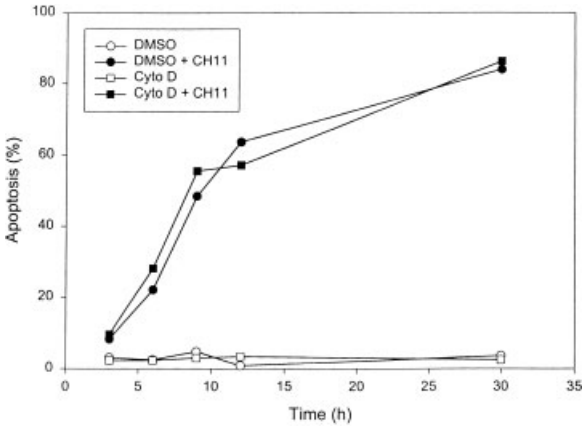

Fig. 5. Treatment of Jurkat T cells with cytochalasin D did not affect the time course or degree of apoptosis in response to CD95 (Fas) ligation. Jurkat T cells were treated with 0.02% DMSO (circles) or 1 μM cytochalasin D (squares) and incubated in media alone (open) or with anti-CD95 (Fas) Ab CH11 (closed) for the indicated times. Cells were then fixed and stained with Hoechst for quantification of apoptosis by nuclear morphology. Results are representative of four experiments.

even at 8 h a minimal effect of cytochalasin D was occasionally (Fig. 6), but not reproducibly (data not shown) observed. After 10 or more hours of cytokine withdrawal, a greater proportion of cells could not be rescued by the readdition of rhIL-2 in cytochalasin D-treated compared with DMSO-treated cells. This suggests that the depolymerization of the actin cytoskeleton, like actin stabilization [Posey and Bierer, 1999], altered the time of commitment to apoptosis.

Overexpression of the Anti-Apoptotic Protein Bcl-x_L did not Abrogate the Effect of Cytochalasin D

The overexpression of Bcl-x_L has been shown to delay apoptosis of cells following cytokine deprivation [Boise et al., 1996] and we have previously demonstrated that Bcl-x_L overexpression restored the rate of apoptosis of jasplakinolide-treated cells [Posey and Bierer, 1999]. At varying times following IL-2 deprivation, CTLL-2 cells overexpressing Bcl-x_L and treated with cytochalasin D (1 μM) or DMSO (0.02%) were assessed for apoptosis (Fig. 7). As expected, Bcl-x_L overexpression retarded the appearance of apoptotic cells, as less than 3% of Bcl-x_L-CTLL-2 cells had undergone apoptosis after 22 h of IL-2 deprivation (Fig. 7), compared to more than 20% of CTLL-20 cells that had undergone apoptosis after 19 h of similar deprivation (Fig. 3). However, at all time points studied, treatment with cytochalasin D increased the percentage of apoptotic cells. This effect differed from that of jasplakinolide [Posey and Bierer, 1999], and is consistent with previous observations [Levee, 1996] that actin depolymerization affected the execution stage of apoptosis.

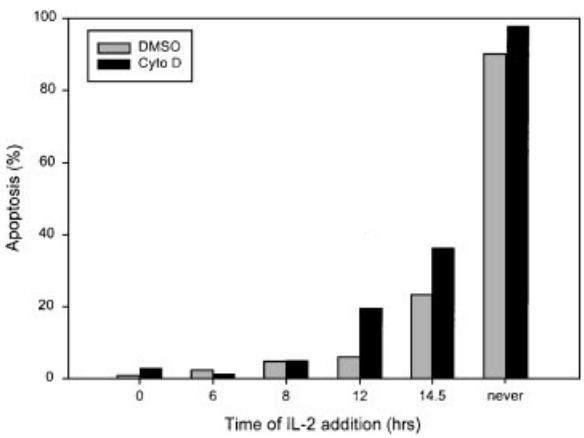

Fig. 6. Actin depolymerization altered the time course of commitment to apoptosis. CTLL-20 cells treated with 1 μM cytochalasin D or 0.02% DMSO were deprived of IL-2 for the indicated periods of time after which 100 U/ml rhIL-2 was added back to the cultures. All samples were harvested 25 h after the beginning of the incubation and fixed for staining with propidium iodide for quantification of apoptosis by nuclear morphology. Data are shown as percent apoptotic cells and are representative of two independent experiments.

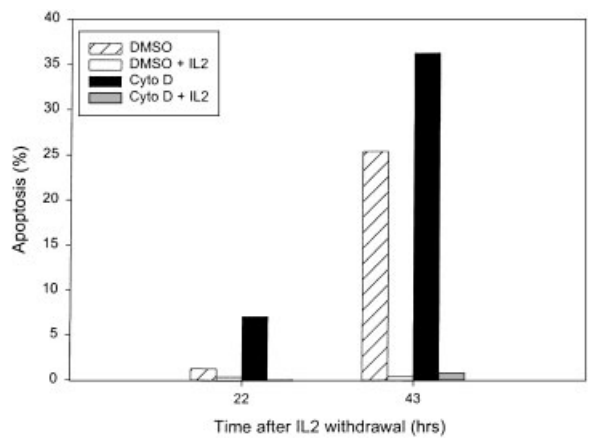

Fig. 7. Treatment with cytochalasin D enhanced apoptosis upon cytokine deprivation of CTLL-2 cells overexpressing Bcl-x_L. Bcl-x_L-CTLL-2 cells were treated with 0.02% DMSO (open bars) or 1 μM cytochalasin D (gray and black bars) in the absence or presence of rhIL-2 (100 U/ml) as indicated. Data shown are percent apoptotic cells at 22 and 43 h, determined by Hoechst counting. The experiment is representative of four independent experiments.

DISCUSSION

The polymerization state and stability of the actin cytoskeleton has been shown to vary with the state of cell differentiation and with cellular transformation in non-lymphoid systems [Nagata et al., 1980; Meyer and Howard, 1983; Antecol et al., 1986; Holme et al., 1986; Katsantonis et al., 1994; Hemstreet et al., 1996; Stournaras et al., 1996; Rao et al., 1997]. Additional studies support a role for the actin cytoskeleton in the coordinated control of cell cycle events [Vojtek et al., 1991; Gomez et al., 1997] and demonstrate that actin-related events such as adhesion, receptor clustering, or receptor internalization can affect mitogenic or survival signals [Rao, 1982; Brock and Chrest, 1993; Caplan and Baniyash, 1995; Caplan et al., 1995; Rozdzial et al., 1995; Valitutti et al., 1995]. Little work, however, has examined the effects of actin modulation on apoptotic signals.

While the fungal metabolite cytochalasin D has previously been used to demonstrate that the actin cytoskeleton plays a role in the terminal phases of apoptosis, at the time of membrane blebbing and cytoskeletal collapse [Ghibelli et al., 1995; Huot et al., 1998], the role of actin in the induction of apoptotic signaling, independent of a requirement for adhesion, has been less clear. In this report, we have used cytochalasin D to demonstrate that actin filament depolymerization shortened the time to and enhanced the rate of apoptosis of cell lines (CTLL-20 and Ba/F3) following cytokine deprivation. Treatment with cytochalasin D appeared to sensitize the cells to cytokine deprivation commitment to apoptosis and modified the growth factor requirement for survival. The effect of actin depolymerization appeared to depend upon the stimulus for cell death, as CD95-mediated cell death was not enhanced by actin depolymerization. However, it does remain possible that the onset of cell death after Fas ligation was sufficiently rapid that an effect of cytochalasin D on the apoptotic signaling process was obscured. Our data therefore support a role for actin depolymerization in early signaling to cell death in a model of cytokine withdrawal-induced cell death, prior to the involvement of the apoptotic machinery, as well as a role in the effector phase of apoptosis. The suggestion that the actin cytoskeleton can directly participate in signaling to apoptosis is further supported by a recent observation that actin disruption by cytochalasin D accelerated apoptosis in response to actinomycin D treatment in CMK-7 cells [Yamazaki et al., 2000].

The molecular mechanisms by which the actin cytoskeleton participates in transducing an apoptotic signal depend upon the specific death stimulus. Cell death that depends upon phagocytosis of pathogenic bacteria can be inhibited by cytochalasin D, secondary to the ability of drug to inhibit the phagocytic process [Rodrigues et al., 1999]. More importantly, disruption of the actin cytoarchitecture by cytochalasin D leading to the loss of adhesion of anchorage-dependent cells initiates the cell death program, suggesting that an intact cytoskeleton is required for transduction of survival signals in adherent cells [Sauman and Berry, 1993; Frisch and Ruoslahti, 1997]. In suspension, anchorage-independent cells, high concentrations (100 μM) of, and prolonged exposure (24 h) to cytochalasin D alone have been shown to promote apoptosis in a protein-synthesis independent, but caspase-3 dependent, fashion [Suria et al., 1999]. The concentrations of drug required in the report of Suria et al. [1999] were 100-fold greater than those used here and far above those required for effective depolymerization of the actin cytoskeleton [Kovbasnjuk et al., 1998]. Recent reports also indicate that in CHO [Subauste et al., 2000] and CEM [Parlato et al., 2000] cells, apoptosis induced by Fas ligation can be inhibited by actin depolymerization [Parlato et al., 2000; Subauste et al., 2000]. We did not see an inhibition of Fas-induced death in Jurkat T cells by cytochalasin D, a difference that may be secondary to cell type studied.

Anti-IgM treatment of normal B cells or B cell lines was shown to stimulate rapid assembly of F-actin and subsequent caspase-dependent apoptosis [Melamed and Gelfand, 1999]. Preincubation of the cells with cytochalasin D prevented both BCR-mediated G- to F-actin changes and cell death. The authors argued that that actin polymerization (conversion of G- to F-actin) was required for the induction of apoptosis [Melamed and Gelfand, 1999]. While it is possible that the role of the actin cytoskeleton in apoptosis differs fundamentally between B and T cells, or between the different mechanisms of induction of apoptosis, we suggest that the data could also be interpreted in terms of actin dynamics and not actin assembly (or disassembly).

Our data demonstrating that apoptosis induced by cytokine deprivation can be enhanced by both actin stabilization, induced by jasplakinolide [Posey and Bierer, 1999], and by actin depolymerization, induced by cytochalasin D treatment (reported here), suggests a more complex role for actin regulation in this death signal, such that a perturbation in actin dynamics is sufficient to accelerate the onset of apoptosis. We have previously shown that the actin-stabilizing compound jasplakinolide enhanced the induction of apoptosis upon cytokine deprivation. It is noteworthy that either jasplakinolide, a cyclic peptide that stabilizes F-actin and promotes actin polymerization, or cytochalasin D, a compound that promotes depolymerization, enhanced the rate of commitment to apoptosis. This suggests that rearrangement of the actin cytoskeleton dynamically regulates apoptosis, such that a redistribution of F-actin or alteration in the distribution of G- and F-actin (i.e., a change in the monomer/polymer actin ratio) affects sensitivity to the cell death signal.

In this system, overexpression of the anti-apoptotic protein Bcl-x_L delayed the onset of apoptosis induced by cytokine deprivation, as has been previously demonstrated [Boise et al., 1996], but did not completely abrogate the enhancement induced by cytochalasin D. In contrast, Bcl-x_L overexpression completely reversed the effects of jasplakinolide at all early time points (< 40 h). The inability of Bcl-x_L to reverse the effects of cytochalasin D implies that the depolymerization of the actin cytoskeleton by drug affected the effector phase, downstream of commitment to apoptosis. This interpretation is in concordance with other reports that demonstrate that actin rearrangement and cleavage are requisite for the effector phase of apoptosis [Huot et al., 1998; Mashima et al., 1999]. The comparison between the results using jasplakinolide [Posey and Bierer, 1999] and cytochalasin D reported here would imply that depolymerization but not stabilization of actin accelerated the effector phase. Whether actin depolymerization affects the rate of morphological changes (membrane blebbing, nuclear condensation) alone or other intracellular machinery remains to be explored.

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