[CANCER RESEARCH 57, 1835–1840, May 15, 1997]

Advances in Brief

Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis[1]

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Abstract

Although apoptosis and necrosis are morphologically distinct manifestations of cell death, apoptosis and some necroses share common features in the death signaling pathway involving functional steps of death-driving interleukin 1β-converting enzyme family proteases and anti-cell death protein Bcl-2. One evident physiological difference in cells undergoing apoptosis *versus* necrosis is in intracellular levels of ATP. In this study, we specifically addressed the question of whether apoptosis depends on intracellular ATP levels, since longer incubation under ATP-depleting conditions results in necrotic cell death. Incubation of cells in glucose-free medium with an inhibitor of mitochondrial F_0F_1-ATPases reduces intracellular ATP levels and completely blocks Fas/Apo-1-stimulated apoptosis. ATP supplied through glycolysis or oxidative phosphorylation restores the apoptotic cell death pathway. ATP depletion also leads to a block in Fas-induced activation of CPP32/Yama(-like) proteases, and when ATP is depleted after the activation of the proteases, subsequent apoptosis is significantly blocked. Thus, ATP-dependent steps exist both upstream and downstream of CPP32/Yama(-like) protease activation in apoptotic signal transduction. Treatment with the calcium ionophore induces apoptosis under ATP-supplying conditions but induces necrotic cell death under ATP-depleting conditions, indicating that ATP levels are a determinant of manifestation of cell death.

Introduction

Apoptosis accounts for most physiological cell death and is morphologically distinguishable from necrosis (1, 2). Apoptotic cells are defined by fragmented nuclei with condensed chromatin, fragmented or condensed cytoplasm, and formation of apoptotic bodies, whereas necrotic cells are characterized by electron-lucent cytoplasm, mitochondrial swelling, and loss of plasma membrane integrity without severe damage to nuclei.

Apoptosis requires tightly regulated cell death pathways. Cysteine proteases encoded by *Caenorhabditis elegans ced-3* (3) and its mammalian homologue represented in the ICE[3] (caspase-1) gene family, including CPP32/Yama (caspase-3, also called apopain), appear to play a key role in driving apoptosis (4, 5). On the other hand, the proto-oncogene *bcl-2* (6–8), whose *C. elegans* homologue is *ced-9* (9), has the ability to inhibit apoptosis induced by a variety of stimuli (10, 11), indicating that Bcl-2 negatively regulates a key event in a common pathway of apoptosis.

We recently showed that chemical hypoxia achieved by inhibiting the mitochondrial respiratory chain and glycolysis induces necrotic cell death (12), yet the same cells undergo apoptosis when other stimuli are used, including Fas/Apo-1 stimulation, tumor necrosis factor, DNA-damaging reagents, Ca^{2+} ionophore, and serum depletion (13, 14). Both apoptosis and chemical hypoxia-induced necrotic cell death were inhibited by the presence of Bcl-2 or CrmA (caspase inhibitor encoded by cowpox virus), or by treatment with the tetrapeptide ICE inhibitor, Ac-YVAD-CHO, indicating some common steps in the death signaling pathway (12, 13). Similarly, hypoxic hepatocytes underwent necrotic cell death, which was associated with increased activity of both ICE(-like) and CPP32/Yama(-like) proteases and was inhibited by tetrapeptide ICE inhibitor Ac-YVAD-CHO or CPP32/Yama inhibitor Ac-DEVD-CHO (15). Because Bcl-2 and Bcl-x_L function upstream of the ICE family protease cascade, including ICE(-like) and CPP32/Yama(-like) proteases (13, 16), some step(s) downstream of the ICE family protease cascade or activation of different members of the ICE protease family might determine the cell death fate, apoptosis or necrosis. One evident physiological difference in cells undergoing apoptosis *versus* necrosis is in intracellular levels of ATP, which are rapidly decreased in chemical hypoxia-induced necrotic cell death but only later in apoptotic cell death (14). Because necrotic cell death occurs in the absence of ATP (see below), we attempted to determine whether apoptosis depends on intracellular ATP levels. We show here that reduced intracellular ATP levels completely block Fas/Apo-1-stimulated apoptosis, and that ATP-dependent steps exist both upstream and downstream of CPP32/Yama(-like) protease activation in apoptotic signal transduction. Treatment with the calcium ionophore induces apoptosis and necrosis under ATP-supplying and ATP-depleting conditions, respectively, indicating that ATP levels are a determinant of manifestation of cell death.

Materials and Methods

Cell Lines and Culture. Jurkat, a human T-cell line, and HeLa cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. ATP depletion was achieved by incubating cells in the presence of 10 μM oligomycin in glucose-free DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 mM malic acid, 2 mM glutamate, 1 mM sodium pyruvate, 10 mM HEPES/Na^+ (pH 7.4), 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% dialyzed fetal bovine serum (Life Technologies, Inc.). The absence of glucose contamination was verified with an amperometric detector of hydrogen peroxide (E502 with a platinum electrode, IRICA, Inc., Tokyo, Japan) combined with an immobilized glucose oxidase column. Under these conditions, oligomycin inhibits mitochondrial F_0F_1-ATPases (17) and the depletion of glucose halts glycolysis so that no ATP-producing machinery operates.

Death-inducing Treatments. Jurkat cells were treated with 0.1 μg/ml of the agonistic anti-human Fas mAb CH11 (Medical and Biological Laboratories, Nagoya, Japan) for various periods. HeLa cells were treated with 1 μg/ml anti-Fas antibody, and all detached and attached cells were collected after treatments. Cell death was also induced by treating Jurkat cells with 1 μM calcium ionophore (A23187), 50 μM etoposide (VP16), or 400 μg/ml dexamethasone. Apoptotic and necrotic cell death were distinguished by a method using fluorescence microscopy with Hoechst 33342 and propidium iodide, which we have recently developed (18): collected cells were stained with Hoechst 33342 (10 μM) and propidium iodide (10 μM) for 3 min and analyzed under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) with exci-

Received 1/13/97; accepted 3/12/97.

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[1] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas and for Center of Excellence Research from the Ministry of Education, Science and Culture, Japan.

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[3] The abbreviations used are: ICE, interleukin 1β-converting enzyme; mAb, monoclonal antibody; Ac, acetyl; YVAD, L-tyrosyl-L-valyl-L-alanyl-L-asparatic acid; CHO, aldehyde; DEVD, L-aspartyl-L-glutamyl-L-valyl-L-asparatic acid; MCA, 4-methyl-coumaryl-7-amide.

tation at UV (360 nm). Because Hoechst 33342 stains all nuclei and propidium iodide stains nuclei of cells with a disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells, which were confirmed by electron microscopy and confocal fluorescence microscopy (18), were observed as blue round nuclei, pink round nuclei, and fragmented blue or pink nuclei, respectively, under a fluorescence microscope. More than 1000 cells were counted.

Measurement of Activity of CPP32/Yama(-like) Proteases. The activities of CPP32/Yama(-like) proteases were measured as described (13, 19). Briefly, at the indicated times, cells were washed three times with PBS and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. After addition of 10 μM digitonin, cells were incubated at 37°C for 10 min. Complete cell lysis was verified by the amount of released lactate dehydrogenase activity. Lysates were clarified by centrifugation at 15,000 rpm for 3 min, and cleared lysates containing 40 μg of protein were incubated with 50 nmol of enzyme substrate Ac-DEVD-MCA (Peptide Institute, Osaka, Japan) at 37°C for 30 min. Levels of released 7-amino-4-methylcoumarin were measured using a spectrofluorometer (F-3000; Hitachi, Tokyo, Japan) with excitation at 380 nm and emission at 460 nm.

Results and Discussion

Requirement for ATP in Fas-induced Apoptosis. To elucidate the dependence of apoptosis on ATP, intracellular ATP levels were manipulated by incubating cells in glucose-free medium with 10 μM oligomycin (an inhibitor of mitochondrial F_0F_1-ATPase) to inhibit the production of ATP from both glycolysis and oxidative phosphorylation (17). Under the conditions, intracellular ATP levels were rapidly reduced within 60 min in Jurkat cells, whereas the addition of glucose or omission of oligomycin maintained the levels of ATP (Fig. 1*A*). Similar results were obtained using HeLa (D98/AH2) cells, although the recovery of ATP levels by omission of oligomycin was not obvious (Fig. 1*B*), suggesting that HeLa cells are more dependent on glycolysis for ATP. ATP-depleting treatments did not alter Fas-antigen expression on the cell surface of either cell line (Fig. 1, *C–H*). Fluorescence microscopy of Jurkat cells treated with an agonistic anti-Fas mAb under ATP-depleting conditions revealed only a few apoptotic and necrotic cells (Fig. 2, *A*, *B*, and *G*), whereas a large number of apoptotic cells appeared when ATP was supplied from glycolysis by the addition of glucose (Fig. 2, *C*, *D*, and *G*). The number of apoptotic cells after ATP restoration was comparable to that found in cultures without oligomycin in the presence of glucose (data not shown). The large number of apoptotic cells in cultures where ATP was supplied from mitochondria by omitting oligomycin in glucose-free medium (Fig. 2, *E–G*) argues against the possibility that the apoptosis might be glucose dependent but not ATP dependent. Necrotic death of Jurkat cells increased gradually regardless of Fas stimulation when incubation was continued under ATP-depleting conditions for more than 24 h (data not shown). Similar results were obtained with HeLa cells (Fig. 2*H*), although in the absence of glucose and oligomycin, a significant fraction of cells underwent apoptosis without anti-Fas antibody treatment, probably due to limited ATP

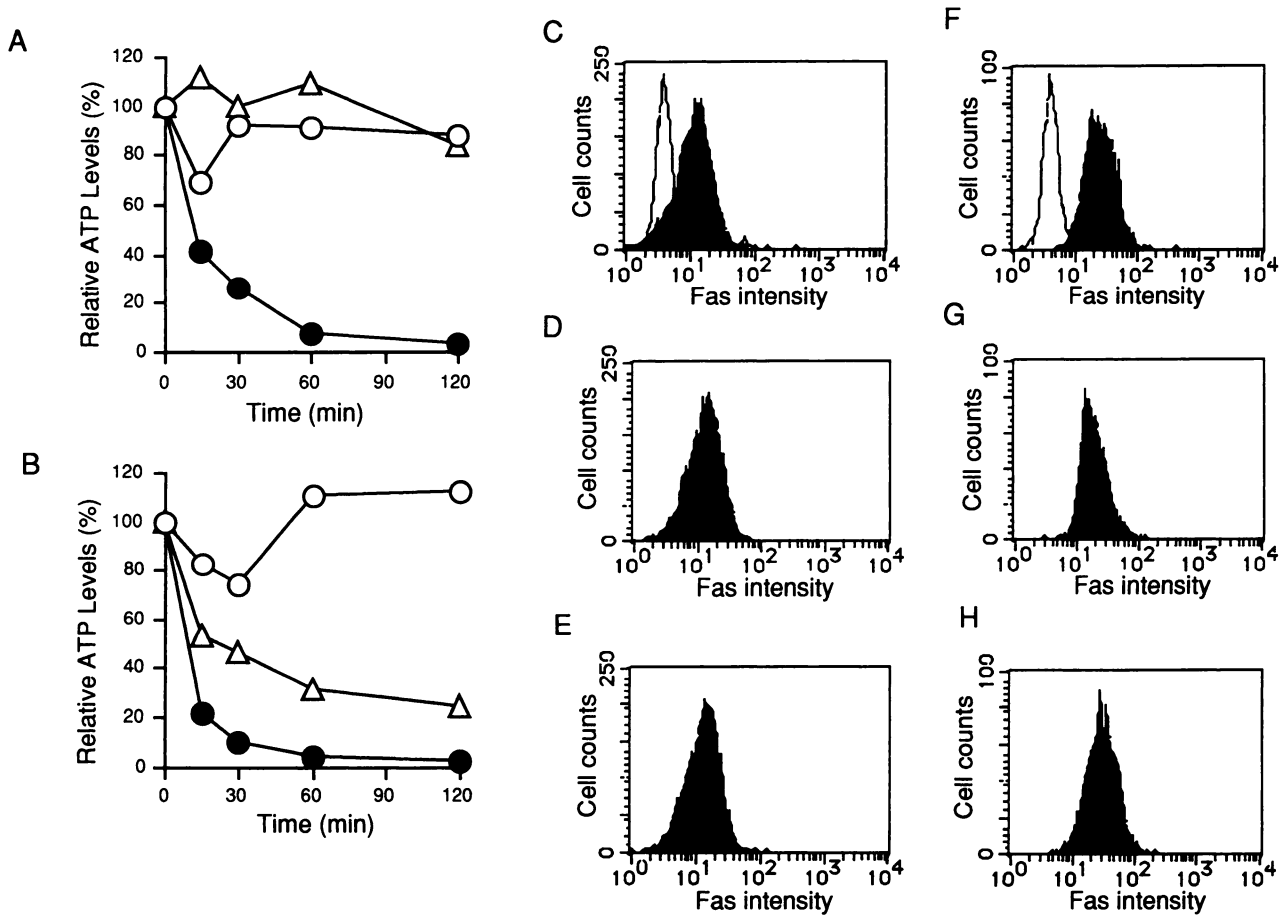

Fig. 1. ATP depletion of cells and expression of Fas antigen. *A* and *B*, Intracellular ATP levels of Jurkat cells (*A*) and HeLa cells (*B*) were determined using the luciferin-luciferase (Sigma Chemical, St. Louis, MO) method (31) after cells were incubated for the indicated periods with 10 μM oligomycin in glucose-free DMEM (●), with 10 μM oligomycin in 0.35% glucose-containing DMEM (○), and without oligomycin in glucose-free DMEM (△). *C–H*, Expression of Fas antigen in Jurkat (*C–E*) and HeLa cells (*F–H*) under various conditions (*filled curves*) was verified by fluorescein-activated cell sorting analysis using FITC-conjugated antihuman Fas antibody clone UB2 (MBL) essentially as described elsewhere (32). Cells were treated with 10 μM oligomycin in glucose-free DMEM (*C* and *F*), with 10 μM oligomycin in 0.35% glucose-containing DMEM (*D* and *G*), or in glucose-free DMEM without the addition of oligomycin (*E* and *H*) for 2 h. Fluorescence-activated cell-sorting profiles from a mouse B cell line which does not express human Fas (*open curves*) are also shown in *C* and *F*.

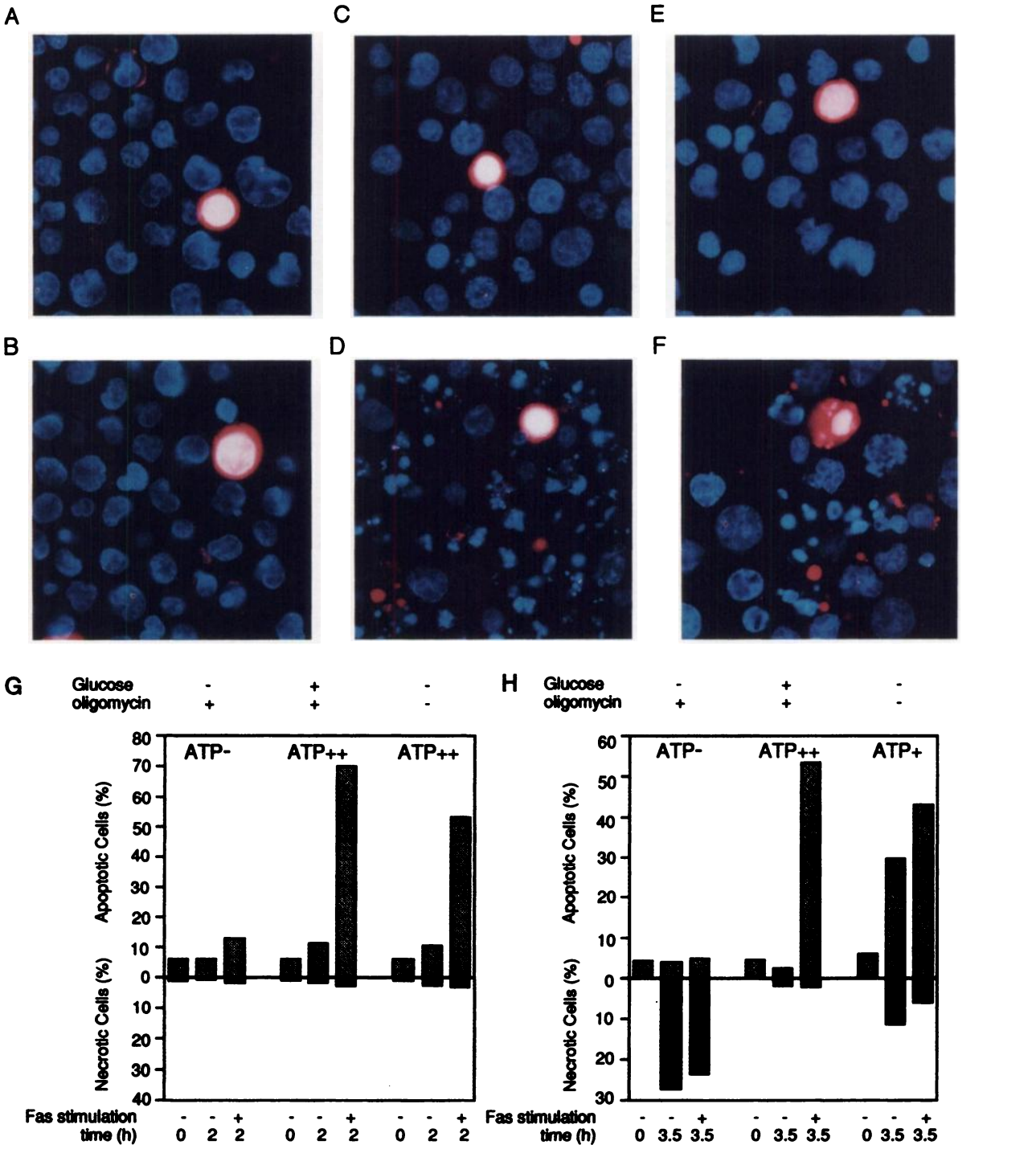

Fig. 2. ATP dependence of Fas-induced apoptosis. *A–F*, Morphology of Jurkat cells cultured under ATP-supplying or ATP-depleting conditions and treated with 0.1 μg/ml anti-Fas mAb CH11 was analyzed by fluorescence microscopy after double staining of cells with Hoechst 33342 (*blue*) and propidium iodide (*pink*) for 3 min. Cells were incubated with 10 μM oligomycin in glucose-free DMEM (*A* and *B*), with 10 μM oligomycin in 0.35% glucose-containing DMEM (*C* and *D*), or in glucose-free DMEM without the addition of oligomycin (*E* and *F*) for 1 h, and further incubated with (*B*, *D*, and *F*) or without (*A*, *C*, and *E*) anti-Fas antibody for an additional 2 h under the same conditions. *G* and *H*, Jurkat cells (*G*) and HeLa cells (*H*) were preincubated under the conditions indicated (glucose or oligomycin) for 1 h (time 0) and further incubated with or without anti-Fas antibody for 2 h (*G*) and 3.5 h (*H*) under the same conditions. The extent of cell death was assessed by counting viable, necrotic, and apoptotic cells under a fluorescence microscope. More than 1000 cells were counted.

supply from mitochondria. Necrotic death of HeLa cells was induced under ATP-depleting conditions regardless of Fas stimulation (Fig. 2*H*). From these results, we concluded that Fas-induced apoptosis is ATP dependent, although we cannot exclude the possibility that depletion of ATP generates some anti-apoptotic signals.

ATP-dependent Steps in Fas-induced Apoptotic Signaling Pathway. In an effort to localize the ATP-dependent steps within the death signaling pathway, we examined the activity of CPP32/Yama(-like) proteases, which are essential in driving Fas-induced apoptosis (19–21). In both Jurkat and HeLa cells treated with anti-Fas antibody, the activity of CPP32/Yama(-like) proteases increased under ATP-supplying conditions, but the increase is greatly reduced under ATP-depleting conditions (Fig. 3, *A* and *B*). The activation of CPP32/Yama(-like) proteases in Fas-stimulated HeLa cells incubated in the absence of glucose and oligomycin was not observed (Fig. 3*B*), probably due to limited ATP supply from mitochondria (Fig. 1*B*), and

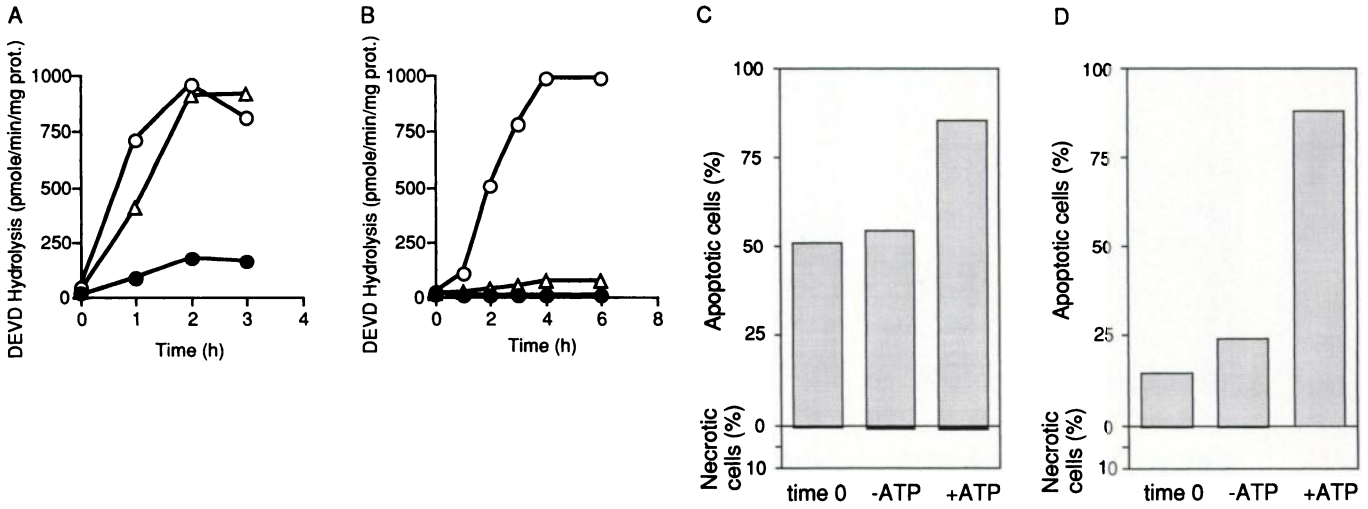

Fig. 3. ATP-dependent steps in death signaling pathways. *A* and *B*, ATP-dependent steps upstream of the activation of CPP32/Yama(-like) proteases. Jurkat (*A*) and HeLa (*B*) cells were preincubated for 1 h under the conditions described below, treated with anti-Fas antibody under the same conditions, and activity of CPP32/Yama(-like) proteases was measured at the indicated times. Cells were stimulated with anti-Fas antibody in glucose-free DMEM with 10 μM oligomycin (−ATP; ●), in 0.35% glucose-containing DMEM with 10 μM oligomycin (+ATP from glycolysis; ○), or in glucose-free DMEM without addition of oligomycin (+ATP from mitochondria; △). Enzyme activity was expressed by the amount of Ac-DEVD-MCA hydrolysis per min per mg of protein at 37°C. *C* and *D*, ATP-dependent steps downstream of the activation of CPP32/Yama(-like) proteases. Jurkat (*C*) and HeLa (*D*) cells were treated as described above with anti-Fas antibody in glucose-free DMEM for 1.5 h (*C*) or in the presence of 0.35% glucose and 10 μM oligomycin for 3 h (*D*) to ensure more than 75% activation of CPP32/Yama(-like) proteases (time 0). Intracellular ATP levels were reduced by adding 10 μM oligomycin (Jurkat) or by changing the medium to glucose-free DMEM supplemented with 10 μM oligomycin without anti-Fas antibody (HeLa; −ATP), or by maintaining cells in the same medium (Jurkat) or changing the medium to include 0.35% glucose (HeLa; +ATP). The extent of cell death at time 0 and after incubation for an additional 1 h with or without ATP supply was analyzed as described in the legend of Figure 2.

this accounts for only a little enhancement of apoptosis by Fas stimulation under the conditions (Fig. 2*H*). These results strongly suggest the existence of an ATP-dependent step(s) upstream of the activation step of CPP32/Yama(-like) proteases in Fas-induced apoptosis. Treatment of Jurkat cells for 1.5 h and HeLa cells for 3 h with anti-Fas antibody in the presence of intracellular ATP to ensure more than 75% activation of CPP32/Yama(-like) proteases (Fig. 3, *A* and *B*), followed by culture under conditions that reduce intracellular ATP

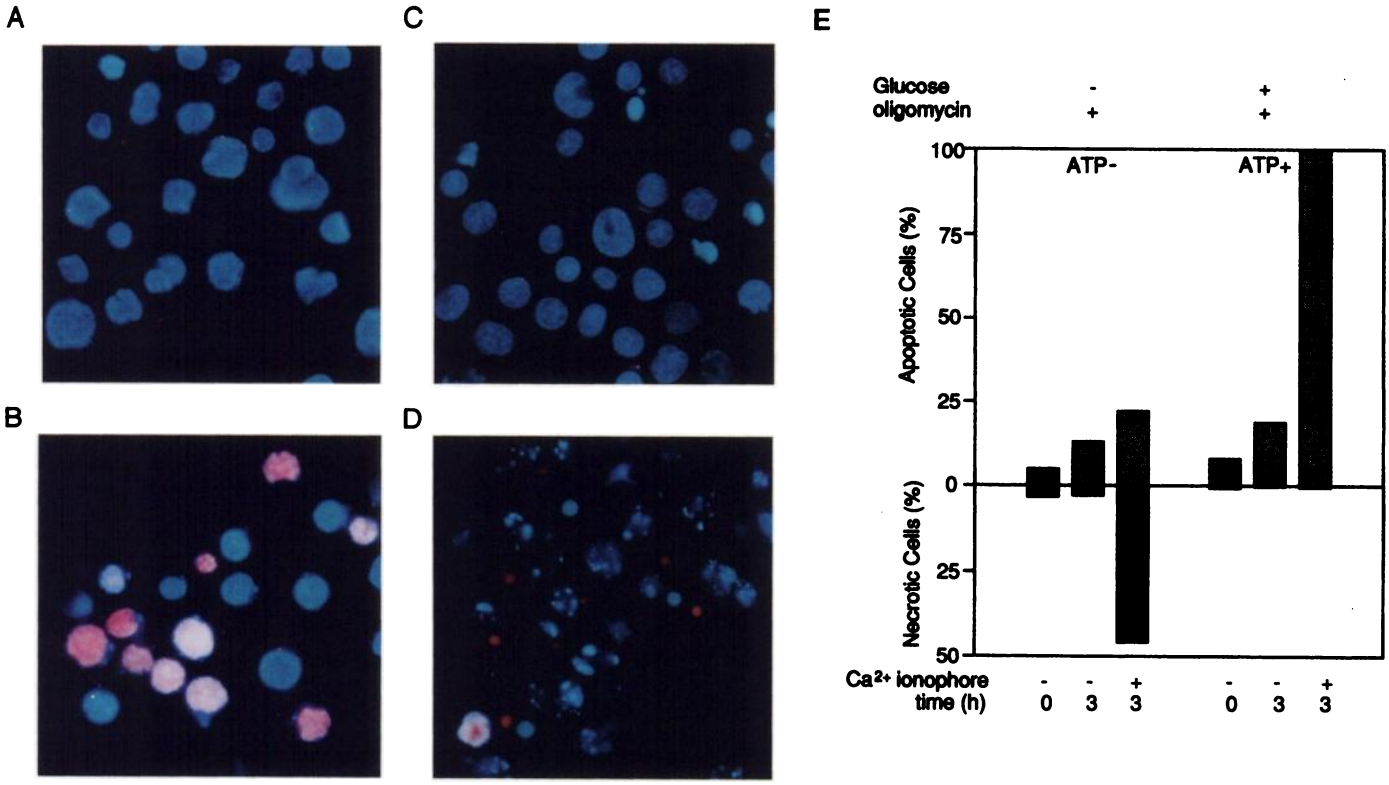

Fig. 4. Induction of necrotic cell death by apoptosis-inducing stimuli under ATP-depleting conditions. *A–D*, Morphology of Jurkat cells treated with 1 μM calcium ionophore A23187 in the presence or absence of intracellular ATP. Jurkat cells were preincubated under the conditions described below for 30 min, treated with (*B* and *D*) or without (*A* and *C*) A23187 for 3 h, and morphology was analyzed as described in the legend to Figure 2. *A* and *B*, Cells were treated in the presence of 10 μM oligomycin in glucose-free DMEM. *C* and *D*, Cells were treated in the presence of 10 μM oligomycin in 0.35% glucose-containing DMEM. *E*, Jurkat cells were preincubated under the conditions indicated (glucose or oligomycin) for 30 min (time 0), treated with 1 μM A23187, and assessed for the extent of cell death after a 3 h-incubation as described in the legend of Figure 2.

levels, greatly reduced the percentage of apoptotic cells during the next 1 h as compared with the extent of apoptosis observed in the presence of ATP supply (Fig. 3, *C* and *D*). Thus, there is also an ATP-dependent step(s) that functions downstream of the activation step of CPP32/Yama(-like) proteases.

Conversion of Apoptotic Signals to Necrotic Signals. Because the apoptotic death signaling pathway contains several ATP-dependent steps, some of which might determine whether cell death occurs by apoptosis or necrosis, we tested the possibility that apoptosis-inducing treatments under ATP-depleting conditions induce necrotic cell death and that ATP dependency is not specific for Fas-induced apoptosis by treating Jurkat cells with other typical apoptosis-inducing reagents, calcium ionophore (A23187), etoposide, and dexamethasone (22), by manipulating intracellular ATP levels during the treatments. Treatment with calcium ionophore induced apoptosis under ATP-supplying conditions and necrotic cell death under ATP-depleting conditions (Fig. 4). No activation of CPP32/Yama(-like) proteases or inhibitory effect of tetrapeptide ICE inhibitor and CPP32/Yama inhibitor was observed in necrotic cell death (data not shown). Apoptosis induced by etoposide and dexamethasone was blocked by ATP depletion, but necrosis was not stimulated (data not shown), possibly due to ATP-dependent reactions that might exist at very early steps in the death signaling pathways induced by these treatments. These results strongly suggest that apoptosis is ATP dependent in general and that cell death fate by apoptosis or necrosis is determined by intracellular ATP levels, at least in the case of calcium ionophore treatment.

Although the prevention of apoptosis and chemical hypoxia- and hypoxia-induced necrotic cell death by ICE family protease inhibitors (12–14, 18) might rest in the presence of determinants of cell death fate downstream of the ICE protease cascade, the failure of ATP depletion to convert Fas-induced activation of CPP32/Yama(-like) protease family to necrotic signals raises the possibility that different members of the CPP32/Yama(-like) proteases are activated by the respective cell death stimuli. Alternatively, cell death fate might also depend on additional signals that are coactivated only by some cell death stimuli. The conversion of Ca^{2+} ionophore-induced apoptotic signals to necrotic signals by ATP-depletion without CPP32/Yama(-like) protease activation suggests an alternative pathway of necrosis that involves a protease cascade of other ICE family protease members or perhaps no such cascade.

Stimulation of Fas antigen was recently shown to recruit the novel ICE/CED-3 family protease, MACH/FLICE (caspase-8), to form a Fas-containing complex that transduces Fas-induced death signals (23, 24), suggesting that a Fas-initiated death signal is transmitted directly to the ICE protease cascade. Because ATP depletion blocked the activation of CPP32/Yama(-like) proteases that is essential for Fas-induced apoptosis (19–21), the activation step of MACH/FLICE and/or the signaling pathway from MACH/FLICE to the ICE protease cascade might involve ATP-dependent reactions. These observations suggest that the ICE protease cascade is regulated by ATP-dependent reactions and that the protease cascade might not proceed in an autonomous fashion.

Because apoptosis is characterized by morphological changes in the nucleus, the apoptotic death signals are probably transmitted from the cytoplasm to the nucleus, and the ATP-dependent step(s) functioning downstream of the activation step of CPP32/Yama(-like) proteases might be an active nuclear transport mechanism which requires ATP hydrolysis (25, 26). Indeed, we recently showed that active nuclear transport is involved in apoptotic change of the nuclei (27). Kinases and ATP-dependent proteases and the regulation of the cytoskeleton might also play important roles in apoptosis.

The present findings could explain the frequent appearance of necrotic cells mixed with apoptotic cells in pathological areas *in vivo*, such as the center of solid tumors (28, 29) and ischemic nervous tissues (30). In areas where blood flow is limited, there is rapid exhaustion of intracellular ATP due to insufficient oxygen and rapid consumption of glucose, inhibiting apoptosis and inducing necrotic cell death. Thus, even *in vivo*, intracellular ATP levels appear to be a factor determining cell death fate by apoptosis and necrosis.

Acknowledgments

We are grateful to M. Hoffman for editorial assistance.

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Cancer Res 1997;57:1835-1840.