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Characterization of Caspase Proteases in Cytokine-Dependent Myeloid Progenitor Cells Using Enzyme Affinity Labeling

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Abstract Bone marrow-derived myeloid progenitor cells are dependent on the presence of cytokines such as interleukin-3 (IL-3) for their survival. The withdrawal of IL-3 from IL-3-dependent myeloid progenitors results in death via an apoptotic program. Previous studies have shown that IL-3 withdrawal induces the activities of caspase proteases. However, the molecular identities of myeloid progenitor caspases have not been determined. In this study, we used an affinity labeling reagent (biotin-YVAD-acyloxymethyl ketone) that binds to processed active caspase subunits, to study caspase activation in 32D and FDCP-1 myeloid progenitor cells. After IL-3 withdrawal, we detected affinity labeling of caspase subunits of 20, 17, and 16 kDa in both cell lines. Surprisingly, affinity labeling of the 20- and 17-kDa proteins, but not the 16-kDa protein, was also detected in healthy cells maintained in the presence of IL-3. By contrast, in cytokine-independent cell lines, affinity labeling of caspase subunits was detected only after treatment with an apoptotic stimulus. Immunoblotting experiments showed that caspase-3 constitutes at least a portion of the 20- and 17-kDa affinity-labeled proteins detected in the myeloid progenitor cell lines. Taken together, these data provide direct evidence of caspase activation in cytokine-dependent myeloid progenitors, and suggest that unique apoptotic pathways may exist in these cells. J. Cell. Biochem. 73:79–89, 1999.

Key words: apoptosis; caspase; protease; myeloid; cytokine

Apoptosis is a genetically defined program of cell suicide that is critically important in developing and adult organisms [Ellis et al., 1991; Thompson, 1995; Jacobson et al., 1997]. In adult mammals, apoptosis is important for eliminating damaged or infected cells, eliminating cells in involuting tissues, and maintaining homeostasis of cell numbers [Jacobson et al., 1997]. Although all apoptotic cells appear to activate certain common pathways, unique pathways of apoptosis are also activated in different cell types and in response to different apoptotic stimuli.

In the myeloid system, apoptosis plays an essential role in regulating the number of circulating mature myeloid cells [Arai et al., 1990]. Mature myeloid cells are derived from progenitor cells in the bone marrow [Metcalf, 1989, 1991]. Cytokines such as interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) promote the proliferation, survival, and differentiation of myeloid progenitors [Arai et al., 1990; Metcalf, 1991; Johnson, 1998]. By contrast, depletion of essential cytokine from cytokine-dependent myeloid progenitors results in rapid apoptotic cell death [Wyllie et al., 1984; Williams et al., 1990; Rodriguez-Tarduchy et al., 1990]. The establishment of IL-3-dependent murine myeloid progenitor cell lines, including 32D [Greenberger et al., 1983a,b] and FDCP-1 [Williams et al., 1990], has provided good models for studying this form of apoptosis. When deprived of IL-3, 32D and

Abbreviations used: bio-YVAD-amk, biotin-YVAD-acyloxymethyl ketone; z-VAD-FMK, z-VAD-fluoromethyl ketone; z-DEVD-FMK, z-DEVD-fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase, PBS, phosphate-buffered saline; kDa, kilodaltons.

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FDCP-1 cells exhibit hallmark changes associated with apoptosis, including cell shrinkage, membrane blebbing and formation of apoptotic bodies, and activation of nuclear DNases and degradation of genomic DNA [Wyllie et al., 1984; Ellis et al., 1991]. However, the precise biochemical mechanisms of apoptosis that are activated in IL-3-deprived myeloid progenitors remain unclear.

In other models of apoptosis, cell death has been shown to be associated with, and dependent on, the activation of caspase proteases. Caspases comprise a family of cysteine proteases that are activated in response to a variety of apoptotic stimuli [Cohen, 1997; Salvesen and Dixit, 1997]. At least 10 members of the caspase family have been described [Alnemri et al., 1996]. Activation of a caspase involves proteolytic processing of an inactive precursor molecule to two smaller subunits, which then associate into a heterotetrameric active enzyme complex [Cohen, 1997; Salvesen and Dixit, 1997]. For example, activation of caspase-3, a centrally important caspase, requires cleavage of a 32-kDa proenzyme form to 17- and 10-kDa subunits [Nicholson et al., 1995; Tewari et al., 1995]. Once activated, caspases cleave specific cellular substrate proteins after aspartate residues, leading to the destruction of the cell. Inhibition of caspase proteases with peptide inhibitors [Thornberry et al., 1992, 1995; Nicholson, 1996] or cowpox virus CrmA protein [Pickup et al., 1986; Ray et al., 1992; Komiyama et al., 1994] inhibits apoptosis caused by FAS or TNF receptor stimulation [Tewari and Dixit, 1995; Enari et al., 1995, 1996; Los et al., 1995, Tewari et al., 1995; Schlegel et al., 1996; Hasegawa et al., 1996; Armstrong et al., 1996], neurotrophic factor withdrawal [Gagliardini et al., 1994], detachment from extacellular matrix [Boudreau et al., 1995; Frisch et al., 1996], or treatment with chemotherapy drugs [Antoku et al., 1997].

Recent evidence indicates that withdrawal of IL-3 from myeloid progenitor cells also leads to the activation of caspase proteases [Johnson, 1998]. Experiments by Kumar [1995] have shown that FDCP-1 cells express caspase-2 mRNA and that expression of antisense caspase-2 in these cells modestly enhances survival after IL-3 withdrawal. Other studies have shown that withdrawal of IL-3 from myeloid progenitors leads to cleavage of the caspase substrate protein poly(ADP-ribose) polymerase (PARP). IL-3 withdrawal-induced PARP cleavage and apoptotic cell death are inhibitable by the caspase peptide inhibitors z-VAD-fluoromethyl ketone (z-VAD-FMK) or z-DEVD-fluoromethyl ketone (z-DEVD-FMK), but not by CrmA protein [Barge et al., 1997; Ohta et al., 1997; Antoku et al., 1998]. In addition, extracts from IL-3-deprived myeloid progenitors can cleave fluorogenic peptides containing the DEVD recognition sequence for caspase-3 [Barge et al., 1997; Ohta et al., 1997]. Unfortunately, immunoblotting experiments that would directly demonstrate processing and activation of caspases in myeloid progenitors have been difficult to perform, as antibodies recognizing murine caspases are not readily available.

An alternative method for visualizing activated caspase proteases uses the technique of enzyme affinity labeling [Nicholson et al., 1995; Takahashi et al., 1996; Faleiro et al., 1997; Keane et al., 1997; Martins et al., 1997]. Affinity labeling of caspases is performed using biotinylated peptides that contain a caspase recognition sequence [Nicholson et al., 1995; Thornberry et al., 1994]. One such labeling reagent is biotin-YVAD-acyloxymethyl ketone (bio-YVAD-amk). This affinity label binds selectively to processed, active caspase subunits. Moreover, when used at high concentrations, bio-YVAD-amk binds to subunits of multiple different caspases [Nicholson, 1996; Faleiro et al., 1997]. Enzymes that are affinity labeled with bio-YVAD-amk can be detected by Western blotting using anti-biotin detection reagents. In this report, we performed affinity labeling with bio-YVAD-amk to study caspase activation in IL-3-deprived 32D and FDCP-1 cells. In addition, we used anti-murine caspase-3 polyclonal antisera to demonstrate expression and activation of caspase-3.

MATERIALS AND METHODS

Cell Lines and Reagents

32D (clone 23) [Greenberger et al., 1983a,b] and FDCP-1 [Williams et al., 1990] murine myeloid progenitor cell lines were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 10% WEHI-3B conditioned media (as a source of IL-3), penicillin, streptomycin, and L-glutamine. WEHI-3B myelomonocytic leukemia cells, YAC-1 T lymphoma cells, and Jurkat T leukemic cells were grown in this same media, except that 10% WEHI-3B conditioned media was not included. All media and media supplements were from Gibco-BRL (Gaithersburg, MD). Cells

were grown in a conditioned atmosphere of 5% CO_2 and 37°C.

The affinity labeling reagent, biotin-YVAD-acyloxymethyl ketone, was purchased from Calbiochem (Cambridge, MA) and dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). NeutrAvidin and horseradish peroxidase (HRP)-conjugated biotin (bio-HRP) were obtained from Molecular Probes (Eugene, OR) and dissolved in phosphate-buffered saline (PBS) (pH 7.4). The caspase inhibitory peptides z-VAD-FMK and z-DEVD-FMK were purchased from Kamiya Biochemical Co. (Tukwila, WA) and dissolved in DMSO. The chemotherapeutic drug VP-16 was obtained from Sigma and dissolved in DMSO. Agonistic anti-Fas monoclonal antibody (clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY) and anti-murine caspase-3 polyclonal antisera was generously provided by Dr. Rafick-Pierre Sekaly (University of Montreal, Quebec, Canada). All resuspended reagents were aliquoted and stored at −20°C.

IL-3 Withdrawal

At 24 h before IL-3 withdrawal, cells were plated at a density of 1.5–3.0 × 10^5 cells/ml in RPMI media containing 10% FCS, 10% WEHI-3B conditioned media, and 5 ng/ml recombinant murine IL-3 (PeproTech, Rocky Hill, NJ). For removal of IL-3, cells were pelleted and washed a total of five times in RPMI media alone. After these washes, the cells were plated at a density of 1 × 10^6 cells/ml in RPMI containing 10% FCS. At various timepoints thereafter, aliquots of the IL-3-deprived cells were removed for determination of cell viabilities or preparation of cell extracts for analysis by Western blotting or affinity labeling. Cell viabilities were determined by trypan blue exclusion

Treatment With Anti-Fas or VP-16

For induction of apoptosis with anti-Fas monoclonal antibody, Jurkat cells were first plated at a density of 5 × 10^6 cells/ml in RPMI media containing 10% FCS. Anti-Fas antibody (clone CH-11) was then added to a final concentration of 200 ng/ml. For control incubations, Jurkat cells were incubated with an equivalent volume of the antibody diluent, PBS.

For induction of apoptosis with VP-16, WEHI-3B cells were first plated at a density of 2.5 × 10^5 cells/ml in RPMI media containing 10% FCS. VP-16 was then added from a 20 mM stock solution to a final concentration of 20 µM. As a control, additional cells were incubated with RPMI/10% FCS containing 0.1% DMSO.

Affinity Labeling and Detection of Affinity-Labeled Caspases

Affinity labeling experiments were performed according to the method of Faleiro et al. [1997], with minor modifications. Briefly, for each labeling reaction 5 × 10^6 cells were pelleted and washed in 1 ml of cold PBS. The cell pellets were gently resuspended in 250 µl of modified KPM buffer (50 mM KCl, 50 mM PIPES [pH 7], 1.92 mM $MgCl_2$, 10 mM EGTA, 1 mM DTT, 10 µg/ml cytochalasin B, 0.1 mM PMSF, and 2 µg/ml each of aprotinin, leupeptin, and pepstatin), allowed to sit for 1 min at room temperature, then pelleted for 10 s in a microfuge. The cell pellet (approximately 30 µl) was frozen on dry ice and stored at −80°C. For affinity labelings, 30 µl of a 20-µM bio-YVAD-amk stock (diluted in modified KPM) was added to each 30-µl cell pellet (5 × 10^6 cells) to achieve a final concentration of 10 µM of the labeling reagent. The resuspended cells were then lysed by subjecting them to five cycles of rapid freeze/thawing. After lysis, labeling reactions were allowed to proceed for 15 min at 37°C before addition of 60 µl of sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. One-half (2.5 × 10^6 cells) of each labeling reaction was then electrophoresed on 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose.

To detect affinity-labeled proteins, nitrocellulose filters were first incubated for 1 hr at room temperature in TNT (20 mM Tris [pH 7.4], 150 mM NaCl, 0.02% Tween-20) containing 1% BSA. The filters were then washed two times in TNT, and incubated for 1 hr at room temperature in 1 µg/ml NeutrAvidin diluted in TNT/1% BSA. After five washes in TNT, the filters were incubated for 1 h at room temperature in 25 ng/ml bio-HRP in TNT/1% BSA. The filters were then washed five times in TNT and developed using an enhanced chemiluminescence (ECL) kit from Amersham (Arlington Heights, IL).

Immunoblotting

For immunoblotting experiments, aliquots of affinity labeling reactions (representing 4 × 10^5 cells) were electrophoresed on a 14% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then incubated for 1 h at room temperature in PBST (PBS,

0.05% Tween-20) containing 5% nonfat dry milk. After this blocking step, the membrane was washed two times for 5 min in PBST. Rabbit anti-murine caspase-3 polyclonal antisera (kindly provided by Dr. Rafick-Pierre Sekaly), diluted 1:5,000 in PBSTB (PBST containing 1% BSA), was then incubated with the membrane for 1 h at room temperature. After washing the membrane five times in PBST, HRP-conjugated goat anti-rabbit antibody (Promega, Madison, WI), diluted 1:2,500 in PBSTB, was incubated with the membrane for 1 h at room temperature. A final series of five PBST washes was then performed, followed by ECL development.

RESULTS

Affinity Labeling of Activated Caspases in IL-3-Dependent Myeloid Progenitors

In an effort to characterize caspase activation in cytokine-dependent myeloid progenitor cell lines, activated caspase subunits were affinity labeled with bio-YVAD-amk. Previous studies have shown that bio-YVAD-amk binds to the processed, active forms of caspase proteases [Thornberry et al., 1994; Faleiro et al., 1997]. Although the YVAD tetrapeptide motif is based on a caspase-1 cleavage site in pro-IL-1β [Thornberry et al., 1992], when used at high concentrations, the bio-YVAD-amk labeling reagent will bind to multiple members of the caspase family [Faleiro et al., 1997]. For this reason, we used bio-YVAD-amk at a concentration of 10 μM. Labeling reactions were performed for 15 min at 37°C, and affinity-labeled proteins were detected by Western blotting as described under Materials and Methods.

For Figure 1A, 32D (lanes 1–5) and FDCP-1 (lanes 6–9) murine myeloid progenitor cell lines were deprived of IL-3 for varying lengths of time, followed by preparation of cell lysates and affinity labeling with bio-YVAD-amk. The viabilities of the IL-3-deprived cells are shown in Figure 1B. As depicted, affinity-labeled species of 20, 17, and 16 kDa were detected in both 32D and FDCP-1 cells. The molecular weights of these affinity-labeled proteins are similar to those reported for the large subunits of processed, active caspases [Cohen, 1997]. Additional proteins of approximately 70 and 110 kDa were also detected after Western blotting (all lanes). However, the 70- and 110-kDa proteins were observed even when bio-YVAD-amk was excluded from incubations (Fig. 1A, lane 1), indicating that these proteins are recognized by the probing reagents and do not represent affinity-labeled species.

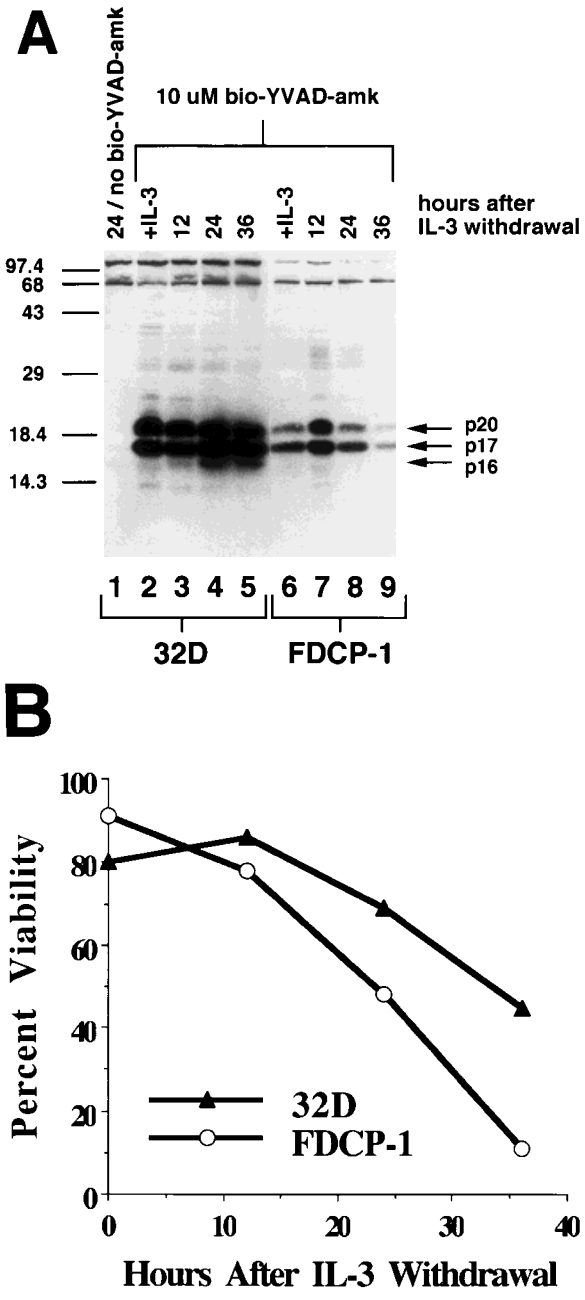

Fig. 1. **A:** Affinity labeling of activated caspases in interleukin-3 (IL-3)-deprived 32D and FDCP-1 cells. 32D (**lanes 1–5**) and FDCP-1 (**lanes 6–9**) myeloid progenitor cells were maintained in IL-3 or deprived of IL-3 for varying lengths of time. At various time points, aliquots of cells (5×10^6 cells/harvest) were harvested and subjected to affinity labeling by incubation with 10 μM bio-YVAD-amk as described in Material and Methods (lanes 2–9). As a control, extract from 32D cells deprived of IL-3 for 24 h was incubated in the absence of bio-YVAD-amk (lane 1). After affinity labeling, one-half of each extract (2.5×10^6 cell equivalents/lane) was electrophoresed on a 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to nitrocellulose, then probed with NeutrAvidin (1 μg/ml), followed by bio-HRP (25 ng/ml). The blot was developed using enhanced chemiluminesence. Arrows, locations of affinity labeled caspase species of 20, 17, 16 kDa. **B:** Viability of IL-3-deprived 32D and FDCP-1 cells. The viabilities of cells analyzed in A were assessed by trypan blue exclusion.

Interestingly, the p20 and p17 affinity-labeled proteins were observed in lysates from cells grown in the presence of IL-3 (lanes 2, 6), as well as lysates from IL-3-deprived cells (lanes 3–5 and 7–9). By contrast, the p16 affinity-labeled protein was detected only in IL-3-deprived cells, appearing prominently in 32D cells 24–36 h after IL-3 withdrawal (lanes 4, 5). The presence of p20 and p17 affinity-labeled proteins in healthy (+IL-3) cells suggests that caspases could be constitutively active in these cells. Alternatively, caspases in these cells may be "primed" for rapid activation, resulting in activation during the harvest or affinity labeling procedure. In experiments with peripheral blood lymphocytes, Zapata et al. [1998] have found that inclusion of 2% SDS in the lysis buffer prevents artificial caspase activation during harvest procedures for immunoblotting experiments. We found that inclusion of 2% SDS in the lysis buffer and affinity labeling reactions abrogated affinity labeling of all proteins in +IL-3 cells and resulted in affinity labeling of only the p17 protein in extracts from cells deprived of IL-3 for 24 h (data not shown). While this suggests that caspases in the +IL-3 cells shown in Figure 1A may have been artificially activated during harvest or affinity labeling, these data are somewhat difficult to interpret, because SDS may disrupt association of bio-YVAD-amk with active caspase subunits.

To further verify that the p20, p17, and p16 affinity-labeled proteins were caspase protease subunits, competitive binding experiments were performed (Fig. 2). For these experiments, cells lysates were preincubated for 15 min at 37°C in the absence (lanes 1, 6) or presence of varying concentrations of the caspase peptide inhibitors z-VAD-FMK (lanes 2, 3, 7, 8) and z-DEVD-FMK (lanes 4, 5, 9, 10). After the preincubation period, affinity labeling reactions were performed and analyzed. As shown in Figure 2, both z-VAD-FMK and z-DEVD-FMK efficiently inhibited affinity labeling by bio-YVAD-amk. These data strongly support the conclusion that the p20, p17, and p16 proteins are caspase subunits. In addition, z-DEVD-FMK appears to be more effective than z-VAD-FMK at blocking the affinity labeling (cf. lanes 4, 5 and lanes 2, 3). The z-DEVD-FMK inhibitor, based on a caspase-3 cleavage site in PARP protein [Lazebnik et al., 1994], is more specific for caspase-3, while z-VAD-FMK is a more general "pan" caspase inhibitor. Thus, although the precise identities of p20, p17, and p16 are unknown, we

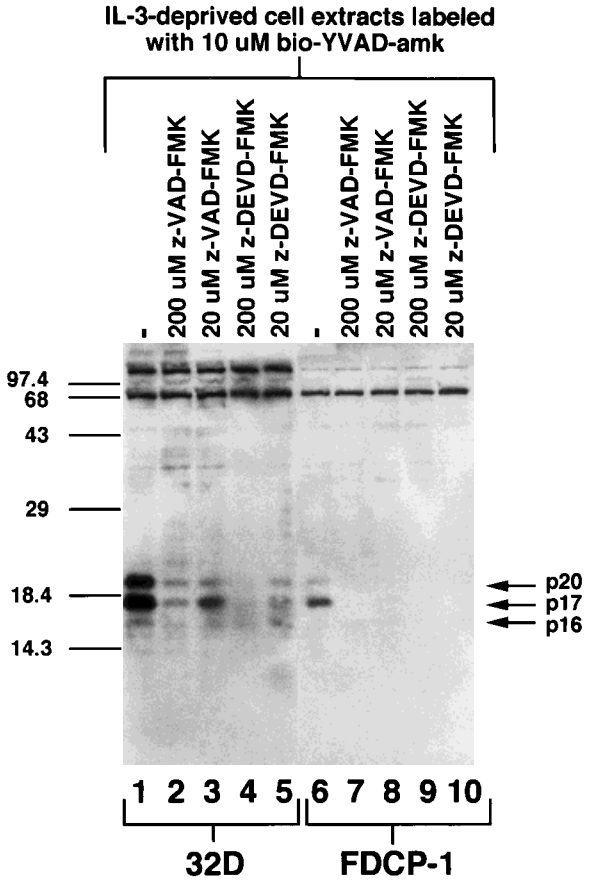

Fig. 2. Inhibition of affinity labeling by peptide inhibitors of caspase proteases. 32D (**lanes 1–5**) and FDCP-1 (**lanes 6–10**) cells were deprived of IL-3 for 24 h, and extracts were prepared for affinity labeling. Before labeling, the extracts were preincubated for 15 min at 37°C in the absence (lanes 1, 6) or presence of varying concentrations of z-VAD-FMK (lanes 2, 3, 7, 8) or z-DEVD-FMK (lanes 4, 5, 9, 10). Affinity labeling reactions were then performed with 10 μM bio-YVAD-amk and affinity-labeled proteins were identified as described for Fig. 1A. Arrows, locations of affinity-labeled p20, p17, and p16 species.

reasoned that they may represent caspase-3-like proteases.

With certain caspases, processing to a final active form is known to require multiple proteolytic steps involving the production of intermediate enzyme forms. For example, in the case of caspase-3, the large subunit is first processed to a 20-kDa polypeptide, followed by further processing to a 17-kDa form [Schlegel et al., 1996]. Thus, it is possible that the p20, p17, and p16 affinity-labeled proteins we observed in IL-3-deprived cells represent different processed forms of a single caspase member. Alternatively, the different affinity-labeled proteins may represent different caspase members. In Figure 3 we examined affinity labeling patterns obtained with different concentrations of bio-YVAD-amk. We found that affinity labeling of

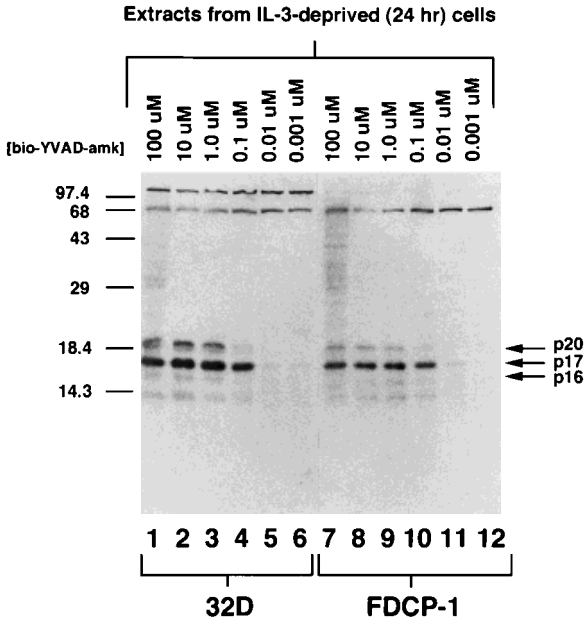

Fig. 3. Concentration dependence of bio-YVAD-amk affinity labeling. 32D (**lanes 1–6**) and FDCP-1 (**lanes 7–12**) cells were deprived of interleukin-3 (IL-3) for 24 h and extracts were prepared for affinity labeling. Labeling reactions were performed for 15 min at 37°C, using varying concentrations of the bio-YVAD-amk labeling reagent.

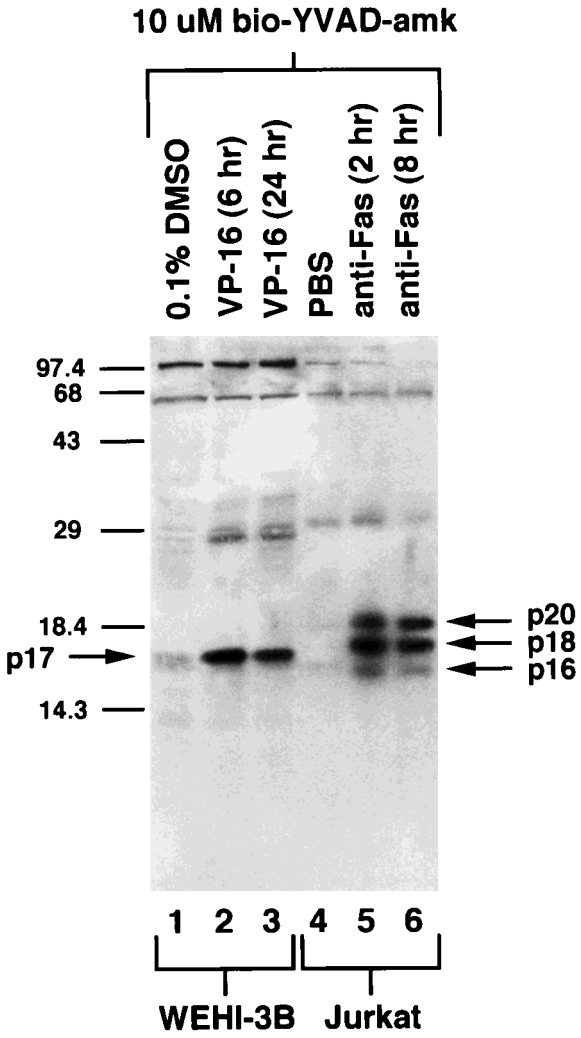

Fig. 4. Affinity labeling of caspases in VP-16-treated WEHI-3B cells and anti-Fas-treated Jurkat cells. WEHI-3B cells were induced to undergo apoptosis by treatment with 20 μM VP-16 for 6 or 24 h (**lanes 2, 3**), and Jurkat cells by treatment with 200 ng/ml anti-Fas antibody for 2 or 8 h (**lanes 5, 6**). As controls, WEHI-3B cells were treated for 24 h with drug diluent (dimethylsulfoxide [DMSO]) (**lane 1**), and Jurkat cells were treated for 8 h with antibody diluent (phosphate-buffered saline [PBS]) (**lane 4**). At the indicated times, cell extracts were prepared and affinity labeled using 10 μM bio-YVAD-amk. Arrows, locations of affinity-labeled caspases from the WEHI-3B (p17) and Jurkat (p20, p18, p16) cells.

the p20 protein decreased markedly when bio-YVAD-amk was reduced from 1.0 μM to 0.1 μM (lanes 3, 4, 9,10). By contrast, affinity labeling of p17 remained constant within the 0.1- to 1.0-μM range, and did not decrease significantly until bio-YVAD-amk was reduced to 0.01 μM (lanes 5, 11). This dose response experiment demonstrates that the two most prominently labeled proteins, p20 and p17, can be distinguished not only on the basis of their size, but also on the basis of their functional ability to bind bio-YVAD-amk.

Affinity Labeling of Caspase Subunits in Cytokine-Independent Cells

To determine whether similar patterns of caspase activation would be observed in cytokine-independent cell lines, affinity labeling experiments were performed with murine WEHI-3B leukemia cells and human Jurkat T leukemic cells (Fig. 4). These cell lines are not dependent on added cytokine for sustained survival. WEHI-3B cells were induced to undergo apoptosis by treatment with 20 μM VP-16 and Jurkat cells by treatment with 200 ng/ml anti-Fas antibody. Figure 4 shows that a single 17-kDa protein was affinity labeled in WEHI-3B cells treated for 6 or 24 h with VP-16 (lanes 2, 3). A single affinity-labeled caspase subunit of 17 kDa was also detected in murine YAC-1 T lymphoma cells treated with 20 μM VP-16 (data not shown). By contrast, three affinity-labeled bands of 20, 18, and 16 kDa were detected in Jurkat cells treated for 2 or 8 h with anti-Fas antibody (lanes 5, 6). The sizes of the affinity-labeled caspases detected in Jurkat cells corresponded closely with those seen in IL-3-deprived 32D and FDCP-1 cells. The different

labeling pattern seen with WEHI-3B and YAC-1 cells suggests that different cell types express different repertoires of caspases, or that processing of caspases differs between cell types.

Figure 4 also shows that little, if any, affinity labeling of caspases was detected in WEHI-3B or Jurkat cells treated with drug solvent (DMSO) or antibody diluent (PBS) alone (lanes 1, 4). This finding was in marked contrast to what was seen with 32D and FDCP-1 cells, where affinity labeling of p20 and p17 was prominent even in the absence of apoptotic stimulus (Fig. 1A, lanes 2, 6). Thus, there appear to be unique biochemical differences between cytokine-dependent myeloid progenitor cells and cytokine-independent cell lines. Perhaps the caspases in cytokine-dependent myeloid cells are poised for rapid activation and are easily activated during the harvest/affinity labeling procedures.

Caspase-3 Subunits Comigrate With Some Affinity-Labeled Caspase Species in IL-3 Deprived 32D and FDCP-1 Cells

Caspase-3 has been shown to be activated by diverse apoptotic stimuli and appears to be important in the apoptotic execution of many cells types [Schlegel et al., 1996; Datta et al., 1996; Jacobson et al., 1996; Stefanis et al., 1996]. Processing of the large subunit of caspase-3 leads to the production of a 20-kDa protein, followed by the production of a 17-kDa form [Schlegel et al., 1996; Cohen, 1997]. To determine whether processed forms of caspase-3 are expressed in IL-3-deprived 32D and FDCP-1 cells, we used a polyclonal antisera directed against murine caspase-3 (generously provided by Dr. Rafick-Pierre Sekaly). This antisera recognizes precursor and processed forms of caspase-3 on immunoblots of murine cells [Alam et al., 1997]. For Figure 5, 32D and FDCP-1 cells were deprived of IL-3 for 24 h, and WEHI-3B cells were treated with VP-16 for 24 h, followed by lysate preparation and affinity labeling with bio-YVAD-amk. Aliquots of the affinity-labeled samples were then subjected to Western blotting for detection of affinity-labeled proteins (lanes 1–3) or anti-murine caspase-3 immunoreactive proteins (lanes 4–6). In both 32D and FDCP-1 cells, prominent caspase-3 bands of 20 and 17 kDa were observed that comigrated with affinity-labeled species from these cells. Thus, at least a portion of the affinity-labeled p20 and p17 proteins in 32D and FDCP-1 cells represent caspase-3 subunits. In the same fashion, in VP-16-treated WEHI-3B cells, a portion of the lone p17 affinity-labeled protein also appears to represent caspase-3 (cf. lanes 3 and 6). Unfortunately, we were unable to use the anti-caspase-3 antisera for immunoprecipitations, and so were unable to determine the percentage of the p20 and p17 affinity-labeled 32D and FDCP-1 proteins that is constituted by caspase-3. In contrast to p20 and p17, the p16 affinity-labeled protein faintly detected in 32D and FDCP-1 cells was not recognized by the anti-murine caspase-3 antisera in several experiments. Therefore, the p16 affinity-labeled caspase subunit that is induced by IL-3 deprivation likely represents a caspase other than caspase-3.

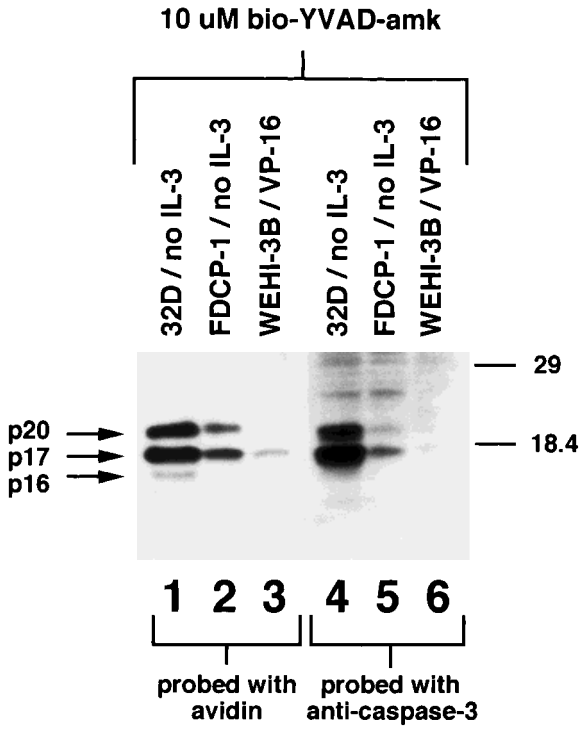

Fig. 5. Processed caspase-3 subunits comigrate with some affinity-labeled caspase species. 32D (**lanes 1, 4**) and FDCP-1 (**lanes 2, 5**) cells were deprived of IL-3 for 24 h, and WEHI-3B cells (**lanes 3, 6**) were treated with 20 M VP-16 for 24 h before extract preparation and affinity labeling with 10 μM bio-YVAD-amk. The affinity-labeled extracts were then electrophoresed on a 14% sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose filter. The filter was probed with NeutrAvidin/bio-horseradish peroxidase (HRP) to detect affinity-labeled caspase species (lanes 1–3), or with anti-murine caspase-3 polyclonal antisera to detect processed caspase-3 subunits (lanes 4–6).

DISCUSSION

Cells in the myeloid lineage play an important role in host defense during an inflamma-

tory response [Arai et al., 1990]. During the process of inflammation, activated T cells synthesize and secrete cytokines, including IL-3 [Metcalf, 1989; Arai, 1990; Metcalf, 1991]. IL-3 acts to promote the proliferation, survival, and differentiation of bone marrow-derived myeloid progenitor cells, giving rise to mature myeloid cells [Arai et al., 1990; Metcalf, 1991; Johnson, 1998]. As an inflammatory response attenuates, the numbers of mature myeloid cells decline rapidly, quickly reaching low basal levels. The rapid loss of mature myeloid cells during attenuation of an inflammatory response and the maintenance of low circulating levels of these cells under normal conditions appears to be tightly regulated by apoptotic cell death. Apoptosis in the myeloid lineage can be caused by induction of differentiation, or by depletion of essential cytokine (ex. IL-3).

Previous studies of IL-3-dependent murine myeloid progenitor cells have shown that IL-3 withdrawal leads to the cleavage of caspase substrate proteins [Barge et al., 1997; Ohta et al., 1997; Antoku et al., 1998]. The induced cleavage activities, as well as ultimate cell death, can be inhibited by peptide inhibitors of caspase substrates [Barge et al., 1997; Ohta et al., 1997; Antoku et al., 1998]. These results indicate that caspase proteases are intimately involved in the apoptotic death of IL-3-deprived myeloid progenitors. Unfortunately, direct demonstration of caspase processing and activation in murine myeloid progenitors has been hampered by unavailability of antisera directed against murine caspases.

In the work described in this report, we used enzyme affinity labeling to directly demonstrate caspase processing and activation in IL-3-dependent 32D and FDCP-1 myeloid progenitor cell lines. Using an affinity labeling reagent, bio-YVAD-amk, that binds to active caspase subunits we detected affinity labeling of caspase subunits of 20, 17, and 16 kDa in extracts from both cell lines. Curiously, only affinity labeling of the 16-kDa species was induced by IL-3 withdrawal. Affinity labeling of p20 and p17 was detected in both the presence and absence of IL-3. This pattern of labeling was dramatically different from what we observed in the cytokine-independent cell lines WEHI-3B, YAC-1, and Jurkat, where essentially no affinity labeling was detected in the absence of an apoptotic stimulus. Thus, we observed considerable differences in the activation of caspases in IL-3-dependent myeloid progenitors when compared with cytokine-independent cell lines.

Our finding that p20 and p17 are affinity labeled even when cells are maintained in the presence of IL-3 may suggest that caspases are constitutively activated in these cells. While most studies have shown that caspase activation is dependent on the presence of an apoptotic stimulus, it has also been reported that caspase-3 can be activated in non-apoptotic T lymphocytes [Miossec et al., 1997; Wilhelm et al., 1998]. The observed activation of caspase-3 in non-apoptotic cells, however, has been questioned by Zapata et al. [1998], who suggest that caspases in some cell types can be artificially activated during harvest procedures. This group reported that formulating lysis buffers to include 2% SDS abrogated artificial activation of caspase-3 and -7 during harvest of non-apoptotic T cells [Zapata et al., 1998]. When we included 2% SDS in our lysis buffer and affinity labeling reactions, we failed to detect any affinity labeling in +IL-3 cells, and only detected affinity labeling of p17 in IL-3-deprived cells. Perhaps this suggests that caspases (or at least the p17 subunit) may be artificially activated during harvest and labeling of the IL-3-dependent myeloid progenitors. Nonetheless, regardless of the interpretation, a fundamental difference in caspase activation was observed between the IL-3-dependent myeloid progenitors and the other cytokine-independent cell lines tested. It appears that caspases in the myeloid progenitors are "primed" or poised for activation.

It is interesting to note that neutrophils, derived by granulocytic differentiation of myeloid progenitors, exhibit short life spans, dying via apoptosis within a few hours after reaching maturity [Savill et al., 1989; Martin et al., 1990; Colotta et al., 1992]. By contrast, monocytic differentiation of myeloid progenitors gives rise to cells (monocytes/macrophages) with considerably longer life spans. Thus, there are lineage-specific differences in susceptibility to apoptosis. Perhaps the "primed" status of caspase activation in myeloid progenitors carries over to the granulocytic lineage and accounts for the rapid apoptotic death of neutrophils. At the same time, there may be mechanisms for down-regulating this "primed" status during differentiation along the monocyte/macrophage pathway. Lineage-specific differences in apoptotic mechanisms have been reported by Sanz et al. [1997], who have observed that the antiapop-

totic protein, Bcl-X_L, is downregulated during granulocytic differentiation, but remains elevated during differentiation along the monocyte/macrophage lineage.

We also demonstrate, using anti-murine caspase-3 polyclonal antisera, that processed caspase-3 subunits of 20 and 17 kDa are expressed in IL-3-deprived 32D and FDCP-1 cells. The observed caspase-3 subunits comigrate with affinity-labeled p20 and p17, indicating that at least a portion of these affinity-labeled species represent active caspase-3. By contrast, affinity-labeled p16 does not appear to be comprised of a caspase-3 subunit. Caspase-3 is expressed in a variety of different cell types [Krajewska et al., 1997] and is known to be processed and activated in response to drug treatment [Chinnaiyan et al., 1996; Datta et al., 1996; Jacobson et al., 1996; Antoku et al., 1997] or stimulation of FAS or TNF receptors [Tewari et al., 1995; Armstrong et al., 1996; Chinnaiyan et al., 1996; Schlegel et al., 1996]. Mice that are deficient in caspase-3 die at about 1–3 weeks of age, and exhibit markedly reduced apoptosis in neurons of the brain [Kuida et al., 1996]. Thus, caspase-3 is important for normal development of the brain. Our data indicate that caspase-3 also may be important in regulating the survival of cells in the myeloid lineage.

While caspase-3 is clearly expressed by IL-3-dependent myeloid progenitors, other caspases (most notably the p16 affinity-labeled caspase) are also likely to be expressed by these cells. Recent studies have shown that different cell types express different repertoires of caspases. In addition, different hierarchies of caspase activation are observed with different apoptotic stimuli [Salvesen and Dixit, 1997]. Additional work is necessary to identify all the caspases that are activated in myeloid progenitor cells and to determine their order of activation in response to cytokine withdrawal.

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