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Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents

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ABSTRACT **Apoptosis induced in myeloid leukemic cells by wild-type p53 was suppressed by different cleavage-site directed protease inhibitors, which inhibit interleukin-1β-converting enzyme-like, granzyme B and cathepsins B and L proteases. Apoptosis was also suppressed by the serine and cysteine protease inhibitor *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), E64, but not by other serine or cysteine protease inhibitors including N^{α}-*p*-tosyl-L-lysine chloromethylketone (TLCK), E64, pepstatin A, or chymostatin. Protease inhibitors suppressed induction of apoptosis by γ-irradiation and cycloheximide but not by doxorubicin, vincristine, or withdrawal of interleukin 3 from interleukin 3-dependent 32D non-malignant myeloid cells. Induction of apoptosis in normal thymocytes by γ-irradiation or dexamethasone was also suppressed by the cleavage-site directed protease inhibitors, but in contrast to the myeloid leukemic cells apoptosis in thymocytes was suppressed by TLCK but not by TPCK. The results indicate that (*i*) inhibitors of interleukin-1β-converting enzyme-like proteases and some other protease inhibitors suppressed induction of apoptosis by wild-type p53 and certain p53-independent pathways of apoptosis; (*ii*) the protease inhibitors together with the cytokines interleukin 6 and interferon-γ or the antioxidant butylated hydroxyanisole gave a cooperative protection against apoptosis; (*iii*) these protease inhibitors did not suppress induction of apoptosis by some cytotoxic agents or by viability-factor withdrawal from 32D cells, whereas these pathways of apoptosis were suppressed by cytokines; (*iv*) there are cell type differences in the proteases involved in apoptosis; and (*v*) there are multiple pathways leading to apoptosis that can be selectively induced and suppressed by different agents.**

Apoptosis can be regulated by various agents. The apoptosis-inducing genes (reviewed in refs. 1–5) include the tumor suppressor gene wild-type p53, but there are also p53-independent pathways of apoptosis (reviewed in refs. 5–8). Activation of apoptosis by wild-type p53 and by certain p53-independent pathways induced by cytotoxic agents can be suppressed by viability-inducing cytokines such as interleukin 6 (IL-6), granulocyte–macrophage colony-stimulating factor (GM-CSF), and interferon γ (IFN-γ) (9–12) and by antioxidants such as butylated hydroxyanisole (BHA) (12). The Ced-3 protein, which is required for programmed cell death during development in the nematode *Caenorhabditis elegans* (13), is a homologue of the mammalian cysteine-protease interleukin-1β-converting enzyme (ICE) (14) and has a similar proteolytic activity (15). Further experiments showed the existence of a family of ICE-related proteases, which can cleave different intracellular proteins and are activated during induction of apoptosis (reviewed in refs. 16–21), and that apoptosis can be suppressed by different protease inhibitors including synthetic cleavage-site-directed inhibitors of ICE-like proteases (17–19, 21). We have now studied the possible involvement of proteases in apoptosis induced by wild-type p53, cytotoxic agents, and viability-factor withdrawal from 32D cells, by using cleavage-site directed peptidyl inhibitors of ICE-like proteases and other protease inhibitors.

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MATERIALS AND METHODS

Cells and Cell Culture. The cells used were: M1 myeloid leukemic cells that do not express p53, transfected with plasmids containing the neomycin resistance gene (M1-neo) or both the neo gene and a temperature-sensitive mutant p53 gene (M1-t-p53) (9, 11, 12); 7-M12 myeloid leukemic cells (22); the interleukin 3 (IL-3)-dependent non-malignant myeloid cell line 32D (23); and normal thymocytes obtained from 1.5-month-old CD-1 mice. The temperature-sensitive p53 codes for a protein [Val-135], which behaves like a tumor suppressing wild-type p53 at 32°C and like a mutant p53 at 37°C (24). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% heat inactivated (56°C, 30 min) horse serum (GIBCO) in a 10% CO_2/90% air atmosphere at 37°C, unless otherwise stated. The IL-3-dependent 32D cells were cultured as above with the addition of 50 μM mercaptoethanol and 10% conditioned medium from WEHI-3B cells as a source of IL-3. When transferred to 32°C, the M1-t-p53 cells expressing the Val-135 temperature-sensitive p53 protein undergo apoptotic cell death (9, 11, 12).

Compounds. The compounds used to determine their effect on induction of apoptosis were the antioxidant BHA (Sigma); the apoptosis-inducing compounds cycloheximide, doxorubicin, dexamethasone (Sigma) and vincristine (Teva Pharmaceutical, Jerusalem); the protease inhibitors chymostatin, E64, pepstatin A, N^{α}-*p*-tosyl-L-lysine chloromethylketone (TLCK), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK) (Sigma), benzyloxycarbonyl-(*Z*) Ala-Ala-Asp-chloromethylketone (Z-AADcmk), Z-Phe-Ala-fluoromethylketone (Z-FAfmk), and Z-Val-Ala-Asp-fluoromethylketone (Z-VADfmk) (Enzyme Systems Products, Dublin, CA); and the recombinant mouse cytokines IL-3 (PeproTech, Rocky Hill, NJ), IL-6 (obtained from J. Van Snick, Ludwig Institute for Cancer Research, Brussels), GM-CSF (Immunex), and IFN-γ (Genzyme).

Assays for Apoptosis and Cell Viability. Apoptosis was induced by culture of M1-t-p53 cells at 32°C (9, 11, 12) and in the other cells used by γ-irradiation at 400R (Co^{60} source, 63R/min, source-sample distance 120 cm) or by addition of the different apoptosis-inducing compounds mentioned above. The percent of apoptotic cells was determined on May–Grünwald–Giemsa-stained cytospin preparations by counting 400 cells. Apoptotic cells were scored by their smaller size,

Abbreviations: BHA, butylated hydroxyanisole; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon γ; IL-3 and IL-6, interleukins 3 and 6, respectively; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethylketone; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; Z-AADcmk, benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone; Z-FAfmk, benzyloxycarbonyl-Phe-Ala-fluoromethylketone; Z-VADfmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; ICE, interleukin 1 β-converting enzyme.

condensed chromatin and fragmented nuclei compared with non-apoptotic cells (9–12). Non-apoptotic and apoptotic cells exclude trypan blue, but the apoptotic cells then undergo additional changes including staining with trypan blue and loss of internal morphology. The percent of cell viability was determined from the ratio of the number of trypan blue excluding and non-apoptotic cells divided by the total number of cells (including trypan blue-stained cells) as described (12).

RESULTS

Protection Against Wild-Type p53-Induced Apoptosis by Z-VADfmk, an Inhibitor of ICE-Like Proteases. Expression of wild-type p53 in M1 myeloid leukemia cells induces apoptotic cell death, which can be suppressed by certain cytokines such as IL-6 and IFN-γ and by antioxidants such as BHA (6–9, 11, 12). In view of the role of ICE-like and other proteases in induction of apoptosis in different cell types (16–21), we examined the possible involvement of such proteases in induction of apoptosis by wild-type p53. As shown previously (9, 11, 12), transfer of M1 cells transfected with the Val-135 temperature-sensitive p53 (M1-t-p53 cells) to the permissive temperature 32°C decreased cell viability (Fig. 1) due to apoptotic cell death (9, 11, 12). Addition of 200 μM Z-VADfmk, a cleavage-site directed peptidyl-fluoromethylketone inhibitor of ICE and other ICE-like proteases to M1-t-p53 cells at the time of transfer to 32°C, suppressed apoptosis and thus increased the percent of viable cells measured at 16–40 hr after cell transfer to 32°C (Fig. 1). This apoptosis-suppressing and therefore viability-promoting effect of Z-VADfmk was dose-dependent both at an earlier (23 hr) or later (40 hr) time after transfer to 32°C (Fig. 2), with a similar degree of protection at 200 and 100 μM Z-VADfmk (Figs. 1 and 2). At 40 hr after transfer to 32°C the percent of cell viability was increased from 5 ± 2% without Z-VADfmk to 22 ± 2% with 100 μM Z-VADfmk (Fig. 3). Z-VADfmk thus gave a better protection than 100 μM of the antioxidant BHA (13 ± 3% viable cells), a similar protection as 1 ng/ml IFN-γ (26 ± 5% viable cells) but a lower protection than 10 ng/ml IL-6 (85 ± 5% viable cells). Because 100 μM of Z-VADfmk can inhibit not only the proteolytic activity of ICE itself but also other ICE-like proteases (18) that may act in a protease cascade, the results suggest that ICE and/or other ICE-like proteases are involved in wild-type p53-induced apoptosis.

Protection Against Wild-Type p53-Induced Apoptosis by Other Protease Inhibitors. The serine-protease granzyme B is involved in induction of apoptosis of target cells by natural killer cells and cytotoxic T lymphocytes (25). This enzyme is unique among the serine proteases in its ability to cleave proteins next to an aspartyl residue (25), a property shared

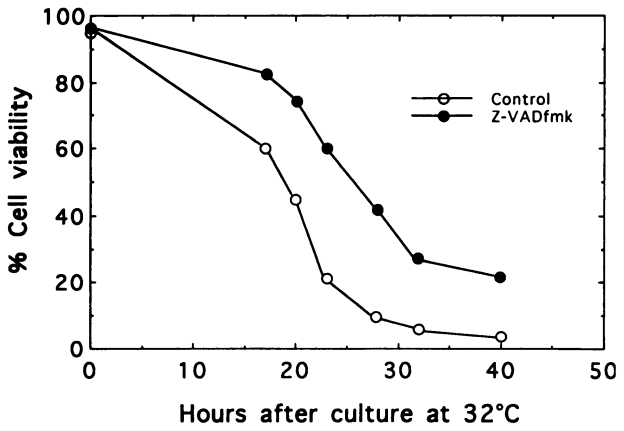

FIG. 1. Time course of protection against wild-type p53-induced apoptosis by the protease inhibitor Z-VADfmk. M1-t-p53 cells were transferred to 32°C to activate wild-type p53 and cultured at 3 × 10^5 cells per ml without or with 200 μM Z-VADfmk. The percent of viable cells was determined at the indicated times after transfer to 32°C.

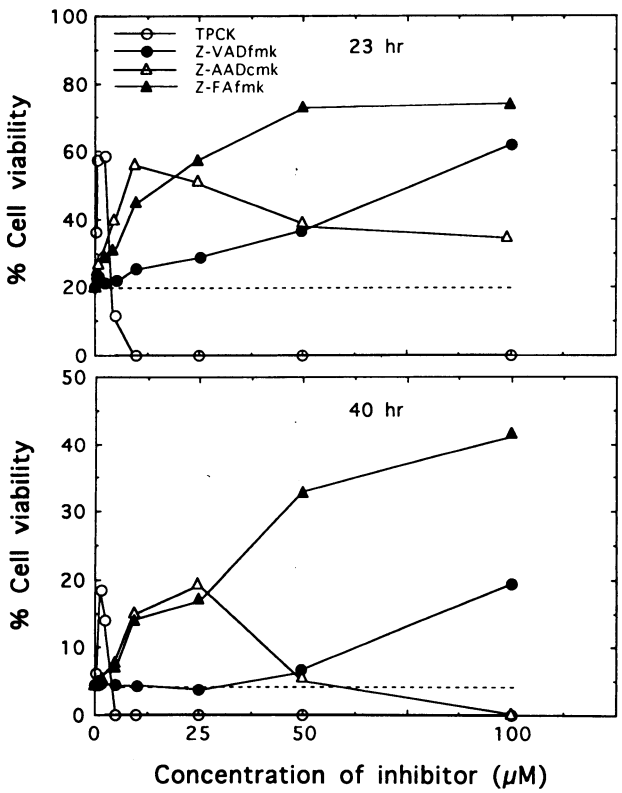

FIG. 2. Dose dependence of the protective effect of different protease inhibitors against wild-type p53-induced apoptosis. M1-t-p53 cells were cultured at 32°C as described in Fig. 1 without any additions (broken line) or with the indicated concentrations of protease inhibitors. The percent of viable cells was determined 23 hr (*Upper*) or 40 hr (*Lower*) after transfer of cells to 32°C.

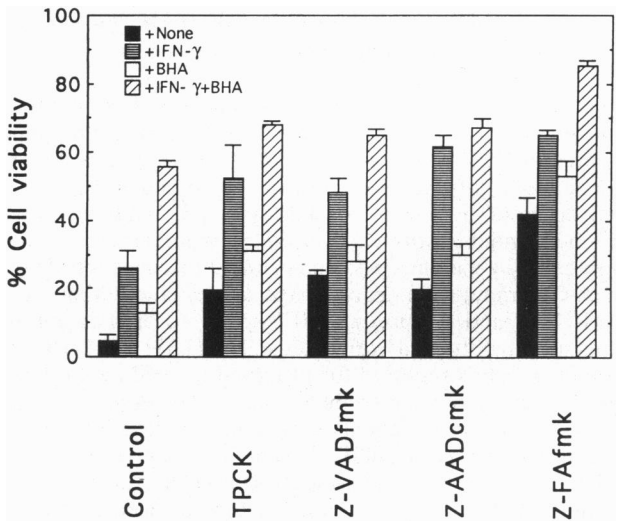

FIG. 3. Cooperative protection of the cytokine IFN-γ, the antioxidant BHA, and different protease inhibitors against wild-type p53-induced apoptosis. M1-t-p53 cells were cultured at 32°C without (control) or with 1 μM TPCK, 100 μM Z-VADfmk, 25 μM Z-AADcmk, or 100 μM Z-FAfmk. The cultures contained either no further additions (None) or were supplemented with 100 μM BHA, 1 ng/ml IFN-γ, or both BHA plus IFN-γ. The percent of viable cells was determined 40 hr after cell transfer to 32°C. When cells were cultured for 40 hr with 10 ng/ml IL-6, the percent of viable cells was 85 ± 5%.

with all ICE-like cysteine proteases (16–21). Granzyme B was also shown to cleave and thus activate the proteolytic activity of other ICE-like apoptosis-inducing proteases (26–30). The use of an active-site-directed peptidyl inhibitor of granzyme B, Z-AADcmk, showed that it was also an effective inhibitor of wild-type p53-induced apoptosis in M1-t-p53 leukemic cells, with an optimum protection at 10–25 μM (Fig. 2). Concentrations of 50 or 100 μM Z-AADcmk gave a lower cell viability (Fig. 2). Although the optimum protective effect of Z-AADcmk was obtained at a 10-fold lower molar concentration than with Z-VADfmk, both protease inhibitors gave a similar degree of protection (Fig. 2). The results indicate that granzyme B or other granzyme B-like enzymes also seem to be involved in wild-type p53-induced apoptosis in M1-t-p53 cells, possibly by activating ICE-like proteases. Z-FAfmk, a cleavage-site-directed peptidyl inhibitor of the lysozomal cysteine proteases cathepsins B and L, also effectively suppressed wild-type p53-induced apoptosis with an optimum activity at 50–100 μM (Fig. 2). Z-FAfmk was even more effective as a suppressor of wild-type p53-induced apoptosis in M1-t-p53 cells than Z-VADfmk or Z-AADcmk (Figs. 2 and 3). But E64, another inhibitor of cathepsins B and L, did not suppress wild-type p53-induced apoptosis (Table 1).

Results obtained with a less specific chloromethylketone containing protease inhibitor, TPCK, which inhibits chymotrypsin-like serine proteases, as well as some cysteine proteases, has also shown a protective effect against wild-type p53-induced apoptosis with an optimum at 1–2 μM and a similar degree of protection as Z-VADfmk and Z-AADcmk (Figs. 2 and 3). Concentrations of 5 μM or higher TPCK were cytotoxic to M1-t-p53 cells. In contrast, the structurally similar protease inhibitor TLCK, which inhibits trypsin-like serine proteases and some cysteine proteases, did not show a statistically significant protection against wild-type p53-induced apoptosis (Table 1). Three other protease inhibitors, E64, which inhibits cysteine proteases, chymostatin, which inhibits chymotrypsins and many cysteine proteases, and pepstatin A, which inhibits acid proteases such as pepsin and cathepsin D, were completely ineffective as antiapoptotic compounds in M1-t-p53 cells even at 100 μM (Table 1). Because TPCK does not inhibit the proteolytic activity of ICE-like proteases (15), the results indicate that ICE-like as well as certain other cellular proteases participate in wild-type p53-induced apoptosis in M1-t-p53 leukemic cells.

Cooperative Protection Against Wild-Type p53-Induced Apoptosis by Protease Inhibitors, IFN-γ, IL-6, and BHA. The results of various experiments (16–21, 26–28, 30–34) have indicated that protease activation during apoptosis may occur in a cascade along a common pathway. According to this suggestion, any protease inhibitor that interferes with such a protease cascade would cause a similar protective effect against apoptosis. There was no increase in protection against wild-type p53-induced apoptosis by using paired combinations of the protease inhibitors Z-VADfmk, Z-AADcmk, Z-FAfmk, and TPCK. Even when all four protease-inhibitors were added together, protection (35 ± 4% viable cells at 40 hr) was similar to protection by the best protective protease inhibitor, Z-FAfmk, by itself (42 ± 5%). This supports the possibility that all these protease inhibitors interfered with a common pathway leading to apoptosis. However, when each of the protease inhibitors tested was combined with the cytokine IFN-γ or the antioxidant BHA, there was a better protection against apoptosis compared with the protective effect of each compound alone (Fig. 3). There was an even better protection when each of the protease inhibitors was combined with both IFN-γ and BHA, increasing M1-t-p53 cell viability at 32°C after 40 hr up to 85 ± 2% with Z-FAfmk (Fig. 3), similar to the protective effect of an optimum concentration (10 ng/ml) of IL-6. Combination of 10 μM Z-AADcmk or 1 μM TPCK with a suboptimal amount (0.5 ng/ml) of IL-6 also gave a better protective effect, 48 ± 3% and 45 ± 4% viable cells at 32°C after 40 hr with Z-AADcmk+IL-6 and TPCK+IL-6, respectively, compared with 20–25% viable cells with each compound alone. These results indicate that cytokines, an antioxidant, and protease inhibitors can act cooperatively to protect cells against induction of apoptosis, presumably by blocking different pathways that lead to apoptosis.

In all of the above experiments, the protective cytokines, BHA, and protease inhibitors were added to M1-t-p53 cells at the time of transfer to 32°C. Because apoptotic cells were not detected up to about 8 hr after transfer of M1-t-p53 cells to 32°C (9), we determined the effect of delayed addition of the apoptosis-inhibiting compounds. Addition of IL-6 or IFN-γ, even as late as 8 hr after transfer to 32°C, still showed the same protection against apoptosis as when added at the time of transfer to 32°C (time 0) (Fig. 4). However, addition of the protease inhibitors or BHA at 4 or 8 hr after cell transfer to 32°C reduced their protective effect compared with their addition at time 0 (Fig. 4). These results also support the suggestion that the cytokines IL-6 and IFN-γ may act by blocking different or additional pathways to apoptosis than those blocked by the protease inhibitors or BHA.

Differential Protective Effect by Protease Inhibitors Against Induction of Apoptosis by Cytotoxic Agents and Viability-Factor Withdrawal. The above experiments were all carried out with M1-t-p53 leukemic cells in which activation of wild-type p53 expression at 32°C induces apoptotic cell death. To

Table 1. Lack of protective effect by some protease inhibitors against wild-type p53 induced apoptosis

Protease inhibitor added	Concentration, μM	Cell viability at 23 hr,* %
None	—	20.5 ± 3.5
TLCK	1–20	27.0 ± 5.4
E64	1–100	17.4 ± 3.6
Pepstatin A	1–100	14.9 ± 3.6
Chymostatin	1–100	15.3 ± 2.5

*M1-t-p53 cells were cultured at 32°C without any additions (None) or in the presence of different protease inhibitors for 23 hr, and the percent of cell viability was determined. The apparent slightly higher percent of viable cells with TLCK compared to the control (None) was not statistically significant.

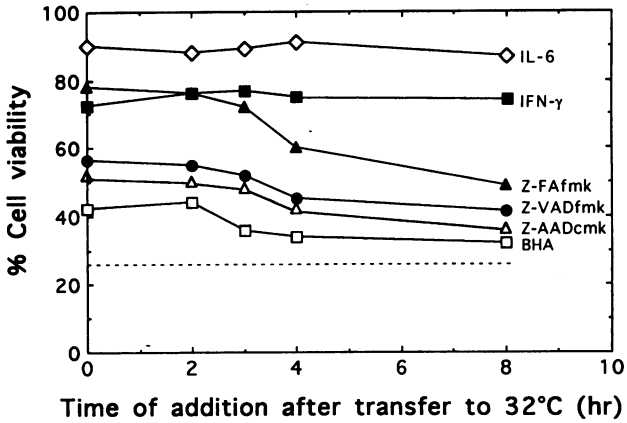

FIG. 4. Effect of delayed addition of cytokines, BHA, and protease inhibitors on protection against wild-type p53-induced apoptosis. M1-t-p53 cells were cultured at 32°C without any additions (broken line) or with 10 ng/ml IL-6, 1 ng/ml IFN-γ, 100 μM BHA, 100 μM Z-VADfmk, 25 μM Z-AADcmk, or 100 μM Z-FAfmk. These compounds were added either at the time of transfer to 32°C (time point 0) or 2, 3, 4, or 8 hr later and percent cell viability was determined 23 hr after cell transfer to 32°C.

Table 2. The ability of GM-CSF, BHA, and different protease inhibitors to protect against induction of apoptosis in 7-M12 cells by γ-irradiation, cycloheximide, and doxorubicin

Material added	Concentration	% apoptotic cells* γ-irradiation (3 hr)	Cycloheximide (2.5 hr)	Doxorubicin (5 hr)
None	—	53.9 ± 6.0	39.7 ± 6.3	34.7 ± 4.8
GM-CSF	5 ng/ml	2.4 ± 1.2†	25.5 ± 5.2†	8.3 ± 2.5†
BHA	100 μM	20.6 ± 4.3†	20.5 ± 3.1†	16.8 ± 4.5†
Z-VADfmk	100 μM	11.3 ± 3.6†	8.4 ± 3.5†	30.7 ± 4.0
Z-AADcmk	25 μM	48.8 ± 6.3	26.6 ± 2.8†	37.5 ± 3.8
Z-FAfmk	100 μM	18.1 ± 3.3†	5.9 ± 2.1†	38.2 ± 4.5
TPCK	1 μM	42.5 ± 4.8‡	29.0 ± 3.5†	34.8 ± 3.6
TLCK	20 μM	55.7 ± 6.3	48.8 ± 5.4	38.0 ± 5.4

7-M12 leukemic cells treated with γ-irradiation at 400R, 0.5 μg/ml cycloheximide, or 1.5 μg/ml doxorubicin were cultured with no additions (None) or with the indicated concentrations of different materials. Percent of apoptotic cells was determined 3, 2.5, or 5 hr after γ-irradiation or addition of cycloheximide or doxorubicin, respectively.
*Four experiments were carried out ($n = 4$) and the P values (Student's t test) given are for the statistical significance of the difference between the added compounds and the control (None) group. †$P < 0.001$; ‡$P < 0.05$.

determine the possible involvement of proteases in apoptosis induced by cytotoxic agents we first used 7-M12 leukemic cells, which accumulate p53 protein followed by apoptosis after treatment with γ-irradiation, cycloheximide, or doxorubicin (12). 7-M12 cells also lack Bcl-2 protein expression (12) and induction of apoptosis in 7-M12 cells is more rapid than in M1 cells. We also used M1-neo leukemic cells, which do not express p53 protein (9) even after treatment with these compounds. As in M1-t-p53 cells cultured at 32°C, induction of apoptosis in 7-M12 cells by cycloheximide was suppressed by Z-VADfmk, Z-AADcmk, Z-FAfmk, and TPCK, but not by TLCK (Table 2). Similar results were obtained with γ-irradiated 7-M12 cells (Table 2). Induction of apoptosis in M1-neo cells by cycloheximide was also suppressed by these protease inhibitors with Z-VADfmk showing the best suppression (Table 3). These results indicate that certain proteases are also involved in apoptosis induced by cycloheximide in myeloid leukemic cells that do or do not express p53. There was also a cooperative protective effect against induction of apoptosis in M1-neo cells by cycloheximide when protease inhibitors were combined with the cytokines IFN-γ or IL-6 (Fig. 5).

Induction of apoptosis in normal thymocytes by γ-irradiation via a p53-dependent pathway or by dexamethasone via a p53-independent pathway (35–37), was effectively suppressed by Z-VADfmk and to a lesser extent by Z-AADcmk and

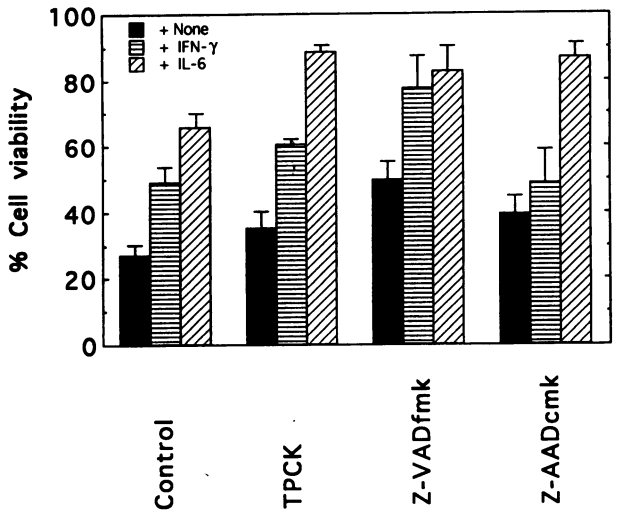

FIG. 5. Cooperative protection of different protease inhibitors and the cytokines IFN-γ and IL-6 against induction of p53-independent apoptosis by cycloheximide. M1-neo cells were cultured at 37°C with 0.2 μg/ml cycloheximide without (control) or with 1 μM TPCK, 100 μM Z-VADfmk or 25 μM Z-AADcmk. The cultures contained either no further additions (None) or were supplemented with 1 ng/ml IFN-γ or 10 ng/ml IL-6. The percent of viable cells was determined 16 hr after addition of the different compounds.

Z-FAfmk, but not by E64, pepstatin A, or chymostatin (Table 4). Apoptosis induced in thymocytes by methylprednisolone or etoposide has previously been reported to be suppressed by Z-VADcmk (38). However, unlike the myeloid leukemic cells, TLCK effectively suppressed apoptosis in thymocytes, but TPCK up to 5 μM did not (Table 4). Higher concentrations of TPCK were cytotoxic. These results indicate that certain proteases are also involved in thymocyte apoptosis and that there are cell type differences in the ability of different protease inhibitors to block apoptosis.

Unlike cytokines such as IL-6, IFN-γ, or CM-CSF (6–11) and the antioxidant BHA (12), none of the protease inhibitors tested suppressed induction of apoptosis in 7-M12 or M1-neo cells by doxorubicin (Tables 2 and 3). These protease inhibitors also did not suppress apoptosis induced in M1-neo cells by vincristine (Table 3) or in the IL-3-dependent 32D cells following IL-3 withdrawal (Fig. 6). The results indicate that the cytokines, BHA, and protease inhibitors have a different ability to suppress induction of apoptosis by different cytotoxic agents (Tables 2 and 3) and presumably block different pathways that lead to apoptosis.

Table 3. The ability of IL-6, IFN-γ, BHA, and different protease inhibitors to protect against induction of p53-independent apoptosis in M1 cells by cycloheximide, doxorubicin, and vincristine

Compound added	Concentration	Cell viability at 16 hr,* % Cycloheximide	Doxorubicin	Vincristine
None	—	26.8 ± 2.9	43.6 ± 5.2	31.0 ± 3.5
IL-6	10 ng/ml	65.5 ± 4.3†	74.5 ± 5.2†	88.5 ± 4.1†
IFN-γ	1 ng/ml	48.9 ± 4.5†	68.2 ± 4.3†	69.6 ± 5.8†
BHA	100 μM	27.2 ± 2.6	64.2 ± 4.3†	30.1 ± 5.0
Z-VADfmk	100 μM	49.5 ± 5.0†	40.3 ± 4.8	23.8 ± 3.1
Z-AADcmk	25 μM	39.1 ± 5.3†	37.5 ± 5.2	15.3 ± 2.6
Z-FAfmk	100 μM	44.7 ± 3.7†	31.8 ± 4.5	20.1 ± 3.1
TPCK	1 μM	35.1 ± 5.2‡	45.7 ± 6.1	27.4 ± 5.2

M1-neo cells were treated with 0.2 μg/ml cycloheximide, 1 μg/ml doxorubicin, or 0.07 μg/ml vincristine and cultured without any other additions (None) or with the indicated concentrations of BHA, IFN-γ, or different protease inhibitors. The percent of cell viability was determined 16 hr later.
*Four experiments were carried out ($n = 4$) and the P values (Student's t test) given are for the statistical significance of the difference between the percent of cell viability in different groups and the control (None) group. †$P < 0.001$; ‡$P < 0.02$.

Table 4. The ability of different protease inhibitors to protect against induction of apoptosis in thymocytes by γ-irradiation and dexamethasone

Compound added	Concentration, μM	Apoptotic cells at 6 hr,* %	
		γ-irradiation	Dexamethasone
None	—	52.0 ± 4.5	51.5 ± 7.8
TPCK	5	47.8 ± 4.7	45.2 ± 10.3
TLCK	20	19.5 ± 6.2†	11.5 ± 4.3†
Z-VADfmk	100	3.5 ± 1.6†	3.2 ± 1.0†
Z-AADcmk	25	29.2 ± 3.8†	24.3 ± 0.6†
Z-FAfmk	100	35.1 ± 8.5‡	40.7 ± 5.3§
E64	100	52.3 ± 5.2	50.8 ± 4.5
Pepstatin A	100	55.8 ± 6.5	48.5 ± 4.8
Chymostatin	100	50.6 ± 6.6	53.5 ± 10.5

Thymocytes were γ-irradiated at 400R or treated with 0.1 μM dexamethasone and cultured for 6 hr without any other additions (None) or the indicated concentrations of different protease inhibitors.

*Three experiments were carried out ($n = 3$) and the P values (Student's t test) given are for the statistical significance of the difference between the percent of apoptotic cells in different groups and the control (None) group. †$P < 0.001$; ‡$P < 0.01$; §$P < 0.05$.

DISCUSSION

Programmed cell death in the nematode *C. elegans* is dependent on the Ced-3 protein, which is a cysteine protease with amino acid sequence and substrate specificity related to mammalian ICE-like proteases (13–15). Activation of apoptosis in mammalian cells by the p55 tumor necrosis factor receptor or the related FAS/APO-1 receptor is associated with activation of ICE and other ICE-like proteases (30, 33, 34), and ICE-knockout mice show a defective FAS-induced apoptotic pathway (39). It has also been shown that apoptosis can be suppressed by active-site-directed inhibitors of ICE-like proteases (reviewed in refs. 16–21).

We have now tested the involvement of proteases in apoptosis induced by wild-type p53-dependent and p53-independent pathways. Using active-site-directed peptidyl inhibitors of different proteases we have shown that wild-type p53-induced apoptosis in M1 myeloid leukemic cells was suppressed by Z-VADfmk, Z-AADcmk, and Z-FAfmk, inhibitors of ICE-like, granzyme B, and cathepsins B and L, respectively. The serine and cysteine protease inhibitor TPCK, which does not inhibit the proteolytic activity of ICE-like proteases (15), also suppressed wild-type p53-induced apoptosis. These results indicate that different proteases, including ICE-like proteases, are involved in induction of apoptosis by wild-type p53. However, other protease inhibitors, including E64, which inhibits cysteine proteases, including cathepsins B and L, TLCK, which inhibits trypsin-like proteases, pepstatin A, which inhibits cathepsin D, and chymostatin, which inhibits different chymotrypsins and some cysteine proteases, did not suppress apoptosis. Since both Z-FAfmk and E64 inhibit cathepsins B and L but only Z-FAfmk suppressed apoptosis, it will be interesting to determine whether Z-FAfmk also inhibits other proteases. The ability of Z-AADcmk, a granzyme B inhibitor, to protect against wild-type p53-induced apoptosis suggests that granzyme B or related proteases are involved in this pathway of apoptosis in myeloid leukemic cells. Granzyme B was shown to cleave and activate several ICE-like proteases (26–30), which could produce a cascade of protease activation during apoptosis. Our experiments have shown that different protease inhibitors can protect against wild-type p53-induced apoptosis. In addition, combinations of different protease inhibitors did not improve the protection against wild-type p53-induced apoptosis. These data support the suggestion (31, 32) that apoptosis is associated with a cascade of activated proteases that may act in a common pathway to apoptosis.

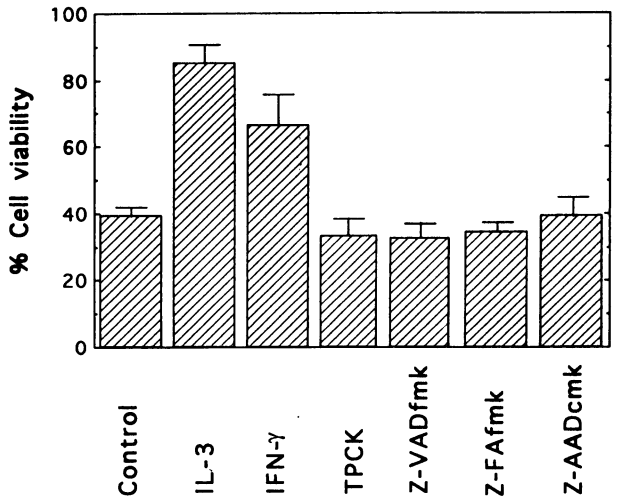

FIG. 6. Lack of protective effect of protease inhibitors against induction of apoptosis in IL-3-dependent 32D cells following IL-3 withdrawal. 32D cells were washed twice to remove IL-3 and cultured without any additions or with 5 ng/ml IL-3, 1 ng/ml IFN-γ, 0.5 μM TPCK, 50 μM Z-VADfmk, 50 μM Z-FAfmk, or 10 μM Z-AADcmk. The percent of viable cells was determined 23 hr after adding the different compounds. When these protease inhibitors were added at a higher concentration, as used for the myeloid leukemic cells, the percent of viable cells was lower; 26.2 ± 5.3%, 20.1 ± 4.8%, 18.9 ± 3.4%, and 12.6 ± 4.1% with 1 μM TPCK, 100 μM Z-VADfmk, 100 μM Z-FAfmk, or 25 μM Z-AADcmk, respectively.

We have previously shown that the cytokine IL-6 can give almost full protection against wild-type p53-induced apoptosis in M1 cells (9, 11, 12), whereas antioxidants (12), IFN-γ (11, 12), and the protease inhibitors in the present results, only partially protect against apoptosis. However, a combination of IFN-γ, the antioxidant BHA, and protease inhibitors gave almost full protection against wild-type p53-induced apoptosis. The protease inhibitors also enhanced the protective effect of a suboptimal concentration of IL-6 against wild-type p53-induced apoptosis and cooperated with IFN-γ and IL-6 to give better protection against induction of apoptosis in M1-neo cells that do not express wild-type p53. This suggests that IFN-γ, IL-6, BHA, and protease inhibitors act by suppressing different pathways leading to apoptosis in these cells. The results indicate that BHA and the protease inhibitors suppress some early steps in the apoptotic process following activation of wild-type p53 so that their delayed addition resulted in a lower ability to suppress apoptosis. In contrast, delayed addition of the cytokines IL-6 or IFN-γ showed the same effectiveness even when added as late as 8 hr after activation of wild-type p53. This also supports the suggestion of a different mechanism of suppression of apoptosis by the cytokines compared with BHA and the protease inhibitors. It will be interesting to determine to what extent the higher effectiveness of IL-6 as an anti-apoptotic agent in this system may be due to its ability to suppress protease activation, as well as activation of some other pathways. It will also be interesting to determine whether the effect of BHA involves a block of protease activation.

In p53-independent apoptosis in M1 leukemic cells, the protease inhibitors suppressed induction of apoptosis by cycloheximide but not by doxorubicin or vincristine. The protease inhibitors also suppressed induction of apoptosis in the p53 expressing 7-M12 leukemic cells by γ-irradiation or cycloheximide but not by doxorubicin, and they did not suppress apoptosis induced by viability-factor withdrawal in the IL-3 dependent 32D cells. The data indicate that certain pathways leading to apoptosis induced by some agents may require activation of proteases that are not inhibited by the protease

Table 5. Summary of the protective effect of cytokines, an antioxidant, and protease inhibitors against induction of apoptosis in M1 myeloid leukemic cells

Protective compound	Protection against apoptosis induced by			
	Wild-type p53*	Cyclo-heximide	Doxo-rubicin	Vin-cristine
Cytokines				
IL-6, IFN-γ	+	+	+	+
Antioxidant				
BHA	+	—	+	—
Protease inhibitors				
Z-VADfmk, Z-AADcmk, Z-FAfmk, TPCK	+	+	—	—

*Wild-type p53-induced apoptosis was determined in M1-t-p53 cells and induction of apoptosis by cycloheximide, doxorubicin, or vincristine was determined in M1-neo cells, which do not express p53.

inhibitors used in the present experiments, or may not require protease activation. The experiments with thymocytes have shown that while proteases are involved in wild-type p53-dependent and p53-independent pathways of apoptosis, the pattern of effectiveness of different protease inhibitors as anti-apoptotic compounds was different than in the myeloid leukemic cells. This suggests that there are cell type differences in the proteases involved in apoptosis. The different pattern of the ability of cytokines, BHA, and protease inhibitors to suppress induction of apoptosis by different agents (Table 5) and their ability to cooperate in protection against apoptosis indicates that there are multiple pathways leading to apoptosis, which can be selectively activated by different stimuli and selectively suppressed by different anti-apoptotic agents.

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