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Different mechanisms for suppression of apoptosis by cytokines and calcium mobilizing compounds

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ABSTRACT **Overexpression of wild-type p53 in M1 myeloid leukemia cells induces apoptotic cell death that was suppressed by the calcium ionophore A23187 and the calcium ATPase inhibitor thapsigargin (TG). This suppression of apoptosis by A23187 or TG was associated with suppression of caspase activation but not with suppression of wild-type-p53-induced expression of *WAF-1, mdm-2,* or *FAS*. In contrast to suppression of apoptosis by the cytokines interleukin 6 (IL-6) and interferon γ, a protease inhibitor, or an antioxidant, suppression of apoptosis by A23187 or TG required extracellular Ca^{2+} and was specifically abolished by the calcineurin inhibitor cyclosporin A. IL-6 induced immediate early activation of *junB* and *zif*/268 (*Egr-1*) but A23187 and TG did not. A23187 and TG also suppressed induction of apoptosis by doxorubicin or vincristine in M1 cells that did not express p53 by a cyclosporin A-sensitive mechanism. Suppression of apoptosis by A23187 or TG was not associated with autocrine production of IL-6. Apoptosis induced in IL-6-primed M1 cells after IL-6 withdrawal was not suppressed by A23187 or TG but was suppressed by the cytokines IL-6, IL-3, or interferon γ. The results indicate that these Ca^{2+}-mobilizing compounds can suppress some pathways of apoptosis suppressed by cytokines but do so by a different mechanism.**

Wild-type p53 overexpression induces apoptosis in M1 myeloid leukemic cells (1), and this gene is required for certain pathways of apoptosis induced by radiation and DNA-damaging compounds in normal thymocytes (2–4) and bone marrow myeloid precursor cells (2). Wild-type p53 also participates in apoptosis pathways induced by viability factor withdrawal from normal myeloid precursors (2) and certain factor-dependent cell lines (5, 6), and there are p53-independent pathways of apoptosis (2–4). Our previous studies with p53-expressing or nonexpressing cells have shown that different cytokines, including interleukin (IL) 6, IL-3, interferon γ (IFN-γ), and granulocyte–macrophage colony-stimulating factor, can suppress apoptosis induced by overexpression of wild-type p53 and by cytotoxic compounds (for review, see refs. 7 and 8). Some apoptotic pathways were also suppressed by certain antioxidants (9) and protease inhibitors (10, 11). The apoptosis-suppressing cytokines, antioxidants, and protease inhibitors suppressed activation of interleukin 1β converting enzyme-like cysteine proteases (caspases), showing that these apoptosis suppressors act upstream of caspase activation (11).

Different cell membrane tyrosine kinase and G protein-linked receptors transduce mitogenic signals through activation of inositol triphosphate, which generates intracellular calcium signaling mechanisms (for review, see ref. 12). Some of these mitogenic signals can be mimicked by sustained increase of intracellular Ca^{2+} concentration through the use of Ca^{2+}-mobilizing compounds such as calcium ionophores (for review, see ref. 13). The calcium ionophore A23187 and the calcium ATPase inhibitor thapsigargin (TG), which cause sustained increase of intracellular Ca^{2+} concentration (14, 15), can promote cell viability by suppressing apoptosis in some cells (16–19) but can induce apoptosis in other cells (20–22). The present experiments, using myeloid leukemic cells with or without wild-type p53, were carried out to determine how far Ca^{2+}-mobilizing compounds can mimic cytokine suppression of apoptosis induced in different ways. The results show that A23187 and TG can suppress wild-type-p53-dependent and some p53-independent pathways of apoptosis and that the mechanism of this suppression differs from that used by apoptosis-suppressing cytokines.

MATERIALS AND METHODS

Cells and Cell Culture. The cells used were mouse M1 myeloid leukemic cells, which do not express p53, transfected with plasmids containing the neomycin-resistance gene (M1-neo) or both the neomycin-resistance gene and a temperature-sensitive mutant p53 gene (M1-t-p53) (1). In M1-t-p53 cells, the temperture-sensitive p53 gene codes for a protein, [Val-135]p53, that behaves like a tumor-suppressing wild-type p53 at 32°C and like a mutant p53 at 37°C (23). The cells were cultured in DMEM (GIBCO/BRL) with 10% heat-inactivated (56°C, 30 min) horse serum (GIBCO/BRL) in a 10% CO_2/90% air atmosphere at 37°C or 32°C. In some experiments, cells were cultured in calcium-free DMEM with 10% dialyzed horse serum.

Compounds. The compounds used to induce apoptosis were doxorubicin (Dox) (Sigma) and vincristine (VCR) (Teva, Jerusalem). The compounds used to suppress apoptosis were the antioxidant butylated hydroxyanisole (BHA; Sigma); the protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma); the calcium ionophore A23187 (Sigma); the calcium ATPase inhibitor TG (Sigma); and the recombinant mouse cytokines IL-6 (obtained from J. Van Snick, Ludwig Institute for Cancer Research, Brussels), IL-3 (PeproTech, Rocky Hill, NJ), and IFN-γ (Genzyme). Tumor necrosis factor (Genzyme) and the calcineurin inhibitor cyclosporin A (CsA; Sandoz Pharmaceutical) were also used.

Assays for Apoptosis and Cell Viability. Apoptosis was induced in M1-t-p53 cells seeded at 3×10^5 cells per ml and cultured at 32°C (1, 9–11, 24) and in M1-neo cells by addition of Dox or VCR. The percent apoptotic cells was determined on May–Grünwald–Giemsa-stained cytospin preparations by counting 400 cells. Apoptotic cells were scored by their smaller size, condensed chromatin, and fragmented nuclei compared

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Abbreviations: BHA, butylated hydroxyanisole; CsA, cyclosporin A; Dox, doxorubicin; IFN-γ, interferon γ; IL, interleukin; MDR, multidrug resistance; Nedd-2, *n*eural precursor cell-*e*xpressed *d*evelopmentally *d*own-regulated gene 2; PARP, poly(ADP-ribose) polymerase; TG, thapsigargin; VCR, vincristine; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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with nonapoptotic cells (1, 9–11, 24–26). The percent cell viability was determined from the ratio of the number of viable cells (trypan blue excluding and nonapoptotic) divided by the total number of cells (including trypan blue stained and apoptotic cells), as described (9).

Determination of Protease Activation and Western Blotting. Activation of caspases during apoptosis in M1 cells is associated with intracellular cleavage of specific proteins, including poly(ADP-ribose) polymerase (PARP) and the proenzyme of the caspase Nedd-2 (*n*eural *p*recursor cell-*e*xpressed *d*evelopmentally *d*own-regulated gene 2) (11). Cleavage of these two proteins was determined by SDS/PAGE of 100 μg of whole-cell extract proteins, followed by Western blotting using antibody to the amino-terminal portion of mouse PARP (A20) or the p12 carboxyl-terminal portion of mouse Nedd-2 (C20) (Santa Cruz Biotechnology) (11). Degradation of IκBα was determined by Western blotting using an anti-IκBα antibody (C-21, Santa Cruz Biotechnology). Blots were washed, incubated with horseradish peroxidase-conjugated anti-IgG secondary antibody (Santa Cruz Biotechnology), and developed with the enhanced chemiluminescence detection kit (ECL; Amersham), and bands corresponding to the appropriate proteins or protein fragments were visualized after exposing the blots to Fuji RX medical x-ray film, as described (11).

RNA Preparation and Northern Blot Hybridization. Cultured cells (10×10^6 cells) were lysed in 0.65% Nonidet P-40 in buffer A (10 mM Tris·HCl, pH 7.5/150 mM NaCl/1.5 mM $MgCl_2$). Nuclei were removed by centrifugation at $800 \times g$, and an equal volume of buffer B (10 mM Tris·HCl, pH 7.5/10 mM EDTA/350 mM NaCl/1% SDS/7 M urea) was added to the supernatant. Cytoplasmic RNA was extracted with phenol/chloroform and precipitated with ethanol. Twenty micrograms of RNA was loaded in each lane of a 1% agarose gel containing 3% formaldehyde, electrophoresed, blotted to a GeneScreen-*Plus* membrane, and hybridized under stringent conditions to [^{32}P]dCTP-labeled (random-priming NEBlot kit, New England Biolabs) cDNA probes for mouse *junB* (27), *zif*/268 (*Egr-1*) (28), *WAF-1* (29), *FAS* (30), and *mdm-2* (31) and a rat genomic probe for cytoplasmic β-actin (32). Blots were exposed to Fuji RX medical x-ray film with an intensifying screen at −80°C.

RESULTS

Suppression of Wild-Type p53 Induced Apoptosis by A23187 and TG. Culture of M1-t-p53 cells at 32°C, when the p53 protein behaves as wild-type p53, induces apoptosis with 32 ± 5% morphologically identified apoptotic cells after 23 hr (1, 9). This resulted in a decrease of cell viability (trypan blue-excluding and nonapoptotic cells) to 24.6 ± 3% (Fig. 1 and Table 1). Addition of 500 nM A23187 or 10 nM TG (the optimum amounts, Fig. 1 *Upper*) at the time of cell transfer to 32°C, decreased the frequency of apoptotic cells to 4.7 ± 1.1% and 9.4 ± 2.8% and thus increased overall cell viability to 73.5 ± 3.5% and 76.8 ± 4.3%, respectively (Fig. 1 and Table 1). As with IL-6 and IFN-γ (10), addition of A23187 or TG even 8 hr after cell transfer to 32°C still gave the same degree of protection against apoptosis. Suppression of apoptosis with A23187 or TG was stronger than with IFN-γ, TPCK, or BHA (Fig. 2 *Upper*) but weaker than with IL-6 (Fig. 1 *Lower*).

The calcium ionophore A23187 transports Ca^{2+} across the cell surface membrane (14) and the endoplasmic reticulum calcium-ATPase inhibitor TG depletes Ca^{2+} from internal stores, which leads to Ca^{2+} influx and, thus, to a sustained intracellular increase in Ca^{2+} concentration (15). To determine the requirement for extracellular Ca^{2+} for the apoptosis-suppressing effect of A23187 or TG, the Ca^{2+} chelator EGTA was added at 2 mM, or the cells were washed and cultured in Ca^{2+}-free DMEM supplemented with 10% dialyzed horse serum. The results with either treatment showed

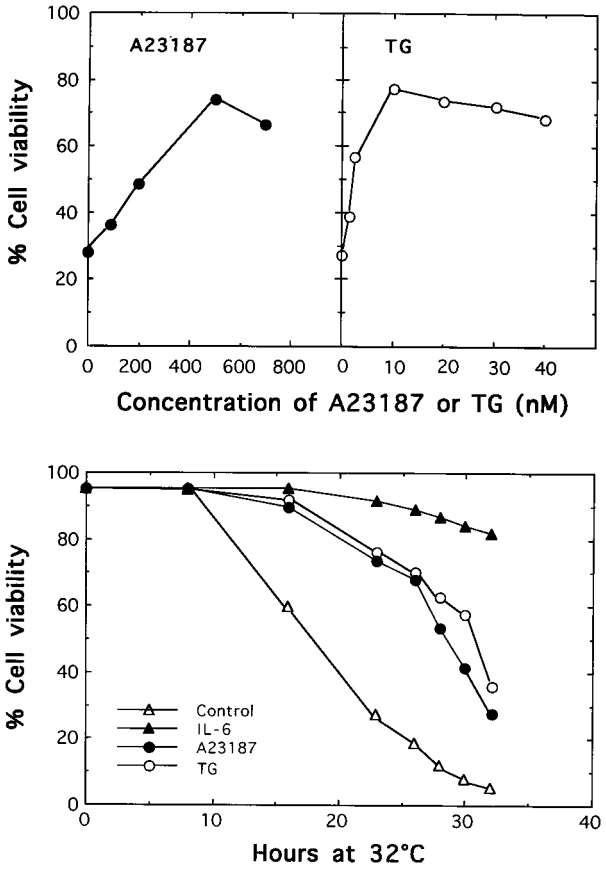

FIG. 1. Concentration- and time-dependent suppression of wild-type-p53-induced apoptosis by Ca^{2+}-mobilizing compounds. M1-t-p53 cells were cultured at 32°C for 23 hr with various concentrations of A23187 or TG (*Upper*) or for various times with no additions (control) or with IL-6 (10 ng/ml), 0.5 μM A23187, or 10 nM TG (*Lower*).

that suppression of wild-type p53 induced apoptosis by A23187 or TG was completely abolished in the absence of extracellular Ca^{2+} (Fig. 2). Although the concentration of Ca^{2+} in DMEM is 1.8 mM, addition of only 300 μM extracellular Ca^{2+} to Ca^{2+}-free DMEM was sufficient to fully restore the apoptosis-suppressing effect of A23187 or TG (Fig. 2 *Lower*). In contrast, addition of 2 mM EGTA had no effect on the apoptosis-suppressing ability of IL-6, IFN-γ, TPCK, or BHA (Fig. 2 *Upper*). There was also no inhibition of the antiapoptotic effect of IL-6 (Fig. 2 *Lower*), IFN-γ, TPCK, or BHA in cells cultured in Ca^{2+}-free medium. Cells cultured in the presence of TG or A23187 in DMEM with 2 mM EGTA or in Ca^{2+}-free DMEM were protected by IL-6, IFN-γ, BHA, or

Table 1. Specificity of the inhibitory effect of CsA on A23187 and TG suppression of wild-type-p53-induced apoptosis

	% cell viability		
Addition (μM)	None	+ A23187	+ TG
None (−)	24.6 ± 3.0	73.5 ± 3.5	76.8 ± 4.3
CsA (0.2)	28.1 ± 4.6	22.1 ± 1.0	20.8 ± 3.1
OA (0.05)	20.6 ± 4.2	75.1 ± 4.9	79.3 ± 6.5
Ver (5)	23.5 ± 3.5	68.0 ± 5.7	73.5 ± 4.6
Flun (40)	22.1 ± 3.6	75.0 ± 5.0	78.2 ± 4.8
dBcAMP (100)	24.9 ± 5.1	76.0 ± 7.1	79.6 ± 5.7
Dex (1)	24.2 ± 4.0	80.1 ± 6.8	82.0 ± 7.1

Cell viability of M1-t-p53 cells was determined 23 hr after culture at 32°C without any additions (None) or in the presence of 500 nM A23187, 10 nM TG, and the indicated concentrations of CsA, okadaic acid (OA), verapamil (Ver), flunarizine (Flun), dibutyril cAMP (dBcAMP), or dexamethasone (Dex).

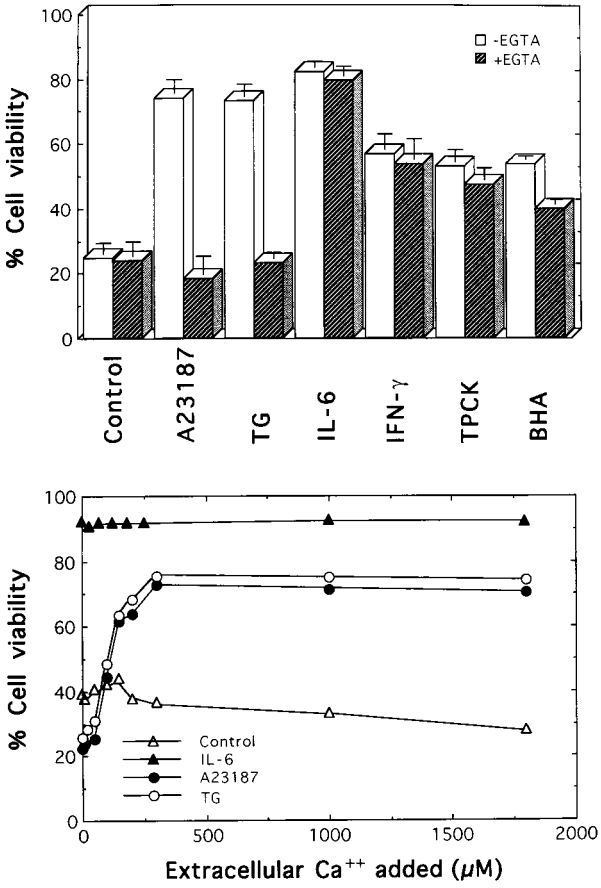

FIG. 2. Requirement for extracellular Ca^{2+} for suppression of apoptosis by A23187 and TG but not for suppression by IL-6, IFN-γ, TPCK, or BHA. M1-t-p53 cells were cultured at 32°C for 23 hr in DMEM without or with 2 mM EGTA (*Upper*) or in calcium-free DMEM (*Lower*). The concentrations of the compounds added were 500 nM A23187, 10 nM TG, IL-6 (10 ng/ml), IFN-γ (2 ng/ml), 1 μM TPCK, or 100 μM BHA.

TPCK to the same extent as cells cultured without TG or A23187. The results indicate that extracellular Ca^{2+} is absolutely required for the apoptosis-suppressing action of A23187 or TG against wild-type p53 apoptosis but is not necessary for apoptosis suppression by cytokines, a protease inhibitor, or an antioxidant.

As shown (11), culture of M1-t-p53 cells at 32°C induces caspase activation during apoptosis. This is shown by a decrease in the amount of uncleaved 116-kDa PARP and 51-kDa pro-Nedd-2 caspase substrates and an increase in the 30-kDa PARP and 12-kDa Nedd-2 fragments (Fig. 3, lane 2). Protection against wild-type-p53-induced apoptosis by IL-6, IFN-γ, TPCK, or BHA is associated with suppression of this caspase activation (11). The present results show that A23187 and TG also suppressed wild-type-p53-induced cleavage of PARP and pro-Nedd-2 (Fig. 3). This indicates that like the other apoptosis-suppressing compounds (11), A23187 and TG also act upstream of caspase activation to suppress wild-type-p53-induced apoptosis.

Wild-Type-p53-Induced Gene Expression in Cells Treated with A23187. Wild-type p53 can transcriptionally activate various genes (for review, see refs. 33 and 34). Expression of wild-type p53 in M1 cells induced *WAF-1, mdm-2,* and *FAS*. We have shown (24) that IFN-γ did not inhibit wild-type p53 activation of *WAF-1*. Addition of 500 nM A23187 or IL-6 at 10 ng/ml did not inhibit the p53-induced activation of any of these three genes (Fig. 4*A*). Similar results were obtained with 10 nM TG. These results indicate that suppression of the apoptosis-

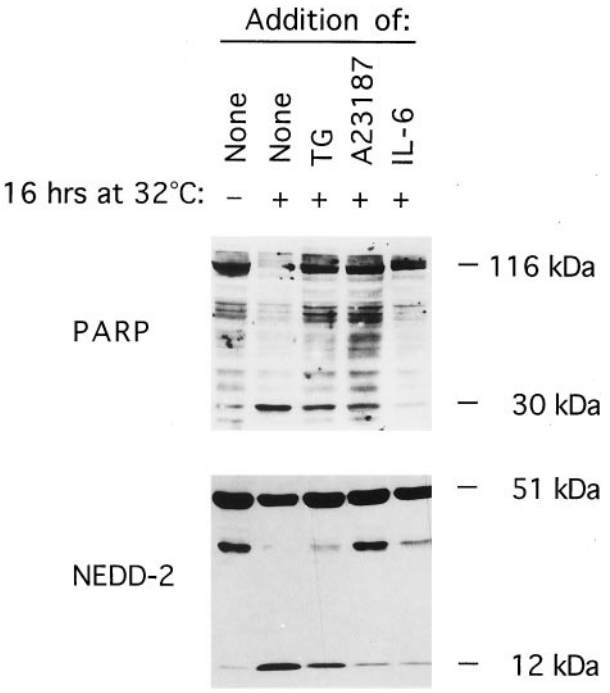

FIG. 3. Suppression of wild-type-p53-induced caspase activation by A23187 and TG. M1-t-p53 cells were cultured at 37°C (lane –) or 32°C (lanes +) for 16 hr with no additions (none) or with 10 nM TG, 500 nM A23187, or IL-6 (10 ng/ml), and cleavage of PARP and pro-Nedd-2 was determined.

inducing effect of wild-type p53 by A23187 or TG is not caused by a general inhibition of wild-type-p53-mediated transcriptional gene activation.

Different Mechanisms of Suppression of Wild-Type p53-Induced Apoptosis by Ca^{2+}-Mobilizing Compounds and IL-6. Many of the biological effects of sustained elevated levels of intracellular Ca^{2+} are mediated by the calcium/calmodulin-activated phosphatase calcineurin. These effects can be blocked by calcineurin-inhibiting immune-suppressing drugs, including CsA, through interaction with the endogenous CsA binding protein cyclophilin (for review, see ref. 35). Addition of CsA suppressed the ability of A23187 or TG to protect M1 cells from wild-type-p53-induced apoptosis, with complete suppression at 100 nM CsA (Fig. 5 *Upper*). Delayed addition of CsA even 10 hr after addition of A23187 or TG to cells cultured at 32°C still effectively suppressed the viability-promoting effect of A23187 or TG (Fig. 5 *Lower*). Without A23187 or TG, CsA had no effect on the viability of M1-t-p53 cells cultured at 32°C. In contrast to the results with A23187 and TG, CsA did not suppress the viability-promoting effect of IL-6 (Fig. 5 *Upper*), IFN-γ, TPCK, or BHA.

Addition of a different potent phosphatase inhibitor, okadaic acid, that inhibits phosphatases PP1 and PP2A but not calcineurin (PP2B) did not inhibit the viability-promoting effect of A23187 or TG (Table 1). CsA can inhibit multidrug resistance (MDR) P-glycoprotein activity (for review, see ref. 36). However, unlike CsA, the Ca^{2+} channel blockers verapamil and flunarizine that also inhibit MDR activity did not suppress the viability-promoting effect of A23187 or TG (Table 1). This indicates that suppression of MDR activity was not involved in the ability of CsA to suppress the protective effect of A23187 or TG against wild-type-p53-induced apoptosis. Activated T cells produce IL-2, which can be suppressed by CsA and also by other compounds including activators of cAMP-dependent kinase and glucocorticoid hormones (for review, see ref. 37). However, unlike CsA, neither dibutyryl cAMP nor dexamethasone suppressed the antiapoptotic effect of A23187 or TG (Table 1).

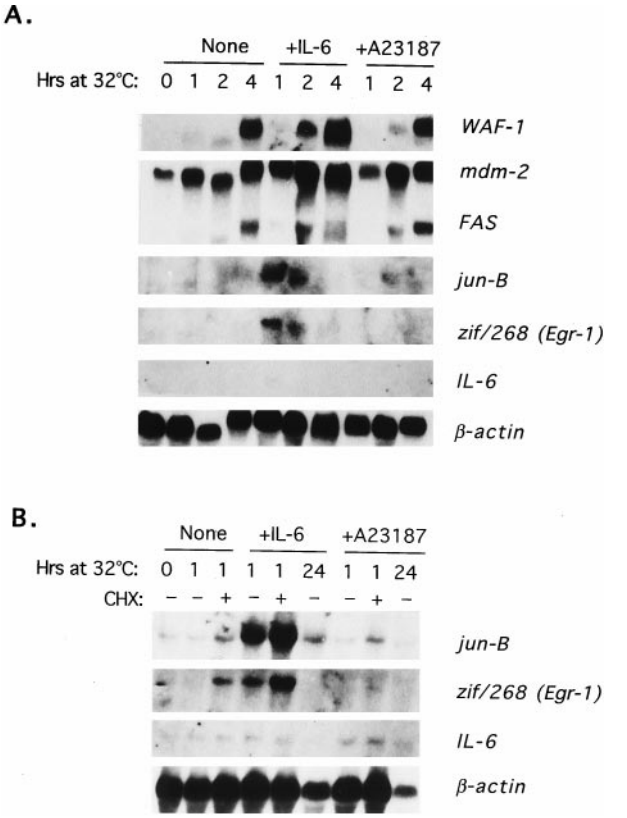

FIG. 4. Northern blot analysis of the effect of IL-6 and A23187 on gene expression in wild-type-p53-expressing cells. (*A* and *B*) M1-t-p53 cells were cultured at 37°C (lanes 0) or at 32°C for the indicated times without (None) or with IL-6 (10 ng/ml) or 0.5 μM A23187. In *B*, cycloheximide (CHX; 20 μg/ml) was added 30 min before RNA extraction (lanes +).

Analyzis of immediate early gene activation has shown that IL-6 activated *jun B* and *zif*/268 (*Egr*-1) but A23187 did not (Fig. 4). Results with TG were similar to those with A23187. The differential requirement for extracellular Ca^{2+}, specific inhibition by CsA, and differential immediate early activation of *jun B* and *zif*/268 (*Egr*-1) show that A23187 and IL-6 suppress wild-type-p53-induced apoptosis by different mechanisms that involve a Ca^{2+}-activated CsA-sensitive pathway by A23187 and TG but not by IL-6.

The Antiapoptotic Effect of A23187 or TG Was Not Mediated by IκB Degradation or Autocrine Induction of IL-6. The calcium ionophore ionomycin can activate in certain cells the transcription factor NF-κB (38), which has been implicated in some cells with an antiapoptotic function (for review, see ref. 39). Analysis of NF-κB activation, which follows degradation of the inhibitory subunit IκBα (for review, see ref. 40), has shown that neither A23187 nor IL-6 induced IκBα degradation in M1-t-p53 cells (Fig. 6). Tumor necrosis factor, which rapidly but transiently induced IκBα degradation in these cells (Fig. 6), does not protect M1 cells from wild-type-p53-induced apoptosis. These results indicate that NF-κB activation was not involved in the antiapoptotic effect of A23187 or IL-6 against wild-type-p53-induced apoptosis.

Activation of the calcineurin pathway in T cells can activate different genes including cytokines (for review, see ref. 37). We, therefore, determined whether A23187 and TG stimulated IL-6 production in M1-t-p53 cells and, thus, protected the cells from wild-type-p53-induced apoptosis by an autocrine mechanism. The results showed no detectable induction of IL-6 mRNA by A23187 even after 24 hr at 32°C (Fig. 4). Addition of neutralizing mAb against mouse IL-6 (41, 42), which completely neutralized the antiapoptotic effect of IL-6

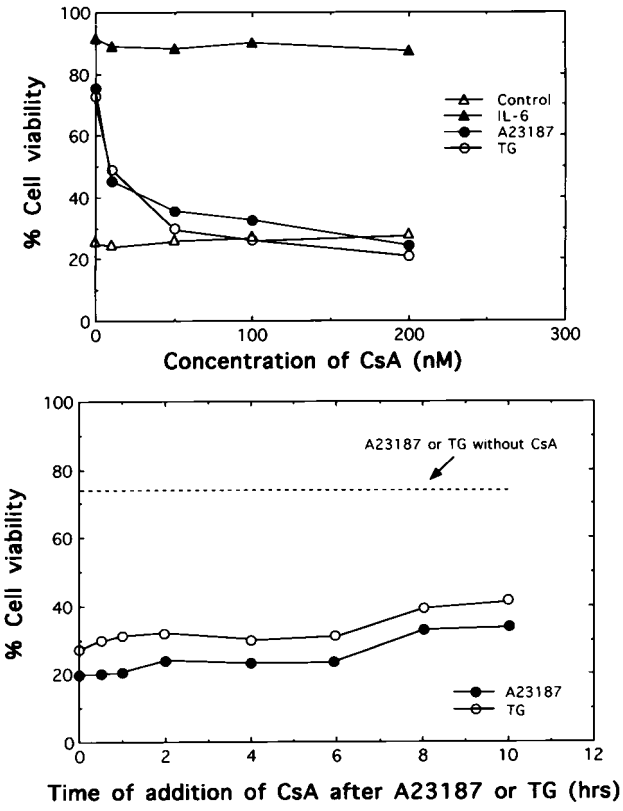

FIG. 5. Suppression of the antiapoptotic effect of A23187 and TG by CsA. M1-t-p53 cells were cultured at 32°C for 23 hr with no additions (none) or with IL-6 (10 ng/ml), 500 nM A23187, or 10 nM TG with various concentrations of CsA added at the same time (*Upper*) or with 100 nM CsA added at various times after adding A23187 or TG (*Lower*).

(89 ± 5% and 27 ± 2% cell viability without or with antibody at 5 μg/ml, respectively), did not show any inhibition of the antiapoptotic effect of A23187 or TG. We also did not detect IL-6 biological activity in conditioned medium of A23187- or TG-treated M1-t-p53 cells even after 24 hr, and dexametha-

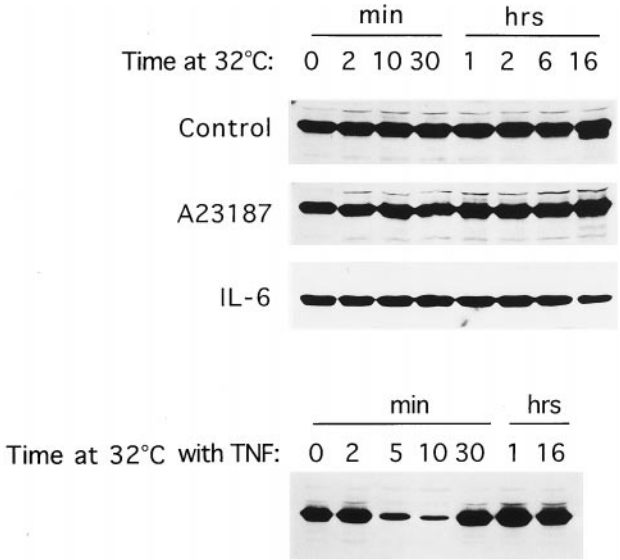

FIG. 6. Antiapoptotic effect of A23187 or IL-6 was not associated with degradation of IκBα. M1-t-p53 cells were cultured at 32°C without (Control) or with 500 nM A23187, IL-6 (10 ng/ml), or tumor necrosis factor (100 units/ml), and IκB α was determined by Western blotting with anti-IκB α antibody.

Table 2. Suppression by A23187 and TG of VCR- and Dox-induced apoptosis in p53-nonexpressing M1-neo cells and its inhibition by CsA

	% cell viability					
	VCR			Dox		
Addition	None	+ CsA	+ Ver (5 μM)	None	+ CsA	+ Ver* (1 μM)
None	30.6 ± 6.9	21.3 ± 4.2	20.6 ± 3.5	44.0 ± 3.3	25.5 ± 1.1	21.1 ± 3.6
IL-6	75.0 ± 5.8	65.9 ± 4.6	63.8 ± 5.2	65.1 ± 5.3	44.6 ± 2.5	30.8 ± 3.1
A23187	67.8 ± 4.8	23.5 ± 3.3	65.2 ± 4.9	70.1 ± 3.5	22.1 ± 3.3	43.1 ± 5.1
TG	67.0 ± 3.1	31.3 ± 2.9	57.3 ± 6.0	72.5 ± 4.1	27.2 ± 3.5	40.2 ± 4.8

M1-neo cells were cultured without (None) or with the apoptosis inhibitors IL-6 (10 ng/ml), A23187 (0.4 μM), or TG (10 nM). Percent cell viability was determined 26 hr after addition of 0.82 μM VCR or 1.84 μM Dox in the absence (None) or presence of 0.1 μM CsA or 1 μM or 5 μM verapamil (Ver) and the apoptosis inhibitors above. Results with 20 μM flunarizine were similar to those with 5 μM verapamil.
*Verapamil was reduced to 1 μM because addition of 5 μM verapamil with 1 μM Dox resulted in less than 1% viable cells.

sone, which can effectively block IL-6 production, did not suppress the viability-promoting effect of A23187 or TG (Table 1). The results thus indicate that A23187 or TG did not exert their protective effect against wild-type-p53-induced apoptosis in M1 cells by autocrine production of IL-6.

Suppression of p53-Independent VCR- and Dox-Induced Apoptosis by A23187 and TG. M1-neo cells that do not express p53 (1) can be induced to undergo apoptosis by cytotoxic compounds such as VCR or Dox (refs. 10 and 11 and Table 2). Induction of apoptosis by both compounds in M1-neo cells was suppressed by A23187 and TG to an extent that was similar to the suppression by IL-6 (Table 2). CsA and verapamil, presumably because of their MDR blocking activity, increased the induction of apoptosis by VCR and Dox. However, only CsA, but not verapamil, suppressed the viability-promoting effect of A23187 and TG (Table 2). The results indicate that Ca^{2+}-mobilizing compounds can also protect these cells by a CsA-sensitive mechanism from induction of apoptosis by cytotoxic compounds acting through a p53-independent pathway.

Apoptosis Induced by Withdrawal of IL-6 in Primed M1 cells Was Not Suppressed by A23187 or TG. Unlike normal myeloid precursors, M1 myeloid leukemia cells are cytokine-independent for viability and growth but can be induced to regain such cytokine dependence by pretreatment for 2 or 3 days with IL-6 (priming; for review, see ref. 7). The IL-6-primed M1 cells then undergo apoptosis after IL-6 withdrawal, unless IL-6 or some other cytokines are added (7, 43–45). The M1-t-p53 cells primed by culture with IL-6 at 10 ng/ml for 24 hr at 32°C undergo apoptosis after IL-6 withdrawal and culture at 37°C (Table 3). Readdition of IL-6 or its replacement with IL-3 or IFN-γ protected these cells from apoptosis at 37°C (Table 3). However, unlike all the above experiments with unprimed M1-t-p53 or M1-neo cells, addition of 400 nM A23187 or 10 nM TG failed to protect IL-6-primed M1-t-p53 cells from apoptosis after IL-6 withdrawal (Table 3). M1-neo cells primed with IL-6 (10 ng/ml) at 37°C for 2 days followed by removal of IL-6 also died by apoptosis showing 57.2 ± 5.1% cell viability at 24 hr. This induction of apoptosis was also prevented by addition of IL-6 (5 ng/ml), IL-3 (5 ng/ml), or IFN-γ (2 ng/ml) (88.9 ± 4.6, 89.2 ± 4.7 or 90.6 ± 3.3% cell viability, respectively) but not by 400 nM A23187 or 10 nM TG (55.2 ± 3.1 or 47.3 ± 4.8% cell viability, respectively). These results indicate that some pathways of apoptosis can be suppressed by cytokines but not by the Ca^{2+}-mobilizing compounds.

Table 3. Apoptosis induced by IL-6 withdrawal in IL-6-primed M1-t-p53 cells was not suppressed by A23187 or TG

Addition	No. of viable cells ($\times 10^{-4}$/ml)	% cell viability
None	18.1 ± 2.4	26.6 ± 5.0
IL-6	48.2 ± 4.1	78.6 ± 5.4
IL-3	50.6 ± 3.2	79.8 ± 6.1
IFN-γ	44.3 ± 4.5	75.3 ± 4.2
A23187	12.8 ± 3.1	17.9 ± 2.9
TG	9.5 ± 2.5	15.1 ± 3.2

M1-t-p53 cells were primed by preincubation of 5×10^5 cells per ml for 24 hr at 32°C with IL-6 (10 ng/ml). Cells were then washed twice, seeded at 3×10^5 cells per ml, and cultured at 37°C with no other additions (None) or with IL-6 (5 ng/ml), IL-3 (5 ng/ml), IFN-γ (2 ng/ml), 400 nM A23187, or 10 nM TG. Number of viable cells and percent cell viability were determined 24 hr after culture at 37°C.

DISCUSSION

The present experiments have shown that the Ca^{2+}-mobilizing compounds A23187 and TG effectively antagonized induction of apoptosis by overexpression of wild-type p53 or by cytotoxic compounds in p53 nonexpressing myeloid leukemic cells and that this suppression of apoptosis was dependent on extracellular Ca^{2+}. But apoptosis-suppressing cytokines (IL-6 and IFN-γ), an antioxidant (BHA), and a protease inhibitor (TPCK) did not require extracellular Ca^{2+} for apoptosis suppression. The Ca^{2+}-mobilizing compounds, therefore, do not truly mimic the antiapoptotic mechanism of IL-6, IFN-γ, or the other compounds in M1 cells but activate alternative pathways that converge on the apoptosis machinery. As with cytokines and the other compounds (11), the Ca^{2+}-mobilizing compounds suppressed activation of caspases that are an important part of the apoptotic machinery (for review, see ref. 46). Activation of *WAF-1*, *mdm-2,* and *FAS* induced by overexpression of wild-type p53 were not inhibited by IL-6, A23187, or TG. These apoptosis suppressors, therefore, do not act by a general inhibition of p53-induced transcriptional gene activation. This suggests that the apoptosis suppressors exert their antiapoptotic function either independently of expression of these genes or at a point downstream from them. It will be interesting to determine whether expression of other genes strongly induced (47) or suppressed by wild-type p53 may be specifically modulated by the apoptosis-suppressing cytokines or Ca^{2+}-mobilizing compounds.

Biological effects of Ca^{2+}-mobilizing compounds mediated by the phosphatase calcineurin can be suppressed by calcineurin inhibitors such as CsA through interaction with the CsA binding protein cyclophilin (for review, see ref. 35). Our experiments have shown that the antiapoptotic effect of A23187 or TG was completely suppressed by CsA but not by another phosphatase inhibitor, okadaic acid, that does not block calcineurin activity. CsA also suppressed the antiapoptotic effect of A23187 or TG in VCR- or Dox-treated M1-neo cells that do not express p53. The results have also indicated that this suppressive effect of CsA was not mediated by its anti-MDR activity. In contrast to A23187 or TG, CsA had no effect on the antiapoptotic activity of IL-6, IFN-γ, BHA, or TPCK. A23187 and TG also differed from IL-6 in their inability to activate immediate early expression of *junB* and

zif/268 (*Egr*-1). The results on the differential requirement for Ca^{2+}, suppression by CsA, and activation of immediate early genes thus show that the Ca^{2+}-mobilizing compounds act by a different mechanism than the apoptosis-suppressing cytokines and some other compounds.

Elevated intracellular Ca^{2+} can activate transcription of different genes including genes for transcription factors (for review, see ref. 48). One transcription factor reported to be activated by a calcium ionophore is NF-κB (38), which has been implicated in an antiapoptotic mechanism in certain cells (for review, see ref. 39). However, neither A23187 nor IL-6 activated NF-κB in M1 cells. Calcium-induced mitogenesis in lymphocytes is associated with activation of genes for cytokines (37) that can thus protect cells against induction of apoptosis by an autocrine mechanism. TG has been reported to protect IL-3-dependent cells from apoptosis after IL-3 withdrawal by autocrine activation of IL-4 and this antiapoptotic effect was blocked by neutralizing antibody to IL-4 (19). A23187 and TG also stimulated IL-6 gene expression in monocytes and macrophages (49, 50). However, A23187 and TG did not activate autocrine expression of IL-6 in M1 cells and the antiapoptotic function of these compounds was not blocked by neutralizing antibody to IL-6. The results indicate that the Ca^{2+}-mobilizing compounds do not act by an autocrine induction of IL-6 in M1 cells.

Although effectively suppressing wild-type-p53-induced and p53-independent apoptosis induced by cytotoxic compounds in M1 cells, the Ca^{2+}-mobilizing compounds failed to suppress apoptosis in IL-6-primed M1 cells after withdrawal of IL-6. In contrast, the cytokines IL-6, IL-3, and IFN-γ effectively protected these IL-6-primed cells from apoptosis. The results show that not all pathways of apoptosis that can be suppressed by cytokines can also be suppressed by Ca^{2+}-mobilizing compounds. Different pathways of apoptosis can thus be differentially suppressed by cytokines and Ca^{2+}-mobilizing compounds.

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