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Thyrsiferyl 23-Acetate and its Derivatives Induce Apoptosis in Various T- and B-Leukemia Cells

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Abstract—Thyrsiferyl 23-acetate (TF-23A), a cytotoxic compound from marine red alga, induced a rapid cell death in various leukemic T- and B-cell lines. During incubation of Jurkat cells with TF-23A, condensation and fragmentation of nuclei occurred and clusters composed of uneven small cellular particles were formed. Concentration- and time-dependent DNA fragmentation was also induced by the incubation of the cells with TF-23A. These results demonstrate that the TF-23A-induced cell death follows a typical apoptotic process. The TF-23A-induced apoptosis was prevented by fetal calf serum and insulin, but not by EGF or PDGF. TF-23A and its several analogous compounds showed apoptosis-inducing activity. However, only TF-23A out of these compounds showed an inhibitory activity for protein phosphatase 2A, PP2A. These results strongly suggest that a structure of TF-23A involved in induction of apoptosis is different from that involved in the PP2A inhibition.

Introduction

Many important biological events such as development, homeostasis, immune regulation, and aging are controlled by a balance between cell division and physiological cell death.[1–4] The physiological cell death is a process to eliminate unwanted host cells, and occurs through a pathway commonly known as apoptosis characterized by several morphological and biochemical events such as membrane blebbing, chromatin condensation, DNA fragmentation, formation of apoptotic bodies, and non-inflammatory removal of the cells from the tissue. Apoptosis is induced not only by physiological stimulations through Fas,[5,6] TNF receptor,[7] TGFβ1 receptor,[8] TCR/CD3 complex[9–12] and glucocorticoid receptor[13,14] but also by pathological stimulations with immune cell killing, irradiation, certain toxins, and withdrawal of growth factors. Despite the importance of apoptosis, the signal transduction pathways responsible for apoptosis are poorly understood. DNA damage caused by irradiation leads to increased levels of p53.[15] Moreover, deregulated expression of c-Myc can cause apoptosis in certain cells.[16] Apoptosis induced in T-cells by activation through their antigen receptors leads to expression of c-Myc. ICE is thought to be essential for Fas-induced apoptosis.[17]

Previously, Suzuki et al. isolated a novel cyclic ether containing a squalene carbon skeleton, thyrsiferyl 23-acetate (TF-23A), from the red alga *Laurencia obtusa* as a cytotoxic compound.[18] We reported that TF-23A is a potent and specific inhibitor of serine/threonine protein phosphatase 2A, PP2A.[19] In this paper, we demonstrate that TF-23A induces apoptosis in various leukemic cells under serum-deprived conditions. A molecular mechanism for the apoptosis is discussed in relation to the PP2A inhibitory activity of TF-23A.

Results

Thyrsiferyl 23-acetate (TF-23A) was isolated from the marine red alga *L. obsusa* as a strong cytotoxic compound[18] (Fig. 1). To elucidate molecular mechanism(s) for the cytotoxicity, we analyzed effects of TF-23A on viability of various leukemic cells under serum-deprived conditions. As shown in Table 1, during incubation of cells with 10 μM TF-23A for 24 h, viability of T- and B-cell lines was dramatically decreased except Daudi cells, while two macrophage-like cell lines, J774.1 and PU5-1.8, showed moderate resistance and two myelocytic leukemia cell lines, HL60 and K562, were strongly resistant to TF-23A, showing 100 and 92% of viability, respectively.

Figure 2(a) and (b) shows that the viability of Jurkat cells progressively decreases with incubation times and increasing concentrations of TF-23A, respectively.

Key words: Cytotoxins; enzyme inhibitors; kinetics; natural products.
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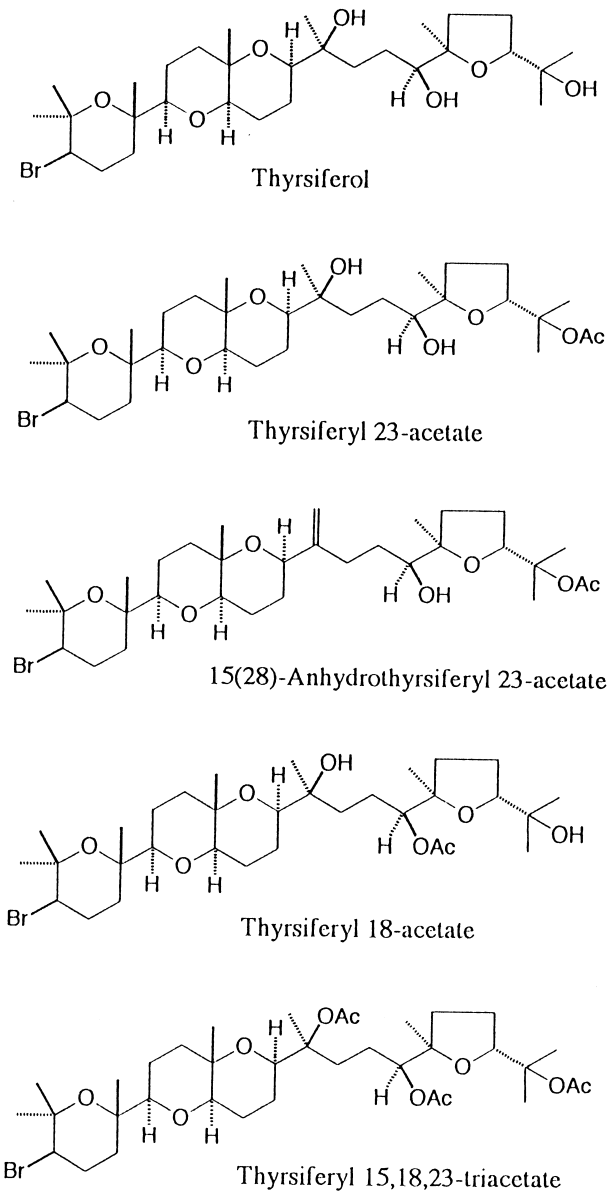

Figure 1. Structure of thyrsiferyl 23-acetate (TF-23A) and its analogous compounds.

Figure 3 shows morphological features of Jurkat cells after incubation with 10 μM TF-23A or 1 μg/mL anti-Fas for 3 h. During incubation of Jurkat cells with TF-23A, condensed and fragmented nuclei began to appear at 1 h, and then the cells became clusters composed of uneven small cellular particles at 3 h. These morphological features induced by the incubation with TF-23A were indistinguishable from those of the typical apoptosis induced with anti-Fas antibody.

Figure 4 shows the concentration- and time-dependent DNA fragmentation induced by incubation of the cells with TF-23A. DNA ladder formation was increased with increasing incubation time and concentration of TF-23A.

From these results, it was concluded that the TF-23A-induced cell death follows a typical apoptotic process.

Table 1 Effects of TF-23A on various leukemic cell lines

Cells	Animal[a]	Type[b]	TF-23A (μM)	% viable cells	
				3 h	24 h
Jurkat	H	T	0.1	91.8	74.3
			1	56.9	55.5
			10	6.6	0
Sup-T13	H	T	0.1	76.3	47.0
			1	56.0	25.8
			10	9.0	0
ILT-Mat	H	T	0.1	97.7	82.3
			1	93.0	14.8
			10	34.5	0
CTLL-2	M	T	0.1	84.5	0
			1	92.3	0
			10	0	0
KV33-12	M	T	0.1	94.8	87.3
			1	94.8	80.5
			10	42.3	0
EL-4	M	T	0.1	93.7	96.3
			1	96.7	93.0
			10	75.0	0
Daudi	H	B	0.1	100	95.0
			1	95.0	79.5
			10	58.3	42.7
WEHI-231	M	B	0.1	100	95.5
			1	62.8	60.0
			10	2.5	0
Bal-17	M	B	0.1	87.0	75.8
			1	57.5	50.8
			10	1.2	0
J774.1	M	MF	0.1	98.0	90.8
			1	99.5	64.5
			10	70.8	12.2
PU5-1.8	M	MF	0.1	95.0	100
			1	90.5	100
			10	71.5	57.5
HL-60	H	MM	0.1	94.5	97.3
			1	96.3	100
			10	92.3	100
K562	H	MM	0.1	99.7	100
			1	100	100
			10	97.0	92.0

Serum-deprived cells (1×10^6/mL) were incubated with the indicated concentrations of TF-23A for 3 and 24 h and viable cells were estimated by trypan blue dye exclusion test. Under control conditions without TF-23A, over 90% of cells were viable and the cell numbers were almost constant. Values are average of three separate experiments. [a] Animals are: H, human; M, mouse. [b] Cell lines are: T, T-cell line; B, B-cell line; MF, macrophage line; MM, myelomonocytoid cell line.

It should be noted that the TF-23A-induced apoptosis was much more sensitive in T- or B-cells compared with that in macrophage-like cells or myelocytic leukemic cells.

It has been reported that c-Myc can cause an apoptosis in certain cells at low concentrations of serum and that the apoptosis is prevented by high concentrations of serum.[16,21,22] Then we examined effects of fetal calf serum (FCS) on the TF-23A-induced apoptosis. As shown in Figure 5, the apoptosis was prevented by FCS in a concentration dependent manner and completely blocked by 5% FCS.

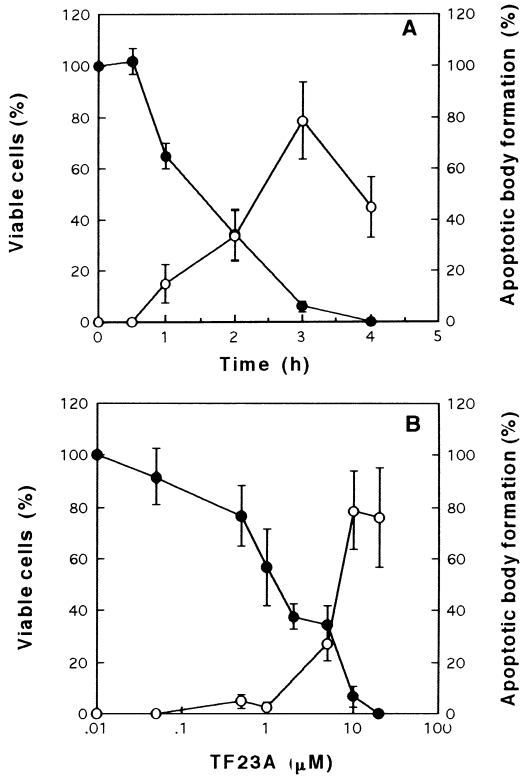

Figure 2. Dose- and time-dependent Jurkat cell death induced by TF-23A. Cells were incubated with 10 μM TF-23A for the indicated times (A) or concentrations of TF-23A for 3 h (B). Viable cells were estimated by trypan blue dye exclusion test and apoptotic cells were counted under phase-contrast microscopy.

To elucidate an involvement of c-Myc in the TF-23A-induced apoptosis, we then analyzed the c-Myc levels by Western blotting at various times after the incubation of Jurkat cells with TF-23A. As shown in Figure 6, the protein levels of c-Myc began to increase at 1 h, reached to the maximum at 2 h, and then decreased. However, four other analogous compounds of TF-23A (Fig. 1) did not show any effect on the c-Myc levels under these conditions. The meaning of c-Myc in the TF-23A-induced apoptosis is described in Discussion.

Then, we examined effects of various effectors for protein kinases and protein phosphatases on the TF-23A-induced apoptosis. As shown in Table 2, only phorbol esters such as PMA and PDBu, activators of protein kinase C, prevented the apoptotic cell death of Jurkat cells induced by TF-23A. Figure 7 shows the effects of concentrations of phorbol esters on the prevention of apoptosis. At the concentrations higher than 10 nm, the apoptosis was clearly prevented. The protecting effect of PMA was stronger than that of PDBu.

Figure 8 shows effects of EGF, PDGF and insulin on the TF-23A-induced apoptosis of Jurkat cells. Insulin showed a protecting effect as increasing the concentrations, whereas EGF and PDGF showed no effects on the apoptosis.

Figure 9 shows effects of TF-23A and its analogous compounds (Fig. 1) on inhibition of PP2A (Fig. 9A) and apoptosis (Fig. 9B). Out of 5 compounds, only TF-23A showed a potent inhibitory activity for PP2A, whereas all of the 5 compounds showed similarly potent apoptosis-inducing activity. We found that TF-23A is very labile and lost gradually its inhibitory activity for PP2A during storage at 4 °C. However, even after complete loss of the PP2A inhibitory activity, the degraded TF-23A showed the apoptosis-inducing activity clearly. From these results, it was concluded that the apoptosis-inducing activity is not ascribed to the PP2A inhibitory activity of TF-23A molecule.

Discussion

In this paper, we have demonstrated that thyrsiferyl 23-acetate (TF-23A), a cytotoxic compound isolated from marine red alga, induces an apoptosis in various leukemic cells. During incubation of Jurkat cells with TF-23A, morphological features indistinguishable from those of typical apoptosis induced with Fas were observed which include condensation and fragmentation of nuclei and formation of clusters composed of uneven small cellular particles. The DNA ladder formation dependent on the incubation times and concentrations

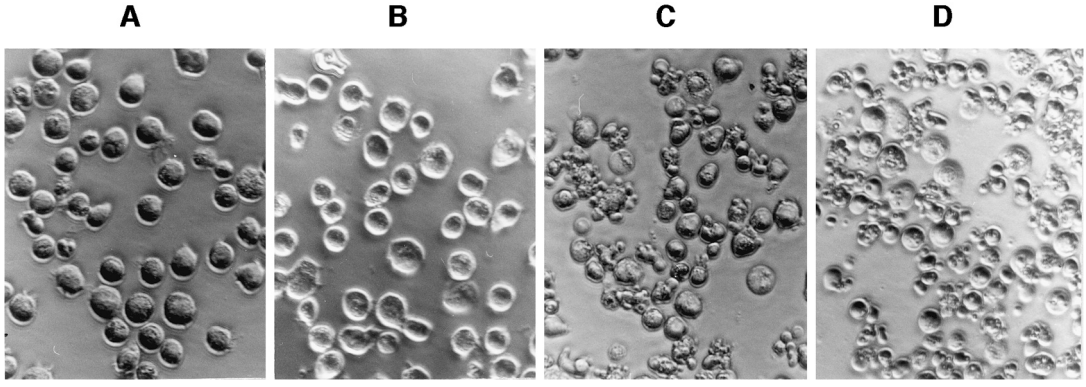

Figure 3. Morphological features of TF-23A-treated Jurkat cells. Jurkat cells (5×10^5) were cultured in the absence of FCS for 3 h (panel A) and 24 h (panel B), or in the medium containing 10 μM TF-23A (panel C) or 1 μg/mL anti-Fas mAb (panel D) for 3 h. Then, the cell suspensions were subjected to phase-contrast microscopy and photographed (original magnification×100).

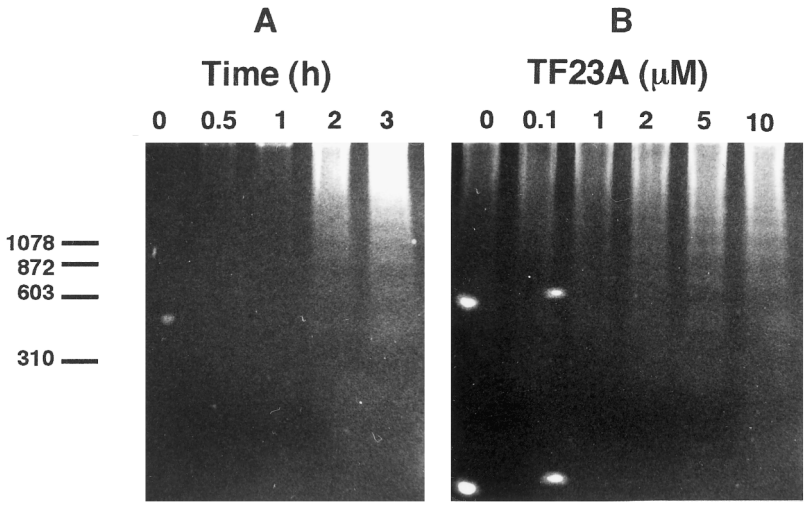

Figure 4. Dose- and time-dependent DNA fragmentation of Jurkat cells induced by TF-23A. Cells were incubated with the indicated concentrations of TF-23A for 3 h (panel A) or 10 μM TF-23A for indicated times (panel B). DNA extracts (1×10^6 cells) were applied to 2.0% agarose gel containing 0.5 μg/mL ethidium bromide. Molecular sizes in kb are indicated to the left of the gel.

of TF-23A was observed. The TF-23A-induced apoptosis was completely protected by 5% FCS or insulin, whereas EGF or PDGF showed no effect.

Recently, the signaling pathways for apoptosis have been extensively elucidated.[17] It should be noted that apoptosis in T- or B-cells was much more sensitive to TF-23A compared to that in macrophage-like cells or myelocytic leukemic cells. The protecting effects of activators of PKC, PMA and PDBu, also support this possibility, because PKC can act as 'survival factor' in the pathway through the antigen receptor complex.[23] Hence the present results suggest a possibility that the signaling pathway from antigen receptor is involved in the TF-23A-induced apoptosis of T-cells.

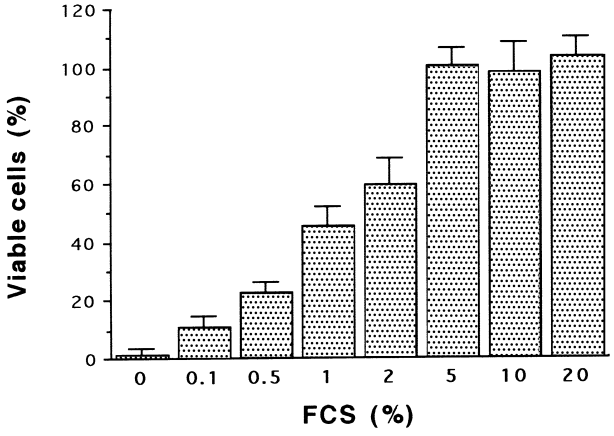

Figure 5. Inhibition of TF-23A-induced apoptosis by FCS in Jurkat cells. Serum-deprived Jurkat cells (1×10^6/mL) were incubated with 10 μM TF-23A in the presence of the indicated concentrations of FCS for 3 h, and viable cells were evaluated by trypan blue dye exclusion test.

We previously reported that TF-23A is a specific inhibitor of PP2A.[19] TF-23A inhibited PP2A activity of not only the purified catalytic subunit but also the holoenzyme form in crude extracts. TF-23A did not show any inhibitory activity for other protein phosphatases such as PP1, PP2B, PP2C or PTP. Therefore, one possibility was thought that the TF-23A-induced apoptosis occurs through the inhibition of PP2A. But this is not the case. TF-23A was labile and gradually lost the PP2A inhibitory activity during storage at 4 °C. However, the degraded TF-23A still showed the apoptosis-inducing activity. Moreover, the four derivatives of TF-23A including TF, 15(28)-anhydro-TF-23A, TF-18A and TF-15A, 18A, 23A did not show any inhibitory activity for PP2A but similarly showed strong apoptosis-inducing activity. Morphological features by these four derivatives were indistinguishable from those induced by TF-23F. It was of interest that expression of c-Myc was

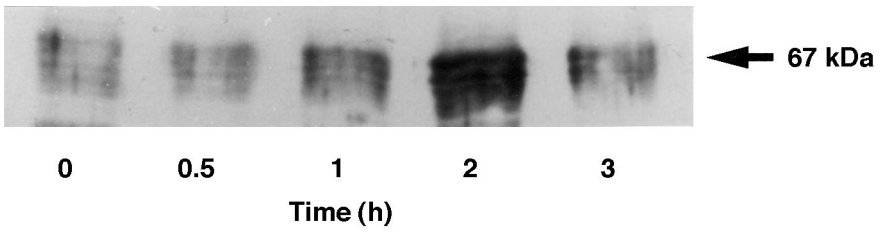

Figure 6. Western blot analysis of c-Myc expression in Jurkat cells during incubation with TF-23A. Serum-deprived Jurkat cells were incubated with 10 μM TF-23A. Incubation was terminated at the indicated times and whole cell lysates were prepared. The lysates were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Immunoblot analysis was carried out by using antibody against c-Myc (C-33) and an ECL Western blotting detection kit.

increased in the TF-23A-induced apoptosis. However, all of the other four analogous compounds of TF-23A did not show any effect on the c-Myc levels despite that they all showed potent apoptosis-inducing activity (data not shown). These facts strongly suggest that the increase in the c-Myc levels induced by TF-23A is not a cause for induction of apoptosis but a result due to the PP2A inhibitory activity of TF-23A.

In any case, these results strongly suggest that the apoptosis-inducing activity of TF-23A is not due to inhibition of PP2A but due to other molecular mechanism(s) which should be further elucidated. Recently, we found that tautomycin has both inhibitory activity for PP1 and PP2A and apoptosis-inducing activity, and that the two activities are ascribed to different moieties of tautomycin molecule.[24] Therefore, the present work provides another new example demonstrating that the PP2A-inhibitory activity and the apoptosis-inducing activity are ascribed to different moieties of a natural compound. The target molecule for the apoptosis-inducing activity should be elucidated further.

Table 2 Effects of various effectors of protein kinase and phosphatase on TF-23A-induced apoptosis

Additions	Viable cells (%) ± SD
None	100
10 μM TF-23A	8.5 ± 3.8
10 μM TF-23A + 1 μM staurosporine	0
+ 100 μM H-7	7.4 ± 2.2
+ 50 μM W-7	0
+ 10 μg/mL genistein	0
+ 10 nM FK506	3.1 ± 0.9
+ 10 nM rapamycin	5.1 ± 2.7
+ 0.1 mM vanadate	0
+ 1 μM A23187	0
+ 1 mM $ZnCl_2$	6.1 ± 3.6
+ 20 nM PMA	72.5 ± 9.9
+ 100 nM PDBu	59.5 ± 7.0

Serum-deprived Jurkat cells (1×10^6/mL) were incubated with 10 μM TF-23A in the presence of the indicated concentrations of various effectors for 3 h, and viable cells were evaluated by trypan blue dye exclusion test.

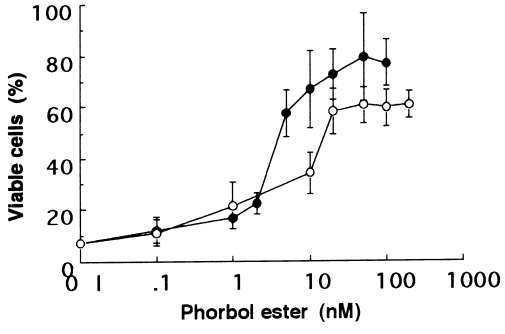

Figure 7. Inhibition of TF-23A-induced apoptosis by phorbol esters in Jurkat cells. Serum-deprived Jurkat cells (1×10^6/mL) were incubated with 10 μM TF-23A in the presence of the indicated concentrations of PMA (closed circle) or PDBu (open circle) for 3 h, and viable cells were evaluated by trypan blue dye exclusion test.

Materials and Methods

Materials

Thyrsiferol (TF),[18] thyrsiferyl 23-acetate (TF-23A)[18] and 15(28)-anhydrothyrsiferyl 23-acetate (15(28)-anhydro-TF-23A)[20] were obtained from the red alga *L. obtusa* (Hudson) Lamouroux. Thyrsiferyl 18-acetate (TF-18A) was prepared by treatment of thyrsiferol with acetic anhydride and pyridine. Furthermore, thyrsiferyl 15, 18, 23-triacetate (TF-15A, 18A, 23A) was prepared by treatment of thyrsiferol with acetic anhydride and 4-dimethylaminopyridine. These compounds were dissolved in dimethyl sulfoxide at 5 mM and used for experiments. The anti-Fas mAb (2D1) was a generous gift from Dr. S. Takahashi (Sapporo Medical University). Anti-c-Myc mAb (C-33) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cells

Human leukemic T cell lines Jurkat and Sup-13, human Burkitt's B lymphoma Daudi, murine T cell line CTLL-2,

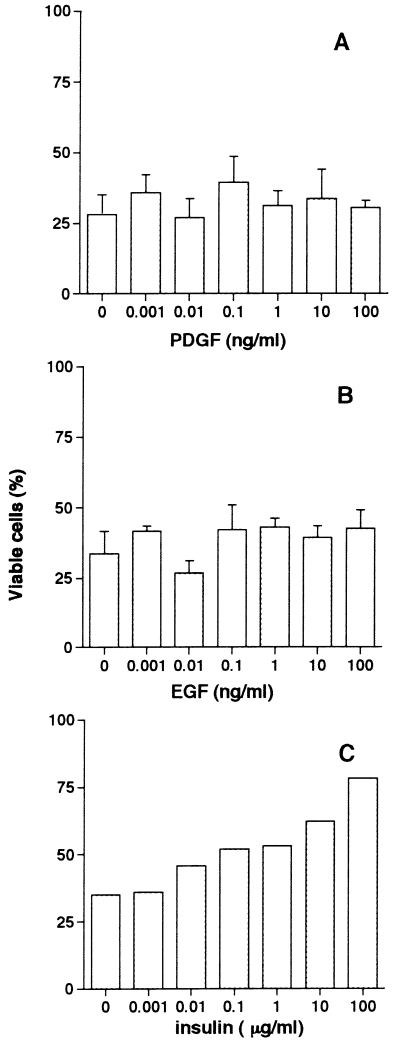

Figure 8. Effects of PDGF, EGF and insulin on the TF-23A-induced apoptosis of Jurkat cells. Jurkat cells (1×10^6/mL) were incubated with 5 μM TF-23A in the presence of the indicated concentrations of PDGF (A), EGF (B) and insulin (C) for 3 h, and viable cells were evaluated by trypan blue dye exclusion test.

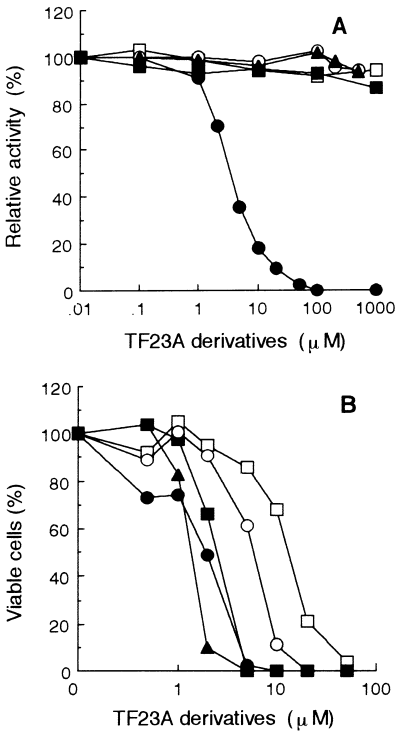

Figure 9. Effects of TF-23A and its analogous compounds on inhibition of PP2A activity and apoptosis. In panel A, 0.015 mU/mL of catalytic subunit of PP2A was preincubated with the indicated concentrations of TF-23A(●), TF (□), 15(28)-anhydro-TF-23A (■), TF-18A(○) and TF-15A, 18A, 23A (▲) for 15 min at 30 °C and was assayed by using ^{32}P-labelled myefin basic protein. In panel B, Jurkat cells (1×10^6/mL) were incubated in the presence of the indicated concentrations of TF-23A and its derivatives for 3 h, and viable cells were evaluated by trypan blue dye exclusion test.

murine thymoma EL-4, murine T cell hybridoma KV33-12, murine B lymphomas WEHI-231 and Bal-17, murine macrophage-like lines J774.1 and PU5-1.8, human promyelocytic leukemia HL-60, and chronic myelogenous leukemia K562 were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, 2 mM L-glutamine. After 2 days of culture, the subconfluent cells were harvested and washed with RPMI-1640 medium three times. The cells were suspended at 1×10^6/mL in the medium and incubated with various concentrations of TF-23A for appropriate times. Cell viability was assayed by the ability to exclude trypan blue dye.

Assay for DNA fragmentation

The TF-23A-stimulated cells (1×10^6) were washed once with PBS, resuspended with 20 μL of lysis buffer A (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.5% SDS, 0.5 mg/mL proteinase K), and incubated for 2 h at 55 °C. Then 10 μL of RNase A (1 mg/mL) was added and the samples were incubated for 1 h at 55 °C. After incubation the samples were heated to 70 °C, and 10 μL of sample buffer (10 mM EDTA, pH 8.0, 0.25% bromophenol blue, 40% sucrose, and 1% low-melting temperature agarose) was added. The samples were loaded into the dry wells of a 2% agarose gel containing 0.5 mg/mL ethidium bromide, electrophoresed at 20 V for 24 h in 2 mM EDTA, 80 mM Tris-phosphate, pH 7.8, and photographed.

Western blot analysis

The cells (1×10^6) were washed once in PBS and lysed in 0.5 mL of lysis buffer B (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 20 mM NaF, 1 mM vanadate, 10 μg/mL aprotinin, 0.5 mM benzamidine, 5 μg/mL leupeptin, and 0.1 mM PMSF) and stirred for 30 min at 4 °C. The lysates were centrifuged at 8,000×g for 10 min and resulting supernatants were used as whole-cell extracts. The extracts were applied on to a 10% polyacrylamide gel and proteins were separated by SDS–PAGE. Separated proteins were transferred to a nitrocellulose membrane, which was blocked in PBS containing 3% (w/v) BSA. After blocking, the membrane was incubated with anti-c-Myc antibody for 1 h. The membrane was washed with 0.1% Tween-20/PBS and incubated with horseradish peroxidase-labeled anti-mouse IgG for 30 min. Detection of the immunoreactive bands was performed with an ECL Western blotting detection kit (Amersham Japan, Tokyo, Japan).

Assays of protein phosphatase PP2A

The activity of PP2A was measured by using ^{32}P-labeled myelin basic protein as a substrate as previously described.[19] One unit (U) of activity was defined as the amount of enzyme that catalyses the release of 1 mmol of phosphate per min.

Acknowledgements

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