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A selective plasmin inhibitor, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-picolyl)tyrosine-octylamide (YO-2), induces thymocyte apoptosis

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Abstract

The treatment of rat thymocytes with YO-2, a novel inhibitor of plasmin, resulted in an increase in DNA fragmentation. DNA fragmentation was also induced by another YO compounds such as YO-0, -3, -4 and -5. These YO compounds are the inhibitor of plasmin activity. On the other hand, YO-1, -6 and -8 that hardly inhibit plasmin activity had no effect on DNA fragmentation. Analysis of fragmented DNA from thymocytes treated with YO-2 by agarose gel electrophoresis revealed that the compound caused internucleosomal DNA fragmentation. In addition, judging from a laser scanning microscopy, annexin V-positive and propidium iodide-negative cells were increased by the treatment of the cells with the compound. Moreover, chromatin condensation was observed in thymocytes treated with the compound. These results demonstrated that YO-2 induces thymocyte apoptosis. There seemed to be some correlation between the apoptosis induced by YO compounds and their plasmin inhibitory effect. However, because the other protease inhibitors including pepstatin A, leupeptin, AEBSF, DFP and E-64-d did not affect DNA fragmentation, YO compounds are likely to have unique mechanism on plasmin or to show the effect on the other plasmin-like proteases. The plasmin inhibitory activity may have an important role in YO-2-induced apoptosis. Furthermore, the stimulations of caspase-8, -9 and -3-like activities were observed in thymocytes treated with YO-2. These results suggest that YO-2 induces thymocyte apoptosis *via* activation of caspase cascade.

Keywords: YO-2; Plasmin inhibitor; DNA fragmentation; Chromatin condensation; Thymocyte apoptosis; Caspases

1. Introduction

YO-2 is the compound that was found by Okada *et al*. [1] as the selective plasmin inhibitor, which is composed of three parts including tranexamic acid. Plasmin, a serine protease, is thought not only to have function of fibrinolysis, but to interact with many biological processes such as ovulatory process [2,3] and invasive growth and metastasis of tumors [4,5]. It has been found that the levels of urokinase-type plasminogen activator (uPA) [6–12] and uPA receptor [13,14] increase in many malignant tissues and the inhibitor of serine protease including plasmin

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Abbreviations: YO-0, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-2-bromobenzyloxycarbonyl)tyrosine-4-acetylanilide; YO-1, *trans*-aminomethylcyclohexanecarbonyl-L-phenylalanine-4-calboxymethylanilide; YO-2, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-picolyl)tyrosine-octylamide; YO-3, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-2-bromobenzyloxycarbonyl)tyrosine-*n*-octylamide; YO-4, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-2-bromobenzyloxycarbonyl)tyrosine-*iso*-amylamide; YO-5, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-2-bromobenzyloxycarbonyl)tyrosine-*n*-octylester; YO-6, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-2-pyrimidyl)tyrosine-3-carboxyanilide; YO-8, 6-(4-benzyloxycarbonylaminobutyl)-3-(2-benzyloxycarbonylethyl)-5-methyl-2(1*H*)-pyrazinone; AEBSF, 4-(2-aminoethyl)-benzensulfonyl fluoride; DFP, diisopropyl fluorophosphates; PMSF, phenylmethylsulfonyl fluoride; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-α-(4-methyl-cumaryl-7-amide); Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methyl-cumaryl-7-amide); Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-α-(4-methyl-cumaryl-7-amide); Z-VAD-FMK, carbobenzoxy-Val-Ala-Asp(OCH_3)-fluoromethane; Z-DEVD-FMK, carbobenzoxy-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fluoromethane; E-64-d, (L-3 *trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine(3-methylbutyl)amide; DTT, (±)-dithiothreitol; EGTA, ethylene glycol-bis-(b-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidepropyl)dimethylammonio]-1-propanesulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

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inhibitors prevent metastasis [15–17]. Thus, plasmin may play a crucial role in poor prognosis of cancer. In recent study, YO-2 had anti-tumor effect to various human tumor cell lines [1]. Among the other YO compounds beside YO-2, only the plasmin inhibitors had this effect. In addition YO-2 activates caspase-3-like protease activity [1]. Although YO-2 is likely to be useful anticancer agent, the mechanism of this effect is still uncertain.

Apoptosis is the characteristic process of cell death and differs from necrosis, another type of cell death. This type of cell death is thought to play an important role in cell growth, differentiation, tissue development [18–20], and several diseases, including cancer [20,21]. Previous studies have demonstrated that a wide range of anticancer agents, including chemotherapeutic agents, hormones, and various biologicals, induce apoptosis in malignant cells *in vitro* [20,22]. Thus, one of the major modes of action of anticancer agents may be *via* the activation of apoptotic process.

To evaluate the mechanism of anticancer effect of YO-2, we have studied whether thymocyte apoptosis is induced by YO-2, and what relationship it has with the apoptosis inducing effect and the plasmin inhibitory activity. The mechanism of anti-tumor action by YO-2 is also discussed.

2. Materials and methods

2.1. Materials

Various YO compounds including YO-0, -1, -2, -3, -4, -5, -6, -8 and *O*-picolyl tyrosine were synthesized as described previously [23]. Tranexamic acid was obtained from Nacalai Tesque Inc. and *n*-octylamine was from Tokyo Kasei Kogyo Co. Ltd. Pepstatin A, leupeptin, AEBSF and DFP were obtained from Wako Pure Chemical Industries Ltd. E-64-d, Z-VAD-FMK, Ac-IETD-MCA, Ac-LEHD-MCA and Ac-DEVD-MCA were purchased from Peptide Institute Inc. Z-DEVD-FMK was obtained from Calbiochem, CN Bioscience Inc. Annexin V-EGFP Apoptosis Detection Kit was from Medical & Biological Laboratories Co. Ltd. All other reagents were analytical grade available.

2.2. Cell culture of thymocytes

Sprague–Dawley rats (5 weeks) were obtained from Charles River Japan Inc. Isolated thymocytes from rats were incubated with RPMI 1640 medium supplemented with 10% fetal calf serum at a density of 10×10^6 cells/ml under 5% CO_2 in air immediately after the isolation [24].

2.3. DNA fragmentation

After the incubation of thymocytes in the absence or presence of the compounds, the cells were collected and washed twice with PBS. The intact and fragmented DNA in the cells was assayed as described previously [25]. DNA fragmentation was expressed as the percentage of fragmented DNA in the total DNA (intact plus fragmented DNA).

2.4. Analysis of fragmented DNA

To examine the DNA laddering, we analyzed the fragmented DNA by 1.8% agarose gel electrophoresis as described previously [26].

2.5. Laser scanning microscopy

Annexin V staining was carried out to determine the translocation of phosphatidylserine at the surface of the cell [27,28] using Annexin V-EGFP Apoptosis Detection Kit (Medical & Biological Laboratories Co. Ltd.). According to its protocol the cells were stained after the incubation with YO-2. A laser scanning microscope (Olympus, LSM GB 200) equipped with argon laser and a 40×, water immersion objective was used to visualize annexin V-positive (green fluorescence) cells.

The nuclear structure in thymocytes treated with YO-2 was analyzed according previous report [29].

2.6. Determinations of caspase-like protease activities

The activities of caspase-8, -9 and -3 were determined by fluorescent substrates as described previously [30]. After the incubation of thymocytes in the absence or presence of 30 μM YO-2, cytosolic extracts were prepared by repeated freezing and thawing of cells (100×10^6 cells) in 50 μL extraction buffer containing 50 mM KCl, 50 mM PIPES (pH 7.4), 5 mM EGTA, 2 mM $MgCl_2$, 20 μM cytochalasin B, 1 mM PMSF, 1 mM DTT, 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A and 2.83 μg/ml E-64-d [31]. Cell lysates were then diluted with assay buffer containing 100 mM HEPES–KOH, 10% sucrose, 0.1% CHAPS, 10 mM DTT and 0.1 mg/mL ovalbumin, and were incubated at room temperature with 100 μM Ac-IETD-MCA, Ac-LEHD-MCA and Ac-DEVD-MCA as the substrates of caspase-8, -9 and -3, respectively.

3. Results

3.1. DNA fragmentation of thymocytes induced by various YO compounds

Figs. 1 and 2 show the effects of various YO compounds on DNA fragmentation in rat thymocytes. All YO compounds except for YO-8 contain tranexamic acid in the structures. YO-1 and -6 inhibit plasma kallikrein (PK) selectively, while YO-2, -3 and -5 are the selective inhibitors of plasmin. YO-4 inhibits both PK and plasmin.

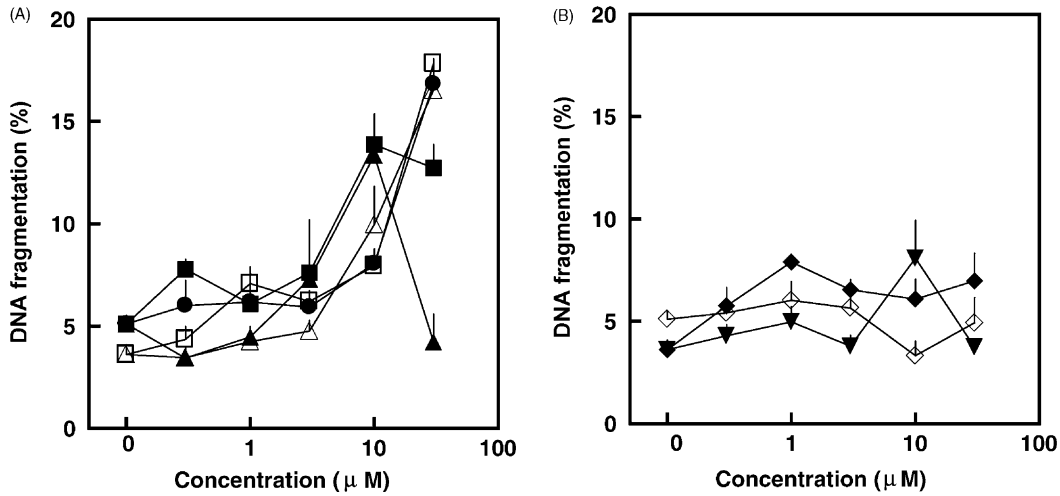

Fig. 1. The effect of various concentrations of YO compounds on DNA fragmentation in rat thymocytes. The thymocytes were incubated with different concentrations of YO compounds for 6 hr. After the incubation, the cells were collected and DNA fragmentation was determined. Values are means ± SEM of 3–4 separate experiments. (A) △, YO-0; ●, YO-2; ▲, YO-3; □, YO-4; ■, YO-5. (B) ▼, YO-1; ◇, YO-6; ◆,YO-8.

YO-8 does not contain tranexamic acid and has no effect on both PK and plasmin activity. YO-0, -2, -3, -4 and -5 increased the DNA fragmentation of rat thymocytes whereas no significant DNA fragmentation was observed by the treatment with YO-1, -6 or -8. A significant increase in DNA fragmentation was observed with 10 or 30 μM (Fig. 1). Since DNA fragmentation by YO compounds were observed more than 2 hr after the incubation, it seems to be necessary for the lag time to increase DNA fragmentation (Fig. 2). These results indicate that only the compounds, which are potent plasmin inhibitor among YO compounds, induced DNA fragmentation of rat thymocytes.

Since DNA fragmentation by several YO compounds including YO-2 were observed, the fragmented DNA by the treatment of YO-2 was analyzed by use of agarose gel electrophoresis. As shown in Fig. 3, the fragmented DNA from cells treated with YO-2 revealed the typical DNA ladder pattern of DNA fragments, indicating that YO-2 caused internucleosomal DNA fragmentation in the cells.

3.2. Morphological characterization of thymocyte apoptosis induced by YO-2

Based on the biochemical observation, YO-2 seems to induce thymocyte apoptosis. In order to clarify YO-2-induced thymocyte apoptosis, annexin V staining in the cells treated with YO-2 was examined. Fig. 4 shows the number of annexin V-positive cells was increased in

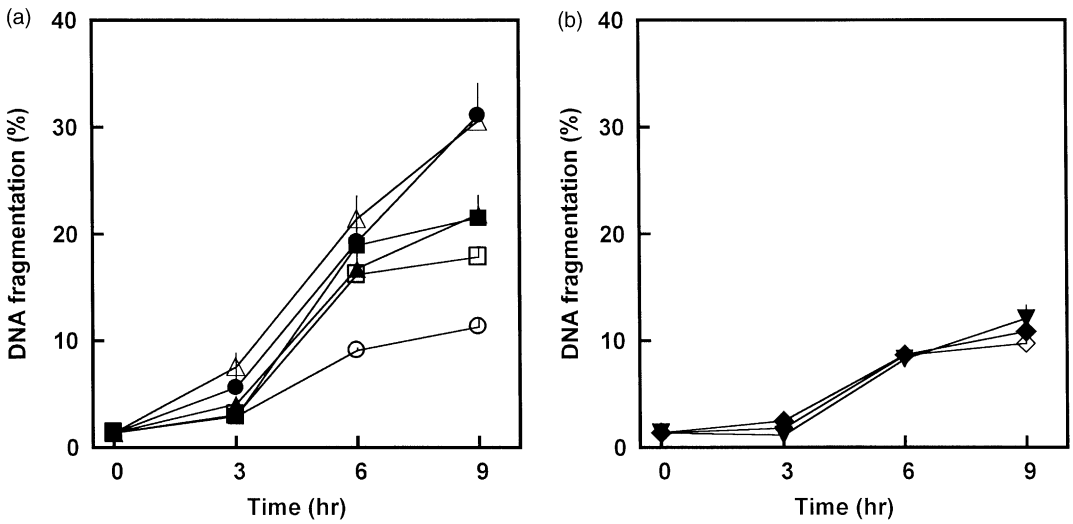

Fig. 2. The effect of incubation time on DNA fragmentation in rat thymocytes. The cells were treated with 30 μM of various YO compounds for indicated times. After the incubation, DNA fragmentation was determined. Values are means ± SEM of 3–4 separate experiments. (A) ○, control; △, YO-0; ●, YO-2; ▲, YO-3; □, YO-4; ■, YO-5. (B) ▼, YO-1; ◇, YO-6; ◆,YO-8.

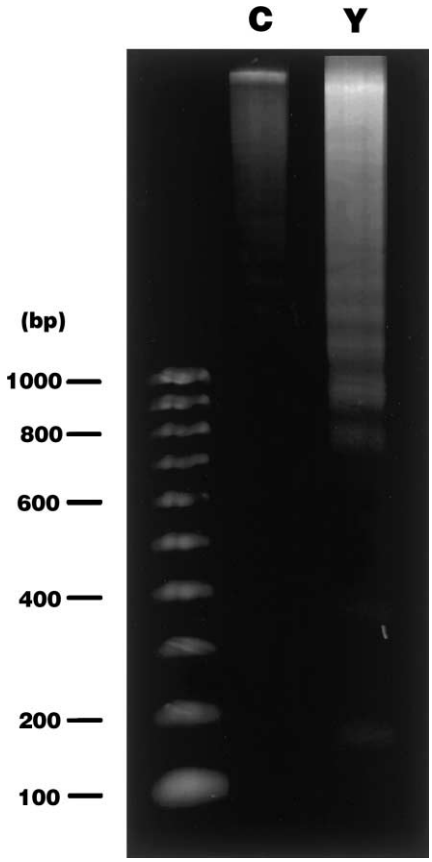

Fig. 3. Electrophoretic analyses of fragmented DNA from the thymocytes treated with YO-2. The cells were treated with 30 μM YO-2 for 6 hr. C, control; Y, YO-2.

the incubation time-dependent manner. The number of annexin V-positive and propidium iodide-negative cells was increased 2-fold by the treatment of the cells with YO-2 for 2 hr, indicating that the translocation of phosphatidylserine, an early event of the apoptotic process, precedes DNA fragmentation. The treatment of thymocytes with YO-2 resulted in a remarkable increase in both annexin V- and propidium iodide-positive cells 6 hr after the incubation. Thus, these results suggested that YO-2 caused apoptosis but not necrosis. The morphological study on the nuclear structure by use of the laser scanning microscope was shown in Fig. 5. The laser scanning microscopic image of control thymocytes showed a core-like structure and unequal contours of fluorescent intensity in most cells, being responsible for the nuclear chromosome. In contrast, the cells treated with YO-2 for 3 hr revealed a peculiar feature of nuclei, having an equal distribution of much more intense fluorescence and lacking of core-like structure or a part of nuclei like a crescent. Their sizes were smaller than the control-like nuclei. The formation of this type of nuclei was dependent on the incubation time and paralleled the increase in DNA fragmentation, thus accounting for the apoptotic nuclei (Fig. 2). These findings prove that YO-2 induced the nuclear condensation, the morphological feature of apoptosis.

3.3. Effect of elements composing YO-2 on DNA fragmentation

YO-2 is composed of three parts—tranexamic acid, *O*-picolyl tyrosine and *n*-octylamine. Tranexamic acid moiety interacts with active center of plasmin and PK, whereas the other two moieties are important with the decision of the selectivity to plasmin. Fig. 6 shows the effect of these compounds on DNA fragmentation. No change in DNA fragmentation was observed by these components.

3.4. Effect of various protease inhibitors on DNA fragmentation

These results suggest that only the YO compounds which have plasmin inhibitory effect causes DNA fragmentation of rat thymocytes. In order to clarify the relationship between inhibition of plasmin and DNA fragmentation inducing activity, effects of the other protease inhibitors on DNA fragmentation were examined. Plasmin is classified into serine protease and this activity is inhibited by serine protease inhibitors such as leupeptin, AEBSF or DFP. However, these plasmin inhibitors showed little effect on DNA fragmentation (Fig. 7). Moreover, pepstatin A and E-64-d, which are acid protease inhibitor and thiol protease inhibitor, respectively, did not induced DNA fragmentation (Fig. 7). In addition, no changes in the nuclear structure were observed in the cells treated with these compounds (Fig. 8).

3.5. Caspase activation in YO-2-treated thymocyte

Since caspases play a crucial role in apoptotic process, the activities of caspases were determined in rat thymocytes treated with YO-2. Fig. 9 represents that the incubation of thymocytes with YO-2 increased caspase-8, -9, -3-like activities in an incubation time-dependent manner. The effect of the inhibitors of caspases on YO-2-induced DNA fragmentation was examined. As shown in Fig. 10, Z-VAD-FMK, a broad inhibitor of caspases, and Z-DEVD-FMK, an inhibitor of caspase-3, prevented DNA fragmentation in the cells treated with YO-2. Thus, the caspase cascade is likely to be involved in YO-2 induced apoptotic cell death.

4. Discussion

Previously we have found that a selective plasmin inhibitor, YO-2, showed anti-tumor effect on M1 (melanoma) cell line and HT29 colon carcinoma cells [1]. In addition to YO-2, the other YO compounds, which inhibit plasmin activity, had the similar action to YO-2. However, the mechanism of anti-tumor effect of YO compound is still uncertain. Since the studies on the molecular mechanism of apoptosis were extensively carried out in the

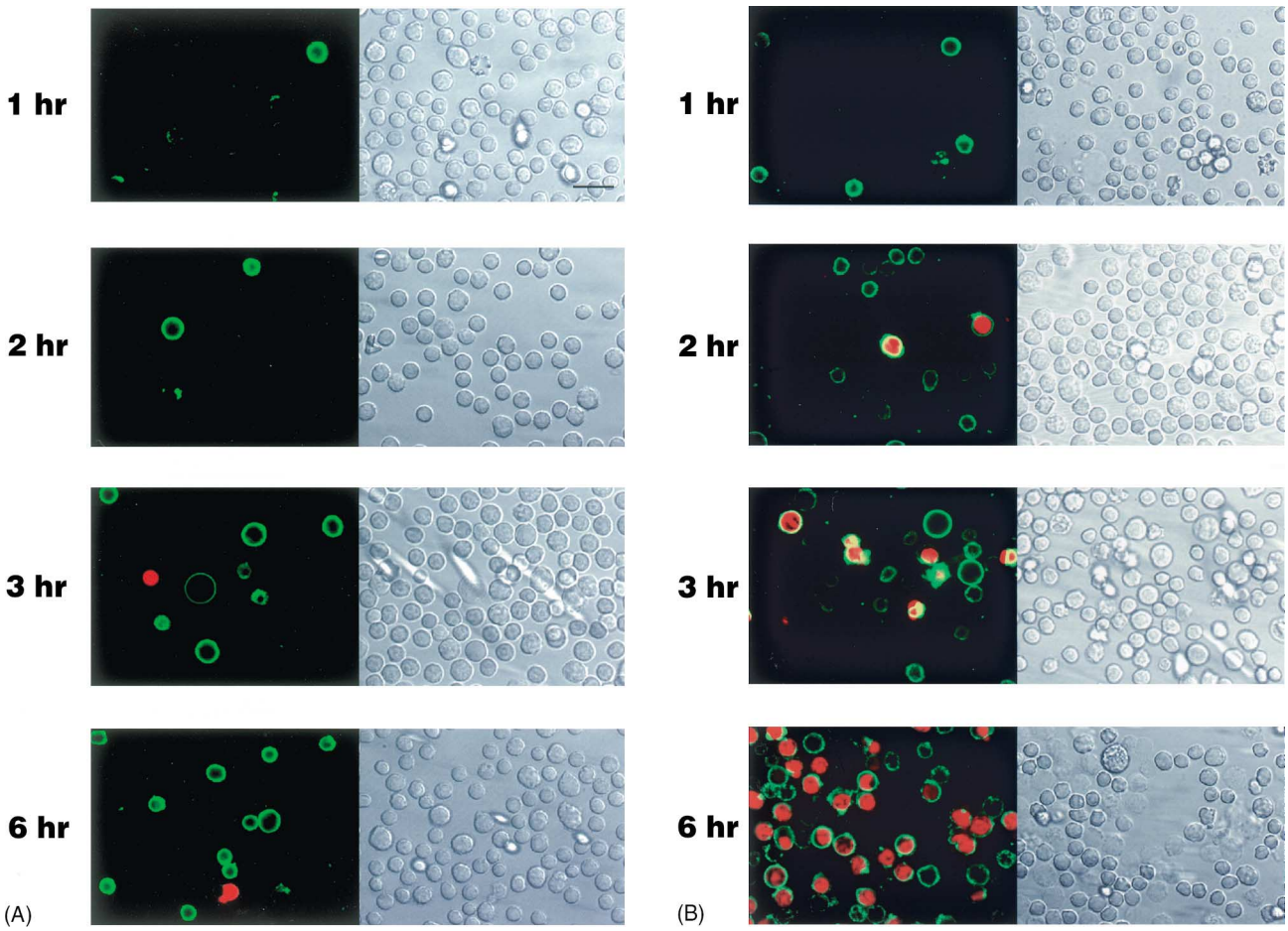

Fig. 4. Annexin V-EGFP and propidium iodide staining of untreated and YO-2 treated thymocytes. The cells were incubated with 30 μM YO-2 for indicated time, then the cells were stained with Annexin V-EGFP (green) and propidium iodide (red) without fixation. The right panels indicated the dioptric images of the same field of vision. (A) control; (B) YO-2-treated. Bar, 10 μm.

thymocytes, we designed this study using thymocytes in order to clear the mechanism of pro-apoptotic action of YO-2.

Generally, it is necessary for evaluation of apoptotic cell death to demonstrate the biochemical and morphological changes in the cells. The data obtained in this study show that the treatment of thymocytes with YO-2 resulted in the internucleosomal DNA fragmentation (Figs. 1 and 2) and the exposure of phosphatidylserine at the surface of the cells (Fig. 4), the biochemical hallmark of apoptosis. Nuclear condensation, the morphological feature of apoptosis, was also observed in the cells treated with YO-2 (Fig. 5). In addition, YO-2 increased caspase-8, -9 and -3-like activities (Fig. 9), YO-2 induced DNA fragmentation was prevented by the inhibitors of caspases (Fig. 10), suggesting that apoptosis induced by YO-2 may be due to stimulation of caspase-like activities. Thus, the present study demonstrated that YO compounds with plasmin inhibitory action induced apoptotic cell death in rat thymocytes. On the other hand, YO compounds that inhibit PK activity, did not induce DNA fragmentation. Therefore, inhibition of plasmin but not PK activity is likely to induce apoptotic cell death in rat thymocytes. To confirm this, the effect of another plasmin inhibitors on DNA fragmentation in the cells was examined. However, neither DNA fragmentation nor nuclear condensation was observed in the cells treated with plasmin inhibitors. At present, the role of cellular plasmin in the induction of apoptosis is unclear.

Characteristic apoptotic changes have been described in tumors after treatment with various chemotherapeutic agents, including cytarabine, 5-fluorouracil, fludarabine doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycine and camptothecin [22]. One of the major modes of action of various anticancer drugs may be *via* the induction of apoptosis. Thus, it was suggested that the anti-tumor effect of YO-2 was caused by the apoptosis inducing activity. A number of studies have suggested the possibility that anticancer drugs trigger apoptosis by inducing the synthesis of FasL [31–38], which then binds to Fas and activates the death receptor pathway. The binding with FasL to Fas causes the activation of caspase-8 and then the activation of downstream caspases, such as caspase-3 and the cells are led to apoptosis. We confirmed that YO-2-treatment on rat thymocytes increased caspase-8, -9 and

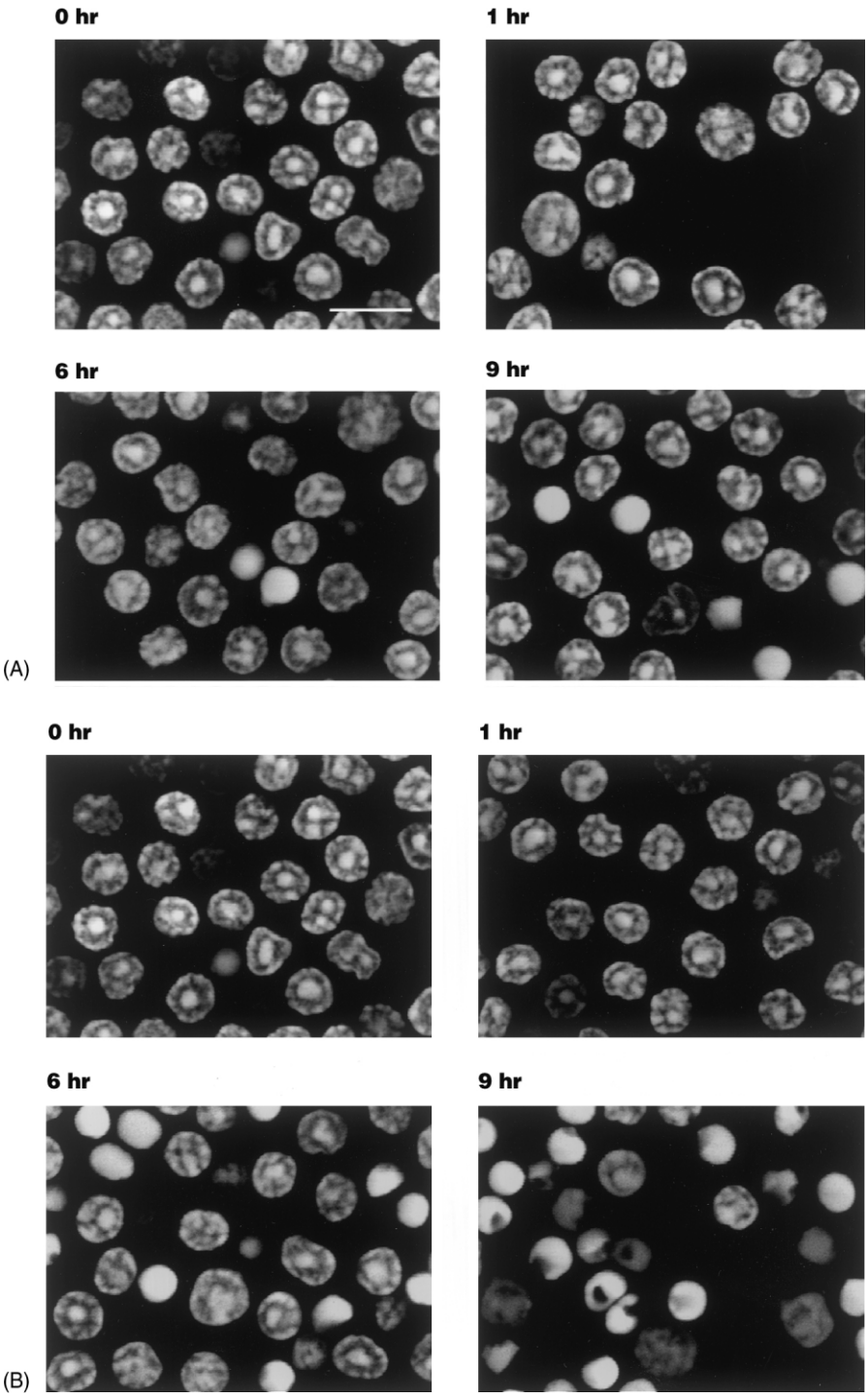

Fig. 5. Laser scanning microscopic images of thymocytes treated with YO-2. The thymocytes were treated with 30 μM YO-2 for indicated times. (A) control; (B) YO-2-treated. Bar, 10 μm.

-3-like activities (Fig. 9) and this fact agreed with the result observed in the tumor cell lines [1]. Moreover, YO-2 induced apoptotic cell death was observed in HL-60 and Jurkat cell lines (data not shown). Thus, the anti-tumor effect of YO-2 may be common to the mechanism of many anticancer drugs partially.

Only the YO compounds which have plasmin inhibitory activity showed anti-tumor effect and apoptosis inducing activity. Plasmin, a potent serine protease, is not only associated with fibrinolysis, but is capable of degrading extracellular matrix protein such as laminin [39], fibronectin [5] and vitronectin [5]. Plasmin also composes the protease cascade, which is called the plasmin cascade [5] and activates dowmstream prometalloproteinases by enzymatic cleavage. uPA and tissue-type plasminogen activator are enzymes that catalyse the conversion on the inactive

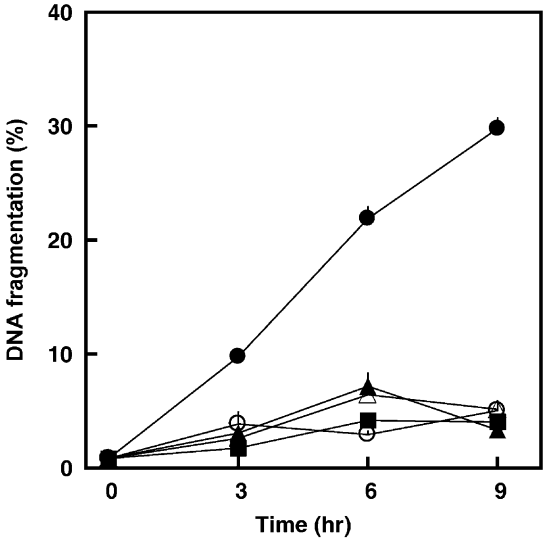

Fig. 6. Effect of elements composing YO-2 on DNA fragmentation. The thymocytes were treated with 30 μM tranexamic acid, *O*-picolyl tyrosine or *n*-octylamine for 6 hr. After the incubation, DNA fragmentation was determined. Values are means ± SEM of four separate experiments. ○, control; ●, YO-2; △, tranexamic acid; ▲, *O*-picolyl tyrosine; ■, *n*-octylamine.

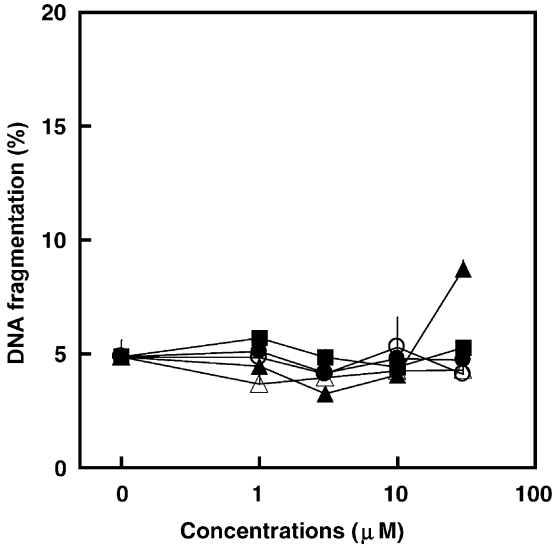

Fig. 7. Effect of other protease inhibitors on DNA fragmentation in rat thymocytes. The cells were treated with various concentrations of protease inihibitors for 6 hr. After the incubation, DNA fragmentation was determined. Values are means ± SEM of four separate experiments. ○, pepstatin A; ●, leupeptin; △, AEBSF; ▲, DFP; ■, E-64-d.

zymogen plasminogen to the active protease plasmin. While uPA generally increases plasmin for extracellular matrix degradation, tissue-type plasminogen activator primarily increases plasmin for thrombolysis [5]. Raised levels of uPA have been found in many malignant tissue types [6–12] and levels of uPA receptor expression in the cell surface also correlate with poor prognosis [13,14,40] such as invasive growth and metastasis of tumors in some cancers. Thus, the plasmin cascade may play a crucial role in invasion and metastasis. Actually, inhibition of serine protease such as plasmin prevents metastasis [15–17]. Combined with the results from this experiment, therefore, it seems that inhibition of metastasis by these inhibitors is due to the induction of apoptosis.

On the other hand, plasmin regulates ovulatory follicular rupture with the localized secretion of tumor necrosis factor (TNF) α and consequent apoptosis [2,3]. TNF α, a cytokine expressed as a 26 kDa integral transmembrane

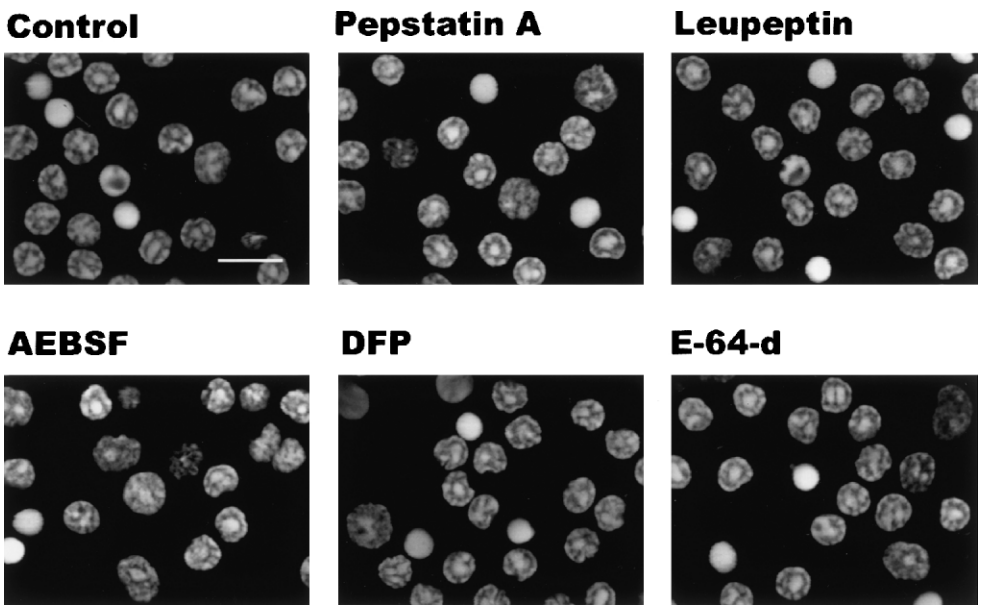

Fig. 8. Laser scanning microscopic images of nuclei in thymocytes treated with various protease inhibitors. The thymocytes were treated with 30 μM of pepstatin A, leupeptin, AEBSF, DFP and E-64-d for 6 hr, and then the cells were stained with Hoechst 33342. Bar, 10 μm.

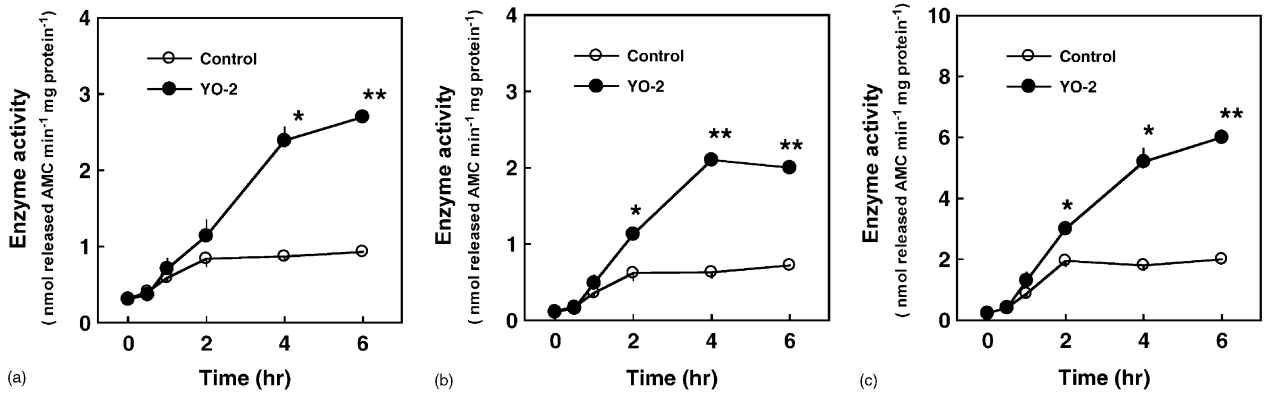

Fig. 9. Caspase activation in YO-2-treated thymocyte. Thymocytes were incubated in the absence or presence of 30 μM YO-2, and then the extracts from the cells were prepared. The resulting extracts were used as the enzyme preparations. The activities of caspase-8 (A), -9 (B) and -3 (C) were determined using the substrates Ac-IETD-MCA, Ac-LEHD-MCA and Ac-DEVD-MCA, respectively. ○, control; ●, YO-2-treated. Results are representative of four independent experiments and are expressed as mean values ± SEM (error bars are smaller than the symbols in many data points). (*) $P < 0.05$ and (**) $P < 0.01$, significant from control (Student's *t*-test).

polypeptide, is a candidate substrate for serine protease, possibly plasmin, attack. Bioactive (soluble) TNF α is generated by enzymatic cleavage of 17 kDa extracellular domain subunit from its membrane anchor. Common cell types, such as leukocytes, smooth muscle, fibroblasts and endothelial cells produce TNF α and plasma membrane receptors for TNF α are present on virtually all nucleated cells. It is apparent that TNF α can convey a signal that results in apoptosis. TNF trimerizes TNFR1 upon binding, inducing association of the receptor's death domain. Subsequently, an adapter termed TNFR-associated death domain (TRADD) binds through its own death domain to the clustered receptor death domains. Fas-associated death domain (FADD) couples the TNFR1–TRADD complex to activation of caspase-8, thereby initiating apoptosis [41]. The plasmin inhibitory activity may have an important role in YO-2-induced apoptosis. However, it remains to be elucidated for the relationship between its plasmin inhibitory action and apoptosis induction. The study on the mechanism of induction of apoptosis by YO-2 is now in progress in our laboratory.

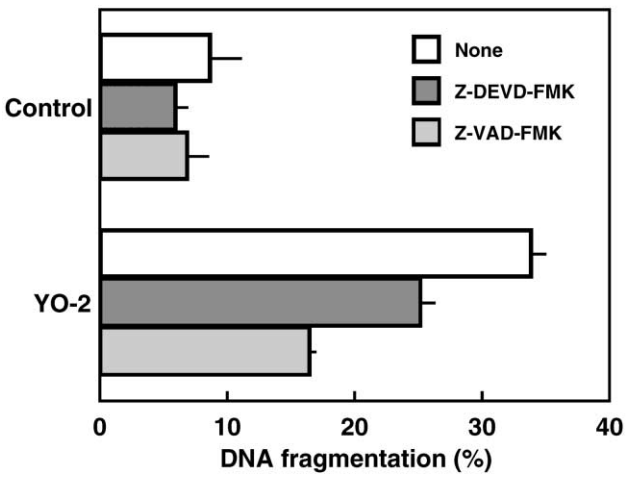

Fig. 10. The effect of caspase inhibitors on DNA fragmentation induced by YO-2. The cells were preincubated in the presence of 50 μM Z-DEVD-FMK and 12.5 μM Z-VAD-FMK for 1 hr. Values are means ± SEM of four separate experiments. Then, YO-2 was added to the cells, and was incubated for 6 hr.

Acknowledgments

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