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YM155 induces caspase-8 dependent apoptosis through downregulation of survivin and Mcl-1 in human leukemia cells

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ABSTRACT

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is highly expressed in various kinds of tumors. In the present study, we investigated the cytotoxic mechanism of YM155, a unique small-molecule inhibitor of survivin, in human myelogenous leukemia cells. YM155 potently inhibited the cell growth of HL-60 and U937 cells with the half-maximal inhibitory concentration (IC_{50}) value of 0.3 nM and 0.8 nM, respectively. YM155 significantly suppressed the levels of mRNA expression and protein of survivin in HL-60 and U937 cells. In addition, we also found that YM155 down-regulated the level of Mcl-1, another critical anti-apoptotic protein, in both HL-60 and U937 cells. Treatment of HL-60 and U937 cells with YM155 induced apoptosis concomitant with the activation of caspase-8 and caspase-3. Interestingly, we have found that caspase-8 inhibitor Z-IETD-FMK strongly inhibited YM155-induced apoptosis in HL-60 and U937 cells. When cells were pretreated with Z-IETD-FMK, the activation of caspase-3 was completely abolished, suggesting that caspase-8 may be involved in the activation of caspase-3 during YM155-induced apoptosis. We demonstrated for the first time that YM155 induces caspase-8 dependent apoptosis through downregulation of survivin and Mcl-1 in human leukemia cells.

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1. Introduction

Apoptosis is regarded as an active suicidal response to various physiological or pathological stimuli including toxic chemicals and anticancer agents [1]. Recently, many molecules have been identified as important regulators of apoptosis [2,3]. Genetic and biochemical data indicate that a family of cysteine proteases with aspartate specificity, known as caspases, plays a pivotal role in apoptosis [4–6]. Caspase-8 is mainly known for its important role within the extrinsic apoptosis pathway, where it signals from proximal death receptor such as Fas, to activation of caspases or mitochondria pathway [7,8]. In contrast, cytotoxic drugs mainly signal via the intrinsic apoptosis signaling pathway, where activated mitochondria release apoptogenic factors such as cytochrome c for cleavage of downstream effector caspases [9,10]. The role of caspase-8 for intrinsic apoptosis signaling has been controversially discussed [11,12].

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and has been implicated in both suppression of apoptosis and regulation of mitosis [13,14]. Survivin is largely undetected or expressed at very low levels in normal tissues [13], whereas it is overexpressed in many malignancies, including leukemia, breast, lung, colon, pancreas, liver, and head and neck cancer [15–19]. Growing evidence suggest that survivin expression is associated with drug resistance in cancer cells [20,21]. Given its aberrant expression in cancer, its ability in antiapoptosis and drug resistance, promotion of cancer cell proliferation, and its association with various clinicopathological parameters, survivin is thought to be a potential therapeutic target in cancers.

YM155, a novel small molecule suppressant of survivin (Fig. 1A) was identified in a survivin gene promoter assay by high throughput screening of chemical libraries [22]. YM155 shows robust anti-proliferative activities against various human cancer cells regardless of p53 status [22]. Moreover, YM155 induces regression of established human hormone-refractory prostate tumor xenografts [22]. Phase I and phase II trials with YM155 have shown its safety and tolerability in patients with unresectable melanoma and advanced refractory non–small cell lung carcinoma [23–25]. Although several clinical studies are ongoing, the exact cytotoxic mechanism of YM155 has remained unclear.

In the present study, we investigated the cytotoxic mechanism of YM155 in human leukemia cells. YM155 exhibits potent

Abbreviation: IC_{50}, half-maximal inhibitory concentration.

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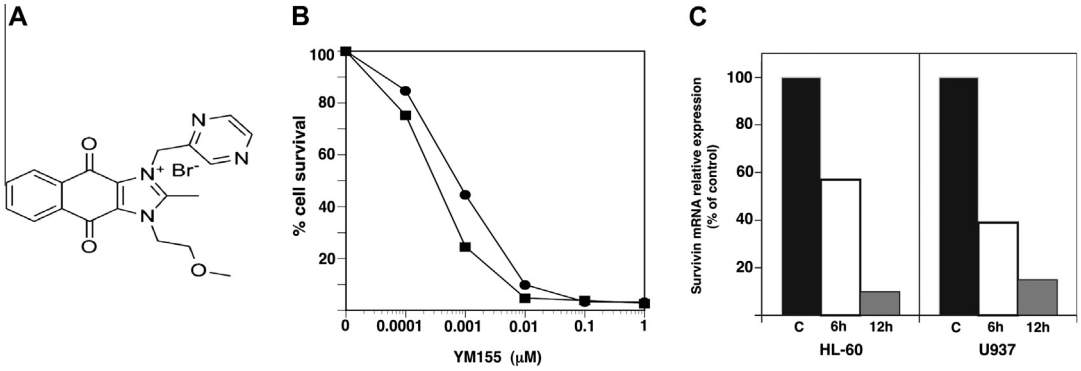

Fig. 1. YM155 exhibits potent antiproliferative activity against human leukemia cell lines. (A) Chemical structure of YM155. Chemical name, 3-(2-methoxyethyl)-2- methyl-4,9- dioxo-1-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho[2,3-d]imidazol-3-ium bromide. (B) Cell growth inhibition by YM155 in human leukemia cell lines. The cells were incubated with various concentrations of YM155 at 37 °C for 72 h. Cell growth inhibition rate was determined by Cell counting Kit as described in Section 2. Point: mean from three separate experiments. (C) YM155 inhibits survivin mRNA expression in human leukemia cells. HL-60 and U937 cells were treated with 1 μM YM155 for indicated time periods (control: 0.1% DMSO). After drug treatment, total RNA was prepared. The relative expression of survivin mRNA was quantitated by real-time RT-PCR analysis as described in Section 2. Columns, mean from three separate experiments.

cytotoxic activity through downregulation of survivin and Mcl-1 protein. Furthermore, we demonstrated for the first time that caspase-8 may play an important role as an up-stream effecter for the activation of caspase-3 during the cell death process by YM155 in human myeloid leukemia cells.

2. Materials and methods

2.1. Cell culture

The human leukemia cell line HL-60 and U937 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% FCS (Sigma, St. Louis, MO) at 37 °C under 5% CO2 in a humidified atmosphere. Exponentially growing cells were exposed to drugs for the indicated time periods.

2.2. Chemicals and antibodies

YM155 was obtained from Selleck chemicals (Houston, TX, USA). Ac-DEVD-MCA and Ac-IETD-MCA were obtained from Peptide Institute (Osaka, Japan). Z-IETD-FMK and Z-VAD-FMK were purchased from BD Biosciences (San Jose, California USA). Antibodies used for immunoblotting were purchased as follows: rabbit anti-human Survivin and Mcl-1 monoclonal antibodies were from Cell Signaling Technology (Danvers, MA); rabbit anti-human actin antibody was from Sigma (St Louis, MO).

2.3. Cytotoxicity assays

Cytotoxicity of YM155 was evaluated using Cell Counting Kit (DOJINDO, Kumamoto, Japan). All assays were performed in triplicate, and all experiments were performed multiple times. After drug treatment, cells were incubated at 37 °C with Cell Counting Kit reagent for 60 min processed according to the manufacturer's instructions (DOJINDO, Kumamoto, Japan).

2.4. Measurement of caspase activity

Activity of caspase-3/7 and caspase-8 was analyzed by the cleavage of the fluorometric substrates Ac-DEVD-MCA and Ac-IETD-MCA, respectively. Cell lysates were prepared by washing cells three times in ice-cold phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) and then incubating the cells for 15 min on ice at a density of 1×10^8/ml in TKM buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl) containing 0.25% Triton X-100 as described previously [26]. The cell lysates were centrifuged (10,000*g* for 15 min at 4 °C) and the pellets were discarded. The supernatants were aliquoted and frozen at −80 °C. Enzyme reactions were performed in 96-well plates with 100 μl of cell lysates, and DEVD-MCA or IETD-MCA. Each sample was seeded in triplicate. After incubated at 37 °C for 90 min, AMC (7-amino-4-methyl coumarin) released from the substrates was measured at excitation and emission wavelengths of 355 nm and 460 nm using a fluorescence micro-plate reader.

2.5. Annexin-V binding assay

Apoptosis was analysed using the annexin-V binding assay (ApoAlert Annexin V-FITC Apoptosis kit, Clontech, Mountain View, CA, USA). Cells were washed with PBS and resuspended in 100 μl binding buffer [10 mM HEPES/NaOH (pH7.4), 140 mM NaCl, 2.5 mM $CaCl_2$. Five microliters of annexin-V FITC were added and incubated for 15 min in the dark at room temperature. The cells were analysed with Becton Dickinson FACScan flow cytometer using the Cells Quest program.

2.6. Flow cytometry for detection of sub-G1 population

The treated cells were washed with PBS, and fixed in 75% ethanol for at least 2 h at 4 °C. Before analysis, the cells were washed once again with PBS, suspended in a cold 0.1% (w/v) sodium citrate solution containing 100 μg/ml RNase A and 50 μg/ml PI, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber; Becton Dickinson, San Jose, CA, USA), and CellQuest software (Becton–Dickinson) was used to determine the relative DNA content based on the presence of PI-positive red fluorescence.

2.7. Immunoblotting

Cells were washed in cold PBS and lysed in Laemmli's buffer containing protease inhibitors (3 mg leupeptin, 3 mg aprotinin and 2 mM PMSF). Cell lysates were electrophoresed at 125 V in 12% SDS–polyacrylamide gels. After transfer to Immobilon-P membranes (Millipore, Bedford, MA), membranes were blocked overnight in PBS-Tween containing 5% non-fat dried milk, probed for 1 h with primary antibody and for 1 h with secondary antibody (1:1000 dilution). Visualization was achieved using enhanced

chemiluminescence (SuperSignal West Pico Substrate, Pierce, Rockford, IL) according to the manufacturer's instructions.

2.8. Real-time quantitative polymerase chain reaction (RQ-PCR)

Total RNA was isolated using BIO ROBOT EZ1 (Qiagen, Hilden, Germany). The amount of RNA was measured by photometry. Reverse transcription of total RNA was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative PCR was done by TaqMan real-time PCR methods. The StepOne Plus PCR System and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used for the quantification of all genes according to the manufacturer's instructions. The assay IDs were: BIRC5 (Survivin) Hs03043574_m1; GAPDH, Hs02786624_g1; The relative mRNA expression of Survivin was calculated using the comparative threshold method (Ct-method) with GAPDH for normalization. Human leukemia HL-60 cells were used as positive controls for Survivin.

3. Results

3.1. YM155 exhibits potent antiproliferative activity against human leukemia cell lines

The antiproliferative activity of YM155 against human leukemia cell lines HL-60 and U937 was examined. As shown in Fig. 1B, YM155 inhibited the cell growth of HL-60 and U937 in a dose dependent manner. The IC_{50} values of YM155 were 0.3 nM and 0.8 nM for HL-60 and U937 cell line, respectively.

3.2. YM155 inhibits the expression of survivin in human leukemia cell lines

We first examined the effect of YM155 on survivin mRNA expression in human leukemia cell lines by real time PCR analysis. As shown in Fig. 1C, exposure of HL-60 or U937 cells to YM155 at 1 μM significantly inhibited survivin mRNA expression in a time-dependent manner. Using anti-survivin antibody, Western blot analysis was done. Survivin protein levels decreased significantly in HL-60 and U937 cells after treatment with YM155 in a time-dependent manner (Fig. 2A and B). In contrast, exposure of these cell lines to YM155 at 1 μM did not affect the expression of another anti-apoptotic protein Bcl-2 (Fig. 2A and B).

3.3. YM155 inhibits the expression of Mcl-1 in human leukemia cell lines

It has been reported that Mcl-1 may play an important role as anti-apoptotic protein in human leukemia cells [27]. Thus, we also investigated the effect of YM155 on Mcl-1 expression in human leukemia cell lines by Western blot analysis. Using anti-Mcl-1 antibody, Western blot analysis was done. Mcl-1 protein levels decreased significantly in HL-60 and 697 cells after treatment with YM155 in a time-dependent manner (Fig. 2C and D). In contrast, exposure of these cell lines to YM155 at 1 μM did not affect the expression of action protein.

3.4. YM155 induces apoptosis in HL-60 and U937 cells

Next, we examined whether YM155 induces apoptosis in human leukemia cells. YM155 was shown to induce a dose-dependent externalization of phosphatidylserine (PS), a hallmark of apoptosis, as detected by Annexin V(+) cells using flow cytometry, in HL-60 cells (Fig. 3A). It is known that activation of caspase-3/7 is an important event during apoptosis. The proportion of apoptotic cells was also assessed by measurement of the percentage of sub G1 peak by flow cytometer. The percentage of sub-G1 peak fraction increased after treatment of HL-60 cells with YM155 in a dose-dependent manner (Fig. 3B). In order to test the ability of YM155 to activate caspase-3/7, cell lysates from YM155-treated HL-60 or U937 cells were prepared and analyzed for caspase-3/7 activity using Ac-DEVD-MCA as substrate. As shown in Fig. 3C, treatment with YM155 induced caspase-3/7 activation in a dose-dependent manner. We also measured the activities of caspase-8 in the cell lysates from YM155 treated cells. As shown in Fig. 3D, activation of caspase-8 was observed both in HL-60 and U937 cells.

3.5. Caspase-8 inhibitor Z-IETD-FMK blocks induction of apoptosis by YM155 through the inhibition of caspase-8 and caspase-3 in HL-60 and U937 cells

We examined the effect of pan-caspase inhibitor Z-VAD-FMK on YM155-induced apoptosis of human leukemia cells. As shown in Fig. 4A and B, Z-VAD-FMK strongly inhibited YM155-induced caspase-8 and caspase-3 activation in HL-60 and U937 cells. Z-VAD-FMK also suppressed YM-155 induced apoptotic cell death (Fig. 4C and D). The effect of Z-IETD-FMK was also tested on YM155-induced caspase-8 activation in HL-60 or U937 cells. As shown in Fig. 4A, YM155-induced caspase-8 activation was blocked by Z-IETD-FMK treatment. In addition, when cells were pretreated with Z-IETD-FMK, the activation of caspase-3 by YM155 was completely abolished (Fig. 4B). Annexin-V binding assay showed that Z-IETD-FMK significantly inhibited apoptosis of leukemia cells incubated with YM155 (Fig. 5C andD). These data indicate that caspase-8 play an important role during YM-155 induced apoptosis in HL-60 and U937 cells.

4. Discussion

In the present study, we found that YM155 potently inhibited the cell growth of HL-60 and U937 cells with subnanomolar IC_{50} value. It was examined that whether YM155 may inhibit the expression of survivin, Bcl-2 and Mcl-1 in human leukemia cells. Our data indicate that YM155 exhibits robust cytotoxic activity through downregulation of survivin and Mcl-1 in human leukemia cells. Recently, Na et al. [28] reported that YM155 also inhibited the expression of EGFR protein in pancreatic cancer cells. Thus, it is quite likely that YM155 can suppress the expression of not only survivin, but also several important molecules in tumor cells.

We observed that biochemical inhibition of caspase-8 activation by the caspase-8 directed inhibitor Z-IETD-FMK inhibited cell death induction by YM155. In addition, when cells were pretreated with Z-IETD-FMK, the activation of caspase-3 was completely abolished. Thus, caspase-8 may represents an important intracellular signaling mediator involved in the activation of caspase-3 for YM155-induced cell death. It has been reported that caspase-8 can be activated downstream of caspases-3 and -6 after drug treatment, independently of death receptor signaling in several experimental system so far examined [29–31]. In contrast, our data suggest that caspase-8 may play an important role as an up-stream effecter for the activation of caspase-3 during the cell death process by YM155 in human myeloid leukemia cells.

Caspase-8 plays a pivotal role for apoptosis induction via the extrinsic apoptosis signaling cascade, although its role for intrinsic apoptosis signaling induced by cytotoxic drugs seems tumor type specific [32,33]. Ehrhardt et al. [31] reported that caspase-8 is crucial for the antileukemic efficiency of cytotoxic drugs in acute lymphoblastic leukemia. Regarding human myeloid leukemia cells, there has been only a few studies, which described that caspase-8 inhibitor Z-IETD-FMK significantly inhibited apoptosis [34,35].

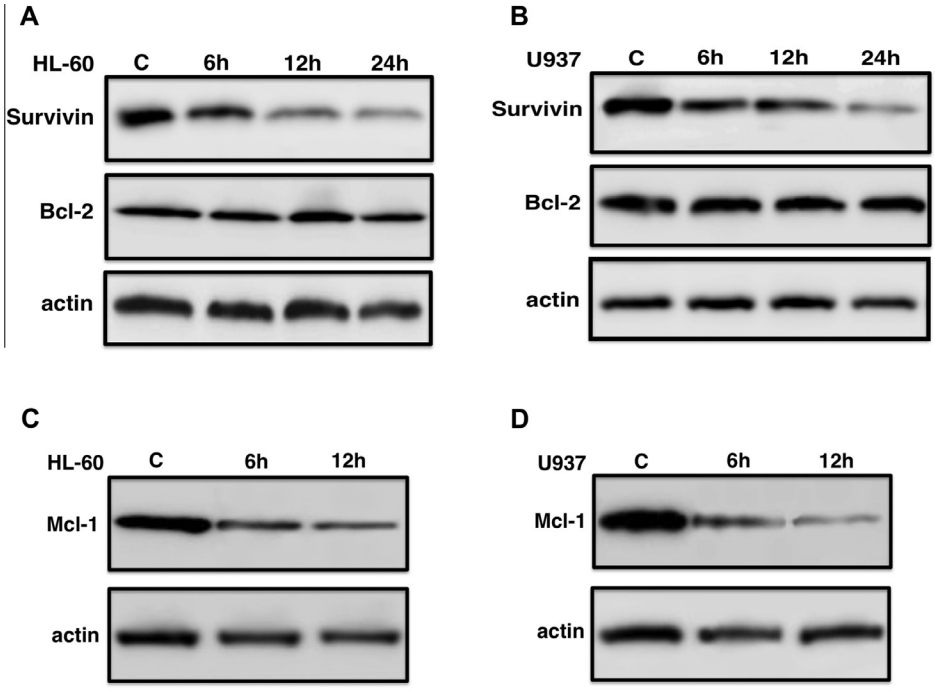

Fig. 2. YM155 suppresses anti-apoptotic protein expression in human leukemia cells. HL-60 {(A) and (C)} or U937 {(B) and (D)} cells were treated with 1 μM YM155 for indicated time periods (control: 0.1% DMSO). Expression levels of survivin, Bcl-2 and Mcl-1 protein were analyzed by Western blot analysis. Western blotting was done as described in Section 2. A representative example of three independent experiments giving very similar results is shown.

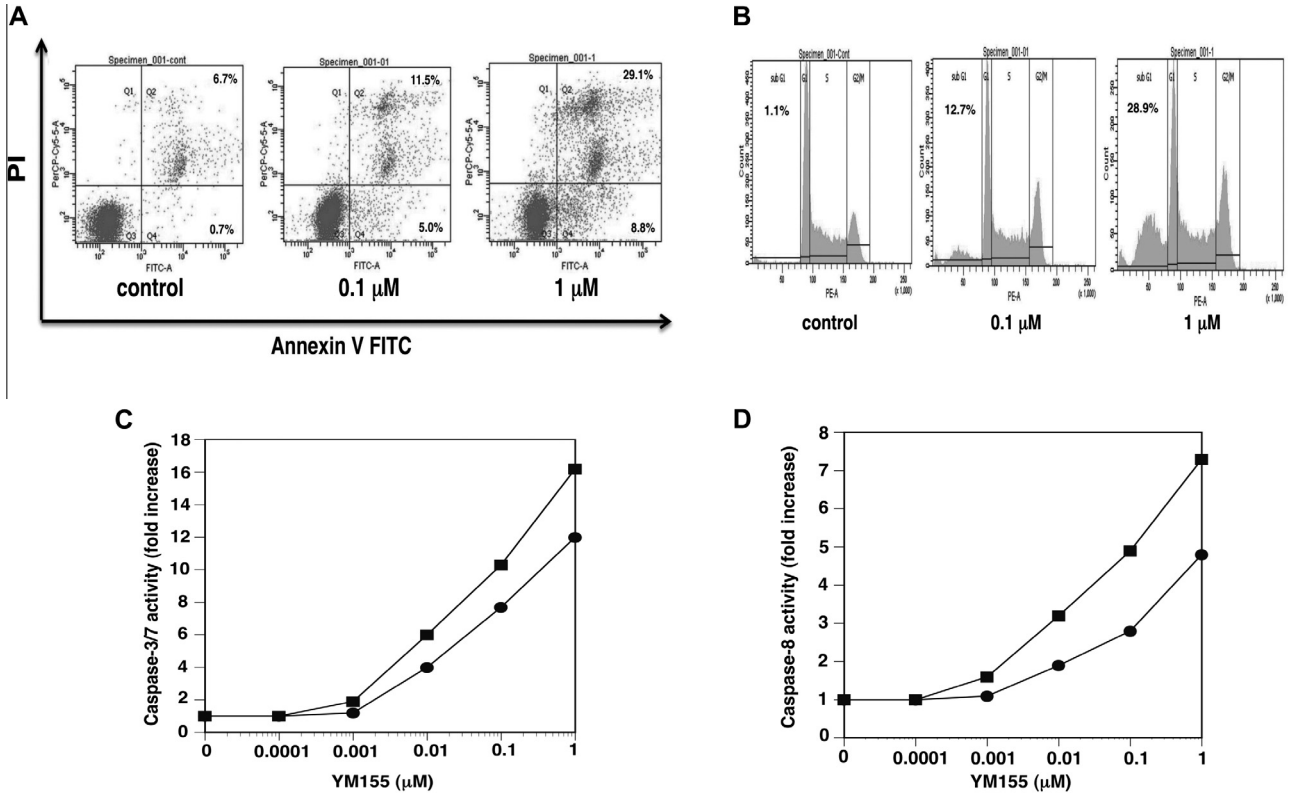

Fig. 3. YM155 induces apoptosis in human leukemia cells. (A) HL-60 cells were incubated with 0.1 or 1 μM YM155 or vehicle (0.1% dimethyl sulfoxide (DMSO) for 8 h. The percentage of apoptotic cells was then determined by Annexin-V binding assay. (B) HL-60 cells were incubated with 0.1 or 1 μM YM155 or vehicle (0.1% dimethyl sulfoxide (DMSO) for 8 h. The percentage of apoptotic cells was also assessed by measurement of the percentage of sub G1 peak by flow cytometer. (C) Dose–response curve of YM155-induced caspase-3/7 activation in HL-60 or U937 cells. HL-60 (●) or U937 (■) cells were incubated with different dose of YM155 for 8 h. (D) Dose–response curve of YM155-induced caspase-8 activation in HL-60 or U937 cells. HL-60 (●) or U937 (■) cells were incubated with different dose of YM155 for 8 h. Activities of casapase-3/7 (C) and caspase-8 (D) in cell extracts were determined by the cleavage of Ac-DEVD-AMC and Ac-IETD-MCA, respectively. Points, mean from three separate experiments.

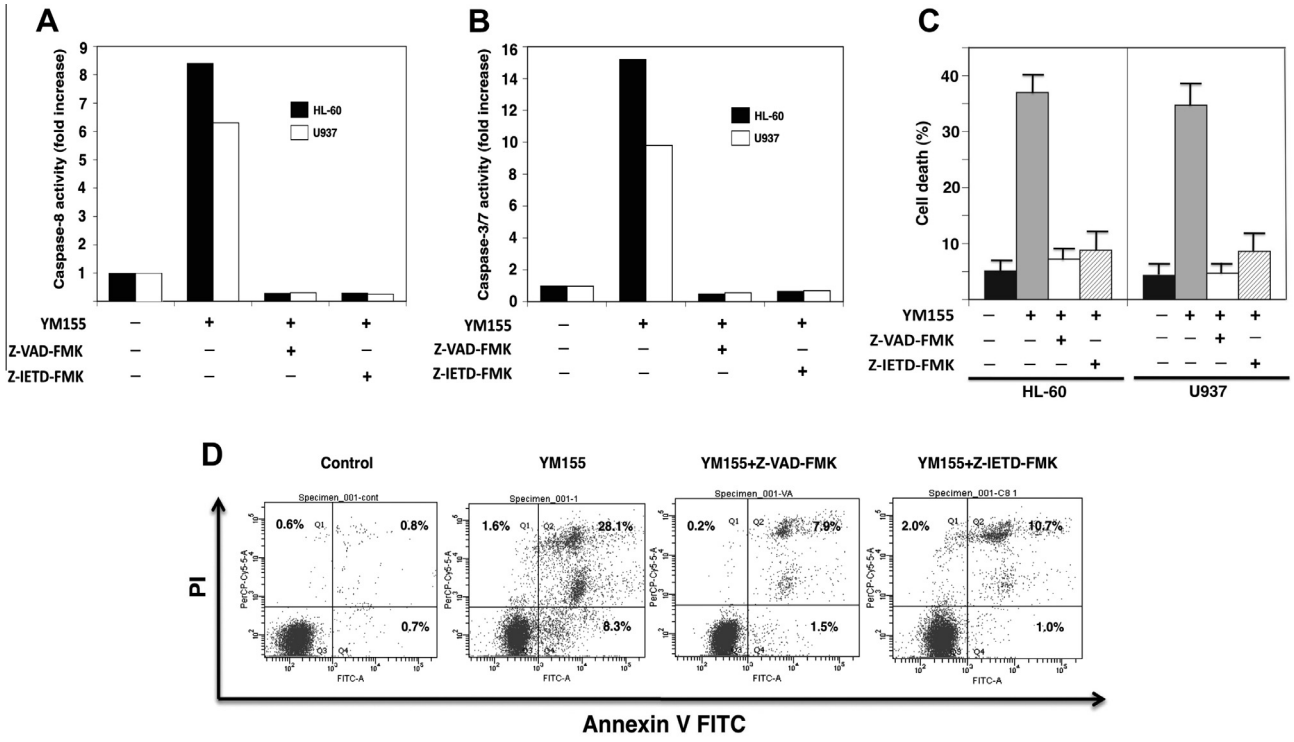

Fig. 4. Effect of caspase-8 inhibitor Z-IETD-FMK on YM155-induced caspase activation and cell death in human leukemia cells. HL-60 or U937 cells were treated with 1 μM YM155 for 8 h in the absence or presence of Z-IETD-FMK (100 μM) for 8 h. Z-IETD-FMK was added 60 min prior to YM155 administration. Activities of caspase-8 (A) and casapase-3/7 (B) in cell extracts were determined by the cleavage of Ac-DEVD-AMC and Ac-IETD-MCA, respectively. Histograms, mean from three separate experiments. (C) The percentage of total cell death (apoptosis + necrosis) induced by YM155 was shown as histogram. The percentage of apoptosis or necrosis was then determined by Annexin-V binding assay. Each experiment was repeated three times. Histograms, mean from three separate experiments. (D) HL-60 cells were incubated with 1 μM YM155 for 8 h in the presence or absence of Z-IETD-FMK. The percentage of apoptotic cells was then determined by Annexin-V binding assay. The vital cells were negative for both annexin-V and PI staining; apoptotic cells positive for annexin-V staining while negative for PI staining, and necrotic cells positive for both annexin-V and PI staining.

Hosokawa et al. [34] reported that treatment of HL-60 cells with the caspase-8 inhibitor Z-IETD-FMK inhibited apoptosis in H_2O_2-treated cells, but not in irradiated cells. The role of caspase-8 for apoptosis induction seems different among various apoptotic stimuli even in the same cell line. Thus, further investigation is required to clarify the importance of caspase-8 in the induction of apoptosis of human myelogenous leukemia cells.

In conclusion, we demonstrated that YM155 exhibits potent cytotoxic activity through downregulation of survivin and Mcl-1 protein. Our data suggest that caspase-8 may play an important role as an up-stream effecter for the activation of caspase-3 during the cell death process by YM155 in human myeloid leukemia cells.

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