Characterization of room temperature induced apoptosis in HL-60

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Abstract We found that exposure to room temperature (RT/21°C) causes apoptosis in HL-60 cells. Here we characterized RT-induced apoptosis in HL-60. After exposure to RT, apoptosis starts within 6 h and more than 80% of the cells underwent apoptosis within 20 h. All cells, however, were committed to apoptosis after 16 h and no viable cells could be recovered. The caspase-1 inhibitor (YVAD-CHO) effectively blocked apoptosis, whereas the caspase-3 inhibitor (DEVD-CHO) did not. About 20% of newly obtained early passage HL-60 cells (passage 10) also underwent apoptosis by RT treatment. These data suggest that some population in HL-60 which responds to RT with apoptosis became dominant during passaging.

Key words: HL-60; Apoptosis; Room temperature; Caspase-1; Caspase-1 inhibitor (YVAD-CHO)

1. Introduction

Apoptosis is an active process of cell death accompanied by cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation and internucleosomal DNA cleavage (DNA ladder) [1].

There seem to exist three phases in the process leading to apoptosis [2]. The first phase is the triggering of signal transduction which is caused by a variety of inducers including an antibody or a ligand to cell surface receptors (Fas/APO-1 or TNF-α) [3] and DNA damaging agents [4] as well as inhibitors of the microtubule function [5]. The activation of intracellular cascades composed of various caspases is the second phase. Since interleukin-1 converting enzyme (ICE) (now renamed caspase-1) [6] was first proposed as a mammalian homologue of *CED-3* of *Caenorhabditis elegans* [7], more than 10 cysteine proteases have been cloned and analyzed [3]. Among these caspases, caspase-3 (CPP32) has been well characterized and seems to be placed in the central pathway of the apoptotic process [8–11]. Because caspase-3 shows the highest homology to *CED-3* [12] and its tetrapeptide inhibitor (DEVD-CHO) often blocks apoptosis induced by a variety of inducers [13,14], it is now thought to be a human equivalent of *CED-3* [12]. The third phase of the apoptotic process is a cleavage of macromolecules including so-called death substrates such as poly(ADP-ribose) polymerase (PARP) [15], U1-small nuclear ribonucleoprotein [16], DNA-dependent protein kinases [17], fodrin [18] and lamins [19]. These death substrates are sequentially cleaved according to the process of apoptosis [20]. It is, however, still unclear if cleavages of these death substrates are crucial for apoptosis.

HL-60 is a myelogenous leukemia cell line [21] widely used for studying the mechanism of apoptosis. A variety of compounds was used for inducing apoptosis including RNA and protein synthesis inhibitors, topoisomerase inhibitors and protein kinase inhibitors [22–26]. Recently, Saeki et al. reported that conditioned medium of confluent HL-60 contained humoral factor(s) which induces apoptosis in HL-60 itself [27]. Faleiro et al. reported that caspases-3 and -6 (Mch2) are major cysteine proteases active in the apoptosis of leukemia cell lines including HL-60 [28]. A recent report by Polverino et al. showed that caspase-3 but not caspase-1 was cleaved to its active form in HL-60 when treated with a protein synthesis inhibitor [23]. Although caspase-3 seems to be more effective than caspase-1 for apoptosis in HL-60, the crucial caspase(s) in each apoptotic event remains to be clarified.

Here we report that simple exposure of HL-60 to room temperature (RT, 21°C) induces apoptosis. Interestingly, apoptosis in HL-60 responding to RT is efficiently blocked by the caspase-1 inhibitor (YVAD-CHO), whereas it is not blocked by the caspase-3 (DEVD-CHO), suggesting that RT treatment could possibly provide a system to study the biological significance of caspase-1 or caspase-1-like protease activity in apoptosis.

2. Materials and methods

2.1. Cell lines and RT treatment

The human myelogenous cell line, HL-60, was from our frozen stock. Since its passage number was unfortunately unclear, additionally a new HL-60 cell line (passage 10) was obtained from the Health Science Research Resources Bank (JCRB0085). Most of the experiments were performed using HL-60 from our frozen stock. Cells were maintained in RPMI 1640 (Life Tech Oriental) supplemented with 10% fetal calf serum (Bio Whittaker, MA) at 37°C in 5% CO_2. To expose cells to RT, 2 ml of $2–4\times10^5$/ml cells was transferred to culture tubes (Nunc, Nippon Intermed Co.) and incubated at 37°C, until the medium pH was equilibrated. Then, the cap of each tube was tightly closed and the cells were placed at RT. Cells were usually recovered for analysis after 12 h.

2.2. Caspase inhibitors

Caspase-1 and -3 inhibitors were purchased from Wako Co. and Peptide Institute Inc., respectively. The amino acid sequences of inhibitors of caspases-1 and -3 are YVAD-CHO and DEVD-CHO, respectively. Each peptide had formaldehyde on the carboxy-terminal amino acid. Peptides were dissolved in dimethylsulfoxide (DMSO) (Wako Co.) and 100 μM peptide was added to the cell suspension with a final concentration of 0.3% DMSO. As a control, 0.3% DMSO was added to a culture.

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2.3. FACS analysis

FACS analysis has been proposed as a versatile method to detect apoptotic cells as a hypodiploid DNA peak (sub-G1) [29]. In the present study we used FACS analysis for evaluation of apoptotic cells. After exposure to RT, cells were centrifuged at about $200\times g$, washed once in PBS, then resuspended in 70% ice-cold ethanol for fixing. After treatment with RNase A (100 μg/ml) (Sigma, St. Louis, MO) for 30 min at 37°C, DNA was stained with 50 μg/ml of propidium iodide (Sigma). Cell cycle analysis was performed by FACScan (Nippon Becton Dickinson Co., Japan) and the number of cells in the area corresponding to the sub-G1 region was calculated using the Lysis II program (Becton Dickinson Co.).

2.4. Extraction of DNA and analysis of DNA ladder

DNA was extracted according to the method described by Yin et al. [30]. Briefly, about 10^7 cells were washed in PBS, then resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100, pH 7.5). Cells were placed on ice for 10 min, then centrifuged at $16000\times g$ for 10 min. The supernatant was transferred to a new tube and extracted once with a phenol:chloroform:isoamyl alcohol (24:24:1) solution. To the aqueous phase NaCl was added to make the final concentration 300 mM. Nucleic acid was precipitated by 2 volumes of ethanol. After centrifugation, the pellet was washed with 70% ethanol, then resuspended in 50 μl of solution with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. RNA was digested with RNase A (100 μg/ml), then 10 μl of each sample was loaded on agarose gel composed of 1% agarose (Gibco BRL, New York) and 1% NuSieve GTG agarose (FMC, Maine). DNA was visualized by ethidium bromide staining.

2.5. Detection of nuclear fragmentation

Cells were fixed in a solution containing acetone and methanol (3:1), then stained with 4 μg/ml Hoechst 33342 (Sigma). Stained cells were examined with a fluoromicroscope (Olympus). Cells showing nuclear fragmentation were judged to be apoptotic.

2.6. MTT assay

Recovery of cell growth after RT treatment was assayed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma), as described [31]. Cells (2×10^5/ml) were returned to conventional culture conditions (37°C and 5% CO_2) after RT treatment. On day 4 after exposure to RT each 100 μl of cell suspension was incubated with 5 mg/ml MTT at 37°C until MTT formazan was detected as fuzzy crystals. Absorbance at 570 nm was measured using a microplate reader (Model 3550, Bio-Rad).

3. Results

3.1. RT-induced apoptosis in HL-60

FACS analysis detected an increased population corresponding to sub-G1 after RT treatment (Fig. 1A, lower left panel). About 60% of the cell population was detected in this region. In contrast the U937 cell line did not show any increase in the sub-G1 population (Fig. 1A, lower right panel). To determine whether this increment of sub-G1 detected in HL-60 is due to apoptosis or necrosis, DNA-ladder formation in the cells was analyzed. Fig. 1B shows that DNA extracted from RT-treated cells contained DNA ladders. Also Hoechst 33342 staining detected nuclear fragmentation in about 70% of the cells (Fig. 1C, right panel). Trypan blue staining indicated that almost 100% of the cells were viable after RT treatment. Based on these findings we concluded that the increase in the population of the sub-G1 region detected by FACS analysis is due to increased apoptotic cells and not to necrotic cells.

The factors influencing apoptosis were studied. Fig. 2A shows the time course of RT-induced apoptosis. Apoptosis started within 6 h and more than 80% of cells underwent apoptosis within 20 h. The effects of temperature (Fig. 2B) on apoptosis were also studied. About 50 and 30% of the cells were detected as apoptotic cells at 25 and 29°C, respectively. Induction of apoptosis at 33°C was greatly reduced (10%). Apoptosis was not detected at 4°C, suggesting that an appropriate temperature is necessary for the enzyme reaction to complete apoptosis. Next we assayed the ability of cells to recover after exposure to RT. The MTT assay was performed on day 4 after exposure to RT. A representative result is shown in Fig. 2C. The recovery of cells, when exposed for more than 12 h, was greatly reduced. Cells treated for 16 or 24 h did not recover at all, suggesting that 100% of the cells were committed to apoptosis during the first 16 h.

3.2. Effects of caspase-1 and -3 inhibitors on RT-induced apoptosis

To approach the molecular mechanism of RT-induced apoptosis we studied the effects of the inhibitors of caspases-1 and -3. The inhibitors are composed of tetrapeptides modified with formaldehyde on the carboxy-terminal residue. Both peptides, YVAD-CHO and DEVD-CHO, are well accepted as specific inhibitors to caspase-1 [3] and caspase-3 [12], respectively. A representative result of three independent experiments is shown in Fig. 3A. DMSO used as a solvent of peptide inhibitor slightly reduced the sub-G1 population from 63% to 43% (Fig. 3A, upper and lower left panels). The effect of YVAD-CHO peptide was striking, reducing the sub-G1 population to 11% (Fig. 3A, upper right panel). In an independent experiment the effect of different doses of the peptide was studied. When 25, 50 and 100 μM peptide were used, the population of sub-G1 was reduced to 34, 22 and 10% from 46% observed in RT-treated HL-60 (data not shown). This result indicates that RT-induced apoptosis is sensitive to YVAD-CHO peptide in a dose-dependent manner.

On the other hand, DEVD-CHO did not change the number of apoptotic cells (Fig. 3A, lower right panel). To exclude the possibility that the DEVD-CHO used in the present study had no biological activity, the same peptide was incubated in

Table 1
Effects of the caspase-1 and -3 inhibitors on apoptosis of HL-60 induced by etoposide and ionomycin

Caspase inhibitor	Treatment		
	Etoposide[a]	Ionomycin[b]	Room temperature
YVAD-CHO	+	−	+
DEVD-CHO	−	−	−

[a]HL-60 cells were preincubated for 30 min in the presence of etoposide (5 μg/ml) (Sigma), then returned to normal culture conditions. After 6 h, cells were fixed and subjected to FACS analysis. After etoposide treatment about 23% of cells were detected in the sub-G1 region. Addition of caspsase-1 inhibitor decreased the population in the sub-G1 region to about 6%.
[b]Cells were incubated for 8 h in the presence of ionomycin (0.5 μg/ml) (Sigma), then subjected to FACS analysis. About 30% of cells were detected in the sub-G1 region after treatment with ionomycin.

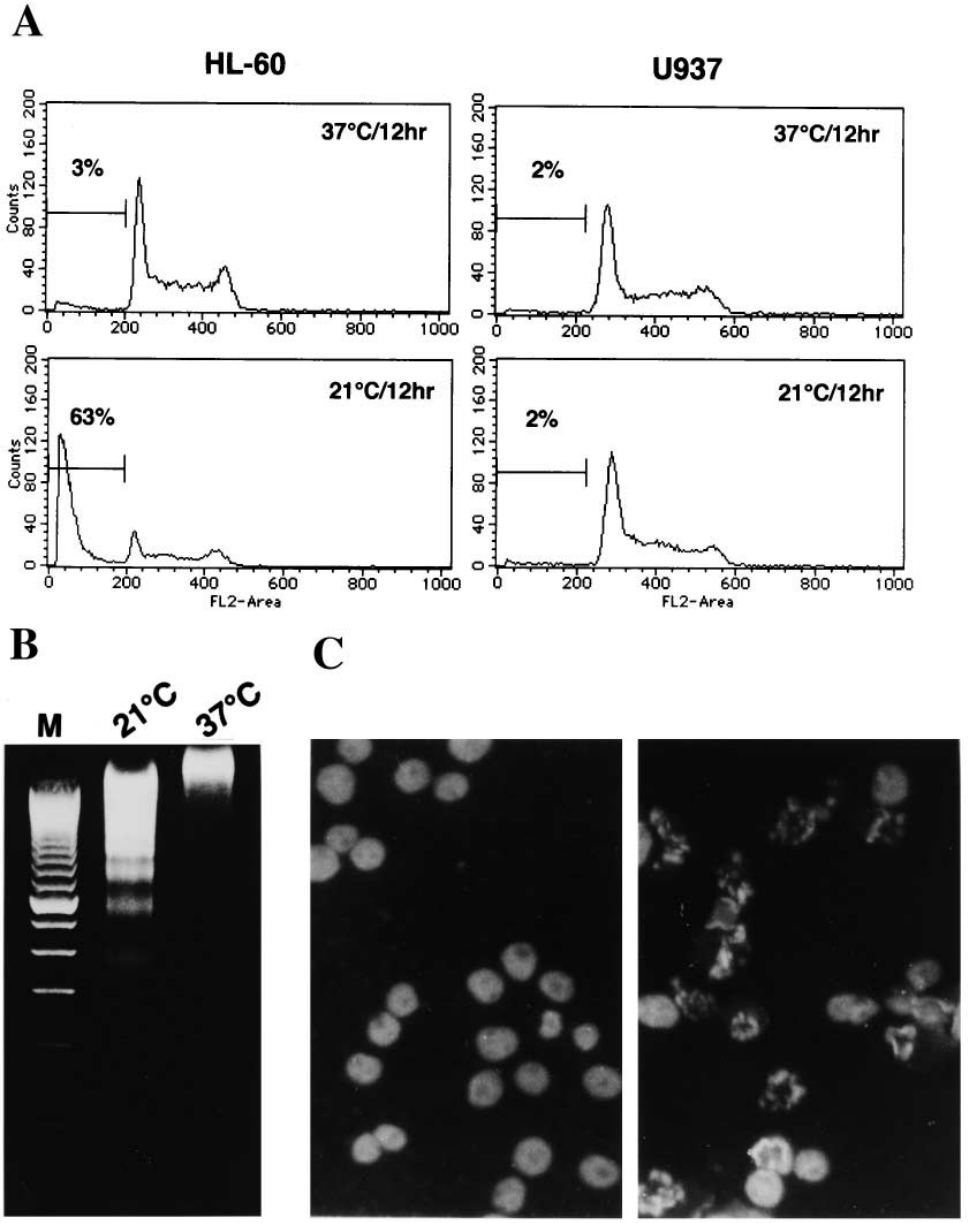

Fig. 1. RT-induced apoptosis of HL-60. FACS analysis of HL-60 and U937 after RT treatment for 12 h (A). The population in the sub-G1 region is indicated. DNA-ladder formation in HL-60 cells treated with RT (B). DNA extracted from cells placed at RT (left panel) and 37°C (right) was analyzed. 'M' denotes the molecular weight marker of 100 bp ladders (Gibco). Demonstration of nuclear fragmentation in HL-60 after RT treatment (C). Morphology of HL-60 cultured at 37°C (left panel) and RT (right panel) is shown.

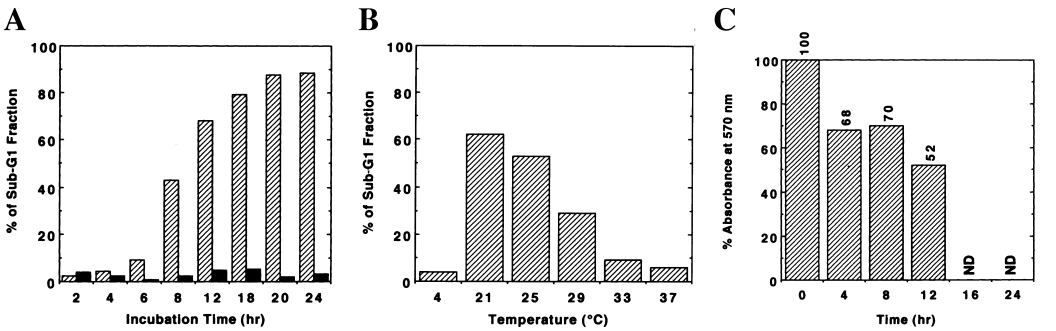

Fig. 2. Factors influencing RT-induced apoptosis. Time course of apoptosis (A). Hatched and solid boxes indicate apoptosis of cells cultured at RT and at 37°C, respectively. The population in the sub-G1 region detected by FACS analysis was plotted as a function of the incubation period. The effects of various temperatures on the induction of apoptosis (B). The method was similar to that described in A. The recovery of cell growth after treatment of RT (C). On day 4 after RT treatment, the cell number was calculated using the MTT assay. Absorbance at 570 nm of the control sample was considered 100% and % recovery was calculated. ND, not detected.

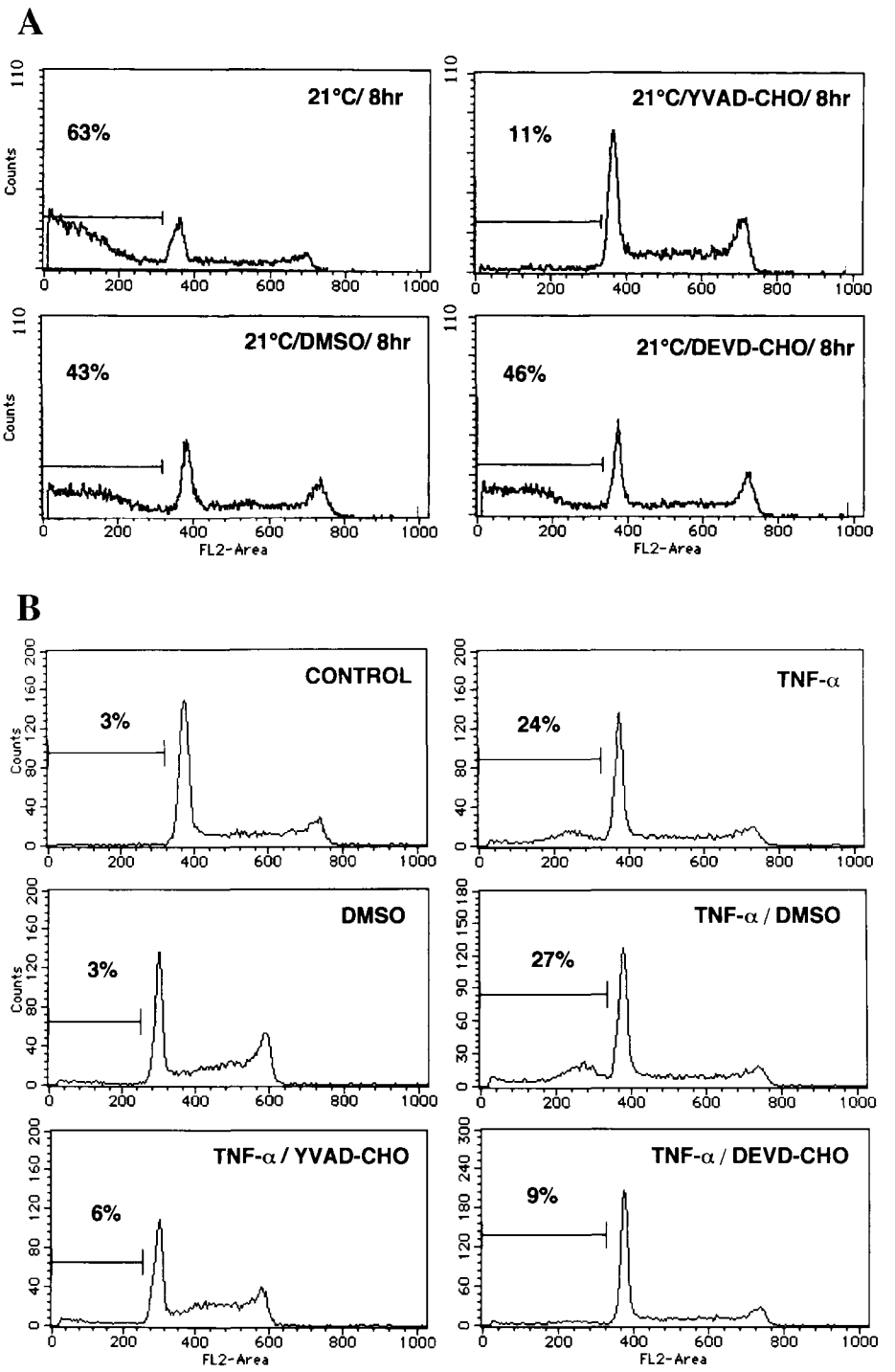

Fig. 3. The effects of caspase inhibitors on RT-induced apoptosis. The change in apoptosis by caspase-1 and -3 inhibitors (A). The effect of 100 μM of peptide inhibitor on RT-induced apotosis was studied. As a control, the effect of 0.3% DMSO used as a solvent of each peptide was also studied (lower left panel). In this experiment cells were exposed to RT for 8 h. The effect of the peptide inhibitor to caspase-3 on apoptosis of U937 induced by TNF-α (B).

a culture of U937 cells incubated with TNF-α (1 ng/ml). As shown in Fig. 3B, TNF-α increased the sub-G1 population from 3% to 24% (Fig. 3B, upper left and right panels). The caspase-1 and -3 inhibitors reduced the population in the sub-G1 region to 6 and 9%, respectively (Fig. 3B, lower left and right panels). This finding is consistent with a previous report that TNF-α-induced apoptosis involves both caspases-1 and -3 [3]. The result clearly indicates that DEVD-CHO peptide is biologically active and that RT-induced apoptosis is insensitive to the caspase-3 inhibitor.

To clarify the biological significance of caspases-1 and -3 in HL-60 apoptosis, we studied the effects of these inhibitors on apoptosis caused by other agents. The agents included etoposide, a DNA damaging agent [4], and ionomycin, a calcium ionophore [32]. The results are summarized in Table 1. Treatment with etoposide caused about 23% of HL-60 cells to

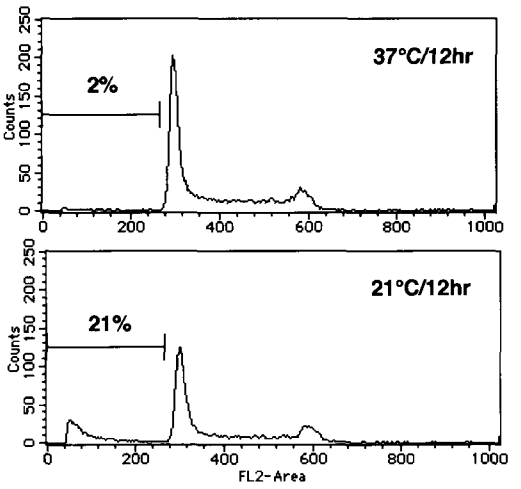

Fig. 4. RT-induced apoptosis in a new HL-60 cell line at passage number 10. Cells were treated at RT for 12 h (lower panel).

undergo apoptosis. Addition of YVAD-CHO decreased the population in the sub-G1 region to about 6%. On the other hand, addition of DEVD-CHO did not change the population in the sub-G1 region. In ionomycin-induced apoptosis both caspase-1 and -3 inhibitors had no effects. These results suggest that the caspase-1 or caspase-1-like protease was involved in the apoptotic process induced by etoposide as well as RT treatment. The signals induced by RT treatment and etoposide possibly share a common cascade in the apoptotic process. Although caspase-3 does not seem to work as a central player in the apoptosis induced by RT treatment, etoposide and ionomycin, it has been reported that anisomycin, a protein synthesis inhibitor, induced apoptosis in HL-60 with the activation of caspase-3 [22]. It is likely that a variety of sets of caspases are involved according to the agents triggering apoptosis.

We tried to study directly whether caspases-1 and -3 were involved in RT-induced apoptosis. It has been reported that the 45 kDa precursor protein of caspase-1 is processed to p20 and p10 by activation [33]. Also the 32 kDa protein of caspase-3 is cleaved to its active forms composed of p12 and p17 subunits [12]. Western blot analysis was performed on the cell extract obtained after 12 h treatment at RT and the blots were probed with antibodies to caspases-1 and -3. Activated forms of these caspases, however, were not detected (data not shown).

3.3. RT-induced apoptosis in a newly obtained HL-60 cell line

To exclude the possibility that RT-induced apoptosis is observed only in our HL-60 stock, we obtained the same cell line from a cell bank and studied the apoptosis by RT treatment. As shown in Fig. 4, the newly obtained HL-60 also showed the increase of population in the sub-G1 region (lower panel). About 20% of the cells were located in the sub-G1 region after a 12 h exposure to RT. Microscopic examination after RT treatment confirmed the generation of membrane blebbings and apoptotic body formation (data not shown). The frequency of cell death was rather low compared to the HL-60 of our stock, suggesting that during passaging populations responding to RT with apoptosis have become dominant.

4. Discussion

In the present report we showed a simple and efficient RT-induced apoptosis of HL-60 which is sensitive to the caspase-1 inhibitor but not to the caspase-3 inhibitor. Western blot analysis could not detect the processing of caspase-1, suggesting that caspase-1-like protease is involved in RT-induced apoptosis. Using this system it may be possible to characterize a novel caspase. Also the physiological role of caspase-1-like protease could be analyzed.

Now we cannot point out a causative factor of RT-induced apoptosis. As one of the candidates it is possible to note that the difference of oxygen consumption at RT generates reactive oxygen. There are reports that reactive oxygen acts as an inducer of apoptosis [34–36]. Peroxynite, a physiological product produced by the reaction of superoxide and nitrite oxide, produces superoxide anion inducing apoptosis in HL-60 [35]. Generation of reactive oxygen was proposed as a crucial step also in dexamethasone-induced apoptosis [36]. In the present work, DMSO, a hydroxy radical scavenger [37,38], slightly suppressed RT-induced apoptosis (Fig. 3A), suggesting the involvement of reactive oxygen in RT-induced apoptosis. We are focusing on the role of reactive oxygen production in RT-induced apoptosis which seems to be a quick biochemical response to RT treatment.

Here, for the first time, we characterized RT-induced apoptosis in a single cell line of HL-60. It is likely, however, that other cell lines also respond to RT. So far we have studied RT-induced apoptosis in more than 10 human cell lines and found that a T-cell lineage underwent apoptosis by RT treatment. Interestingly, the mode of sensitivity to caspase inhibitors in this cell line is different from that of HL-60 (M. Shimura, unpublished data). Using these two cell lines it is possible to clarify the redundant pathways of the apoptotic process induced by the same trigger. Such information would be useful for understanding the complexity of the apoptotic process. More importantly, there might be leukemia cases in which leukemic cells undergo apoptosis when exposed to RT. The simple procedure of RT treatment might be a new strategy to eliminate leukemic cells in such cases.

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