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Effects of Poly (ADP-ribose) polymerase Inhibitors and NAD on the Apoptosis induced by Actinomycin D in Human Leukemia Cells HL-60

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Abstract

We investigated the effects of poly (ADP-ribose) polymerase (PARP) inhibitors and NAD on the apoptosis induced by Actinomycin D in human promyelomonocytic leukemia cells HL-60. As the results, the apoptosis was repressed by various PARP inhibitors especially by 3-aminobenzamide (3-ABA), which is a potent inhibitor for PARP reaction. On the contrary, 3-ABA was no more inhibitory in a cell-free apoptosis system. This result suggests that the inhibitory effect of 3-ABA is effective only before apoptotic cytosol is made up in the cells, *i.e.*, in an early stage of the apoptotic process. But NAD inhibited the apoptosis in the same system depending on NAD concentration. So the inhibition of apoptosis by NAD may be concerned in a later step of apoptosis.

Key Words: poly (ADP-ribose) polymerase • NAD • apoptosis • Actinomycin D • HL-60

Introduction

Apoptosis has diverse and very important functions in organisms, *e.g.* regulation of developmemt, immunity, hormone, *etc.* and repression of carcinogenesis. Indeed the concept of apoptosis was introduced by Kerr *et al.* already in 1972[1], but the molecular and enzymic cascades during the execution phase of apoptosis have been elucidated only in the last couple of years. It was proved that one of the important substances in apoptotic process is ICE-like proteases and various enzymes belonging to them, *e.g.* CPP32[2], Yama[3], and apopain[4], have been found so far. Recently these enzymes are suggested to call "caspase". Several substrates for such enzymes were found and one of them, which was proved by Lazebnik *et al.* in 1994[5], was poly (ADP-ribose) polymerase (PARP). It was found that PARP was activated by DNA damage and nuclear proteins were poly (ADP-ribosyl) -ated by this enzyme using NAD as a substrate, and was concerned in DNA repair[6]. However, it is not known yet what kind of signal activates ICE-like proteases after an apoptosis-inducing stimulus nor the way in which DNA is fragmented after activation of these enzymes.

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PARP, which was first found by Chambon *et al.* in 1966[7], has been highly purified from animal organs and cultured cells until now, and its properties are known, *e.g.* molecular weight is 116 kDa and rich in basic amino acids[8]. PARP is activated after DNA damage by alkylating agents, ultraviolet light, X-ray, *etc.* The activated PARP transfers ADP-ribose moiety of a substrate NAD to various target proteins and dozens of ADP-ribose moieties are polymerized, and finally high molecular weight poly (ADP-ribose) is formed on target proteins and their functions are regulated[9]. There are three domains in a PARP molecule[8]. DNA-binding domain of 46 kDa in N-terminal has two zinc fingers as frequently found in many DNA-binding proteins. In the center of the molecule there is an auto modification domain of 22 kDa and PARP itself is poly (ADP-ribosyl) ated on it. The active site of the enzyme, *i.e.* NAD-binding domain of 54 kDa, is found in the C-terminal. The function of PARP is suggested that it helps DNA-repair enzymes to approach damaged region in DNA and promotes DNA repair[10]. Recently knockout mouse of PARP gene depletion was made and the function of PARP was investigated[11]. No apparent abnormality was found in this mouse at birth and, needless to say, PARP activity was not detected and poly (ADP-ribosyl) ation was not observed. Contrary to their expectations, however, the ability of DNA repair was normal at early stage, and then abnormality on the skin of about 30% mouse was observed after 5 to 6 months after birth. It was reported that this meant impossibility of skin protection against stimuli from outside and that obese was finally observed.

It is suggested that one of the functions of PARP is to check the degree of DNA damage and when the damage is serious it converts the signal to start apoptosis. The following two phenomena occur after activation of PARP by DNA damage: regulation of nuclear proteins by combining poly (ADP-ribose) chain and decrease of NAD content in cells. These two phenomena may possibly become the signal for starting apoptosis.

However, the detailed functions of PARP are not well known at present. Moreover the endonuclease which hydrolyzes DNA at apoptosis is negatively controlled by PARP and it is suggested that when PARP is hydrolyzed by CPP32/Yama/apopain, endonuclease is activated, and then apoptosis is promoted[12], or the decrease of NAD level in cells after poly (ADP-ribosyl) ation leads to cell death[13]. Thus the relationship between apoptosis and PARP is thought to be complicated.

In this study we investigate the effects of various PARP inhibitors, *e.g.* 3-aminobenzamide (3-ABA), nicotinamide (NAm), and related compounds, on the apoptosis induced by anticancer antibiotic Actinomycin D (AcD) in human promyelomonocytic leukemia cell line HL-60 to know the effect of poly (ADP-ribosyl) ation on apoptosis *in vivo*. As already mentioned above, NAD level in the cells decreases after poly (ADP-ribosyl) ation. To investigate directly the effect of NAD on the apoptosis, we employed the cell-free apoptosis system. The cell-free apoptosis system was originated from the report by Lazebnik *et al.* in 1993[14]. They found condensation of nuclei and fragmentation of DNA can be seen after adding cell extract from synchronously cultured chicken hepatoma cells to the nuclei from HeLa cells. It was reported that the cell extract (cytosol) from apoptotic cells induced by Fas antibody or ultraviolet light causes DNA fragmentation and specific cleavage of PARP in intact nuclei extracted from normal cells[15,16]. By using cell-free apoptosis system it becomes possible to know that which component in apoptotic cell extract induces DNA fragmentation and to observe easily the effect of high molecular weight compounds, which is not permeable into cell membrane, on apoptosis. To apply this advantage to our research, we want to know the effect of NAD on apoptosis and to find clue for elucidating the role

of PARP in apoptosis with the data from *in vivo* experiments concerning the inhibition of PARP by various compounds.

Materials and Methods

Reagents

Various reagents used in this research were purchased from the companies described as follows: Mitomycin C, RNase A, Proteinase K, AEBSF (Pefabloc SC), and ALLN from Boehringer Mannheim GmbH. 3-Aminobenzamide, MTT, ethidium bromide, Cytochalasin B, Aprotinin, and Pepstatin A from Sigma Chemical Co. Nicotinamide, 2-aminobenzamide, 4-aminobenzamide, benzamide, and leupeptin from Nacalai Tesque Inc. 1,5-Isoquinolinediol from Research Biochemicals International. RPMI 1640, fetal bovine serum, and 1 kb DNA Marker from GIBCO BRL Co. 0.4% Trypan Blue from Flow Laboratory Co. Agarose from Takara Shuzo Co. Ltd. (LO3 "TAKARA"). λ/HindⅢ from Stratagene. Actinomycin D, spermidine and spermine from Wako Pure Chemical Industries Co. Other general reagents of the highest purity obtainable were Nacalai Tesque Inc. and/or Wako Pure Chemical Industries Co.

Cell Culture

HL-60 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37℃ in CO_2 incubator under 5% CO_2. Trypan Blue exclusion method was used for counting viable cells.

Effects of PARP Inhibitors on the Apoptosis induced by Actinomycin D

Induction of apoptosis by AcD was carried out according to the protocol reported by Tanuma *et al*[17]. Each PARP inhibitor was added to cells, whose concentration was previously adjusted to $3 \sim 6 \times 10^5$ cells /mℓ, and incubation was carried out for 30 min. Then AcD (final concentration was 1 μg/mℓ) or Mitomycin C (MMC, final concentration was 10 μg/mℓ)was added as an apoptosis inducer. After incubation, DNA fragmentation was analyzed by agarose gel electrophoresis. Microphotographs of the cells were taken and the number of normal cells and bubbling cells were counted, and the ratio of each cells was calculated.

Analysis of DNA Fragmentation

DNA fragmentation was analyzed by agarose gel electrophoresis after enzymatic degradation of proteins and RNA in cells[17,18] as described below. After induction of apoptosis, the cells ($3 \sim 6 \times 10^5$) were collected by centrifugation at 200 x *g* for 10 min. Then 20 μℓ of lysis buffer (50 mM Tris-HCl, pH 7.8 containing 10 mM EDTA-Na and 0.5 % (w/v) sodium-*N*-lauroylsarcosinate) was added and mixed well. To this mixture 1 μℓ of RNase solution (10 mg/mℓ) was added and incubated at 50 ℃ for 30 min and 1 μℓ of Proteinase K solution (10 mg/mℓ) was also added and incubated at 50℃ for 60 min.

Detection of DNA Fragmentation by Agarose Gel Electrophoresis

Agarose gel (2%) was prepared by ordinary method. Electrophoresis was carried out with DNA molecular weight marker (λ/HindⅢ or 1 kb DNA marker) at 50 V. DNA and its fragments were stained with ethidiumbromide and the picture of stained gel was taken by Image Master VDS (Pharmacia Biotech) under UV irradiation.

Counting of Viable Cells by MTT Method

The number of viable cells was counting by using MTT-reducing method reported by Tanuma *et al*[17].

Cell-free apoptosis

The methods reported by Martin *et al.*[15] Enari *et al.*[16] and Liu *et al.*[19] were modified and used for cell-free apoptosis in this study as described below. Mice (ddY strain, 4 to 5 weeks old) were purchased from Japan SLC and livers were removed and rinsed with ice-cold PBS. Livers were cut into small pieces with scissors on ice and homogenized with 4 volumes (v/w) of "nuclei isolation buffer" in a Teflon-glass homogenizer with three strokes. Component of the nuclei isolation buffer was as follows: 10 mM Hepes-KOH, pH 7.6; 15 mM KCl; 2mM sodium EDTA, pH 8.0; 0.2 mM spermine; 0.5 mM spermidine; 1 mM DTT; 0.5mM AEBSF (Pefabloc SC); and 2.3 M sucrose. Nuclei isolation buffer was put into a centrifuge tube until half of the capacity and equal volume of the liver homogenate was layered on it, and centrifuged at 37,000 x *g* for 1 hr at 4℃. Nuclei pellets were rinsed with "nuclei storage buffer" and nuclei concentration was adjusted to 2×10^8 nuclei/mℓ with nuclei storage buffer containing 50% glycerol. This final preparation was served as nuclei stock for cell-free apoptosis. Component of nuclei storage buffer was as follows: 10mM Pipes, pH 7.4; 80mM KCl; 20mM NaCl; 5 mM sodium EGTA, pH 8.0; 0.2mM spermine; 0.5 mM spermidine; 1 mM DTT; 1 mM AEBSF (Pefabloc SC); 250mM sucrose; and 50% glycerol. The nuclei stock was kept at -80℃ until use.

Preparation of Cell Extract from Apoptotic Cell for Cell-free Apoptosis

Suspension of apoptosis-induced or not-induced cells (about 5×10^7 cells in 50 mℓ) were centrifuged at 200 x *g* for 10 min at 4℃ to collect cell pellets. These pellets were rinsed twice with ice-cold PBS and then rinsed once with "cell extraction buffer". Component of the cell extraction buffer was as follows: 20 mM Hepes-KOH, pH 7.5; 10mM KCl; 1.5mM $MgCl_2$; 1.5 mM sodium EGTA, pH 8.0; 1.5 mM sodium EDTA, pH 8.0; lmM DTT; 0.1 mM AEBSF (Pefabloc SC); 5 μM cytochalasin B; 5 μg/mℓ pepstatin A; 10 μg/mℓ leupeptin; 2 μg/mℓ aprotinin; and 2.5 μg/mℓ ALLN (Calpain inhibitor I). The pellets were suspended in five volumes of cell extraction buffer. This suspension was freezed at -80℃ and then thawed at 4℃. This manipulation was successively repeated three times to break cell membrane and the extract was centrifuged at 9,600 x *g* for 10 min at 4℃. The supernatant obtained was centrifuged again at 9,600 x *g* for 40 min at 4℃. The final supernatant was used as soon as possible for cell-free apoptosis. Protein concentration of this preparation was 5～6 mg/mℓ. The mixture containing 20 μℓ of the final supernatant described just above, 4 μℓ of various reagent solution dissolved in 1 mM $MgCl_2$, and 1 μℓ of the nuclei stock solution (2×10^5 nuclei) was incubated at 37℃ for fixed times. After the incubation 3.5 μℓ of the reaction mixture was withdrawn and mixed with 10 mM Hoechst 33258, and kept overnight at 4℃. Morphological change of cells were checked with fluorescence microscope. The remaining sample was centrifuged at 4,400 x *g* for 5 min and lysis buffer was added to the pellets, and mixed well. Then 1 μℓ of ProteinaseK solution (10 mg/mℓ) was added and kept at 50℃ for 90 min. Finally 1 μℓ of RNase A solution (10 mg/mℓ) was added and kept at 50℃ for 60 min. This final preparation was served to agarose gel electrophoresis for determination of the DNA fragmentation.

Results

1. Effects of PARP Inhibitors on the *in Vivo* Apoptosis induced by Actinomycin D in HL-60 Cells

Bubbling in HL-60 cells was gradually observed at 2 hr after addition of AcD at a final concentration of 1 μg/mℓ and at 4 hr bubbling was observed in 80% cells as shown in Fig. 1. The cells were changed

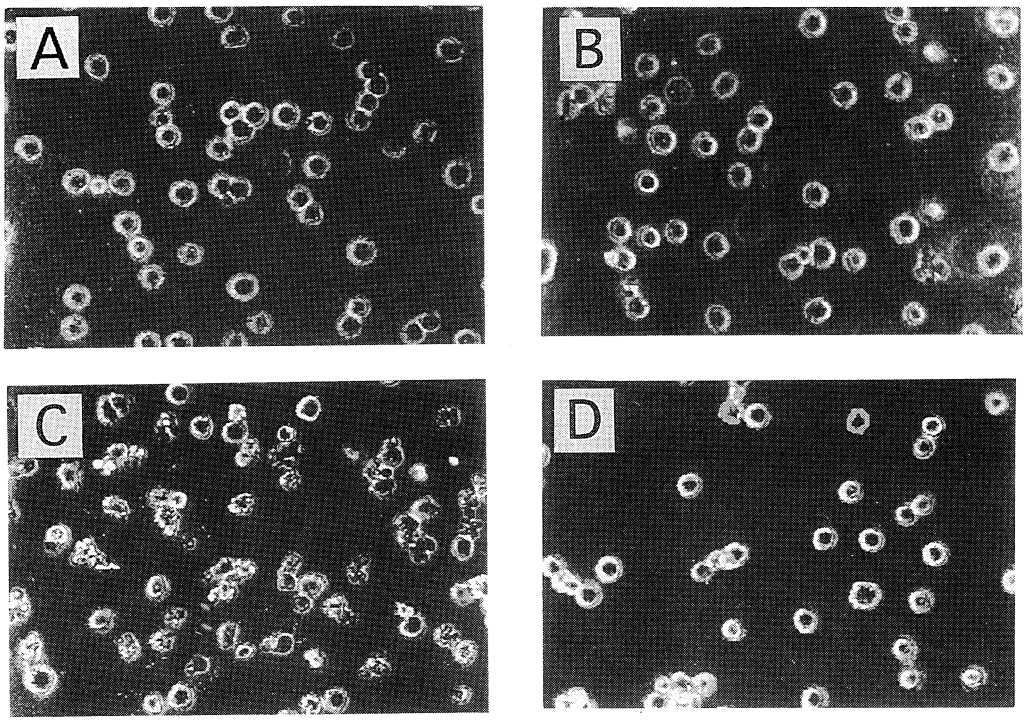

Fig. 1. Morphological Changes of HL-60 Cells after Treatment with Actinomycin D.
After incubation for the indicated times, microphotographs of the cells were taken by a phase contrast microscope.
A, 1 hr; B, 2 hr; C, 4 hr after addition of Actinomycin D; D, none addition (4 hr)

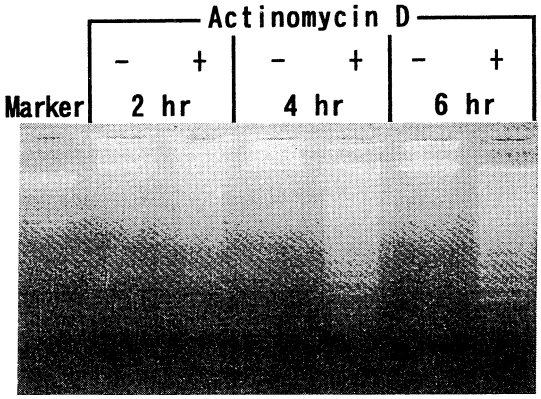

Fig. 2. Internucleosomal DNA Fragmentation of HL-60 Cells treated with Actinomycin D during the Execution Phase of Apoptosis.
DNA fragmentation was analyzed by agarose gel (2%) electrophoresis.
DNA molecular weight marker: λ/Hind Ⅲ or 1 kd DNA marker
Details are written in the text.

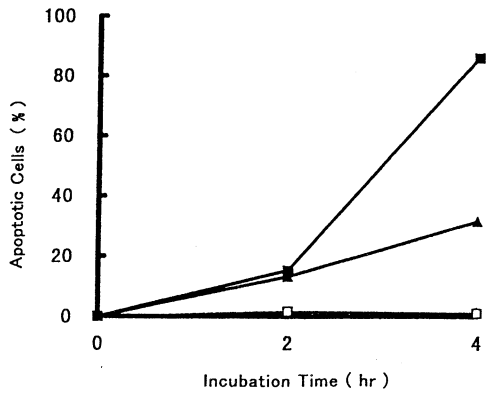

Fig. 3. The Effect of 3-Aminobenzamide on Morphological Change of HL-60 Cells during the Execution Phase of Apoptosis.
○, none addition; □, 3-aminobenzamide; ■, Actinomycin D; ▲, Actinomycin D + 3-aminobenzamide

into small bubbles called apoptotic body and scattered at more than 4 hr after addition of AcD (data not shown). Fig. 2 shows the result of analysis of DNA fragmentation by agarose gel electrophoresis. DNA fragmentation was not observed in the cells not treated with AcD even when they weve incubated for 6 hr. On the contrary DNA fragmentation was observed in AcD-treated cells after 2 hr, and a typical DNA ladder structure of apoptosis was observed after 4 hr. It was also observed after 6 hr and the pattern was almost the same as that of after 4 hr. Naora *et al.* reported that the ladder was appeared on the agarose gel within 6 hr after addition of AcD to U937 cells and Jurkat cells derived from blood cells like HL-60[20]. These data show that AcD has rapid apoptosis-inducing ability to blood cells.

The effect of PARP inhibitors on AcD induced apoptosis in HL-60 was investigated. 3-Aminobenzamide (3-ABA), nicotinamide (NAm), and related compounds were used as PARP inhibitors. The bubbling appeared at 4 hr after addition of AcD was repressed by 3-ABA at 5 mM (data not shown). Fig.3 shows the effect of 3-ABA on the bubbling. The bubbling found in nearly 90% cells at 4 hr after addition of AcD were repressed to about 30% cells. NAm and benzamide (BA) also repressed the bubbling but not so strong as 3-ABA. Effect of 2-ABA and 4-ABA, which are structural analogues of 3-ABA and weak inhibitors of PARP, was also investigated. The bubbling was weakly repressed by these compounds. There were no difference in the rate of apoptotic cells between control and 3-ABA treatment, therefore both symbols are overlapped in Fig. 3. Comparison of inhibitory power of various PARP inhibitors was already reported by Ueda and Banasik[21]. The degree of repression of apoptosis and the inhibitory power against PARP is found to be parallel, therefore 3-ABA inhibited the DNA fragmentation a little but NAm, 2-ABA, and 4-ABA had almost no effect on it (data not shown). To confirm more precisely the relationship between the inhibition of PARP and the repression of apoptosis, we used one of the most powerful inhibitors for PARP, *i.e* 1,5-isoquinolinediol (IQD), which IQD is about 100 times powerful than 3-ABA when IC_{50} is compared[21]. The bubbling induced in HL-60 cells by AcD was repressed by IQD at 0.05 mM or 0.1mM (data not shown). The bubbling ratio was decreased from about 90% to about 35% in the cells induced by AcD at 4 hr after addition. The rate of repression by

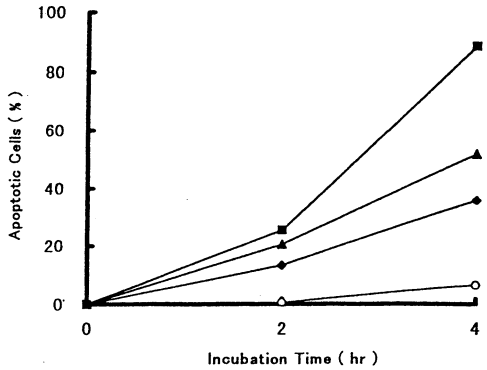

Fig. 4. The Effect of Isoquinolinediol on Morphological Change of HL-60 Cells during the Execution Phase of Apoptosis.
○, none addition: ■, Actinomycin D;
▲, Actinomycin D + isoquinolinediol 0.05 mM;
◆, Actinomycin D + isoquinolinediol 0.1 mM

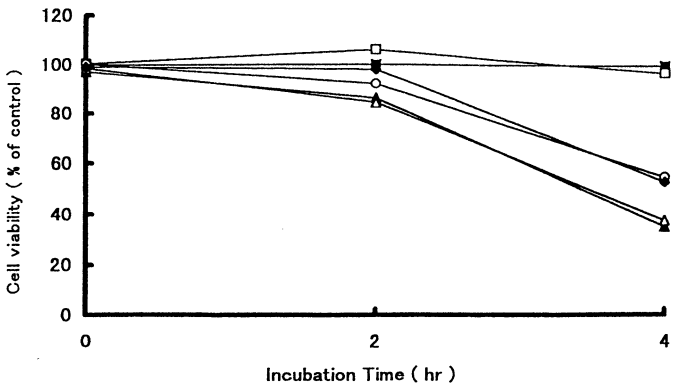

Fig. 5. The Effect of Poly (ADP-ribose) polymerase Inhibitors on Cell Viability of HL-60 Cells detected by MTT Method.
■, none addition; □, 3-aminobenzamide; ▲, Actinomycin D;
○, Actinomycin D + 3-aminobenzamide;
△, Actinomycin D + 4-aminobenzamide;
◆, Actinomycin D + nicotinamide

IQD was dependent on the concentration of this compound as shown in Fig. 4. The apoptosis was repressed by not only 3-ABA and its related compounds but also IQD, whose structure is quite different from 3-ABA. From these data it is suggested that the apoptosis induced by AcD is repressed by the inhibition of PARP.

The effect of PARP inhibitors on apoptosis was also investigated by MTT assay method. Enzyme activity in mitochondria can be determined directly by this method. Therefore this is a useful method for counting viable cells. The mitochondrial activity was decreased by AcD treatment, but it was recovered by the addition of 3-ABA *etc.* as shown in Fig. 5.

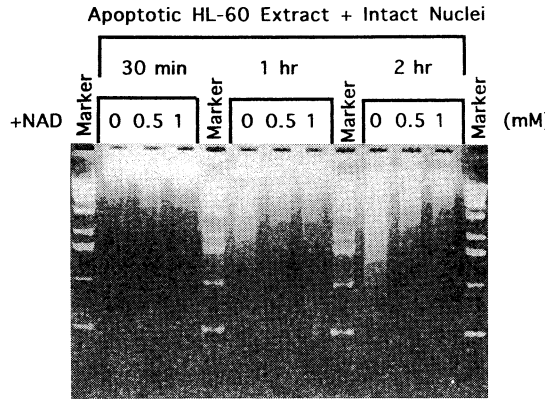

Fig. 6. The Effect of NAD on the Cell-Free Apoptosis induced in Normal Mouse Nuclei by the extract from Apoptotic HL-60 Cells.
The mixture containing the cell-free extract, NAD solution and the nuclei suspension (2×10^5 nuclei) was incubated for indicated times. The DNA fragmentation was analyzed by agarose gel electrophoresis. Datails were written in the text.

2. Effects of Various Compounds on the Cell-free Apoptosis

DNA fragmentation was observed after incubation of nuclei from normal mouse for 30 min with the cell-free extract from apoptotic cells of HL-60. DNA ladder could be seen after 1 hr incubation (data not shown). When the nuclei was incubated with the extraction buffer instead of the cell-free extract from apoptotic cells, not only the ladder but also smear could not be observed during incubation for 2 hr. When the nuclei was incubated with cell-free extract from intact HL-60 cells, which was not treated with AcD, DNA fragmentation was not observed but smear was observed after 1 hr or 2 hr incubation (data not shown). The incubated cells were stained by Hoechst 33258 and observed with fluorescence microscope. The nuclei incubated with the extraction buffer or cell-free extract from HL-60 without AcD treatment maintained the structure and nothing special morphological change was detected. However, "chromatin condensation" was observed in the nuclei incubated with the cell-free extract from apoptotic cells, *i.e.* chromatin lost normal structure and was condensed near nuclear membrane (data not shown). From these data it is suggested that the important substance for executing apoptosis is present in cytosol but not in nuclei. And it is also suggested that the cells received apoptosis-inducing stimuli lead to apoptosis by the change of content in cytosol, because the cell-free extract from normal cells have no apoptosis-inducing ability.

3-ABA at 2 or 5 mM had no effect on the DNA fragmentation in such cell-free apoptosis system. Morphological change in the nuclei was not detectable with fluorescence microscope. Once the switch for apoptosis is turned on, PARP inhibitors are no more inhibitory for DNA fragmentation. PARP is an enzyme which catalyzes poly (ADP-ribosyl) ation to nuclear proteins using NAD as a substrate. So when this enzyme is activated, NAD level in cells decreases rapidly. The effect of NAD on the cell-free apoptosis was investigated at 0.5 and 1 mM in the incubation mixture. As a result DNA fragmentation was repressed by NAD in a manner of concentration dependently as shown in Fig. 6. From these data it is suggested that NAD has inhibitory effect on cell-free apoptosis, which is *in vitro* experiment system.

Discussion

We investigated the effect of PARP inhibitors on apoptosis in human leukemia cells of HL-60. Among such inhibitors 3-ABA showed repressive effect on the apoptosis. Especially bubbling of the cells were very clear. It is well known that AcD inhibits DNA dependent RNA polymerase. Recently the alteration of PARP arrangement in nuclei when inhibitors for RNA polymerase including AcD were added to cells was reported by Desnoyers *et al*[22]. From these data direct effect of AcD on PARP may be suggested and the relationship between inhibition of PARP and repression of apoptosis induced by AcD should be considered. However, similar repression of apoptosis was observed with MMC (data not shown), whose action mechanism is different from that of AcD. Moreover, various apoptosis induced by UV, cisplatin, and nitrogen monoxide is also repressed by PARP inhibitors including 3-ABA[12,23]. From these data it is suggested that the repression of apoptosis by PARP inhibitors is common phenomenon among apoptosis induced by various stimuli.

3-ABA, which was used as a PARP inhibitor in this experiment, has another effect reported by Tiozzo *et al*[24]. They showed that 3-ABA had inhibitory effect on cell proliferation at higher than 10 mM. This effect may be due to the inhibition of rearrangement of cell skeleton. In our experiments we used IQD in addition to 3-ABA and related compounds. IQD has a quite different structure from that of 3-ABA and its inhibitory effect on PARP is very strong. By this compound the bubbling, which is a representative morphological change in apoptosis, was repressed. From this result, it can be concluded that the repression of apoptosis is caused by the inhibitor of PARP.

The apoptosis induced by AcD was observed at 4 hr after addition of it and bubbling of cells and DNA fragmentation was proceeded simultaneously. However, the effect of PARP inhibitors on bubbling and on DNA fragmentation were not coincided, and the effect on bubbling was much stronger. A paper reporting the repression of DNA fragmentation by PARP inhibitor is found[12]. Bubbling of cells and DNA fragmentation during the process of apoptosis may not always proceed simultaneously via same pathway. Further investigation is required to confirm this.

The cell death caused by apoptosis can be judged by MTT assay method. The change of cell function during cell death is determined by assaying mitochondrial activity by this method. Tetrazolium salt, MTT is changed into the blue colored formazan by viable cells. It should be noted that the results become positive also in the case of necrosis.

To know the relationship between PARP inhibitors and apoptosis, cell-free apoptosis system of *in vitro* experiment was used. This system was originated from the report by Lazebnik *et al.* in 1993[14]. They reported that nuclei condensation and DNA fragmentation could be reproduced when cell-free extract prepared from chicken hepatoma cells was added to nuclei of HeLa cells. DNA fragmentation and specific cleavage of PARP was observed in nuclei prepared from other cells when the cell-free extract (cytosol) from apoptotic cells induced by Fas antibody or UV. We used the cell-free extract from fully apoptotic cells at 4 hr after AcD treatment. However, 3-ABA had no effect on the cell-free apoptosis. From this result it is suggested that 3-ABA is not concerned during the process of DNA fragmentation in nuclei caused by the cell-free extract from apoptotic cells. Shimizu and Pommier carried out cell-free apoptosis by using cell-free extract from apoptotic cells induced by topoisomerase I inhibitor, camptothecin[25]. They reported that 3-ABA had almost no effect on this cell-free apoptosis. This result

is similar to our data.

We considered that similar result can be obtained in the case of PARP inhibitors. Namely, the repressive effect of PARP inhibitors to apoptosis may participate in the stage before apoptotic cytosol is formed. One of the effects of poly (ADP-ribosyl) ation to cells is decrease of NAD level in cells. Hence, the effect of NAD on cell-free apoptosis system was investigated. As the result, DNA fragmentation was repressed by NAD. From this result, it is suggested that some relationship may exist between NAD level in cells and progress of apoptosis.

In conclusion, apoptosis was repressed by PARP inhibitors including 3-ABA. It is suggested that such repression was attributable to maintain NAD level in cells because of inhibition of PARP. Zhang *et al.* reported that PARP was activated by nitrogen monoxide, then NAD level in cells decreased and cell death was induced[13]. However, it is suggested that this cell death is due to necrosis at present[26].

Coppola *et al.* carried out an experiment of apoptosis using the same cells with different NAD levels. They reported that apoptosis was difficult to find in the cells with high NAD level when the apoptosis was tried to induce by active oxygen and also in the cells previously treated with NAm to raise NAD level in the cells[26]. From these data it is suggested that there is some relationship between apoptosis and NAD content in the cells. Moreover, negative effect on apoptosis is thought when NAD content in the cells is high.

Yoon *et al.* suggested that PARP was activated by apoptotic stimuli, then histone in nuclei was poly-(ADP-ribosyl) ated, and finally the endonuclease, which catalyzed the DNA fragmentation reaction, was activated and apoptosis was induced[12]. Therefore, the effect of PARP inhibitors on apoptosis can be explained not only by decrease of NAD level in the cells but also poly (ADP-ribosyl) ation of nuclear proteins. These two phenomena of the decrease of NAD level and poly (ADP-ribosyl) ation of nuclear proteins may exert complex influences on apoptosis. ATP is necessary during the process of apoptosis and various compounds in mitochondria are related to it. It is interesting to know the effect of NAD on such process. Further studies are required to solve the problem.

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