Biochemical Pathways of Apoptosis: Nicotinamide Adenine Dinucleotide-deficient Cells Are Resistant to Tumor Necrosis Factor or Ultraviolet Light Activation of the 24-kD Apoptotic Protease and DNA Fragmentation

By Susan C. Wright,* Qi Sheng Wei,* David H. Kinder,‡ and James W. Larrick*

*From the *Palo Alto Institute of Molecular Medicine, Mountain View, California 94043; and the ‡College of Pharmacy, Ohio Northern University, Ada, Ohio 45810*

Summary

The function of nicotinamide adenine dinucleotide (NAD) and adenosine diphosphate (ADP) ribosylation reactions in the mechanism of apoptotic cell death is controversial, although one theory postulates an essential role for NAD depletion by poly-ADP-ribose polymerase. The present study examined the role of intracellular NAD in tumor necrosis factor (TNF) and ultraviolet (UV) light–induced activation of the 24-kD apoptotic protease (AP24) leading to internucleosomal DNA fragmentation and death. Our results demonstrate that nutritional depletion of NAD to undetectable levels in two leukemia lines (U937 and HL-60) renders them completely resistant to apoptosis. This was attributed to a block in the activation of AP24 and subsequent DNA cleavage. Normal cells show an elevation of ADP-ribosyl transferase (ADPRT) in both the cytosol and nucleus after exposure to TNF, but before DNA fragmentation. ADPRT activity as well as cell death was suppressed by an inhibitor specific for mono-ADPRT. Nuclei from NAD-depleted cells were still sensitive to DNA fragmentation induced by exogenous AP24, indicating a selective function for NAD upstream of AP24 activation in the apoptotic pathway. We confirmed a requirement for intracellular NAD, activation of ADPRT, and subsequent NAD depletion during apoptosis in KG1a, YAC-1, and BW1547 leukemia cell lines. However, this mechanism is not universal, since BJAB and Jurkat leukemia cells underwent apoptosis normally, even in the absence of detectable intracellular NAD. We conclude that TNF or UV light–induced apoptotic cell death is not due to NAD depletion in some leukemia cell lines. Rather, NAD-dependent reactions which may involve mono-ADPRT, function in signal transduction leading to activation of AP24, with subsequent DNA fragmentation and cell death.

Apoptosis is the normal physiological process of cell death essential for the maintenance of homeostasis (for reviews see references 1–3). Because of its important role in development and recent evidence implicating that abnormal regulation of this process underlies various pathological conditions (4), there has been intense interest in the biochemical mechanism of apoptosis. Work in our laboratory has analyzed the apoptotic pathway using the human monocytelike U937 leukemia as a model system. Our studies indicate that both TNF and UV light initiate similar biochemical processes culminating in internucleosomal DNA fragmentation. A key event in this pathway is the activation of a serine apoptotic protease of 24 kD (AP24)[1]. This enzyme was purified from apoptotic U937 cells after exposure to UV light and was shown to initiate internucleosomal DNA fragmentation in nuclei isolated from normal U937 cells (5). Since protein synthesis is not required for apoptosis in this system (6), AP24 must be expressed in an inactive form or else is sequestered from its substrate in normal U937 cells. The upstream signaling events leading to activation of AP24 are not well understood. Since we previously have shown that inhibitors of ADP-ribosylation reactions block TNF-induced apoptosis (6), the present study was undertaken to investigate the potential role of ADP-ribosyl transferase (ADPRT) in activation of AP24 and apoptotic cell death.

[1] *Abbreviations used in this paper:* ADP, adenosine diphosphate; ADPRT, ADP-ribosyl transferase; AP24, apoptotic protease of 24 kD; DK120, carbobenzoxy-ala-ala-borophe; MAAPV, methoxysuccinyl-ala-ala-pro-val *p*-nitroanilide; NAD, nicotinamide adenine dinucleotide; pADPRp, poly-ADP-ribose polymerase.

There are two types of enzymes that catalyze the transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to proteins (for a review see reference 7). Several different eukaryotic mono-ADPRTs have been described that transfer a single ADP-ribose moiety to acceptor proteins. Although widely distributed, the biological function of these enyzymes is not understood. Poly-ADP-ribose polymerase (pADPRp) catalyzes the transfer of multiple ADP-ribose moieties to itself and other receptor proteins. This predominantly nuclear enzyme is activated by DNA strand breaks and is thought to function in DNA repair.

The role of ADP-ribosylation reactions in apoptosis is still controversial. Evaluation of enzyme activity in cells undergoing apoptosis and the effects of inhibitors of ADPRT have indicated that in some systems, ADP-ribosylation promotes cell death (6, 8–12), whereas in other models it is inhibitory (13–15). According to one hypothesis, hyperactivity of pADPRp will deplete cellular levels of NAD, and subsequently ATP, which is ultimately the cause of cell death due to impaired energy metabolism (16, 17). It is possible to nutritionally deplete some types of cells of NAD by growth in medium lacking nicotinamide, the biosynthetic precursor of NAD. This technique was used previously to demonstrate the function of pADPRp in DNA repair, which was defective in NAD-depleted cells (18). We reasoned that if pADPRp consumption of NAD is the cause of apoptotic death, then cells nutritionally depleted of NAD should still be sensitive (or even more sensitive) to TNF or UV light–induced apoptosis. If, on the other hand, ADP-ribosylation reactions function to transmit apoptotic signals, then depletion of this enzyme's essential substrate, NAD, should render cells relatively resistant to apoptosis. The present study demonstrates that the latter hypothesis holds true for TNF or UV light–induced apoptosis in U937 cells.

Materials and Methods

Cell Lines. The human histiocytic lymphoma, U937, the human myeloid leukemias HL-60 and KG1a, the murine T cell leukemias, YAC-1 and BW5147, the human B cell leukemia, BJAB, and the human T cell leukemia, Jurkat, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in antibiotic-free RPMI 1640 supplemented with 10% FCS and L-glutamine (2 mM). Cell lines were routinely tested for mycoplasma and were always found to be negative according to the Mycotect kit (GIBCO BRL, Gaithersburg, MD).

To deplete NAD, cells were cultured in RPMI 1640 that was formulated without nicotinamide and 10% dialyzed FCS. It usually required 5–7 d of culture to deplete NAD to levels undetectable in the NAD assay. After 4 wk of culture in the absence of nicotinamide, cell viability decreased, and the cells were no longer used in experiments. The viability of NAD-depleted cells for all experiments reported here was always ≥95% by trypan blue exclusion.

Reagents. Purified human rTNF-α (sp act, 10^7 U/mg) was purchased from R&D Systems, Inc. (Minneapolis, MN).

Quantitative Assay of DNA Fragmentation. The assay we routinely employ to quantitate DNA fragmentation was described in detail previously and found to produce results similar to other standard DNA fragmentation assays (6). The assay was set up in triplicate in a flat bottom 96-well microtiter plate in RPMI 1640 with 2.5% FCS in a total volume of 0.1 ml. Target cells were suspended at 10^6/ml and 0.05 ml was added to each well. Wells for total counts received an additional 0.05 ml of medium, whereas experimental wells received 0.05 ml of the appropriate concentrations of TNF plus 0.5 μg/ml cycloheximide. This concentration of cycloheximide alone was nontoxic. Assays were incubated for the indicated length of time at 37°C depending on the particular experiment. The assay was terminated by the addition of 150 μl 10 mM EDTA, and 0.3% Triton X–100 to each well. Plates were harvested using a harvester (Packard Instrument Co., Inc., Meriden, CT) and radioactivity was counted using a beta counter (model Matrix 96; Packard). The percent DNA release was calculated as follows:

$$\text{percent DNA release} = [(\text{total cpm} - \text{experimental cpm})/\text{total cpm}] \times 100.$$

Using this technique, the percent spontaneous DNA release varied from 0 to 5% over a 5-h incubation period and did not exceed 10% release in a 20-h incubation. Correcting for these low levels of spontaneous release did not significantly alter the results, and therefore was not performed. Standard deviations were always <15% of the mean of the triplicate values.

ADPRT Assay. ADPRT activity was assayed as described previously (19). U937 cells (10^6 total) were given various treatments as indicated for each experiment. Cells were then pelleted, the supernatant removed, and the pellet resuspended in assay buffer (56 mM Hepes, pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.25 μCi [^{3}H]NAD) containing 0.01% digitonin to permeabilize the cells. After incubation for 10 min at 37°C, protein was precipitated with 0.2 ml of 50% TCA, pelleted at 12,000 rpm in a microfuge, and washed once with 50% TCA. The pellet was solubilized with 2% SDS in 0.1 M NaOH overnight at 37°C, and counted in a scintillation counter. Results of the treated groups are presented as a percentage of the cpm of the untreated control cells.

In experiments where ADPRT activity was measured separately in the cytosol and nuclear fractions, after the desired treatments, cells were pelleted and resuspended in assay buffer with 0.01% digitonin; the nuclei were then pelleted immediately by spinning them at 6,000 rpm for 2 min in a microfuge. The supernatant containing the soluble cytosol fraction was removed and assayed as described for whole cells. Assay buffer (0.5 ml) was then added to the pelleted nuclei and the assay continued as described for whole cells.

NAD Assay. Intracellular NAD levels were quantitated according to a previously described method (20). Cells (6×10^6 total) were pelleted, the supernatant removed, and the pellet extracted with 0.2 ml of 0.5 M perchloric acid for 15 min at 0°C. After centrifugation at 14,000 rpm in a microfuge for 5 min, the supernatant was adjusted to neutral pH with 1.0 M NaOH. To measure NAD levels, each reaction tube contained 0.2 ml of either cell extract or NAD standards (from 0.16 to 10.0 μM), 1.0 ml of reaction mixture containing 600 mM ethanol, 0.5 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 2.0 mM phenazine ethosulfate, 5.0 mM EDTA, 1.0 mg/ml BSA, and 120 mM *N*-bis glycine, pH 7.8. The reaction was incubated at 37°C in the dark for 20 min and terminated by adding 0.5 ml of 12 mM iodoacetate. The OD was measured at 570 nM and the concentration of NAD in cell extracts determined relative to the

NAD standard curve. The lowest level of NAD detectable in this assay was 10 pmol/10^6 cells.

Assessment of U937 Apoptosis by Various Criteria. U937 cells cultured with and without nicotinamide were exposed to different apoptotic stimuli and responses in terms of apoptotic morphology. DNA fragmentation (measured as described above), cell viability, and inhibition of proliferation were measured at different time points thereafter. Concentrations of inducing agents were as follows: 5.0 ng/ml TNF plus 0.5 μg/ml cycloheximide, 0.1 J/cm^2 UV light, heat–shock at 43°C for 30 min, 100 μM etoposide, 2.0 μg/ml cycloheximide, and 100 nM Calphostin C. The percentage of cells exhibiting an apoptotic morphology was determined by microscopic enumeration at the same time that the DNA fragmentation assay was harvested. This time point occurred before the appearance of significant numbers of dead cells, and viability was determined by trypan blue exclusion ~2–3 h after the DNA fragmentation assay was harvested. DNA fragmentation assays were harvested 2.5 h after exposure to either TNF or UV light and 5.5 h after exposure to heat–shock, etoposide, cycloheximide, or Calphostin C. In all cases, proliferation was assessed 24 h after exposure to the apoptosis inducers by measuring incorporation of [^{3}H]thymidine added for the last 18 h of culture.

DNA Fragmentation ADPRT Activation, and NAD Depletion in Different Cell Lines Cultured With and Without Nicotinamide. Preliminary studies were performed to establish the optimal conditions to measure DNA fragmentation and alterations in NAD metabolism in various cell lines exposed to different inducers. Since different cell lines undergo apoptosis with different kinetics and show differential sensitivity to various doses of stimuli, we determined the best assay conditions for each cell line. The release of ^{3}H-labeled DNA fragments, the measurement of ADPRT activity, and the quantitation of NAD levels were performed as described for U937 above. The incubation times chosen for YAC-1 and BW5147 cell lines were 5 h to measure DNA fragmentation and 3.5 h to measure ADPRT and NAD. The KG1a cell line was incubated 3 h to measure DNA fragmentation and 2.5 h to measure ADPRT and NAD. BJAB and Jurkat cell lines were incubated 5 h to measure DNA fragmentation and 4 h to assess ADPRT and NAD. In all cases, ADPRT and NAD levels were determined before there were significant numbers of dead (unable to exclude trypan blue) cells in the culture. Preliminary dose–response curves were performed to select concentrations of inducers that fell in the linear portion of the curve for each cell line. The cell lines were stimulated as follows: for KG1a, 1.0 ng/ml TNF plus 0.5 μg/ml cycloheximide, 200 μg/ml cycloheximide alone, 0.1 J/cm^2 UV light; for YAC-1, 0.1 J/cm^2 UV light, 200 nM Calphostin C; for BW5147, 0.2 ng/ml TNF plus 0.5 μg/ml cycloheximide; for BJAB, 100 nM Calphostin C; and for Jurkat, 2 μg/ml anti-Fas.

Affinity Purification and Quantitation of AP24 Activity. The synthesis and characterization of the boronic acid containing amino acid analog protease inhibitor (carbobenzoxy-ala-ala-borophe [DK120]) was described previously (21, 22). AP24 was purified by DK120 affinity chromatography as described in detail previously (5). Briefly, cytosol lysates were prepared from 50 × 10^6 control or UV light–pretreated cells after incubation for 1.5 h at 37°C. At this point, ~50–70% of U937 cells exhibited the typical apoptotic morphology, yet still maintained a viability of >95% by trypan blue exclusion. 1 ml of lysate from 50 × 10^6 normal or UV light–treated cells was chromatographed in an identical fashion on DK120 columns. Bound material was eluted with 0.1 M HCl, neutralized to pH 7.5, and assayed for AP24 using the synthetic chromogenic substrate, methoxysuccinyl-ala-ala-pro-val *p*-nitroanilide (MAAPV). Units of AP24 activity were calculated as the amount or enzyme that hydrolyzes 1 nM substrate per hour, as described previously (5).

DK120 affinity chromatography of apoptotic U937 cell lysates results in a 98–99-fold purification of AP24. Further purification steps revealed the affinity-purified material contained only one protease as measured on the MAAPV substrate (preferred by elastaselike enzymes). Furthermore, purification to homogeneity confirmed that only one protease was present in the affinity-purified material, and that this activity copurified with the nuclear DNA fragmenting activity (5). Thus, one-step affinity purification represents a feasible means to separate AP24 from other intracellular proteases and quantitate its activity in lysates from a reasonable number of cells after various experimental manipulations.

Assay of DNA Fragmentation in Isolated Nuclei. DNA fragmentation in isolated nuclei was assayed as described in detail previously (5). [^{3}H]Thymidine-labeled U937 cells were prepared as described for the whole cell DNA fragmentation assay above. Cells were pelleted and the cytoplasmic membrane lysed by resuspending the cells in assay buffer (50 mM Tris, 250 mM sucrose, 10 mM $MgSO_4$, pH 7.5) plus 0.02% NP-40. Nuclei were then pelleted and resuspended in assay buffer at 10^6/ml. The assay was set up in triplicate in flat bottom microtiter plates under sterile conditions. Nuclei (0.05 ml) were mixed with 0.05 ml of sample diluted in 50 mM Tris, pH 7.5, or buffer alone to determine total counts. Plates were incubated for 5 h at 37°C and then harvested by the addition of 0.1 ml of harvesting buffer (10 mM Tris, 10 mM EDTA, 0.3% Triton X–100, pH 7.5). High molecular weight DNA was collected by filtration onto glass fiber paper and the radioactivity counted on a Packard Matrix 96 beta counter. Percent DNA fragmentation was calculated as described for the whole cell DNA fragmentation assay. The spontaneous release of [^{3}H]thymidine relative to the total counts at the initiation of the assay did not exceed 5%.

Results

NAD-depleted Cells Are Refractory to TNF or UV Light–induced DNA Fragmentation. Initial experiments tested the hypothesis that if ADPRT activity is required for apoptosis, then depletion of intracellular NAD levels should confer resistance to apoptotic cell death. U937 and HL-60 cells cultured in media lacking nicotinamide for 7–10 d contained undectable levels of NAD (greater than five- to sixfold depletion), whereas ATP levels were decreased by only 24–35% (Table 1). The NAD-depleted cells retained a normal morphology as revealed by light microscopy, how-

Table 1. *NAD but not ATP is Severely Deleted in Cells Cultured without Nicotinamide*

Cell line	Cultured +/− nicotinamide	NAD pmol/10^6 cells	ATP pmol/10^6 cells
U937	+	63	135
U937	−	<10	102
HL-60	+	50	143
HL-60	−	<10	93

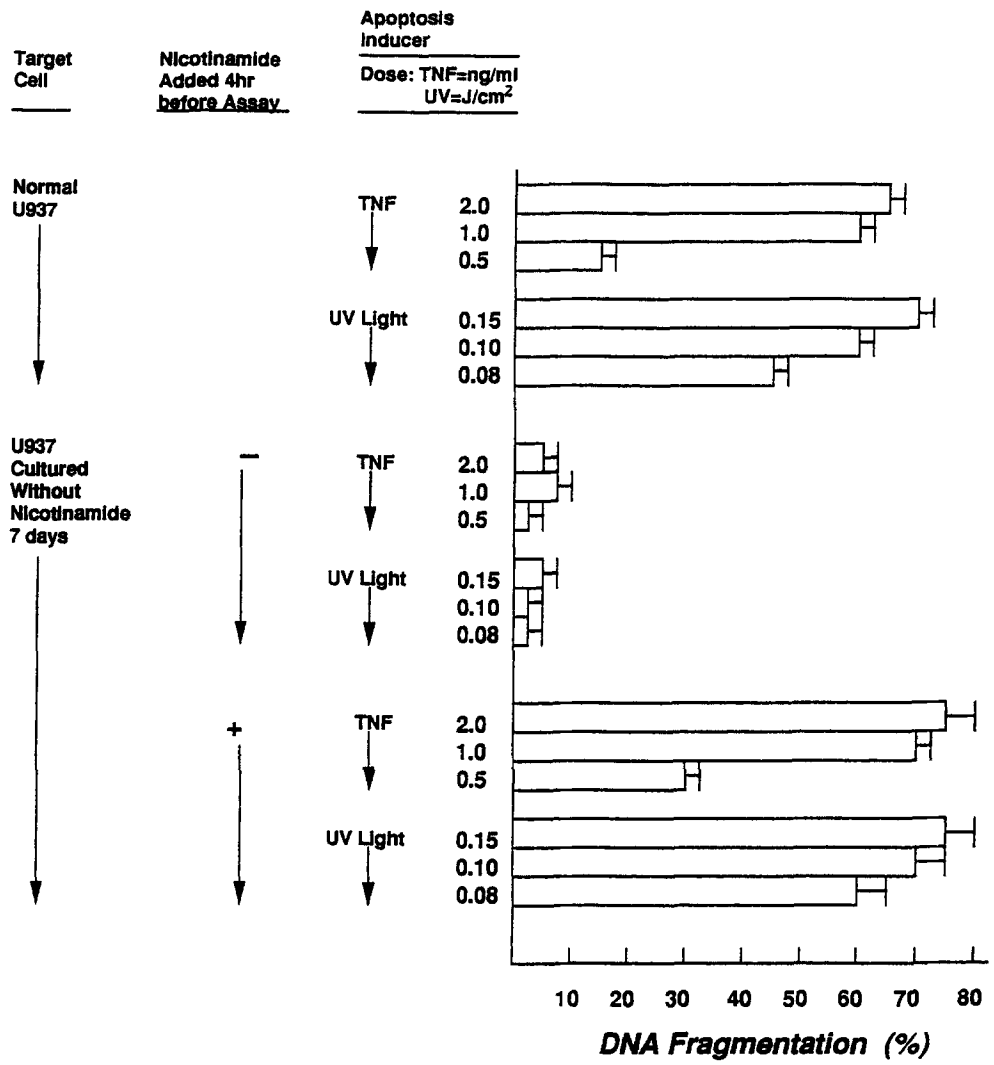

Figure 1. U937 cells depleted of NAD are resistant to TNF or UV light–induced apoptosis. Normal or NAD-depleted (*Cultured Without Nicotinamide*) U937 cells were tested for susceptibility to DNA fragmentation induced by the indicated concentration of TNF plus 0.5 cycloheximide μg/ml or UV light. In some experiments, NAD-depleted target cells were pretreated with nicotinamide to allow resynthesis of NAD and recovery of sensitivity to apoptosis. The assay was incubated for 2 h and percent DNA fragmentation ± SD determined as described in Materials and Methods. This experiment has been repeated on three other occasions with similar results.

ever their growth rate was slightly reduced to ~80% that of normal cells as assessed by [^{3}H]thymidine incorporation (data not shown). The results shown in Fig. 1 demonstrate that NAD-depleted U937 cells were highly resistant to TNF or UV light–induced DNA fragmentation. Microscopic examination revealed that the NAD-depleted cells were >95% viable, even after an additional 24-h incubation (data not shown). However, if the same cells were incubated with nicotinamide at concentrations normally present in cell culture medium (1.0 μg/ml) for 4 h before exposure to TNF or UV light to allow resynthesis of NAD, they regained full sensitivity to apoptosis. We obtained similar findings with NAD-depleted HL-60 cells (Fig. 2), demonstrating that this phenomenon is not limited to U937 cells. These findings support the hypothesis that NAD-dependent reactions are essential for TNF or UV light–induced apoptosis. Furthermore, this evidence indicates that apoptotic death is not the result of NAD depletion in this system.

Since the above experiments used only the release of DNA fragments to assess TNF or UV light–induced apoptosis, we wanted to confirm our findings using other readouts of cell death as well as other inducers of apoptosis. Several stimuli were chosen that show different modes of action including heat-shock, the chemotherapeutic topoisomerase inhibitor, etoposide, high-dose cycloheximide, and the protein kinase C inhibitor, Calphostin C. In all cases, NAD starvation conferred high levels of resistance to apoptosis (Table 2). It also prevented the characteristic morphological alterations (membrane blebbing) exhibited by apoptotic cells before death. Furthermore, NAD-deprived cells did not undergo necrosis, since they were still highly viable by trypan blue exclusion, nor did they show a significant inhibition of proliferation in response to the apoptosis inducers, in contrast to normal U937 cells. For some stimuli, such as etoposide, NAD starvation resulted in relative as opposed to complete resistance to apoptosis. This may indicate that some agents activate both NAD-dependent and -independent apoptotic pathways in a given cell line.

ADPRT Is Activated in the Cytosol and Nucleus of Cells Undergoing Apoptosis. Because previous studies implicated an essential role for ADPRT in some models of apoptosis (6, 8–12), experiments were performed to determine if this

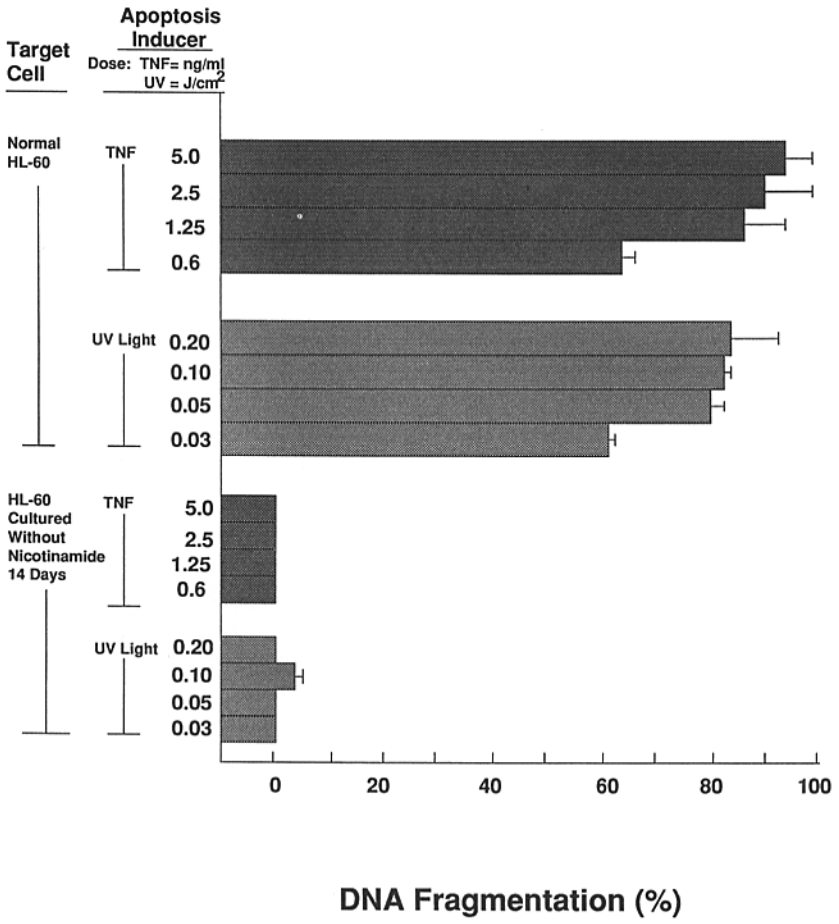

Figure 2. HL-60 cells depleted of NAD are resistant to TNF or UV light–induced apoptosis. HL-60 cells were treated as described for U937 cells in Fig. 1. This experiment has been repeated on two other occasions with similar results.

Table 2. *NAD-depleted U937 is Resistant to Apoptosis Induced by Different Stimuli and Assessed by Various Criteria*

U937 Cultured +/− nicotinamide	Apoptotic stimuli	Percent apoptotic cells	Percent DNA fragmentation	Percent dead cells	Percent inhibition proliferation
				%	
+	—	1	0	0	0
−	—	1	0	1	0
+	TNF 5 ng/ml	78	91 ± 1.5	88	78 ± 7.1
−	TNF 5 ng/ml	0	9 ± 0.4	0	3 ± 0.1
+	UV light	67	61 ± 6.1	75	75 ± 12.8
−	UV light	0	8 ± 0.8	1	17 ± 0.6
+	Heat–shock	ND	48 ± 1.0	84	74 ± 7.4
−	Heat–shock	ND	13 ± 1.1	22	25 ± 1.0
+	Etoposide	ND	46 ± 5.1	88	77 ± 10.7
−	Etoposide	ND	28 ± 2.8	18	32 ± 1.9
+	Cycloheximide	72	73 ± 2.2	69	61 ± 3.1
−	Cycloheximide	1	0	2	0
+	Calphostin C	69	71 ± 1.0	73	68 ± 2.0
−	Calphostin C	0	0	4	0

Methods used to induce and assess apoptosis are described in Materials and Methods.

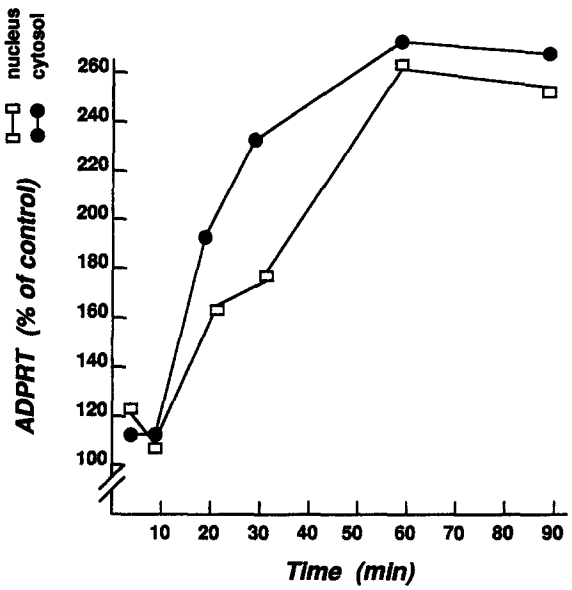

Figure 3. ADPRT is activated in both the cytosol and nucleus of U937 cells treated with TNF. U937 cells were treated with 2 ng/ml TNF plus 0.5 μg/ml cycloheximide, and at the indicated time, cytosol and nuclear extracts were prepared and assayed for ADPRT. This assay has been repeated on two other occasions with similar results.

NAD-dependent enzyme is activated in the U937 model system. ADPRT activity was measured separately in cytosol and nuclear extracts from U937 cells after exposure to TNF. The results in Fig. 3 show that within 30 min of exposure to TNF, there was a significant elevation of ADPRT activity in both the cytosol and nucleus with a further increase to peak activity at 60 min. We also observed a concomitant decrease in intracellular NAD levels to only 30% of control cells at 60 min (data not shown). We obtained similar results with U937 cells undergoing apoptosis in response to UV light (data not shown). Since pADPRp is predominantly a nuclear enzyme, these findings suggest that cytosol activity may be due to mono-ADPRT.

NAD-dependent Reactions Are Involved in Some, but Not all Forms of Apoptosis. Our results demonstrated that for both U937 and HL-60 cells, NAD depletion conferred resistance to either TNF or UV light–induced DNA fragmentation. To further explore the generality of this phenomenon, we tested a series of other types of cell lines exposed to a variety of apoptotic agents. Nutritional depletion of NAD to undetectable levels rendered the human myeloid leukemia, KG1a, and the murine T cell leukemias, YAC-1 and BW5147, relatively resistant to apoptosis induced by agents as diverse as TNF, UV light, high-dose cycloheximide, and Calphostin C. Microscopic examination revealed that NAD-depleted cells were highly viable, with no evidence of necrotic cell death after exposure to apoptosis-inducing agents (data not shown). Additional studies provided further evidence to support a role for altered NAD metabolism in these examples of apoptosis. Normal cells cultured in the presence of nicotinamide were tested for ADPRT activity and NAD content before and after exposure to apoptosis inducers, but before the accumulation of dead cells as described in detail in Materials and Methods.

Table 3. *NAD Depletion of Some but Not All Cell Lines Confers Resistance to Apoptosis*

Target	+/− NAD	Stimulus	Percent DNA Fragmentation	ADPRT activity percent control	NAD content percent control
				%	
KG1a	+	TNF	41 ± 5.3	200	40
KG1a	−	TNF	0	ND	ND
KG1a	+	UV light	43 ± 1.2	234	32
KG1a2	−	UV light	3 ± 0.1	ND	ND
KG1a	+	Cycloheximide	33 ± 2.3	161	59
KG1a	−	Cycloheximide	0	ND	ND
YAC-1	+	UV light	51 ± 7.7	174	51
YAC-1	−	UV light	7 ± 0.2	ND	ND
YAC-1	+	Calphostin C	29 ± 2.3	180	32
YAC-1	−	Calphostin C	7 ± 0.2	ND	ND
BW5174	+	TNF	72 ± 8.6	385	37
BW5174	−	TNF	27 ± 2.7	ND	ND
BJAB	+	Calphostin C	37 ± 1.4	109	112
BJAB	−	Calphostin C	34 ± 1.0	ND	ND
Jurkat	+	Anti-Fas	47 ± 4.7	106	90
Jurkat	−	Anti-Fas	45 ± 1.6	ND	ND

Conditions for induction of apoptosis are described in Materials and Methods.

As shown in Table 3, KG1a, YAC-1, and BW5147 cells undergoing apoptosis all showed increases in ADPRT activity ranging from 161 to 385% that of control cells. Furthermore, there was a concomitant decrease in the intracellular NAD levels that ranged from 32 to 59% that of control cells. These findings support the hypothesis that in these cell lines, the initiation of apoptosis depends on adequate intracellular levels of NAD, which is then consumed during the apoptotic process, probably due to activation of ADPRT.

However, apoptosis is not dependent on NAD in all types of cells. Table 3 shows two such examples. The human B cell leukemia, BJAB stimulated with Calphostin C, and the human T cell leukemia, Jurkat stimulated with antibodies directed against the Fas antigen (anti-Fas), were still sensitive to DNA fragmentation, even after nutritional depletion of NAD. Furthermore, normal BJAB and Jurkat cells undergoing apoptosis did not show any increase in ADPRT or a significant decrease in NAD levels. Therefore, some mechanisms of apoptosis are independent of these alterations in NAD metabolism.

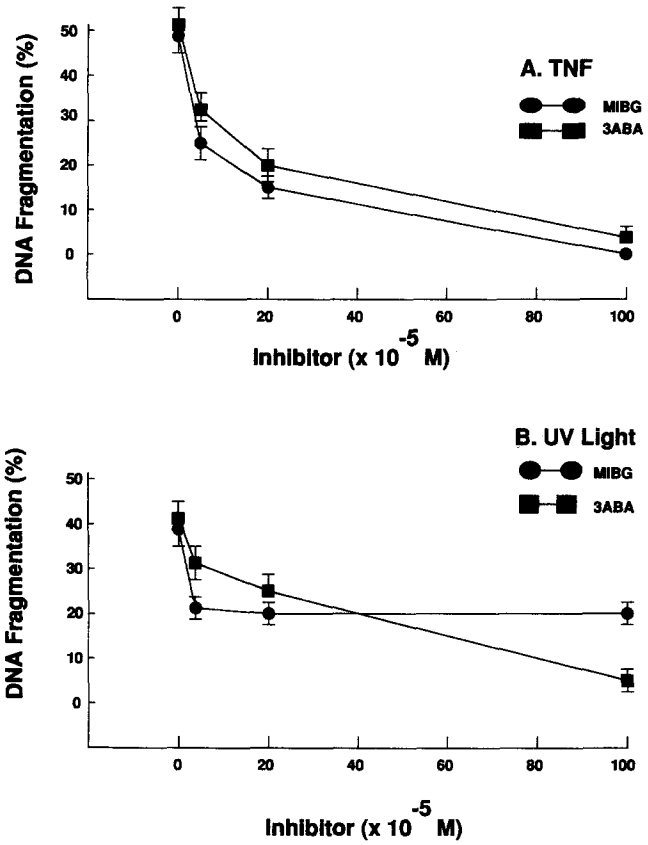

Figure 4. Suppression of TNF (*A*) or UV light (*B*) induced DNA fragmentation by ADPRT inhibitors. U937 cells were pretreated with the indicated concentration of inhibitor for 30 min before exposure to 2 ng/ml TNF plus 0.5 μg/ml cycloheximide (*A*) or 0.1 J/cm^2 UV light (*B*). After a subsequent 2-h incubation, assays were terminated and percent DNA fragmentation determined as described in Materials and Methods. This experiment has been repeated on three other occasions with similar results.

Inhibitors of Both pADPRp and Mono-ADPRT Suppress TNF or UV Light–induced DNA Fragmentation. Previous studies using inhibitors of pADPRp have provided evidence for both positive (8, 11) and negative (19) regulation of apoptosis by this enzyme. 3-aminobenzamide (3ABA) is a compound that inhibits both pADPRp and mono-ADPRT, whereas *meta*-iodobenzylguanidine (MIBG) has been shown to specifically suppress mono-ADPRT activity (23). Both these agents were found to dose dependently inhibit TNF or UV light–induced DNA fragmentation (Fig. 4). These findings are consistent with the involvement of mono-ADPRT, but do not eliminate the possible role of pADPRp in the apoptotic response.

UV Light Does Not Activate AP24 in NAD-depleted Cells, Although Their Nuclei Are Still Sensitive to DNA Fragmentation Induced by Exogenous AP24. We next attempted to localize at what point in the apoptotic pathway NAD is required. Experiments were performed to determine if NAD is needed for UV light–induced activation of AP24 proteolytic activity or for downstream nuclear events involved with DNA fragmentation. Both normal U937 and cells nutritionally depleted of NAD were exposed to UV light, and after 2 h of incubation, cell extracts were prepared and quantitated for proteolytic activity of affinity-purified AP24. The results shown in Table 4 demonstrate that UV light induced over a twofold increase in AP24 activity recovered from normal cells, whereas this protease was not activated in NAD-depleted cells. This indicates that NAD-dependent reactions are essential signaling events leading to AP24 activation, but do not rule out additional involvement in subsequent DNA fragmentation. Therefore, we tested the susceptibility of nuclei from NAD-depleted cells to undergo DNA fragmentation in response to exogenous AP24.

The results in Fig. 5 show that both normal nuclei and those with undetectable levels of NAD were equally susceptible to DNA fragmentation induced by exogenous AP24 purified from normal U937 cells undergoing apoptosis induced by UV light (Fig. 5). It is concluded that normal intracellular levels of NAD are required for upstream

Table 4. *UV Light Does Not Stimulate AP24 Activity in NAD-depleted Cells*

Cell line	+/− UV* light	AP24 Activity‡
		U
Normal U937	−	9.5
Normal U937	+	22.0
NAD-depleted U937	−	8.0
NAD-depleted U937	+	8.0

*U937 cells were exposed to 0.1 J/cm^2 and UV light incubated for 2 h. Cytosol extracts were then prepared and purified by chromatography on DK120 columns.

‡Affinity-purified AP24 activity was quantitated in a kinetic protease assay using the MAAPV substrate as described in Materials and Methods.

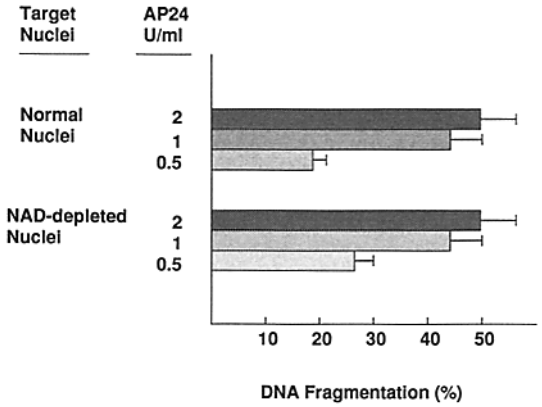

Figure 5. NAD-depleted nuclei are still sensitive to DNA fragmentation by AP24. Nuclei were isolated from normal U937 cells or cells that were nutritionally depleted of NAD to undetectable levels, and tested for sensitivity to AP24-induced DNA fragmentation as descrbed in Materials and Methods. This experiment has been repeated on two other occasions with similar results.

events leading to activation of AP24, but not for subsequent DNA fragmentation initiated by AP24 in the nucleus.

Discussion

The present study examined the role of intracellular NAD in the U937 model system of TNF and UV light–induced apoptosis. Experiments were designed to determine if cell death was due to NAD depletion or if NAD functioned in the signal transduction pathway leading to DNA fragmentation. The demonstration that cells nutritionally depleted of NAD become completely resistant to TNF or UV light–induced apoptosis provides convincing evidence that NAD depletion is not the cause of death in this system. However, because multiple mechanisms of apoptosis exist, it is still possible that NAD depletion is the cause of death in other systems, as proposed previously (16, 17).

In addition to U937 cells, we confirmed that NAD depletion leads to relative resistance to apoptosis in HL-60, KG1a, YAC-1, and BW5147 cells exposed to diverse stimuli including TNF, UV light, cycloheximide, and Calphostin C. Furthermore, in cells with normal NAD content, exposure to apoptosis inducers activated ADPRT and caused a drop in NAD levels before DNA fragmentation and cell death. These findings support an essential role for NAD-dependent signal transduction in apoptosis of these leukemia cell lines. However, dependence on NAD was not universal for all cell lines tested, since NAD depletion of BJAB or Jurkat cells did not confer resistance to apoptosis.

It is now clear that multiple mechanisms of apoptosis exist that vary depending on cell type and apoptotic stimuli. For example, TNF and antibodies directed against the Fas antigen (anti-Fas) have been shown to initiate apoptosis through distinct pathways (24). It is interesting to note that inhibitors of ADP-ribosylation were shown to block cell killing by TNF but not by anti-Fas (25). Thus, dependence on NAD and ADPRT may be a useful criterion to categorize mechanisms of apoptosis.

Although NAD may function as a cofactor for various enzymes, our studies support a role for ADPRT in signaling apoptosis. We confirmed previous findings (6, 11, 12) showing that 3ABA, an inhibitor of both pADPRp and mono-ADPRT, can protect cells from some forms of apoptosis. This, and the fact that pADPRp is activated by DNA strand breaks, led many investigators to focus on the role of pADPRp in apoptosis. Our finding that MIBG, a specific inhibitor of mono-ADPRT (23), suppressed U937 apoptosis, led to the new concept that mono-ADPRT may function in apoptotic signal transduction. This hypothesis is further supported by the finding that cytosolic ADPRT activity is elevated in cells undergoing apoptosis, whereas pADPRp is predominantly a nuclear enzyme.

At present we know little about how the postulated mono-ADPRT and its substrates function to transmit apoptotic signals. However, the present study demonstrates that NAD is required for some upstream signaling event(s) leading to activation of AP24 and subsequent DNA fragmentation. Our preliminary findings suggest that AP24 itself is not the substrate of mono-ADPRT. Instead, our evidence implicates other signal-transducing molecules, which will be the subject of a future communication.

Although ADPRT activity was elevated in the nuclei of apoptotic U937 cells, nuclei of cells depleted of NAD were still susceptible to DNA fragmentation induced by exogenous AP24. Thus, once activated, AP24 induction of DNA fragmentation is independent of normal intracellular levels of NAD, and presumably, of ADPRT reactions. However, in normal cells undergoing apoptosis, pADPRp may be activated by DNA strand breaks as a result of DNA fragmentation rather than a causal event.

The finding that depletion of normal intracellular levels of NAD will confer resistance to apoptosis in some tumor cell lines has important implications for the pathogenesis and treatment of cancer. Acquisition of resistance to apoptosis may promote tumor survival by evasion of host–defense mechanisms, as well as the cytotoxic effects of chemotherapeutic agents (26, 27). A decrease in intracellular NAD levels has been associated with metabolic alterations in transformed cells (28). In this situation, therapeutics designed to restore normal intracellular NAD levels, or to bypass the NAD requirement and activate the apoptotic pathway at downstream events (e.g., AP24 activation) may benefit patients with apoptosis-resistant cancer.

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Address correspondence to Dr. Susan C. Wright, Palo Alto Institute of Molecular Medicine, 2462 Wyandotte Street, Mountain View, CA 94043.

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