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Azidothymidine Induces Apoptosis in Mouse Myeloma Cell Line Sp2/0

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ABSTRACT. Azidothymidine (AZT), which has been extensively used as an antiviral agent in the treatment of AIDS, showed strong inhibition of growth of Sp2/0 cells *in vitro*. AZT-treated cells showed a decrease in viability in a dose-dependent manner. AZT specifically induced typical apoptotic cell death with DNA double-strand cleavage and subsequent formation of apoptotic bodies. The induction of DNA double-strand cleavage into the oligonucleosomal ladder by AZT was protected in the presence of thymidine or uridine. An increase in endonuclease activity from nuclear extract of AZT-treated cells was observed. The enzyme activity was found to be Ca^{2+}- and Mg^{2+}-dependent and was inhibited by zinc acetate. A marked enhancement of PARP activity was observed in AZT-treated cells. These observations show that AZT can trigger both morphological and biochemical changes typical of apoptosis in the mouse myeloma cell line Sp2/0. BIOCHEM PHARMACOL 52;6:857–862, 1996.

KEY WORDS. azidothymidine; apoptosis; endonuclease; poly(ADP-ribose)polymerase

AZT,† in its phosphorylated triphosphate form (AZT-TP), is a potent inhibitor of human immunodeficiency virus (HIV) replication *in vitro* due to its interaction with virus reverse transcriptase [1, 2]. AZT traverses the cell membrane chiefly by nonfacilitated diffusion [3], and is phosphorylated into triphosphate by cellular enzymes [2]. AZT-TP is also capable of inhibiting DNA polymerases α and β [4] and γ [5]. AZT causes bone-marrow toxicity, resulting in anemia and netropenia [6]. AZT brings about toxicity by its incorporation into the DNA of human bone-marrow cells [7]. It has been reported that thymidine suppresses AZT phosphorylation in human mononuclear cells [8] and uridine protects normal human granulocyte-macrophage progenitor cells from AZT cytotoxicity *in vitro* without impairment of antiretroviral activity [9]. Thymidylate kinase is inhibited by AZT-MP, depleting dTTP needed for DNA synthesis [10]. AZT-MP triggers toxicity of AZT by inhibiting exonucleolytic repair of AZT-MP-terminated DNA [11]. AZT induces perturbation of dNTP pools leading to DNA fragmentation in cells [12–14].

Apoptosis is a distinct mode of physiological cell death that has been associated with characteristic biochemical and morphological changes [15]. Typical DNA fragmentation into oligonucleosomal-sized fragments mediated by Ca^{2+}/Mg^{2+} dependent endonuclease [16] is a hallmark of apoptosis. It has been reported that apoptosis may be an important mode of cell death for cells treated with chemotherapeutic agents [17]. In this paper, we present evidence to show that AZT induces morphological and biochemical changes characteristic of apoptosis in the mouse myeloma cell line Sp2/0.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM) was obtained from GIBCO (Grand Island, NY); fetal calf serum was obtained from Boehringer Manheim, Germany; azidothymidine, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt of sodium, phenazine methosulfate, propidium iodide, and RNaseA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{32}P] β-NAD^+ (Sp. Activity, 1000 Ci/mmol) was purchased from Board of Radio Isotope Technology (Bombay, India). All other reagents used were of analytical reagent grade.

Cells and Drug Treatment

Sp2/0, a mouse myeloma cell line derived from BALB/c mice was originally obtained from National Facility for Animal Tissue and Cell Culture (Pune, India). Sp2/0 cells were grown in IMDM supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, penicillin (100 IU/mL), streptomycin (100 μg/mL), gentamycin (20 μg/mL) and mycostatin (5 IU/mL) at

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† *Abbreviations:* AZT, 3′-azido-3′-deoxythymidine; AZT-MP, AZT monophosphate; AZT-TP, AZT triphosphate; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt of sodium; PMS, phenazine methosulfate; PI, propidium iodide; PARP, poly(ADP-ribose)polymerase.

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37°C in a water-jacketed incubator with 5% CO_2. Cells from log phase were harvested, 1×10^6 cells were seeded in milk dilution bottles containing 10 mL medium and incubated at 37°C for 2 hr. Cells were, then, treated with various concentrations of AZT and incubated at 37°C for 48 hr.

Cell Viability

Sp2/0 (2×10^3 cells/well) were grown in flat-bottomed 96-well tissue culture plates at 37°C for 2 hr. Cells were treated with different concentrations of AZT and incubated at 37°C for 60 hr. Cytotoxicity of the AZT was determined using tetrazolium reagent XTT as described by Scudiero *et al.* [18]. The percentage viability was calculated using the formula [(experimental – blank)/(control – blank)] × 100.

Phase Contrast Microscopy

Sp2/0 (1×10^4 cells/well) were grown in 96-well plates and treated with various concentrations of AZT at 37°C for 48 hr. Morphological changes occurring in the cells were observed under a phase-contrast microscope and photographed.

Fluorescence Microscopy

Sp2/0 cells (1×10^6) were grown in milk-dilution bottles in the presence of various concentrations of AZT at 37°C for 48 hr. Cells were collected by centrifugation at 500 *g*, washed in PBS, and fixed in 70% ethanol in PBS. The cells were washed, resuspended in PBS, and a drop was taken on a glass slide and air dried. The cells were stained with hypotonic fluorochrome solution containing propidium iodide (PI) (50 μg/mL), 0.1% sodium citrate, 0.1% Triton X-100, and RNase A (100 μg/mL) and observed under a fluorescence microscope.

Extraction of DNA and Gel Electrophoresis

DNA was isolated from AZT-treated Sp2/0 cells as described by Miller *et al.* [19], with modifications. Sp2/0 cells (1×10^6) were incubated separately with various concentrations of AZT, uridine and thymidine, and in combination of AZT with thymidine and AZT with uridine at 37°C for 48 hr. The drug-treated cells (2×10^6) were washed in PBS, centrifuged at 500 *g*, and the pellet was resuspended in 1.5 mL of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 400 mM NaCl, and 2 mM Na_2 EDTA. The cell lysate was digested overnight at 37°C with 0.25 mL of proteinase K solution (0.1 mg proteinase K in 1% SDS containing 2 mM Na_2 EDTA) and 0.1 mL of 10% SDS. After completion of digestion, 0.5 mL of saturated NaCl was added to each tube and centrifuged at 9000 *g* for 25 min. The supernatant containing the DNA was precipitated with 2 volumes of ethanol at −70°C overnight. The precipitated DNA was then collected by centrifugation, washed in cold 70% ethanol, and dried at room temperature. The DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, and 0.2 mM Na_2 EDTA) and was allowed to dissolve at 37°C for 2 hr. RNA was removed from this solution by treating with RNase A (25 μg/mL) at 37°C for 1 hr and the DNA was estimated at 260 nm. DNA (10 μg/lane) was then electrophoresed on 1.5% agarose at 20 V using TBE buffer, pH 8.3, containing 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA. Bromophenol blue was used as a tracking dye. The gel was stained with ethidium bromide (0.5 μg/mL) and photographed.

Endonuclease Assay

This assay was carried out as described by Ucker *et al.* [20] Sp2/0 cells (1×10^5/mL) were incubated with 250 μM AZT at 37°C for 48 hr, harvested, washed with PBS, and nuclei were prepared by disruption in 1 mL of $STEM_1$ buffer (200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EGTA, 0.5 mM DTT, and 0.2% Triton X-100) and kept on ice for 10 min. Nuclei were collected by centrifugation, resuspended in $STEM_2$ buffer (200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM DTT), and incubated at 4°C for 3 hr. The supernatant containing nuclear endonuclease was collected after centrifugation at 13,000 *g* for 15 min. Protein concentration was determined as described by Lowry *et al.* [21].

Endonuclease activity was assayed using supercoiled pUC DNA as substrate. Serial dilutions of supernatant were incubated with a constant amount of pUC DNA (0.4 μg) in $STEM_2$ buffer supplemented with 1 mM $CaCl_2$. Endonuclease was characterized by incubating the assay mixture separately with chelating agents, such as EDTA (37 mM), EGTA (37 mM), and zinc acetate (7.5 mM and 15 mM) at 37°C for 3 hr. The reaction products were separated by electrophoresis on 0.8% agarose gel containing ethidium bromide (0.5 μg/mL) in TBE buffer pH 8.3, and photographed.

Poly(ADP-ribose)polymerase Assay

This assay was carried out as described by Yoshihara *et al.* [22]. Sp2/0 cells (1×10^5/mL) were treated with various concentrations of AZT at 37°C for 48 hr. Cells were harvested, washed in PBS, and 1×10^5 cells were suspended in 90 μL of PBS without divalent cations and disrupted by the addition of 30 μL of 250 mM Tris-HCl, pH 8.0, containing 10 mM DTT, 100 mM $MgCl_2$, 0.5% Triton X-100, and 15 μg of nicked DNA. After the samples were kept on ice for 10 min, 165 μL of 2.91 M ammonium sulphate and 15 μL of [^{32}P] NAD^+ solution containing 1 nmol of NAD^+ were added, and the samples were incubated at 25°C for 1 hr. The reaction was terminated by the addition of 0.7 mL of 10% TCA, and TCA-insoluble radioactivity was monitored.

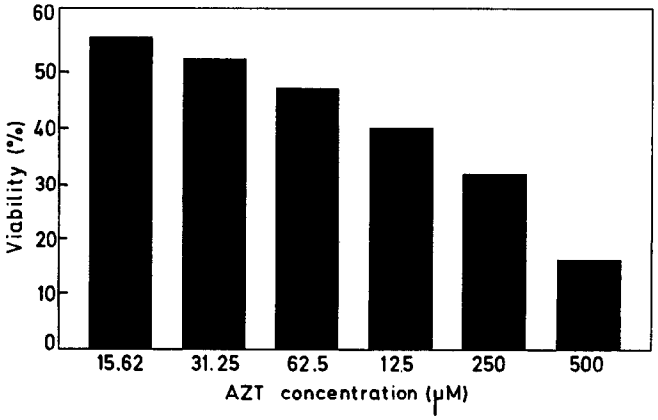

FIG. 1. Effect of AZT on viability of Sp2/0 cells. 2 × 10^3 cells in 200 μL of IMDM containing 5% FCS were plated in each well, treated with various concentrations of AZT ranging from 15 to 500 μM, and incubated at 37°C for 60 hr. Percentage of cell viability was determined using XTT assay. The mean value obtained from 6 replicate cultures was used to calculate percentage viability. The SD was always less than 10% of the mean value.

RESULTS

Effect of AZT on Viability of Sp2/0 Cells

The viability of Sp2/0 cells decreased progressively when treated with increasing concentrations of AZT (Fig. 1). AZT induced a decline in cellular viability as determined by the decrease in the ability of cells to convert XTT to a formazan reaction product.

Effect of AZT on Sp2/0 Cell Morphology

The morphology of Sp2/0 cells revealed the following features associated with apoptosis. After 24 hr of incubation,

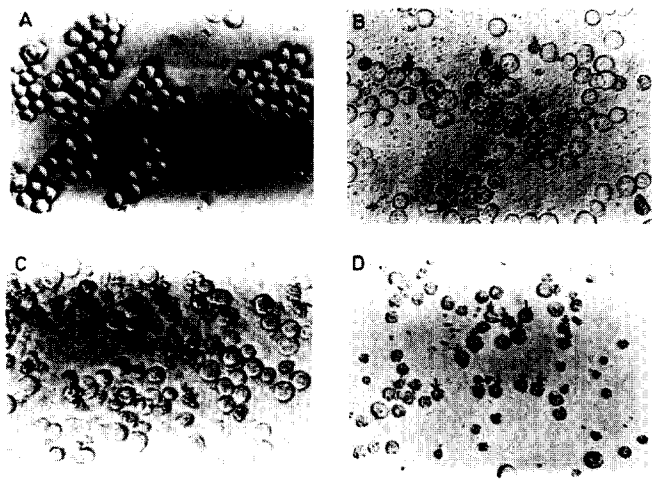

FIG. 2. Effect of AZT on Sp2/0 cell morphology. Sp2/0 cells (1 × 10^4) were cultured in the absence (A) or presence of 25 μM (B), 250 μM (C) and 500 μM (D) AZT for 48 hr and photographed under phase contrast microscope (40 × objective lens). Some of the apoptotic cells are indicated by arrows.

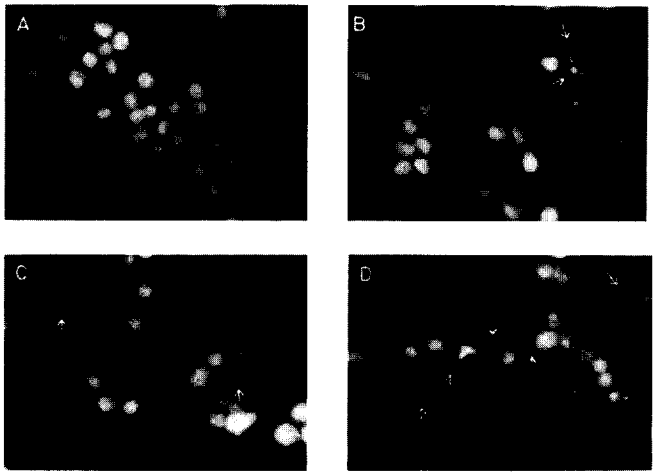

FIG. 3. Effect of AZT on Sp2/0 cell morphology. Sp2/0 cells (1 × 10^6) were cultured in the absence (A) or presence of 25 μM (B), 250 μM (C) and 500 μM (D) AZT for 48 hr. The cells were washed with PBS, ethanol-fixed, PI-stained, and photographed under a fluorescence microscope (40 × objective lens). Drug-treated Sp2/0 nuclei show a marked appearance of apoptotic bodies. Some of the apoptotic cells are indicated by arrows.

cells were found floating in the medium containing 250 and 500 μM AZT. AZT-treated cells were smaller, with blebs in the plasma membrane, and had increased cytoplasmic dense bodies as compared to untreated cells (Fig. 2). These changes were most marked at high concentrations of AZT. When observed under a fluorescence microscope, many apoptotic bodies stained with PI appeared smaller than normal nuclei (Fig. 3).

AZT-Induced DNA Double-Strand Breaks

DNA in the AZT-treated cells showed multiples of oligonucleosomal sized (180–200 bp units) ladder bands corresponding to the internucleosomal unit, which is a characteristic feature of apoptosis (Fig. 4A). In contrast, this type of ladder was not observed in DNA isolated from untreated cells. DNA fragmentation induced by AZT was protected when cells were incubated with AZT (50 μM) in the presence of thymidine (0.5 and 1 mM) or uridine (5 or 7.5 mM) at 37°C for 48 hr (Fig. 4B).

Endonuclease Activity

pUC DNA, when subjected to various concentrations of AZT-treated Sp2/0 cell nuclear extract, gave low-molecular weight DNA (Fig. 5A). When the nuclear extracts (5 μg of protein) from AZT (250 μM)-treated cells were incubated with supercoiled pUC DNA in the presence of EGTA or EDTA, complete inhibition of endonuclease activity was seen (Fig. 5B, Lanes 4 and 5). The endonuclease activity observed during the AZT-induced apoptosis in Sp2/0 cells

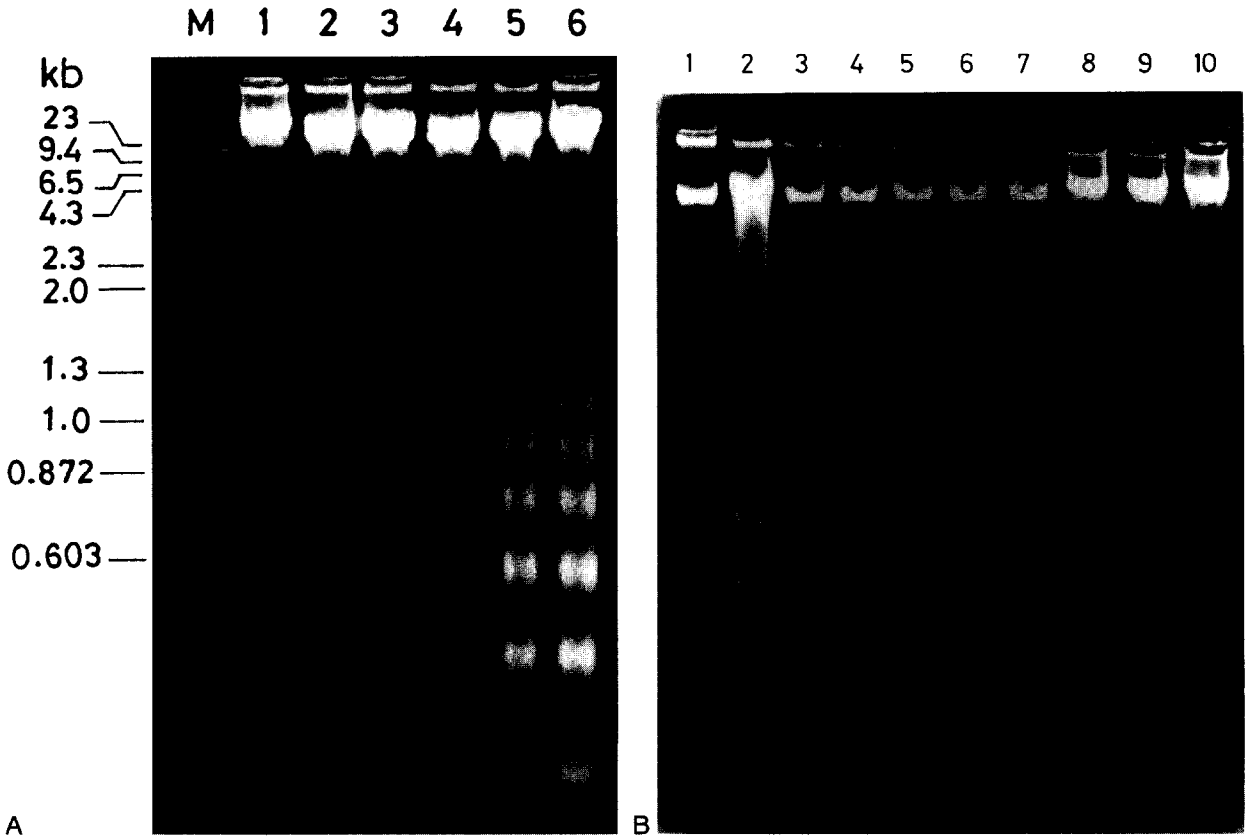

FIG. 4. (A) AZT-induced DNA double-strand breaks. Sp2/0 cells were cultured for 48 hr in the absence (lane 1) or presence of 25 μM (lane 2), 50 μM (lane 3), 100 μM (lane 4), 250 μM (lane 5), and 500 μM (lane 6) AZT for 48 hr. DNA was isolated from the cells and 10 μg was loaded in each lane. Lane (M) indicates molecular weight markers. (B) Sp2/0 cells were cultured for 48 hr in the absence (lane 1) or presence of AZT (50 μM) (lane 2), thymidine 0.5 and 1 mM (lanes 5 and 6), and uridine 5 and 7.5 mM (lanes 9 and 10) separately, or treated with AZT (50 μM) in the presence of thymidine 0.5 and 1 mM (lanes 3 and 4) and uridine 5 and 7.5 mM (lanes 7 and 8).

is Ca^{2+} and Mg^{2+} dependent. When AZT-treated Sp2/0 cell nuclear extract was incubated at 37°C for 3 hr with pUC DNA in the presence of zinc acetate, the endonuclease activity was completely inhibited (Fig. 5B, Lanes 6 and 7). The nuclear extract was found to be free from endogenous DNA (Fig. 5B, Lanes 2 and 3).

Poly(ADP-ribose)polymerase Activity

When Sp2/0 cells were treated with AZT, PARP activity increased with increasing concentrations of the drug (Fig. 6). Increased PARP enzyme activity was observed with a much lower concentration of AZT (10 μM) than that required for ladder formation of DNA. It was observed that increased PARP activity correlates with DNA double-strand breaks caused by AZT in Sp2/0 cells.

DISCUSSION

In the present investigation, we have demonstrated that AZT induces apoptosis in the mouse myeloma cell line Sp2/0 grown *in vitro*. The morphological changes associated with apoptosis are compaction and segregation of the nuclear chromatin with the formation of sharply delineated granular masses and condensation of cytoplasm, followed by production of membrane-bound apoptotic bodies [15]. These characteristic morphological changes were observed in AZT-treated Sp2/0 cells. Distinct apoptotic bodies, a typical feature of cell death, were observed when AZT-treated cells were stained with a DNA-binding fluorescent dye, propidium iodide.

AZT specifically induced DNA double-strand cleavage resulting in an oligo-nucleosomal ladder in Sp2/0 cells. Many chemotherapeutic agents have been reported to cause DNA double-strand cleavage in various cancer cell lines [23]. It was proposed that AZT inhibits thymidylate kinase due to the accumulation of AZT-MP in cells [2], which leads to the reduction of thymidine nucleotide pools [10]. The perturbation of deoxynucleotide pools (decreased dTTP) may lead to fragmentation of DNA [12–14]. It has been suggested that inhibition of AZT phosphorylation by thymidine may be the biochemical basis for the prevention of AZT toxicity [8, 9]. Reversal of AZT cytotoxicity was not observed in the presence of 2′-deoxyuridine, 2′-deoxyadenosine, 2′-deoxyguanosine, or 2′-deoxycytidine [9]. AZT cytotoxicity was reversed by uridine and, to a

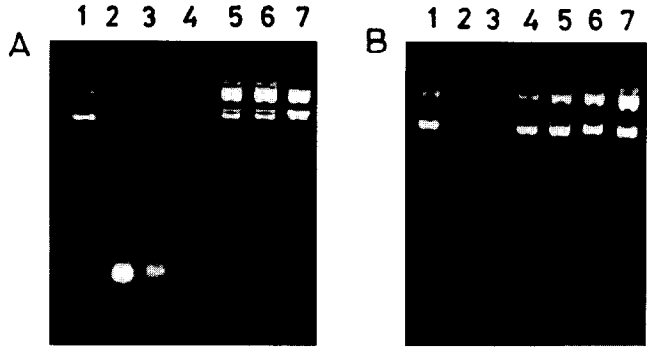

FIG. 5. (A) Endonuclease activity. Various protein concentrations of nuclear extract of AZT-treated Sp2/0 cells containing 0.0 μg (lane 1), 0.6 μg (lane 2), 0.3 μg (lane 3), and 0.15 μg (lane 4) and of AZT-untreated cells containing 0.15 μg (lane 5), 0.3 μg (lane 6), and 0.6 μg (lane 7) were separately incubated with 0.4 μg of supercoiled pUC DNA in the presence of Ca^{2+} and Mg^{2+} at 37°C for 3 hr. The DNA samples were electrophoresed on 0.8% agarose and visualized with ethidium bromide. Undigested DNA was detected as high-molecular-weight bands, whereas digested DNA was observed as lower-molecular-weight bands. (B). pUC DNA (lane 1), untreated (lane 2), and AZT-treated (lane 3) Sp2/0 cells nuclear extracts (5 μg) without plasmid, AZT-treated Sp2/0 cell nuclear extract (5 μg) incubated with pUC DNA (0.4 μg) separately, in the presence of 37 mM EGTA (lane 4), 37 mM EDTA (lane 5), 7.5 and 15 mM zinc acetate (lanes 6 and 7).

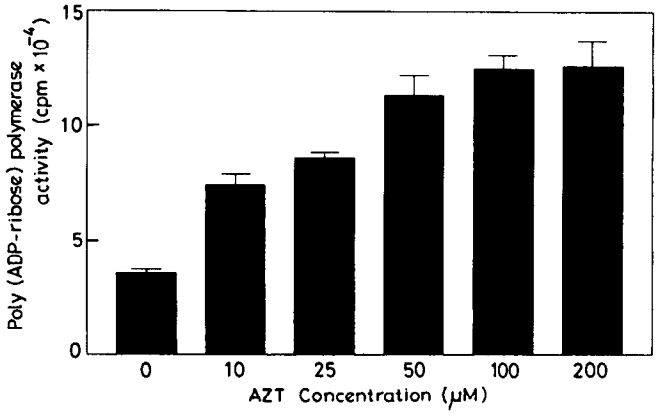

FIG. 6. Poly(ADP-ribose)polymerase activity. Sp2/0 cells were grown in the presence of 0.0, 10, 25, 50, 100, and 200 μM of AZT for 48 hr. The PARP activity was determined. SD was less than 10% of the mean of the triplicate values.

lesser extent, by cytidine in normal human granulocyte-macrophage progenitor cells [9].

Calcium-dependent endonuclease activated by glucocorticoids has been characterized in apoptotic rat thymocytes [24] and in physiological cell death mediated by cytotoxic T lymphocytes [20]. Endonuclease activity associated with high-molecular-weight and internucleosomal DNA fragmentation in apoptosis has been observed in thymocytes, liver, HL-60-, and IL-2-dependent CTLL cells [25]. The endonuclease involved in apoptosis has been characterized as DNase I and shown to be Ca^{2+}- and Mg^{2+}-dependent [26]. Zinc inhibits endonuclease activity and blocks cell death [24]. The enhanced endonuclease activity was observed in this study in the nuclear extract of AZT-treated Sp2/0 cells.

Apoptotic cell death associated with NAD depletion as the result of enhancement of PARP has been reported in some cell lines treated with different apoptotic inducers [27, 28]. Enhanced PARP activity plays a role in stress and adenosine-induced apoptosis in HL-60 and U937 cells [29, 30]. The enzyme is activated through its binding to a DNA strand break site. Enhanced PARP activity was observed in AZT-treated Sp2/0 cells. Cycloheximide was shown to induce apoptosis in HL-60, U937, Molt-4, Daudi, MRC-5, and Raji cells [31]. Apoptosis was induced when Sp2/0 cells were grown for 24 hr in the presence of cycloheximide (0.5 μg/mL) (data not shown). Typical characteristic apoptotic morphology and DNA ladder formation were observed when CaSki (human cervical epidermoid carcinoma) cells were treated with the same concentrations of AZT as used in the case of Sp2/0 cells (data not shown).

The apoptotic cell death brought about by AZT may be due to the following. AZT inhibits DNA synthesis by termination of DNA chain elongation [2]. AZT inhibits the exonucleolytic repair of chain-terminated DNA by its metabolite AZT-MP [11]. AZT depletes the dTTP pool by competing for thymidine kinase and thymidylate kinase and, also, inhibiting thymidylate kinase by its metabolite AZT-MP, thus, inducing perturbation in dNTP pool in the cell [10, 12, 13]. Perturbation of dNTP pools has been reported to trigger DNA fragmentation and cell death in mouse leukemia cells [14], mouse mammary tumor cells FM3A [32], and mouse thymocytes [33]. In this study, we conclude that AZT, which has been extensively used as an antiviral agent against AIDS, is apoptogenic and induces programmed cell death in the mouse myeloma cell line Sp2/0.

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