J Cancer Res Clin Oncol (2001) 127: 570–576
DOI 10.1007/s004320100264

ORIGINAL PAPER

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Thymidine-dependent attenuation of the mitochondrial apoptotic pathway in adenosine-induced apoptosis of HL-60 cells

Received: 14 April 2001 / Accepted: 17 May 2001 / Published online: 6 July 2001

Abstract *Objective*: We previously reported that adenosine-induced apoptosis in HL-60 cells was attenuated by cotreating the cells with pyrimidine nucleosides. The mechanism involved in this adenosine-induced apoptosis by the differential supply of nucleosides is studied here with a particular focus on the regulation of apoptosis-associated mitochondrial events. *Methods*: Time-dependent changes in the mitochondrial membrane potential (MMP) after treatment with adenosine and/or thymidine were monitored. *Results*: The cells did not show any decrease of MMP level up to 2.5 h after 1 mM adenosine exposure, whereas cytochrome c release, caspase-9 and caspase-3 activity, and DNA fragmentation were already activated, suggesting that mitochondrial depolarization is not a prerequisite of other apoptosis-related mitochondrial events. In contrast, the translocation of Bax to mitochondria and the release of cytochrome c began within the first hour of adenosine treatment. *Conclusion*: Thus, it is believed that adenosine-induced apoptosis is mediated by the activation of the caspase cascade by cytochrome c release with concomitant increase of Bax in the mitochondria, which implies that the translocation of Bax might be a leading event in the adenosine-induced apoptosis. Moreover, we found that most of the apoptotic parameters in adenosine-induced cellular changes, such as translocation of Bax, the release of cytochrome c, and the consequent activation of caspase-9 and caspase-3, were attenuated by thymidine supplement, thus indicating that the sensing of a nucleoside or nucleotide balance might be an upstream event of cytochrome c release. Therefore, it can be concluded that thymidine can attenuate adenosine-induced apoptosis by modulating the earliest stage of the mitochondrial apoptotic pathway.

Keywords Adenosine · Thymidine · Mitochondria · Bax translocation · Cytochrome c · Apoptosis

Abbreviations *MMP* mitochondrial membrane potential · *Ac-LEHD-AFC* acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin · *Ac-DEVD-AFC* acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin

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Introduction

It has recently been suggested that mitochondria play a crucial role in apoptosis (Reed 1997; Kroemer et al. 1998) by releasing apoptogenic factors, such as cytochrome c (Liu et al. 1996; Yang et al. 1997; Kluck et al. 1997) and apoptosis-inducing factor (AIF) (Susin et al. 1996) from the mitochondrial intermembrane space into the cytoplasm. Cytochrome c, the major apoptogenic factor, is located within the mitochondrial intermembrane space, where it plays an essential role as a constituent of the respiratory chain. The release of cytochrome c from the mitochondria to the cytosol is an important step in the apoptotic signaling pathway, which activates the caspase cascade (Li et al. 1997), and results in the subsequent proteolysis of mitochondrial substrates and induces various mitochondrial changes, such as a decreased membrane potential (Marchetti et al. 1996), release of proapoptotic factors, and the generation of reactive oxygen species (Buttke and Sandstrom 1994). Once released into the cytosol, cytochrome c forms a multiprotein complex with Apaf-1 and procaspase-9, the apoptosome, which initiates the activation of caspase-3 (Liu et al. 1996). However, the mechanism of cytochrome c release during the early apoptotic phase is not well understood.

Several groups have shown that Bax, Bcl-x_L, and Bcl-2 can create ionic channels in liposomes (Minn et al.

1997; Schendel et al. 1997; Antonsson et al. 1997; Shimizu et al. 1999). It has also been proposed that the proapoptotic Bax protein induces influx of ions into the mitochondria, followed by a swelling of the intermembrane space and the rupture of the outer mitochondrial membrane (Green and Reed 1998). To regulate Bax activity, Bcl-2 can block the channel by dimerizing with Bax (Wolter et al. 1997) or it may create channels working in the opposite direction to compensate for the effects of Bax (Schendel et al. 1997). Another hypothesis is based on the existence of the permeability transition pore (PTP), a large-conductance unselective channel localized in the inner mitochondrial membrane (Green and Reed 1998; Bernardi et al. 1994; Zoratti and Szabo 1995). Until recently, the opening of this channel was considered to be an important apoptotic step involving mitochondria.

In our previous study, we reported that adenosine-induced apoptosis is ameliorated by cotreatment with pyrimidine nucleosides, such as uridine or thymidine (Kim et al. 1998). Moreover, the protective effect of pyrimidine nucleosides upon adenosine-induced apoptosis has been suggested to depend upon the quantitative ratio between purine and pyrimidine nucleosides. However, the molecular mechanism whereby the nucleoside ratio works at the cellular level is unresolved.

Therefore, we analyzed the effect of pyrimidine nucleosides on adenosine-induced apoptosis from the aspect of mitochondrial events, since apoptosis is known to be initiated by the release of cytochrome c from the mitochondria. For this purpose, we studied the effect of adenosine and/or thymidine on the regulation of these apoptosis-associated parameters, and focussed in particular on the modulation of cytochrome c release from mitochondria in HL-60 human promyelocytic leukemia cells.

Materials and methods

Materials

Adenosine, thymidine, dipyridamole, 3-(4,5-dimethylthiazo 1-2-yl)-2,5-diphenylte trazolium bromide (MTT), and propidium iodide (PI) were purchased from Sigma (St. Louis, Mo., USA). RPMI-1640 medium and fetal bovine serum (FBS) were from Gibco-BRL (Gaithersburg, Md., USA). 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) an MMP-sensitive lipophilic cationic dye, was purchased from Molecular Probes (Eugene, Ore., USA). Mouse anti-cytochrome c monoclonal antibody (7H8.2C12) and polyclonal rabbit anti-human Bax antibody were obtained from Pharmingen (San Diego, Calif., USA). Caspase-9 substrate, Ac-LEHD-AFC, and caspase-3 substrate, Ac-DEVD-AFC, were from Bio-Rad (Hercules, Calif., USA). The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham (Bucks., UK), other reagents were obtained from Sigma.

Cell culture and treatment of reagents

Human promyelocytic leukemic HL-60 cells were obtained from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS in a 10-centimetre dish at a density of 1×10^5 cells/ml at 37°C in a humidified, CO_2-controlled (5%) incubator. Adenosine and thymidine were dissolved in RPMI-1640 culture medium and dipyridamole was dissolved in ethanol; the final concentration of ethanol in the culture medium was 0.1%.

Quantification of cytotoxicity by MTT assay

Aliquots of 0.5 ml of cell suspension were transferred into each well of a 24-well plate and MTT assay was carried out. The supernatant was then discarded by centrifugation and 1 ml of dimethylsulfoxide was added to dissolve the blue insoluble MTT formazan. The absorbance of the sample was measured at 570 nm in a spectrophotometer (Kontron Uvikon 930). The cytotoxicities of the reagents were calculated as the relative values of their absorbancies to the control.

Determination of apoptosis by flow cytometric analysis

After the cells had been subjected to the different treatments, they were harvested by centrifugation, washed with ice-cold PBS once, and fixed in 70% ethanol before being stored at 4 °C overnight. The cells were then washed once with ice-cold PBS and treated with 50 μg/ml Dnase-free Rnase A and 50 μg/ml of propidium iodide at room temperature for 30 min. In each sample, 1×10^4 cells were counted and analyzed with FACS Calibur using Cell Quest software (Becton Dickinson, San Jose, Calif., USA).

Analysis of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was determined as described by Reers et al. (Reers et al. 1995) with a slight modification. An MMP-sensitive lipophilic cationic dye, JC-1, was prepared as a stock solution (30 mM) in dimethyl sulfoxide. To prepare a working concentration, the stock solution was diluted to 10 mM with ethanol, and then added to the culture medium. 3×10^5 cells were incubated with 5 μM of JC-1 for 20 min in a culture medium at 37°C and in 5% CO_2. The cells were collected and washed once with ice-cold PBS. 1×10^4 cells were counted in each sample and analyzed using FACS Calibur with Cell Quest software (Becton Dickinson, San Jose, Calif., USA); the excitation wavelength used was 488 nm and emission was observed at 590 nm.

Western blot analysis

HL-60 cells were collected by centrifugation at 500 g for 5 min at 4°C. The cells were then washed twice with ice-cold PBS, pH 7.4. To obtain the cytosol and mitochondria-rich fraction, the cell pellets were resuspended in five volumes of extraction buffer, containing 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 2 mM, $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 68 mM sucrose, 220 mM mannitol, 1 mM PMSF, and protease inhibitors (Complete Cocktail, Boehringer Mannheim) (Bossy-Wetzel et al. 1998). After being incubated for 20 min on ice, the cells were homogenized with a glass Dounce and a tight pestle (Wheaton, Millville, N.J., USA). The cell homogenates were spun at 1,000 g to remove unbroken cells, nuclei, and heavy membranes, and the supernatant was respun at 14,000 g for 30 min to collect the mitochondria-rich (the pellet) and the cytosolic (the supernatant) fractions. The mitochondrial-rich fraction was washed once with the extraction buffer, and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EGTA) containing protease inhibitors. The protein concentration was measured by the BCA method and the samples were mixed with loading dye buffer and stored at –20°C. Fifty micrograms for the cytosolic fraction or 20 μg for the mitochondrial fraction were loaded onto each lane of an 5%/15% SDS-polyacrylamide gel, and

blotted onto a nitrocellulose transfer membrane (Schleicher & Schuell, Germany). Immunodetection of the blot was performed using anti-cytochrome c monoclonal antibody, 7H8.2C12, and polyclonal rabbit anti-human Bax antibody; the immunoreactive bands were visualized using an enhanced chemiluminescence kit as recommended by the manufacturer.

Measurement of caspase activity

The cells were washed once with ice-cold PBS, at pH 7.4, and collected by centrifugation at 1000 g for 5 min at 4°C. They were then resuspended in 100 µl of apopain lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 20 µg/ml leupeptin) and subjected to five freeze and thaw cycles by transferring them from a liquid nitrogen bath to a 37°C water bath. The cell extracts were centrifuged at 14,000 g for 30 min and 4°C and the supernatant was collected to measure the activities of the caspases. The activities of caspase-9 and caspase-3 were measured by the cleavage of the fluorogenic peptide substrates Ac-LEHD-AFC and Ac-DEVD-AFC, respectively, according to manufacture's instructions (Bio-Rad, Hercules, Calif., USA). One unit was defined as the amount of enzyme activity required to cleave 1 nmol of substrate per minute, and was expressed as units/mg of cytosolic protein.

Results

Attenuation of adenosine-induced cytotoxicity and apoptosis

HL-60 cells were incubated for 4 h in the presence of 1 mM adenosine and/or 2–10 mM thymidine. Adenosine (1 mM) reduced the viability of the HL-60 cells by over 55%, and this was dose-dependently attenuated by the addition of thymidine, up to a 50% reduction by 10 mM thymidine (Fig. 1). Figure 2A shows the electrophoregram of total nuclear DNA from HL-60 cells treated with 1 mM adenosine alone for 2 h (lane 3),

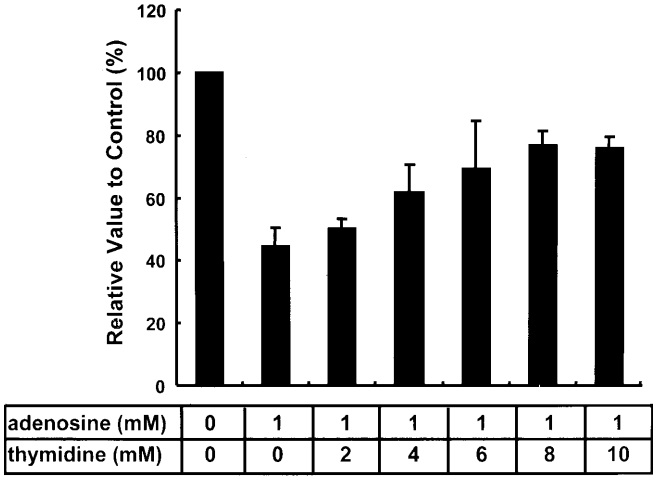

Fig. 1 Effect of adenosine and thymidine on the viability of HL-60 cells, as measured by MTT assay. HL-60 cells were treated with 1 mM adenosine alone (*lane 2*), 1 mM adenosine plus 2–10 mM thymidine (*lanes 3–7*) for 4 h, and with MTT for 2 h. All the results were obtained from three independent experiments, each with triplicate wells for every condition

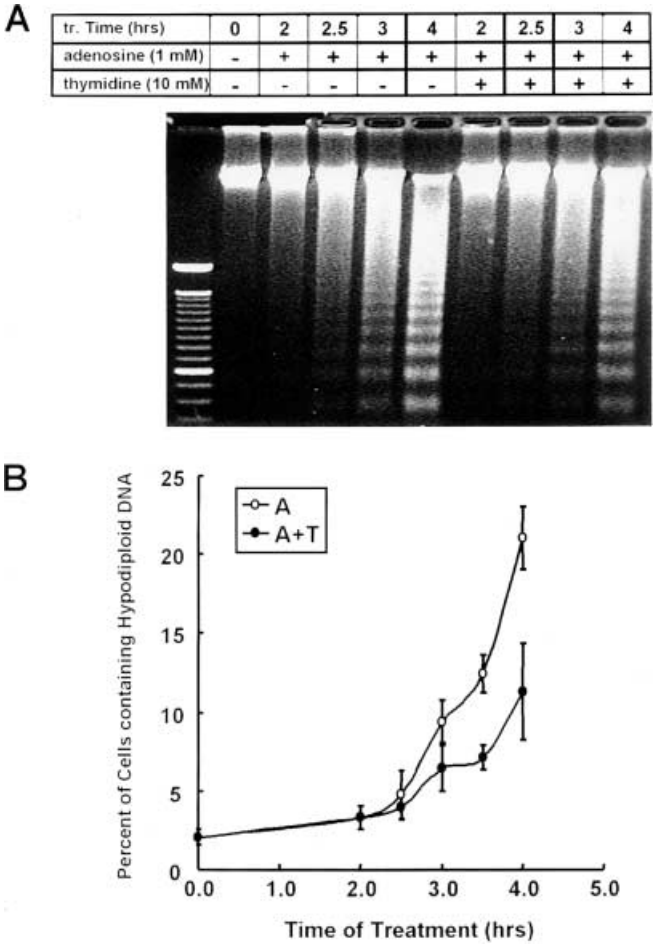

Fig. 2 Effect of adenosine and thymidine on the apoptosis of HL-60 cells. HL-60 cells were treated with 1 mM of adenosine and/or 10 mM thymidine for 0–4 h. Cells were harvested, fixed, stained with propidium iodide, and the rate of apoptosis was quantified by a flow cytometry. *A* 1 mM adenosine, *A* + *T* 1 mM adenosine plus 10 mM thymidine. All the results were obtained from three independent experiments, each with duplicate dishes for every condition

2.5 h (lane 4), 3 h (lane 5), 4 h (lane 6). The adenosine-induced DNA fragmentation increased in a time-dependent manner, which was also attenuated by the addition of thymidine (lanes 7–10). When HL-60 cells were treated with 1 mM adenosine for up to 4 h, a weak sub-diploid peak, below 5% of the total, was observed at 2.5 h, but it increased rapidly to over 21% at 4 h of treatment (Fig. 2). The thymidine (10 mM) supplement reduced the sub-diploid peak to about a half (11.3% of the total), suggesting a protective effect of thymidine in adenosine-induced apoptosis.

Modulation of cytochrome c release and mitochondrial membrane potential

We also examined whether or not the release of cytochrome c is implicated in adenosine-induced apoptosis. HL-60 cells were incubated with adenosine and/or

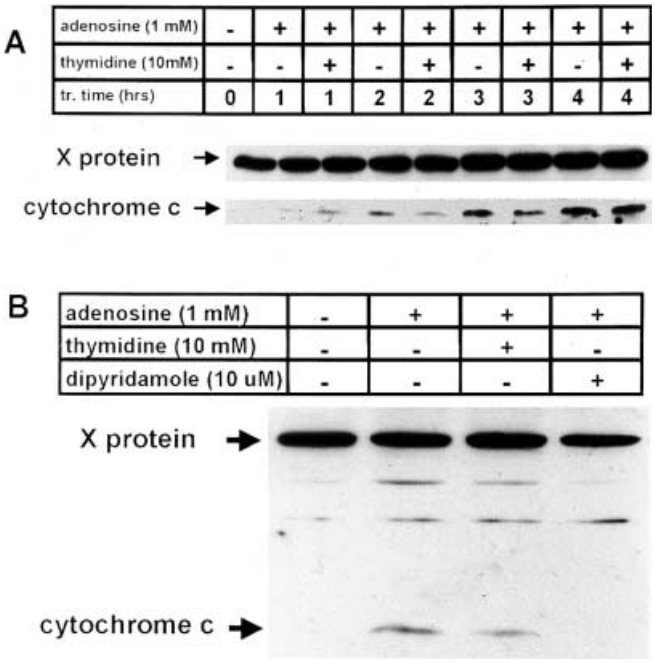

Fig. 3A,B Cytochrome c release in adenosine- and/or thymidine-treated HL-60 cells. **A** Time course analysis of cytochrome c release in adenosine- and/or thymidine-treated cells. HL-60 cells were incubated with 1 mM adenosine alone (*lanes 2*, *4*, *6*, *8*, respectively) and 1 mM adenosine plus 10 mM thymidine (*lanes 3*, *5*, *7*, *9*, respectively) for 1–4 h, respectively; **B** the effect of adenosine, thymidine, and dipyridamole, an inhibitor of the adenosine transporter, on the release of cytochrome c. HL-60 cells were incubated with 1 mM adenosine (*lane* 2), 1 mM adenosine plus 10 mM thymidine (*lane* 3), and 1 mM adenosine plus 10 μM dipyridamole (*lane* 4) for 3 h. The release of cytochrome c into the cytoplasm was determined by Western blot analysis using a specific antibody against cytochrome c, 7H8.2C12. Similar results were obtained from three independent experiments

thymidine for up to 4 h, and the release of cytochrome c into the cytoplasm from the mitochondria was determined by Western blot analysis using a specific antibody to cytochrome c. The cytosolic fraction from the nucleoside-treated cells were subjected to SDS-PAGE, and probed with the anti-cytochrome c antibody, 7H8.2C12. The adenosine treatment caused a release of cytochrome c into the cytoplasm, which was reduced by the thymidine supplement (Fig. 3A). The addition of dipyridamole, an inhibitor of the adenosine transporter, blocked the release of cytochrome c (Fig. 3B), suggesting that the transport of adenosine across the cytoplasmic membrane is a prerequisite for inducing apoptosis. Figure 3A shows the time-dependent increase of cytochrome c release by adenosine treatment. Note that the cytochrome c release was observed only 1 h after the adenosine treatment.

Mitochondrial depolarization and the subsequent release of pro-apoptotic factors have been suggested to be required for the activation of a cell death program in some forms of apoptosis. The time-dependent changes in MMP during apoptosis by adenosine and/or thymidine on HL-60 cells were examined using the MMP-sensitive

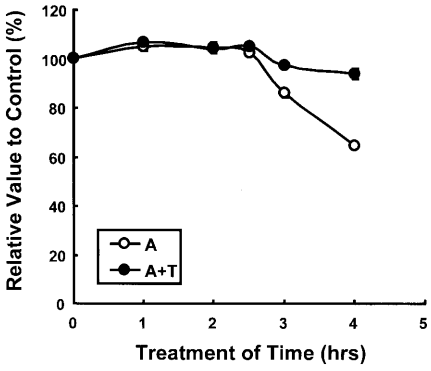

Fig. 4 The time course of changes in mitochondrial membrane potential (MMP) by adenosine and/or thymidine. HL-60 cells were treated with 1 mM of adenosine and/or 10 mM of thymidine for up to 4 h. MMP was analyzed with MMP-sensitive lipophilic cationic dye, JC-1, as described in Materials and Methods. *A* 1 mM adenosine, *A* + *T* 1 mM adenosine plus 10 mM thymidine. All the results were obtained from three independent experiments, each with triplicate dishes for every condition

lipophilic cationic dye, JC-1 as a probe for up to 4 h. Initially, the MMP of the adenosine-treated cells was slightly higher than that of the control cells. However, it reduced rapidly after only 2.5 h of treatment (Fig. 4), that was in contrast to the release of cytochrome c, which was evident after less than 1 h of treatment (Fig. 3A). The adenosine-induced reduction of MMP was inhibited by the thymidine supplement.

Activities of caspases in nucleoside-treated cells

It has been suggested that the release of cytochrome c from the mitochondria to the cytosol can initiate an apoptotic protease cascade (Kroemer et al. 1998; Yang et al. 1997; Kluck et al. 1997; Li et al. 1997; Kluck et al. 1997). In order to confirm that adenosine-induced apoptosis is mediated by this mitochondrial pathway, we measured the activities of caspase-9 and caspase-3 by a fluorometric assay using a caspase substrate, Ac-LEHD-AFC for caspase-9 and Ac-DEVD-AFC for caspase-3. The increased caspase-9 activity in the adenosine-treated cells was attenuated by co-treatment with thymidine after 2.5 h (Fig. 5B). Caspase-3 activity was maintained at the basal level for up to 1 h after the adenosine treatment, however, it then increased rapidly in the 3-h and 5-h-treated groups; this was reduced in the thymidine co-treated groups (Fig. 5A). These results suggest that thymidine supplementation of adenosine treated cells regulates events upstream of the activation of both caspase-9 and caspase-3.

Effect of nucleosides on the translocation of Bax protein

HL-60 cells were cultured for up to 4 h in the presence of adenosine and/or thymidine. A mitochondria-rich frac-

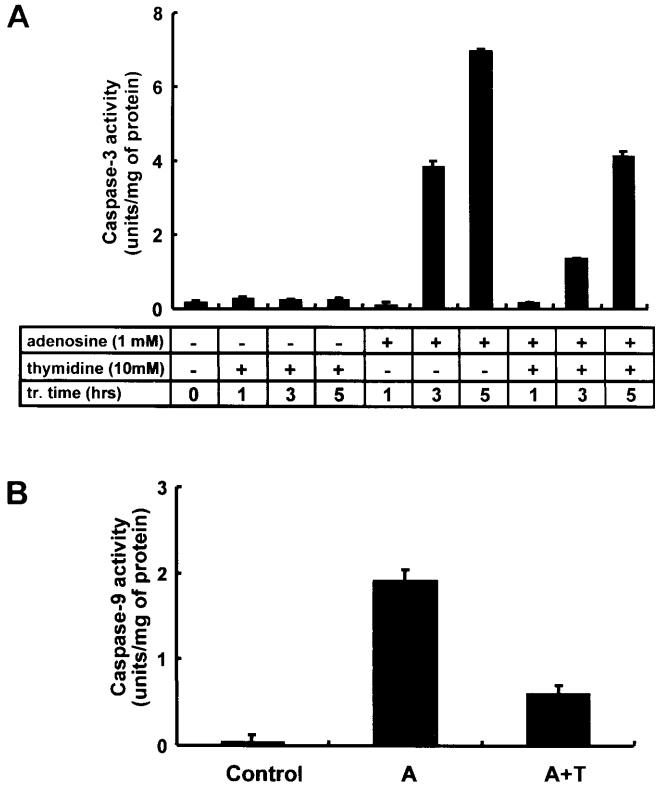

Fig. 5 Caspase activation by adenosine and/or thymidine in HL-60 cells. Caspase-9 activation: HL-60 cells were incubated without any treatment or in the presence of 1 mM of adenosine or 1 mM of adenosine plus 10 mM of thymidine for 2.5 h. Caspase-9 activity was measured by fluorometric assay using caspase-9 substrate, Ac-LEHD-AFC. *Control* mock, *A* 1 mM adenosine, *A* + *T* 1 mM adenosine plus 10 mM thymidine. Time course activation of caspase-3: HL-60 cells were incubated without any treatment or in the presence of 1 mM of adenosine, 10 mM of thymidine, or 1 mM of adenosine plus 10 mM of thymidine for 0, 1, 3, and 5 h, respectively. Caspase-3 activity was measured by fluorometric assay using caspase-3 as substrate, Ac-DEVD-AFC. The results shown are the average values from three independent experiments, each with duplicate dishes for every condition

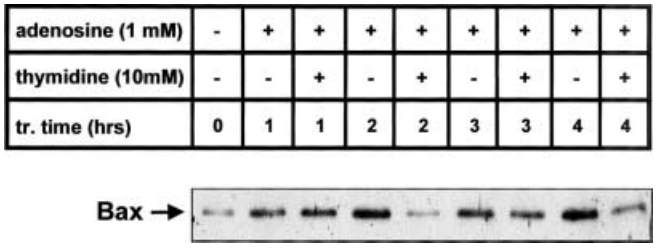

Fig. 6 The effect of adenosine and thymidine on the translocation of Bax protein from the cytosol to the mitochondria in HL-60 cells. HL-60 cells were incubated with 1 mM of adenosine (*lanes 2*, *4*, *6*, and *8*, respectively) or 1 mM of adenosine plus 10 mM of thymidine (*lanes 3*, *5*, *7*, and *9*, respectively) for 1–4 h, respectively. Similar results were obtained from three independent experiments

tion was prepared and subjected to SDS-PAGE. Figure 6 shows the time-dependent changes in mitochondria Bax protein following adenosine and/or thymidine treatment. The translocation of Bax protein was detected in the first hour of nucleoside treatment, which was attenuated by thymidine supplement. Therefore, this finding suggests that adenosine-induced apoptosis can be attenuated by thymidine supplement, probably via upstream regulation of Bax translocation or cytochrome c release.

Discussion

Mitochondria play important roles in the early events of apoptosis. Both mitochondrial membrane depolarization and the release of cytochrome c from the mitochondrial intermembrane space have been proposed to be early central events in apoptotic cell death, although the cause-and-effect relationship between these two events remains to be clarified (Yang et al. 1997; Kluck et al. 1997; Susin et al. 1997). Previously, we showed that extracellular adenosine at a concentration of several hundred micromoles/liter is cytotoxic and induces apoptosis in human promyelocytic leukemia HL-60 cells, which could be effectively attenuated by pyrimidine nucleoside supplementation (Kim et al. 1998). Subsequently, we extended this line of study to understand the molecular mechanism underlying adenosine-induced apoptosis and the attenuating effects of pyrimidine nucleosides, by focusing particularly on apoptosis-related mitochondrial events. Adenosine-induced apoptosis requires cytosolic uptake of adenosine, since the inhibition of adenosine transport by dipyridamole prevents apoptosis (Kim et al. 1998; Tanaka et al. 1994; Pearson et al. 1978; Wakade et al. 1995; Lewis et al. 1997). Adenosine, once transported into the cytoplasm, can be metabolized by several enzymes, including adenosine kinase and adenosine deaminase, into various adenosine derivatives, while an excess intake of adenosine leads to growth inhibition and apoptosis, probably due to the overproduction of dATP (Liu et al. 1996) and the consequent nucleotide imbalance (Weisman et al. 1988; Hugo et al. 1992). As was shown in our previous study (Kim et al. 1998), thymidine protects against adenosine-induced apoptosis in a dose- and time-dependent manner (Figs. 1 and 2). These results suggest that adenosine-induced apoptosis can be regulated by simply adjusting the intracellular purine/pyrimidine nucleoside or nucleotide ratio. However, the mechanism of apoptosis-modulation by adjusting the nucleoside or nucleotide is still not elucidated.

It is currently believed that perturbed mitochondrial function results in a release of cytochrome c, which binds to Apaf-1. Subsequently, the cytochrome c-Apaf-1-caspase-9 complex activates caspase-3, which in turn cleaves DFF, a heterodimeric DNA fragmentation factor, and leads to nuclear DNA fragmentation (Liu et al. 1997). In the present study, we monitored the sequential steps of the apoptotic phenomena including cytochrome c release, caspase-9 and caspase-3 activation, and nuclear DNA fragmentation in response to adenosine and/or thymidine treatment (Figs. 2, 3, and 5). The addition of

thymidine significantly modified all the parameters of apoptosis, induced by adenosine, such as the release of cytochrome c (Fig. 3), the activation of caspase-9 and caspase-3 (Fig. 5) as well as the subdiploid DNA peak (Fig. 2). These results indicate that thymidine affects the earliest steps of the mitochondrial pathway of adenosine-induced apoptosis. Therefore, we studied the regulatory mechanism of cytochrome c release by nucleosides, a prerequisite of the above apoptotic changes.

We examined the time-dependent changes required for the release of cytochrome c and the decrease of mitochondrial membrane potential in response to apoptosis induction. Mitochondrial membrane depolarization was reported to be an early event in apoptosis, but there has been some debate on the timing of the cytochrome c release (Zamzami et al. 1995; Zamzami et al. 1996). In fact, our result showed that adenosine reduced the mitochondrial membrane potential (Fig. 4), only 2.5 h after treatment, while the cytochrome c release was evident already 1 h after treatment (Fig. 3A). Therefore, it is likely that cytochrome c release precedes membrane depolarization in adenosine-induced apoptosis. Moreover, it has been suggested recently that caspases, activated by the cytochrome c, may disrupt the mitochondrial membrane and reduce the membrane potential (Finucane et al. 1999b; Krohn et al. 1999; Bossy-Wetzel and Green 1999; Marzo et al. 1998c). In the present study, both the adenosine-induced decrease of mitochondrial membrane potential and the release of cytochrome c were found to be reduced by thymidine cotreatment (Figs. 3A and 4). The essential role of adenosine in the release of cytochrome c was confirmed by dipyridamole treatment, since the inhibition of adenosine uptake blocked cytochrome c release and consequent apoptosis (Fig. 3B).

It has been proposed that the translocation of Bax, a pro-apoptotic protein, from the cytosol to the mitochondria is a key mechanism for the release of cytochrome c (Wolter et al. 1997; Gross et al. 1998; Marzo et al. 1998a). Therefore, we followed the translocation of pro-apoptotic Bax, implicated in cytochrome c release and caspase activation (Marzo et al. 1998a; Finucane et al. 1999a; Clem et al. 1998). A close correlation was observed between the release of cytochrome c and the translocation of the Bax protein in HL-60 cells after adenosine treatment. As shown in Fig. 6, the translocation of Bax occurred almost concomitantly with the release of cytochrome c (Fig. 3A), indicating the Bax translocation is strongly associated with cytochrome c release.

The release of cytochrome c by mitochondria was reported to be conducted through a permeability transition (PT) pore, which consists of a voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), and cyclophiline D. This PT pore allows the exchange of ATP/ADP, as well as of water and solutes, with the mitochondria (Marzo et al. 1998b). In the early stages of apoptosis, cytochrome c is released by altering the permeability of the PT pores, which is controlled by Bcl-2 family proteins (Finucane et al. 1999a; Narita et al. 1998; Reed et al. 1998). In addition, it has been suggested that pro-apoptotic Bax protein interacts with both the mitochondrial ANT and VDAC (Marzo et al. 1998a), which alters PT pore permeability to cytochrome c, whereas the anti-apoptotic Bcl-x_L was found to play a role in maintaining mitochondrial ATP/ADP exchange, thereby protecting the cells from apoptosis (Vander Heiden et al. 1999). The efficient exchange of ADP for ATP via ANT permits oxidative phosphorylation to be regulated in response to cellular ATP/ADP levels and allows mitochondria to adapt to changes in metabolic demand. Therefore, it is possible that intracellular nucleotide or nucleoside ratio changes affect the efficiency of the permeability transition pore, through a modulation of the mitochondrial translocation of Bax. Moreover, when the functional efficiency of ANT is impaired, the resulting deregulation of adenosine nucleotides may induce reactive oxygen species generation, which would damage the cells. In addition, the recent finding that one type of NDPK (nucleoside diphosphate kinase) namely nm23-H6, is localized in the mitochondrial fraction (Tsuiki et al. 1999) implies that active exchanges of nucleotides occur. These findings suggest the possibility of the active sensing of nucleotides or nucleosides by mitochondrial or epimitochondrial components, which could regulate energetic efficiency for survival or permeability transition pore activity for apoptosis.

In conclusion, we suggest that adenosine-induced apoptosis is modulated by thymidine supplementation, probably through an alteration of the cellular purine/pyrimidine nucleoside and/or nucleotide balance at the earliest stage of the mitochondrial apoptotic pathway.

Acknowledgements The authors would like to thank Mrs. Eun-Kyung Kim, Seoul National University Hospital Clinical Research Institute, for her skillful technical support for FACS. This work has been supported partially by grants from Seoul National University Hospital and the Korea Research Foundation for Health Science.

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