EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 29, No 3, 183-190, September 1997

Active adenosine transporter-mediated adenosine entrance into HL-60 cells leads to the induction of apoptosis through down-regulation of c-Myc

Seong-Hye Hong,[1] Seong-Yun Jeong,[1] Sang-Gun Ahn,[1] In-Sung Hwang[1] and In-Kyung Kim[1,2,3]

1 Department of Biochemistry, Catholic University Medical College, Seoul 137-701, Korea

2 Research Institute of Molecular Genetics, Catholic Research Institutes of Medical Science, Catholic University Medical College, Seoul 137-701, Korea

3 Corresponding author. Department of Biochemistry, Catholic University Medical College, 505 Banpo-Dong, Socho-Ku, Seoul 137-701, Korea

Accepted 5 September 1997

Abbreviations: Ado, adenisine; DPD, dipyridamole; AdoHcy, *S*-adenosylhomocystine; NBTI, S-(4-nitrobenzyl)-6-thioinosine; DZAri, 3-deazaaristeromycin; DZAHcy, 3-deazaadenosylhomocystine; AdoHcyase, AdoHcy hydrolase

Abstract

Although adenosine (Ado) is being recently recognized as a potent inducer of apoptosis, molecular mechanism of apoptosis by Ado remains to be elucidated. In this study we observed that c-Myc was rapidly down-regulated in the apoptosis in human promyelocytic leukemia HL-60 cells treated with Ado. To establish the molecular and biochemical mechanisms of apoptosis, we tested the specific effects of several antagonists of Ado receptors or inhibitors of Ado transporter on the induction of apoptosis. Treatment of dipyridamole (DPD), an Ado transport inhibitor, effectively suppressed both c-Myc reduction and DNA fragmentation, suggesting that the induction of apoptosis and down-regulation of c-Myc is mediated by active Ado transporter. It was another evidence supporting the entrance of Ado into cells undergoing apoptosis that Ado cytotoxicity was potentiated by a addition of methylation cycle intermediates. These results suggest that the active Ado transporter-mediated Ado entrance into HL-60 cells leads to the induction of apoptosis through down-regulation of c-Myc.

Keywords: adenosine, adenosine transporter, apoptosis, c-Myc, HL-60 cells

Introduction

Apoptosis, one of physiological modes of cell death, is an endogenous cell suicide mechanism that is selectively triggered in response to various stimuli (Kerr *et al*., 1972). This is an active, energy-dependent process characterized by stereotypical morphological changes such as cell shrinkage, nuclear condensation, and finally fragmentation of both nucleus and cytoplasm leading to apoptotic bodies (Wadewitz and Lockshin, 1988; Gerchenson and Rotello, 1992). Oncogenes such as *c-myc* and *bcl-2* (Sullivan and Willis, 1989; Hockenbery *et al*., 1990), tumor suppressor genes including *RB* and *p53* (Benveniste and Cohen, 1995; Szekely *et al*., 1992), and proteolytic enzymes (Wyllie, 1980; Arends *et al*., 1990) have been reported to be associated with apoptosis. Among them, c-Myc is one of the most potent mediator for the programmed cell death. However, there are discrepancies in reports showing the relation of apoptosis with the regulation of c-Myc oncoprotein (Onclercq *et al*., 1989; Bertrand *et al*., 1991; Evan *et al*., 1992; Askew *et al*., 1993; Packham and Cleveland, 1995), implicating cell specificity of regulatory mechanism.

Adenosine (Ado) is known to be an important intracellular molecule acting as an oscillator for circadian rhythm and a ubiquitous neuro-modulator in mammalian central nervous system (Sanchez, 1995; Fredholm *et al*., 1994). Since cytotoxicity of abnormally accumulated Ado, first observed in adenosine deaminase deficiency disease and severe combined immunodeficiency disease (SCID) (Giblett *et al*., 1972; Carson and Seegmiller, 1976; Cohen *et al*., 1978), has been known to lead apoptosis, Ado is recognized as a strong inducer of apoptosis in various normal and tumor cells. Its intracellular increase in such diseases leads an idea that an Ado transporter might play an important role in the entrance of Ado into the cells (Kizaki *et al*., 1990; Abbrachio *et al*., 1995). However, several lines of evidence suggest that Ado-induced apoptosis was mediated by Ado specific membrane-bound receptors, like its other functions (Tanaka *et al*., 1994; Wakade *et al*., 1994).

We previously reported that apoptosis was induced by Ado in mouse leukemia L1210 cells with a preceding and transient expression of c-Myc (Kim *et al*., 1994). A human leukemia cell line, HL-60 cells essentially overexpress c-Myc oncoprotein in a basal condition. Thus, the HL-60 cells might be a good model for testing the effects of Ado on apoptosis under c-Myc amplified state.

In this study, we examined the regulation of c-Myc oncoprotein in Ado-treated HL-60 cells as well as the entrance mechanism of Ado into the cells. We have demonstrated here that Ado-induced apoptosis in HL-60

cells, consequently resulting in rapid down-regulation of c-Myc oncoprotein before appearance of morphological apoptotic features, is mediated by active Ado transporter.

Materials and Methods

Reagents

Ado, theophylline, caffeine, dipyridamole (DPD) *S*-adenosylhomocystine (AdoHcy) were obtained from Sigma Chemical Co. (St. Louis, MO) and *S*-(4-nitrobenzy1)-6-thioinosine (NBTI) was purchased from Research Biochemicals International (Natick, MA). 3-Deazaaristeromycin (DZAri) and 3-deazaadenosylhomocystine (DZAHcy) were donated by Dr. Chiang of the Walter Reed Army Institute of Research, Washington DC. Other reagents not described here were from Sigma Chemical Co. and otherwise specified.

Cell cultures and induction of apoptosis

Human promyelocytic leukemia HL-60 cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated (56˚C for 30 min) fetal bovine serum (FBS) (Hyclone, Logan, UT) and 50 μg/ml of gentamycin, 20 mM HEPES, 25 mM sodium bicarbonate in a humidified atmosphere containing 5% (v/v) CO_2 at 37˚C. To examine the effects of Ado in HL-60 cells, cells were plated at a density of 1×10^6 cells/ml in 12-well plates and treated with Ado. To study the signalling pathway of Ado-induced apoptosis, Ado receptor inhibitors (theophylline, 100 μM), Ado active transport inhibitor (DPD, 100 μM) or nucleoside uptake inhibitor (NBTI, 100 μM) were co-treated with Ado or pre-treated for 1 h before Ado addition. And in the test on methylation cycle intermediates were added at non-toxic doses to Ado-treated cells.

DNA fragmentation analysis

After treatment, DNA was extracted from HL-60 cells as described (Lee *et al*., 1995) and 10 μg of extracted DNA was electrophoresed in 1.5% agarose gel. For the quantitative analysis of DNA fragmentation, the amount of intact and fragmented DNA was quantitated by the modified diphenylamine method as described. Cells were lysed with hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100 and lysates were centrifuged at 13,000 *g*. Diphenylamine reagents [0.15 g diphenylamine (Fisher Scientific, Fairlawn, NJ), 0.15 ml sulfuric acid, 0.05 ml acetaldehyde in 10 ml glacial acetic acid] were added to each tube. After color-development overnight, optical densities were read at 570 nm. The degree of DNA fragmentation was calculated as the percentage of fragmented DNA to total DNA (sum of fragmented DNA and intact DNA).

Examination of cell morphology

Ado-treated cells were fixed for 1 h in 4F-1G fixation solution [$NaH_2PO_4H_2O$ 11.6 g, 50% (w/v) glutaraldehyde 20 ml, NaOH 2.7 g, 40% (v/v) formaldehyde 100ml, made up 1L with H_2O]. After dehydration cells were embedded in epon resins and sectioned. The ultrathin sections were mounted on nikel grids, stained in a LKB stainer containing uranyl acetate and lead citrate, photographed with a JEM-1200 EX (Jeol, Tokyo, Japan) electron microscope at an accelerating voltage of 80 kV.

Western blot analysis

Total cellular proteins prepared from cells lysed in lysis buffer (Laemmli, 1975) were separated by 5-15% gradient SDS-PAGE and then electroblotted onto a nitrocellulose membrane. After blocking with 5% non-fat milk in PBS, the membrane was incubated with a mouse monoclonal antibody against human c-Myc protein (Oncogene Science, Cambridge, UK) for 1 h at room temperature. The blots were washed twice with PBS (room temperature for 15 min) and incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G as a secondary antibody (Promega, Madison, WI) for 1 h at room temperature. After washing twice with PBS (room temperature for 15 min), the proteins were visualized by enhanced chemiluminescence system (Amersham, Buckinghamshire, UK). The protein bands developed in X-ray film were quantitated by Image Documentation (Stratagene, La Jolla, CA).

Northern blot analysis

Total RNA from HL-60 cells were extracted with RNAzol B (TEL-TEST, Inc., Friendswood, TX). Equal amount of RNA was separated on a denaturing agarose gel (1% agarose containing 2.2 M formaldehyde) and then transferred to a nylon filter (Boehringer Mannheim, Mannheim, Germany) by capillary action with 20 × standard saline citrate (SSC). After the filter was UV-crosslinked (UV-stratalinker 1800, Stratagene), the filter was prehybridized in high SDS buffer [7% SDS, 50% formamide, 5 × SSC, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 50 mM sodium phosphate pH 7.0, 0.1% (w/v) *N*-lauroylsarcosine] at 50˚C for 2 h, and digoxigenin (DIG)-labelled human *c-myc* probe prepared from specific primer and cDNA fragment (Amplimer sets, Clontech, Palo Alto, CA) by the polymerase chain reaction was added. After hybridization overnight at 50˚C, the nylon membrane was rinsed twice with 2 × SSC, 0.1% SDS for 5 min at room temperature, and then sequentially washed twice with 0.1 × SSC, 0.1% SDS for 15 min at 68˚C. DIG-labelled probes were detected by chemiluminescence after treatment with alkaline phosphatase-conjugated antibody against DIG and chemiluminescent substrate, disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenylphos-phate (CSPD) (Boehringer

Mannheim, Mannheim, Germany).

Results

Adenosine induces apoptosis in HL-60 cells

To assess the apoptotic effects of Ado in HL-60 cells, DNA was extracted from the cells treated with Ado and then analyzed by agarose gel electrophoresis. As shown in Figure 1B, DNA fragmentation into ladder was observed in the sample treated with 1 mM Ado. This effect of Ado for DNA fragmentation was progressively increased as time dependently as shown in Figure 1A. In these data, we demonstrated that Ado induces DNA fragmentation as a time- and dose-dependent manner, and these findings are supported by previous other reports (Kizaki *et al*., 1990; Tanaka *et al*., 1994; Wakade *et al*., 1994; Abbrachio *et al*., 1995). The percentages of DNA fragmentation were analyzed by the modified diphenylamine method described in methods. As the time of Ado treatment was increased, the ratio of DNA fragmentation was augmented to 7% after 2 h, 17.5% after 4 h and 40.1% after 6 h (Figure 1C).

To further characterize the morphological effect of Ado on the HL-60 cells, the cells exposed to 100 μM of Ado were examined by transmission electron microscopy (Figure 2). Untreated cells showed intact chromatin, cellular organelles and plasma membrane (Figure 2A). After 3 h of treatment of Ado, we observed the typical apoptosis in early stage showing margination of condensed chromatin and cells are started to become fragmentation in both nucleus and cytoplasm as typical apoptotic bodies (Figure 2B). Apoptotic bodies were mostly shown in cells treated with Ado cells 5 h (Figure 2C).

In these data, we demonstrated that Ado is a very effective and strong inducer of apoptosis in the HL-60 cells. Judging from this time schedule of apoptosis as above, we determined the most appropriate experimental condition

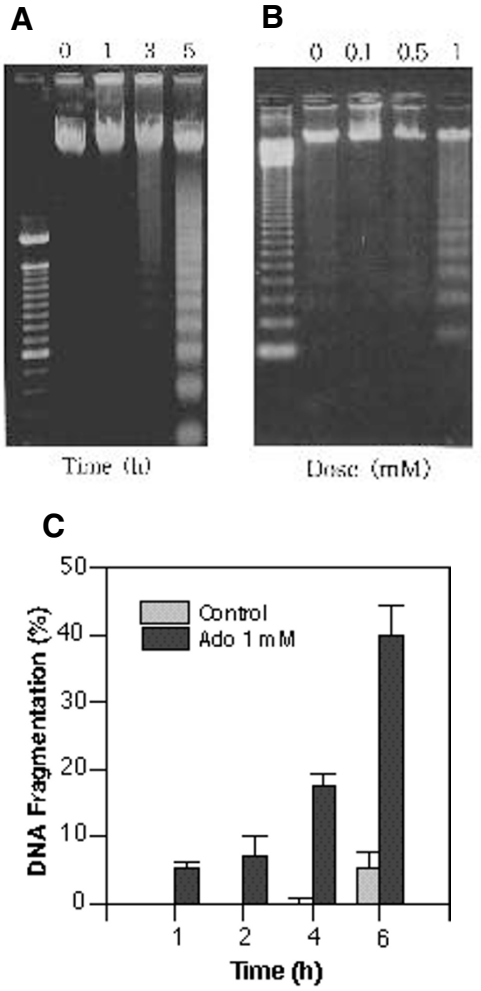

Figure 1. Analysis of DNA fragmentation in HL-60 cells by Ado. Agarose gel electrophoresis of DNA from HL-60 cells treated with 1 mM Ado for various times **(A)** and with various concentrations of Ado for 4 h (B). Lanes 1 shows 123 bp **(A)** and 100bp **(B)** DNA size markers, and lane 2 is untreated control, respectively. **(C)** Time-dependent kinetics of DNA fragmentation following 1mM Ado treatment. Cells (1×10^6/ml) were treated with Ado for the indicated times was analyzed by diphenylamine method.

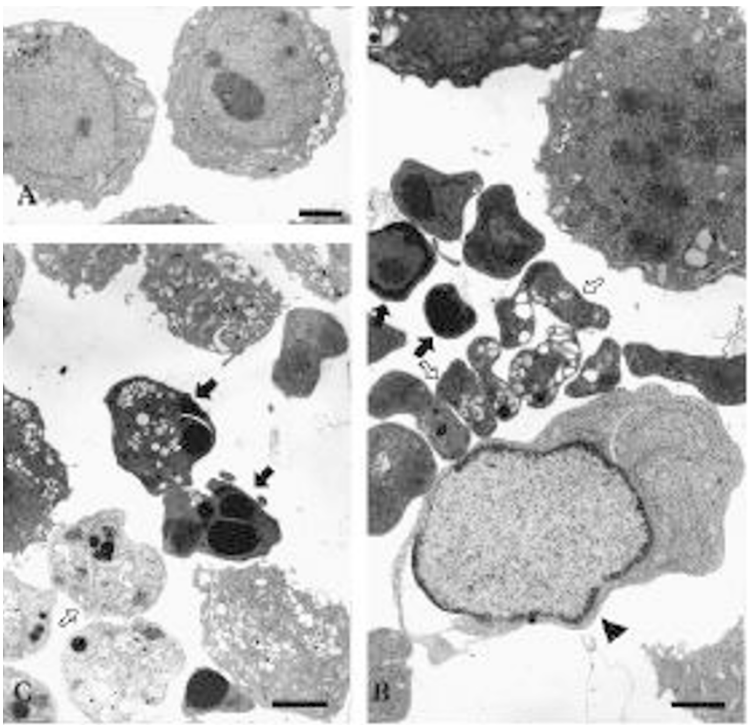

Figure 2. Transmission electron micrographs demonstrating normal (**A**; ×4,800) and various stages of apoptotic HL-60 cells after treatment with 100 μM of Ado for 3 h (**B**; ×4,600) or 5 h (**C**; ×4,600). Margination of condensed chromatin (arrow head) and apoptotic bodies with (closed arrows) or without (open arrows) nuclear fragments were shown. Bars, 2 μm.

for inducing apoptosis as incubation with 1 mM of Ado for at least 3 h, followed by all next experiment in our study.

c-Myc oncoprotein is down-regulated by Ado

Since Ado-induced apoptosis is associated with change of c-Myc oncoprotein level (Packham and Cleveland, 1995), we tested whether the level of c-Myc oncoprotein is modulated by Ado (Figure 3A). The levels of protein production were determined by Western blot analysis using c-Myc antibody. As the incubation time with Ado was increased, the production of c-Myc 1 and 2 oncoproteins were rapidly reduced as a time-dependent manner. Only 54.5% of control level remained after 30 min, 22.7% after 1 h, and undetected levels after 3 h. Next we examined the transcriptional level of c-Myc in Ado-treated HL-60 cells. As shown in Figure 3B, the amount of its mRNA was started to decrease after 10 min of incubation with 1 mM of Ado. While the protein was still detected up to 1 h, the expression of c-myc mRNA was not detected at the same time. This result shows that the level of c-Myc expression is down-regulated at the relatively early stage of apoptosis induced by Ado in HL-60 cells.

Active Ado transport system mediates apoptosis in HL-60 cells

Although several groups reported that the induction of apoptosis by Ado in HL-60 cells was mediated by activation of membrane-bound Ado receptors (Kizaki *et al*., 1990; Abbrachio *et al*., 1995), Ado membrane transporter is known to be responsible for intracellular accumulation of Ado (Thampy and Barnes, 1983), which is the major cause of apoptotic cell death in SCID patients, suggesting that the induction of apoptosis in HL-60 cells may be modulated through Ado membrane transporter. To investigate whether apoptotic cell death occurred in HL-60 cells either by Ado membrane transporter or Ado receptor, we tested the effects of several antagonists of Ado recepter or active Ado transport inhibitors on apoptosis of HL-60 cells treated with Ado. HL-60 cells were treated with 1 mM Ado alone or with Ado and other reagents at 100 μM. The concentration of reagents used in this study did not show any harmful effects on the proliferation or survival of HL-60 cells (data not shown). Theophyline (Figure 4A) and caffeine (data not shown) known as non-specific Ado receptor antagonists such as A1, and cyclopentylxanthine did not have any effects on DNA fragmentation induced by Ado (data not shown). However, 100 μM of DPD, well-known Ado transport inhibitor, or NBTI, a nucleoside uptake inhibitor, effectively suppressed DNA fragmentation caused by treatment of Ado for 3 h (Figure 4A). The blocking ability was not enough to pertain intact chromatin, but showed satisfactory results in protection from fragmentation into low molecular DNA fragments. When DPD was tested for 1 h before Ado addition, its inhibitory effect disappeared (lane 7 in Figure 4B). The results shown in Figure 4 indicate that Ado specific transporter acts as a

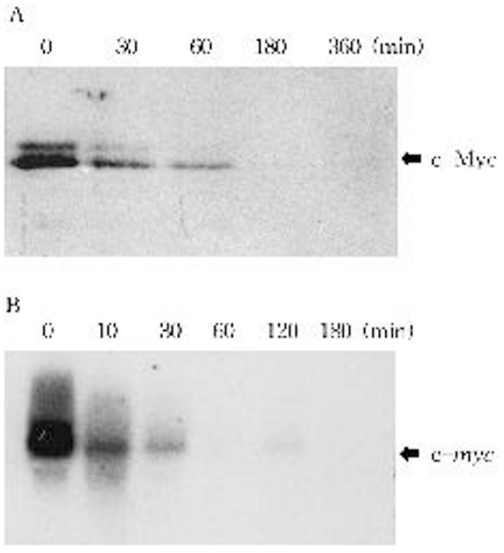

Figure 3. The c-myc expression in Ado-treated HL-60 cells. **(A)** Immunoblot analysis of c-Myc. Cells (1×10^6/ml) were treated with 1mM Ado for indicated times. c-Myc protein was revealed by indirect immuno-peroxidase method using enhanced chemiluminescence detection reagents. **(B)** Northern blot analysis of the expression of *c-myc* proto-oncogene in HL-60 cells. After extraction of total RNA from HL-60 cells treated with 1 mM Ado for the indicated times, 25 μg of them were loaded. *c-myc* mRNA was detected using DIG-labeled probe.

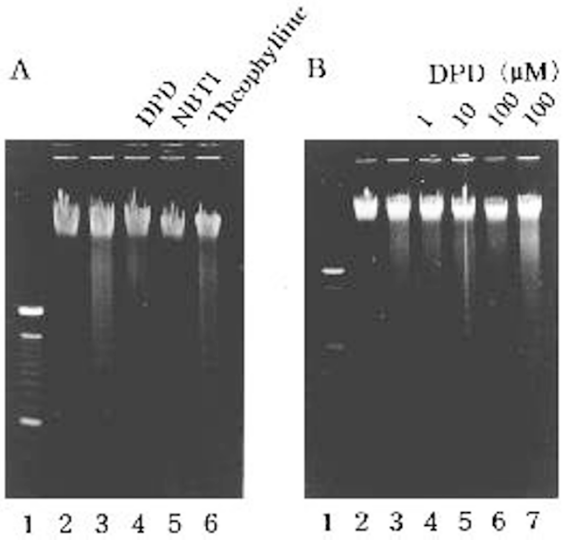

Figure 4. Effects of Ado active transport inhibitors or Ado receptor antagonists. **(A)** Cells (1×10^6/ml) were treated with 1 mM Ado alone (lane3) or co-treated with other agents (lane 4-6, 100 μM respectively) for 3 h. **(B)** Ado (1 mM) was treated alone (lane 3), co-treated with DPD for 3 h (lane 4-6) or treated 1h after DPD treatment (lane 7) at indicated concentrations. Lane 1 is 100 bp DNA size markers and lane 2 is untreated control cells, respectively.

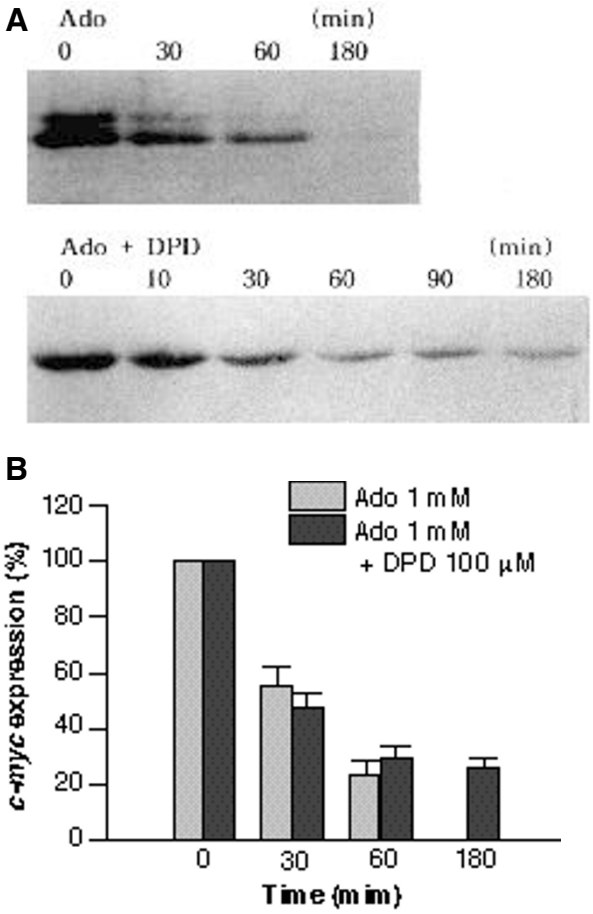

Figure 5. The effect of DPD on the c-Myc expression. After treatment of Ado alone or co-treatment with DPD for indicated times in HL-60 cells (1×10^6/ml), immunoblot analysis was processed with c-Myc monoclonal antibody. The level of c-myc expression was quantitated using image documentation system.

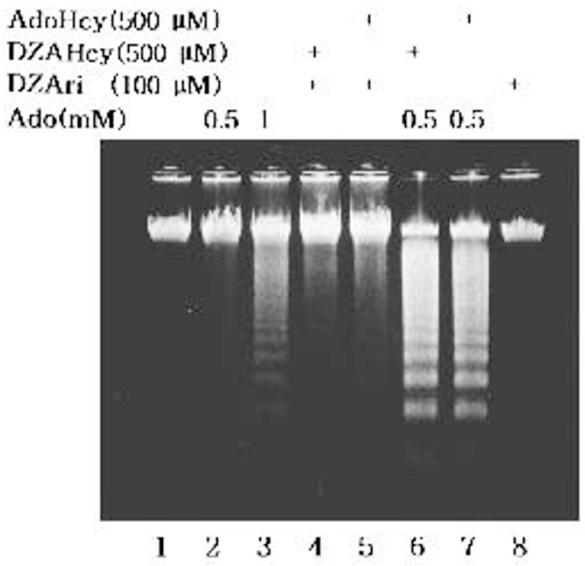

Figure 6. The stimulatory effect of DZAHcy or AdoHcy on Ado-induced apoptosis. HL-60 cells (1×10^6/ml) were treated with Ado alone at the indicated concentrations or co-treated with DZAHcy or AdoHcy of 500 μM for 3 h. In the case of DZAri, it was pre-treated 3 h before other agents addition at 100 μM.

mediator of Ado uptake into the cells, in contrast to the case of Ado receptor-mediated entrance, resulting in the induction of apoptotic cell death in HL-60 cells. Also, it was reported that from other studies that A1 and A2 Ado receptors do not have a major contribution to apoptosis in HL-60 cells (Tanaka *et al*., 1994; Kohno *et al*., 1996).

Our hypothesis inferred from the inhibitory effect of DPD on Ado-induced DNA fragmentation was tested by Western blot analysis of c-Myc oncoprotein after co-treatment of DPD with Ado in HL-60 cells. c-Myc oncoprotein was still expressed as co-treated with DPD at 100 μM, while the production of c-Myc was not observed in the cells treated with Ado alone for 3 h (Figure 5). Results shown in both Figure 4 and 5 indicate that the reduction of c-Myc oncoprotein was performed by Ado which is actively transported into the cells. Additionally, incomplete recovery of c-Myc diminution, enough to relieve from the apoptotic cell death in these cells, be caused by incomplete blocking of Ado entrance by DPD.

AdoHcy has a synergistic effect on DNA fragmentation by Ado

It is well illustrated that Ado reacts with L-homocysteine (Hcy) to form AdoHcy by *S*-adenosylhomocystine hydrolase (AdoHcyase) as the concentration of Ado was increased inside of the cells. The increased concentration of AdoHcy potently inhibits *S*-adenosyl-L-methionine-mediated reactions, which modulate donation of methyl groups to DNA, RNA, and protein (Cantoni *et al*., 1979), resulting in hypersensitivity of the hypomethylated DNA to nucleases (Lewis and Bird, 1991). Loennechen *et al*. (1989) reported that methylation cycle was interrupted by DZAri (Lewis and Bird, 1991), a potent inhibitor of AdoHcyase (Kim *et al*., 1982), DZAHcy, and AdoHcy.

To investigate the effects of methylation intermediates on Ado cytotoxicity in HL-60 cells, we exmined the DNA fragmentation followed by co-treatment of Ado with AdoHcy. In the experiment shown in Figure 6, DNA fragmentation was stimulated at the marginal level when either AdoHcy or DZAHcy was treated with DZAri, a potent inhibitor of AdoHcyase. In cells that Ado was co-treated with AdoHcy or DZAHcy, however, Ado-induced DNA fragmentation was stimulated more than two times that expected if they acted additively. Therefore, these reagents are able to cooperate to produce synergistic stimulation of Ado-induced DNA fragmentation. This observation suggests that the accumulation of AdoHcy

and DZAHcy leads to inhibit to *S*-adenosyl L-methionine mediated DNA to nuclease. Therefore, it seems that apoptosis may be due to the combined action with a methylation-dependent manner of DNA.

Discussion

We have demonstrated that Ado-induced apoptosis in HL-60 cells is mediated through an active Ado transporter, consequently resulting in rapid down-regulation of c-Myc oncoprotein before appearance of morphological apoptotic features. The rapid reduction of c-Myc expression in both mRNA and protein level indicates that the production of c-Myc oncoprotein is regulated in early step of the apoptotic process in response to Ado surpassing threshold level. Our data show that reduction of c-Myc level lasted for 6 h describes that apoptosis is a stochastic process, with individual cells committing to the programmed death over an extended time (Packham and Cleveland, 1995). Human promyelocytic leukemia HL-60 cells are tumor cells that constitutively express large amount of c-Myc proteins (Little *et al*., 1983). It seems that these cells respond to the powerful signals of proliferation which is originated from overexpression of c-Myc and take place as stable tumor cells, perhaps through obtaining circumstance which supplies factors for survival apoptotic suppression, as in the rare reports (Ryan and Birnie, 1996).

Our results that DNA ladder formation was inhibited by DPD, a Ado transporter inhibitor, however, it was not influenced by specific Ado receptor antagonists (Figure 4B) suggest that DNA fragmentation is may be mediated come from membrane-bounded Ado receptors. Also, several lines of evidence in other studies suggested that Ado-induced apoptosis in HL-60 cells was not mediated by Ado-specific A1 or A2 receptor. Tanaka *et al*. (1994) demonstrated that poly-(ADP-ribosyl)ation, one of early event in apoptosis, was effectively reduced at 100 times lower concentration of DPD than that of Ado (10 μM versus 1 mM). However, the concentration of DPD at 10 μM was deficient for demoting Ado effect under threshold level and it was necessary for satisfactory result in blocking the formation of DNA ladder at 10 times higher dose. A recent study that Ado cytotoxicity was almost completely protected in neuronal cells when Ado was treated with either Ado-transporter inhibitors such as dilazep or NBTI in the culture indicates that Ado-induced apoptosis may be mediated through an Ado transporter in sympathetic neurons (Wakade *et al*., 1995). In contrast, Kohno *et al*. (1996) reported that Ado A3 receptor agonist induced apoptosis in HL-60 cells, which is a membrane- mediated pathway. They were still able to detect A3 receptor mRNA in the absence of Ado A3 receptor agonist. One possible explanation proposes that Ado acts differently from its specific receptor analogs in the induction mechanism of apoptosis, although we presently do not know exact action mechanism of apoptotic pathway.

It is likely that affinity of DPD or NBTI to a transporter is much higher than that of Ado, showing that they effectively inhibited DNA fragmentation even at 10 times lower concentration (100 μM versus 1 mM) (Figure 4). Cells that were treated with DPD for 1 h before Ado addition could not neutralize the Ado effect on Ado-induced DNA fragmentation (Figure 4B). The reason for this observation is unclear, but several explanations are possible. First, DPD may be held by Ado transporter and then recycled to plasma membrane. Second, DPD may be rapidly degraded after endocytosis through membrane. Third, DPD has no ability to regulate the programmed cell death like Ado itself. Although DPD gave satisfactory results in blocking DNA fragmentation (Figure 4), it did not provide any meaningful recovery of c-Myc level (Figure 5). From these results, we inferred that blocking of Ado entrance into the cells by DPD was not complete, allowing just weak recovery of c-Myc reduction, which, however, protected successfully DNA fragmentation, we describe here that the role of c-Myc protein was dependent on the amount of its expression level, not on the presence of the oncoprotein.

Other reports that inhibition of the methylation reaction had an important role in SCID pathogenesis (German *et al*., 1983) suggest that Ado-induced apoptosis may be correlated with methylation reactions. In this paper, we tested the effect of AdoHcy or DZAHcy to assess the specific mechanism of Ado-induced apoptosis. AdoHcy, which is accumulated due to inhibition of the *S*-adenosyl-homocysteine hydrolase, and DZAHcy, its stable analog, inhibit the activity of methyltransferase (Garcia *et al*., 1983; Backlund *et al*., 1986). It was demonstrated that DNA from HL-60 cells exposed to DZA and DZAri, a specific AdoHcy hydrolase inhibitor, was hypomethylated (Loennechen *et al*., 1989). It is clear that the methylation is known to influence chromatin structures and nonmethylated DNAs have a higher sensitivity to degradation by nucleases than methylated DNAs (Lewis and Bird, 1991). Although DZAHcy or AdoHcy alone did not have any effects on apoptosis of HL-60 cells, consistent with other studies (Endresen *et al*., 1993; Wakade *et al*., 1995), synergistic activity of DNA fragmentation between these agents and Ado has potential implications for the regulation of Ado-induced apoptosis of HL-60 cells through perturbance of methylation.

Although c-Myc oncoprotein has important roles in cell cycle progression and transformation, its correlation with apoptosis does not have general agreement owing to specificity in cell types and stimuli. Recent studies demonstrate that reduction of its endogenous level had an obligatory role in the induction of apoptotic cell death regardless of constitutive amount of c-Myc oncoprotein (Packham and Cleveland, 1995). HL-60 cells may loose endogenous c-Myc level in apoptosis. However, stimulation of endogenous c-Myc oncoprotein was also reported to trigger apoptosis in other cell types (Evan *et al*., 1992; Kim

et al., 1994). There is no direct evidence that presents requirement of cell cycle progression as c-Myc induces apoptosis and that demonstrates relationship between c-Myc and Ado-induced apoptosis (Kimura *et al*., 1995). The direct evidence for the role of c-Myc in Ado-induced apoptosis of c-Myc amplified HL-60 cells will be supported if apoptosis is inhibited by maintenance of stable expression of c-Myc using potent vehicles in Ado-treated HL-60 cells.

Acknowledgements

This study was supported in part by a grant from the Mission oriented grant from Korea Science and Engineering Foundation (F015534), the Korea Science and Engineering Foundation through the Cancer Research Center at Seoul National University (KOSEF-SRC-56-CRC94-8) and the 1996 Korean National Cancer Control Program, Ministry of Health and Wellfare, R.O.K. We thank Professor Jin Kim (Department of Anatomy, Catholic University Medical College) for his generous help in the preparation of the electron micrographic experiments. A part of this study will be submitted as In-Sung Hwang's doctoral thesis.

References

Abbrachio, M. P., Ceruti, S., Barbieri, D., Franceschi, C., Malorni, W., Biondo, L., Burnstock, G. and Cattabeni, F. (1995) A novel action for adenosine: Apoptosis of astroglial cells in rat brain primary cultures. *Biochem. Biophys. Res commun*. 213: 908-915

Arends, M. J., Morris, R. G. and Wyllie, A. H. (1990) Apoptosis The role of the endonuclease. *Am. J. Pathol.* 136: 593-608

Askew, D. S., Ihle, J. N. and Cleveland, J. L. (1993) Activation of apoptosis associated with enforced myc expression in myeloid progenitor cells is dominant to the suppression of apoptosis by interleukin-3 or erythropoietin. *Blood* 82: 2079-2087

Backlund, P. S., Carotti, D. and Cantoni, G. L. (1986) Effects of the S-adenosylhomocysteine hydrolase inhibitors 3-deazaadenosine and 3-deazaaristeromycin on RNA methylation and synthesis. *Eur.J. Biochem.* 160: 245-251

Benveniste, P. and Cohen, A. (1995) p53 expression is required for thymocyte apoptosis induced by adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. USA* 92: 8373-8377

Bertrand, R., Sarang, M., Jenkin, J., Kerrigan, D. and Pommier, Y. (1991) Differential induction of secondary DNA fragmentation by topoisomerase II inhibitors in human tumor cell lines with amplified c-myc expression. *Cancer Res.* 51: 6280- 8285

Cantoni, G. L., Richards, H. H. and Chiang, P. K. (1979) In *Transmethyl-ation* (Usdin, E., Borchardt, R. T. and Croseling, E. R. eds.) pp. 155-164, Elsevier, Amsterdam, New York

Carson, D. A. and Seegmiller, J. E. (1976) Effect of adenosine deaminase inhibition upon human lymphocyte blastogenesis. *J. Clin. Invest.* 57: 274-282

Cohen, A., Hirschhorn, R., Horowitz, S. D., Rubinstein, A., Polmar, S. H., Hong, R. and Martin, D. W. (1978) Deoxyadenosine triphosphate as a potentially toxic metabolite in adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. USA* 75: 472-476

Endresen, P. C., Eide, T. J. and Aarbakke, J. (1993) Cell death initiated by 3-deazaadenosine in HL-60 cells is apoptosis and is partially inhibited by homocysteine. *Biochem. Pharmacol.* 46: 1893-1901

Evan, G. I., Wyllie, A. H. Gilbest, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. and Hancock, D. C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69: 119-128

Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P. and Williams, M. (1994) Nomenclature and classification of purinoceptors. *Parmacol. Rev.* 46: 143-156

Garcia-Castro, I., Mato,M. J., Vasanthakumar, G., Wiesmann, W. P., Schiffmann, E. and Chiang, P. K. (1983) Paradoxical effects of adenosine on neutrophil chemotaxis. *J. Biol. Chem.* 258:4345-4349

German, D. C., Bloch, C. A. and Kredich, N. M. (1983) Measurements of S-adenosylmethionine and L-homocysteine metabolism in cultured human lymphoid cells. *J. Biol. Chem*. 258: 10997-11003

Gerschenson, L. E. and Rotello, R. J. (1992) Apoptosis: A different type of cell death. *FASEB J.* 6: 2450-2455

Giblett, E. R., Anderson, J. E., Pollara, B. and Meuwissen, H. J. (1972) Adenosinedeaminase deficiecy in two patients with severely impaired cellular immunity. *Lancet* 2: 1067-1069

Guranowski, A., Montgomery, J. A., Cantoni, G. L. and Chiang, P. K. (1981) Adenosine analogues as substrates and inhibitors of S-adenosylhomo-cysteine hydrolase. *Biochemistry* 20: 110-115

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348, 334-336

Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br.J. Cancer* 26: 239-257

Kim, I. K., Aksamit, R. R. and Cantoni, G. L. (1982) Mechanism of the cytostatic activity of 3-deazaaristeromycin, an inhibitor of adenosylhomo-cysteine hydrolase. *J. Biol. Chem.* 257: 14726-14729

Kim, I. K., Copeland, R. L. Jr., Lee, J. H., Kim, H. S., Asafo-Adjei, Brown, N. D., Estrada, J. S., Gordon, R. K., Garcia, G. E. and Chiang, P. K. (1994) Induction of apoptosis and c-myc in L1210 lymphocytic leukemia cells by adenosine. *J. Biomed. Sci.* 1: 154-157

Kimura, S., Maekawa, T., Hirakawa, K., Murakami, A. and Abe, T. (1995) Alterations of c-myc expression by antisense oligodeoxynucleotides enhance the induction of apoptosis in HL-60 cells *Cancer Res.* 55: 1379-1384

Kizaki, H., Suzuki, K., Tadakuma, T. and Ishimura, Y. (1990) Adenosine receptor-mediated accumulation of cyclic AMP-induced T-lymphocyte death through internucleosomal DNA cleavage. *J. Biol. Chem.* 265: 5280-5284

Kohno, Y., Sei, Y., Koshiba, M., Kim, H. O. and Jacobson, K. A. (1996) Induction of apoptosis in HL-60 human promyelocytic leukemia cells by adenosine A_3 receptor agonists. *Biochem. Biophys. Res. Commun.* 219: 904-910

Laemmli, U.K. (1975) Characterization of DNA condensates induced by poly(ethyleneoxide) and polylysine. *Proc. Natl. Acad. Sci. USA* 72: 4288-4292

Lee, J. H., Kim, H. S., Jeong, S. Y. and Kim, I. K. (1995) Induction of p53 and apoptosis by Δ^{12}-PGJ_2 in human hepatocarcinoma SK-HEP-1 cells. *FEBS. Lett.* 368: 348-352

Lewis, J. and Bird, A.(1991) DNA methylation and chromatin structure. *FEBS. Lett.* 285: 155-159

Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A.F. and Minna, J. D. (1983) Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature* 306: 194-196

Loennechen, T., Prytz, P. S. and Aarbakke, J. (1989) DNA-methylation in HL-60 cells treated with 3-deaza-(±)-aristeromycin and 3-deazaadenosine. *Biochem. Pharmacol*. 38: 2748-2751

Onclercq, R., Barbinet, C. and Cremisi, C. (1989) Exogenous c-Myc gene overexpression interferes with early events in F9 cell differentiation. *Oncogene Res.* 4: 293-302

Packham, G. and Cleveland, J. L. (1995) c-Myc and apoptosis. *Biochim. Biophys. Acta* 1242: 11-28

Ryan, K. M. and Birnie, G. D. (1996) Myc oncogenes: the enigmatic family. *Biochem. J.* 314: 713-721

Sanchez, V. C. (1995) Circadian variations of adenosine and of its meta-bolism. Could adenosine oscilator for circadian rhythms? *Can. J. Physiol. Pharmacol.* 73: 339-355

Sellins, K. S. and Cohen, J. J. (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol*. 139: 3199-3206

Sullivan, N. F. and Willis, A. E. (1989) Elevation of c-myc protein by DNA strand breakage. *Oncogene* 4: 1497-1502

Szekely, L. W., Jiang, Q., Bulie-Jakub, F., Rosen, A., Ringerts, N., Klein, G. and Wimman, K. C. (1992) Cell type and differentiation dependent heterogeneity in retinoblastoma protein expression in SCID mouse fetuses. *Cell Growth Differ.* 3: 139-156

Tanaka, Y., Yoshihara, K.,Tsuyuki, M. and Kamiya, T. (1994) Apoptosis induced by adenosine in human leukemia HL-60 cells. *Exp. Cell Res.* 213: 242-252

Thampy, K. G. and Barnes, E. M. Jr. (1983) Adenosine transport by primary cultures of neurons from chick embryo brain. *J. Neurochem.* 40: 874-879

Wadewitz, A. G. and Lockshin, R. A. (1988) Programmed cell death: Dying cells synthesize a co-ordinated, unique set of proteins in two different episodes of cell death. *FEBS Lett.* 241: 19-23

Wakade, T. D., Palmer, K. C, MeCauley, R., Przywara, D. A. and Wakade, A. R. (1995) Adenosine-induced apoptosis in chick embryonic sympa-thetic neurons: A new physiological role for adenosine. *J. physio*l. 488: 123-138

Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284: 555-556