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8-Chloroadenosine Induced HL-60 Cell Growth Inhibition, Differentiation, and G_0/G_1 Arrest Involves Attenuated Cyclin D1 and Telomerase and Up-Regulated $p21^{WAF1/CIP1}$

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Abstract 8-Chloroadenosine, an active dephosphorylated metabolite of the antineoplastic agent 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP), induces growth inhibition in multiple carcinomas. Here we report that 8-chloroadenosine inhibits growth in human promyelocytic leukemia HL-60 cells by a G_0/G_1 phase arrest and terminates cell differentiation along the granulocytic lineage. The mechanism of 8-chloroadenosine-induced G_0/G_1 arrest is independent of apoptosis. The expressions of cyclin D1 and *c-myc* in HL-60 are suppressed by 8-chloroadenosine, whereas the cyclin-dependent kinases inhibitor $p21^{WAF1/CIP1}$ is up-regulated. 8-Chloroadenosine has less effect on the expressions of cyclin-dependent kinase (cdk)2 and cdk4, G_1 phase cyclin-dependent kinases, and only moderately induces the expression of transforming growth factor β1 (TGFβ1) and the mitotic inhibitor $p27^{KIP1}$. Telomerase activity is reduced in extracts of 8-chloroadenosine treated HL-60 cells, but 8-chloroadenosine does not directly inhibit the catalytic activity of telomerase in vitro. Therefore, anti-proliferation of HL-60 cells by 8-chloroadenosine involves coordination of cyclin D1 suppression, reduction of telomerase activity, and up-regulation of $p21^{WAF1/CIP1}$ that arrest cell-cycle progression at G_0/G_1 phase and terminate cell differentiation. J. Cell. Biochem. 97: 166–177, 2006.

Key words: 8-chloroadenosine; HL-60; differentiation; cyclin D1; $p21^{WAF1/CIP1}$; telomerase

Intracellular adenosine 3′,5′-cyclic-monophosphate (cAMP) and its chemical analogues regulate the growth of carcinoma cells. For example, 8-chloroadenosine 3′,5′-cyclic-monophosphate (8-Cl-cAMP), inhibits various type of carcinomas in vitro and in vivo, and has undergone clinical trials as an anti-cancer drug [Ally et al., 1989; Ramage et al., 1995; Tortora et al., 1995, 1997; Cummings et al., 1996; Langdon et al., 1998]. Pharmacokinetic studies have shown that the anti-cancer effect of 8-Cl-cAMP involves an active metabolite, 8-chloroadenosine [Langeveld et al., 1992; Cummings et al., 1994; Halgren et al., 1998; Robbins et al., 2001]. Further studies confirm similar cytostatic effects of these two molecules against tumor cells suggesting the involvement of a common signaling pathway in the anti-neoplastic process [Gandhi et al., 2001].

8-Chloroadenosine induces cell growth inhibition, differentiation, and apoptosis in a spectrum of human carcinomas [Pepe et al., 1991; Carlson et al., 2000; Yin et al., 2001]. Early studies demonstrated that both 8-Cl-cAMP and 8-chloroadenosine regulate the expression of protein kinase A (PKA) regulatory subunit genes, which was proposed as the mechanism for their anti-proliferative effects [Tortora et al.,

Abbreviations used: 8-Cl-cAMP, 8-chloroadenosine 3′,5′-monophosphate; cdk, cyclin-dependent kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NBT, nitro blue tetrazolium; MTT, methylthiazoletetrazolium; RA, retinoic acid; TGFβ1, transforming growth factor β1; TRAP, telomere repeat amplification protocol.

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1989, 1997; Rohlff et al., 1993; Langeveld et al., 1997; Noguchi et al., 1998]. In human promyelocytic leukemia HL-60 cell, 8-Cl-cAMP suppresses PKA RIα expression [Rohlff et al., 1993], inhibits cell proliferation, terminates cell differentiation, and delays cell-cycle progression [Pepe et al., 1991]. However, mechanisms for 8-Cl-cAMP in affecting cell-cycle kinetics, growth inhibition, and differentiation in HL-60 cells are not fully understood. Recently, conflicting data have attributed effects of 8-Cl-cAMP and 8-chloroadenosine on carcinoma cells to the regulations of PKA regulatory subunits expression, and may be unrelated [Carlson et al., 2000; Lamb and Steinberg, 2002]. Microarray data have shown that 8-Cl-cAMP and 8-chloroadenosine are closely parallel in down-regulation of proliferation and transformation genes in human neuroblastoma cells but differ in the up-regulation of differentiation and development genes [Park et al., 2002].

Chemical-induced growth arrest and differentiation in HL-60 cell has been correlated to cell cycle related-cyclins, kinase, and kinase inhibitors [Burger et al., 1994; Jiang et al., 1994; Wang et al., 1996]. G_0/G_1 arrest related—but apoptosis independent—cell differentiation was observed when phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate (TPA)), 1,25-dihydroxyvitamin D3 (1,25D3), retinoic acid (RA), or DMSO were used to maturate HL-60 cells towards granulocyte and/or monocyte phenotype [Bestilny et al., 1996; Wang and Studzinski, 1997]. Further, the expression of telomerase, a critical enzyme involved in maintaining carcinoma cell immortality and proliferation, is also down-regulated in HL-60 cell during the terminal differentiation induced by TPA, RA, and DMSO [Bestilny et al., 1996]. However, these critical proteins in 8-chloroadenosine-treated HL-60 cell are unknown. Here we report that 8-chloroadenosine induce maturation of HL-60 phenotype towards granulocytes and changes of proteins correlated to G_0/G_1 arrest, and reduction of telomerase activity.

MATERIALS AND METHODS

Materials

8-Chloroadenosine was synthesized and provided by Dr. L.H. Zhang. Other chemicals were purchased from Sigma (St. Louis, MO). 8-Chloroadenosine and TPA were dissolved in DMSO. Final concentration for DMSO was 0.1% and was used alone as a vehicle control.

Cell Culture

Human promyelocytic leukemia HL-60 cells were maintained as a suspension in RPMI 1640 medium supplemented with 10% FBS and cultured at 37°C, 5% CO_2/95% air. The viability of cell in culture was monitored by trypan blue exclusion.

MTT Method for Cell Growth

HL-60 cell growth was measured by methylthiazoletetrazolium (MTT) assay [Hirano et al., 1994]. Briefly, 4×10^3 cells/well were seeded to 96-well plates and cultured with different concentrations of 8-chloroadenosine for 7 days or as indicated, and compared to vehicle control (0.1% DMSO). Then, MTT stock solution was added (50 μg/well) and continuously cultured for another 3 h. Suspensions of HL-60 cells were collected by low speed centrifugation and cell pellets were dissolved in DMSO. $OD_{570\ nm}$ was read on microplate reader (Bio-Rad model 450) with $OD_{665\ nm}$ as the reference. A relative growth inhibition rate was calculated as: $OD_{570\ nm}$ Sample $\times$ 100%/ $OD_{570\ nm}$ Control.

NBT Reduction for Cell Differentiation

Morphological assessment of differentiation of HL-60 cell was performed by nitro blue tetrazolium (NBT) reduction as described previously [Newberger et al., 1979; Bestilny et al., 1996]. Briefly, 2×10^4 cells/ml were seeded in 35 mm dishes with 8-chloroadenosine and cultured for 5 days or as indicated. Then, cells were incubated with continuous shaking for 60 min at 37°C with 0.2% NBT in PBS containing TPA (200 ng/ml). Cytospin slides were prepared in Gimsa-Wright staining. The percentage of cells (200 cells for each slide) containing reduced blue-black formazan deposits was determined.

Quantification of Cell Cycle and Apoptosis by Flow Cytometry

HL-60 cells were assessed for cell-cycle analysis and apoptosis as described [Nicoletti et al., 1991]. Briefly, 1×10^6 cells were collected by a centrifugation at 200*g* and fixation in ice-cold 70% ethanol for 45 min. Cells were washed

and re-suspended in 1 ml of PBS. Flow cytometry was performed after addition of propidium iodide (50 μg/ml with 0.1% Triton X-100) for 15 min at room temperature. DNA content was measured with a FACS-scan (Becton Dickinson, SCANVantage SE), and cell-cycle distribution and apoptotic content in sub-diploid were calculated using *CellQuest* software.

DNA Fragmentation

DNA fragmentation was measured using a DNA/histone-complex ELISA kit (Roche) for 10,000 cells/well of HL-60 cultured in 96-wells plate as described [Zhu et al., 2005]. After 24 h, cells were dosed with 8-chloroadenosine and grown for additional 24–120 h. Comparative values of DNA fragmentation between controls and treatments were based on using the same cell number in each assay.

Western Blots Analysis of Cell Extracts

Protein samples in whole cell lysates were prepared from 8-chloroadenosine and TPA (100 ng/ml) treated HL-60 cells and Western blots performed as previously described [Thompson et al., 2002]. Briefly, 100 μg proteins were subjected to 12.5% polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane. Antibodies for cyclin D1 (*SC*-246), *c-myc* (*SC*-042), $p21^{WAF1/CIP1}$ (*SC*-397), $p27^{KIP1}$ (*SC*-528), cyclin-dependent kinase-2 (cdk2) (*SC*-163), cdk4 (*SC*-260), transforming growth factor β1 (TGFβ1) (*SC*-146), β-actin, biotin labeled secondary antibodies, and streptavidin/alkaline phosphatase were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were quantified by densitometry (AlphaEase DIAS, CA) and expressed as percentage of control, mean ± SEM, $n = 4$ for cyclin D1 and $p21^{WAF1/CIP1}$ and $n = 3$ for all others.

Measurement of Telomerase Activity by TRAP-ELISA Method in Cell Extracts

Cell extractions and assays for telomerase activity in HL-60 cells were performed using telomere repeat amplification protocol (TRAP)–PCR–ELISA Kits (Roche Applied Science, Indianapolis, IN). Briefly, 2×10^6 cells after 8-chloroadenosine treatment were collected and washed twice with PBS and then suspended in lysis reagent on ice for 30 min. Cell lysates were centrifuged at 16,000*g* for 20 min at 4°C. The supernatants were collected and protein concentration measured by *Dc* Protein Assay Kits (Bio-Rad, Hercules, CA). Substrate oligonucleotide, biotin 5′-tip labeled P1-TS primer used for the telomerase-catalyzed primer elongation, PCR amplification P2 primer, and cell extracts (3 μg total protein) were added to the reaction mixture (50 μl). Cell extract pre-treated with 1 mg/ml RNase A or isolated from HEK 293 cell was used as negative or positive controls. TRAP reactions were performed in a PCR thermal cycler (GeneAmp 9600, Perkin Elmer) including telomerase-catalyzed primer elongation at 25°C for 20 min, telomerase inactivation for 5 min at 94°C, and PCR amplification for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. Samples were then kept at 72°C for 10 min and maintained at 4°C. The amplification product (5 μl) with denaturation reagent (20 μl) were incubated at room temperature for 10 min before addition of hybridization buffer (225 μl) containing digoxigenin-labeled probe with complementary telomeric repeat sequences. The mixture (100 μl) was added to a streptavidin pre-coated microtiter plate and incubated for 2 h at 37°C in shaking bath (300 rpm). After washing, peroxidase substrate tetramethylbezidine was added and $OD_{655\ nm}$ determined.

RESULTS

8-Chloroadenosine Inhibits HL-60 Cells Growth and Promotes Cell Differentiation Towards Granulocytes

8-Chloroadenosine led to growth inhibition in HL-60 cells after 5–7 days treatment (Fig. 1A) with an IC_{50} value of 1.35 μM at day 7 (Fig. 1B). Prior to maximal growth inhibition, morphological changes of HL-60 cells were observed after 3 days of treatment with 8-chloroadenosine. A granulocyte-like maturation of HL-60 cell was observed with 2–16 μM range of 8-chloroadenosine. These effects were characterized by a decreased cell size and the appearance of less prominent nucleoli along with segment or band nuclei (pictures not shown). Biochemical characterization of granulocytic maturation induced by 8-chloroadenosine in HL-60 cells was further confirmed by measuring reduction of water-soluble dye NBT to formazan deposits [Newberger et al., 1979; Bestilny et al., 1996]. The maximal NBT reduction (up to 40%) for 8-chloroadenosine (16 μM) treated HL-60 cells was observed at day 5–9, with a 50% of

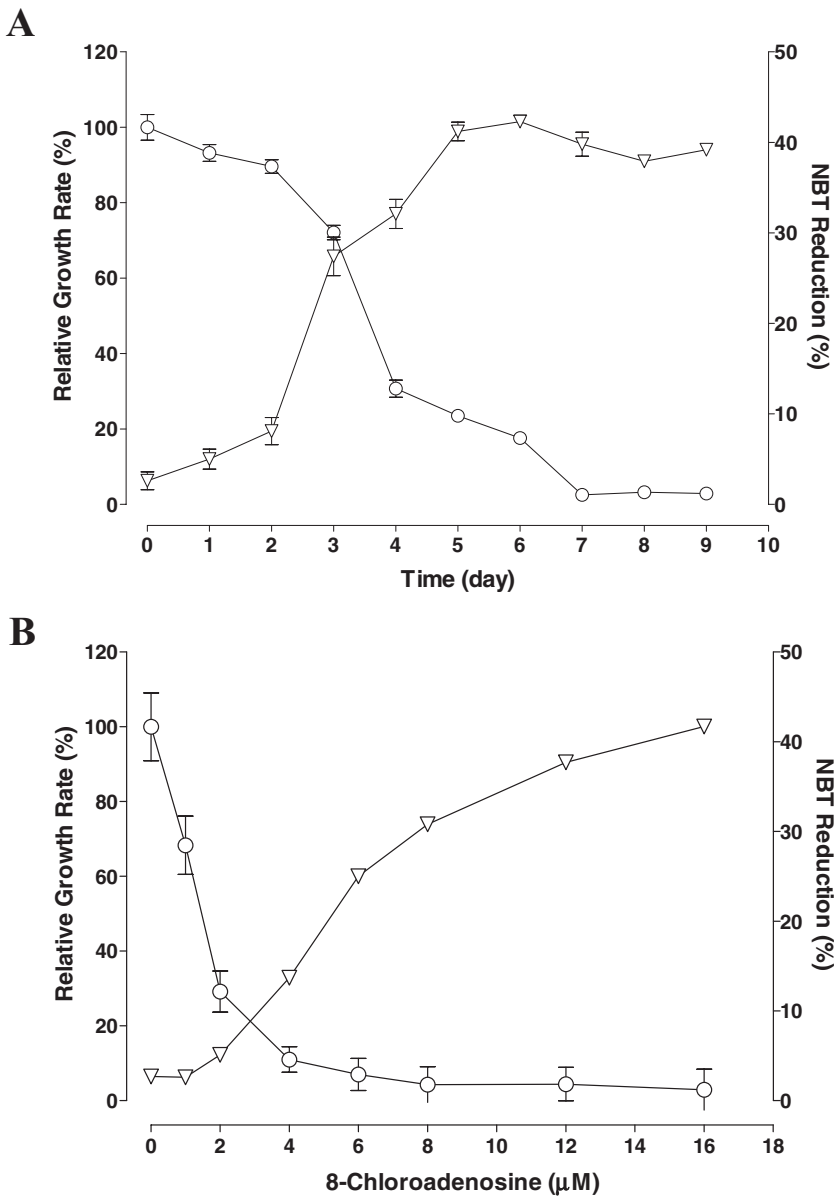

Fig. 1. Growth inhibition and granulocytic differentiation of HL-60 cell by 8-chloroadenosine. **A**: Time course for 8-chloroadenosine inhibited cell growth (○) and nitro blue tetrazolium (NBT) reduction (▽). HL-60 cells were continuously cultured in 8-chloroadenosine (16 μM) for 9 days. **B**: Dose response for 8-chloroadenosine inhibited cell growth (day 7) and NBT reduction (day 5). Cell proliferation and differentiation were measured every day by methylthiazoletetrazolium (MTT) and NBT reduction. Relative growth rate in 8-chloroadenosine treated cells were normalized with vehicle control (0.1% DMSO) at each day, and indicated as 100% for staring at day zero for time course and concentration zero for dose response at day 7. NBT positive cells treated by 8-chloroadenosine were expressed as the percentage of total cells of measurement (200 cells for each sample of slide). NBT positive in vehicle controls at day 0, 3, 5, 7, and 9 were also performed and all less than 5% that were indicated at day 0 and concentration zero for day 5. Values represent the mean ± SEM of five experiments performed in triplicate.

maximum of NBT reduction (EC_{50}) observed at 5.75 μM at day 5 (Fig. 1A,B).

8-Chloroadenosine Induces G_0/G_1 Arrest and Apoptosis

Flow cytometric analysis indicated that 8-chloroadenosine induced a G_0/G_1 phase accumulation, reduction of cells in the S phase and $G_2 + M$ phase after 24 h treatment (Table I). The percentage of cells in the G_0/G_1 phase was increased from $34.5 \pm 2.3\%$ (control) to $65.1 \pm 3.7\%$ (16 μM of 8-chloroadenosine, $P < 0.05$), whereas the percentage of cells in the S phase were decreased from $55.5 \pm 3.2\%$ to $31.3 \pm 1.1\%$

TABLE I. Changes in HL-60 Cell-Cycle Phase Distribution by 8-Chloroadenosine

Group	Concentration (μM)	Cell-cycle phase distribution (%)[a]		
		G_0/G_1	S	G_2+M
Control	—	34.5 ± 2.3	55.5 ± 3.2	10 ± 0.9
8-ClA	2	54.8 ± 4.6*	39.1 ± 2.7*	6.1 ± 1.4*
	4	58.0 ± 2.1*	37.7 ± 1.9*	4.3 ± 0.7*
	8	65.2 ± 4.3*	32.6 ± 3.0*	2.1 ± 1.3*
	16	65.1 ± 3.7*	31.3 ± 1.1*	3.7 ± 1.1*

[a]HL-60 cells were treated with 8-chloroadenosine (8-ClA) or a vehicle control for 24 h. DNA contents were determined by FACS analysis. Data were expressed as the percentage of cells in different phase of cell cycle from one experiment repeated four times (mean ± SEM, n = 3).
*$P < 0.05$ as compared with control.

($P < 0.05$), and in the G_2+M phases were also decreased from 10.0 ± 0.9% to 3.7 ± 1.1% ($P < 0.05$). Similar effects on G_0/G_1 arrest with treatment of 8-chloroadenosine at longer time period of 48–96 h but were not sustained at 120 h (not shown). A lower rate of apoptosis for 8-chloroadenosine was maintained over 120 h at dose of 2 μM (8.24 ± 1.82%) and at 24 h at 16 μM concentration (7.77 ± 1.42%, Table II). The apoptotic rate was increased only when the concentration of 8-chloroadenosine was increased (66–330 μM) above the concentrations to affect growth, differentiation and G_0/G_1 phase arrest in these cells.

DNA fragmentation data confirmed that 8-chloroadenosine-induced apoptosis at 24 h had only occurred at a high dose range, 66 μM (Fig. 2). Such increased rates of DNA fragmentation reached maximum at 72 h and showed a dose-related manner at 8–66 μM concentrations range, and the rate was decreased when the treatment time was prolonged to 96–120 h. At 8 and 16 μM 8-chloroadenosine, the DNA fragmentation was increased 30%–50% of controls at 72–120 h. There was no change of DNA fragmentation for 2 μM 8-chloroadenosine at 24–96 h treatment, and less than a 20% increase was found at 120 h treatment, consistent with those observations by flow cytometry assay.

TABLE II. Apoptosis in 8-Chloroadenosine Treated HL-60 Cell

Group[a]	Treat time (h)	Concentration (μM)	Apoptosis rate (%)
Control	24	—	1.65 ± 0.71
	120	—	2.10 ± 0.94
8-ClA	24	2	5.99 ± 0.53
	24	4	5.59 ± 0.58
	24	8	5.90 ± 1.13
	24	16	7.77 ± 1.42
	24	66	14.1 ± 2.90
	24	330	24.7 ± 2.11
	48	2	5.96 ± 1.02
	72	2	12.3 ± 1.82
	96	2	7.03 ± 1.61
	120	2	8.24 ± 1.82

[a]HL-60 cells were treated with 8-chloroadenosine (8-ClA) or vehicle controls. DNA contents were determined by FACS analysis. Data were expressed as the percentage of cells in sub-diploid area (apoptosis) of the analysis from one experiments repeated four times (mean ± SEM, n = 3).

Reduced Cyclin D1 Expression in 8-Chloroadenosine Treated HL-60 Cells

Western blots indicated that cyclin D1 expression in HL-60 cell was decreased when cells were treated with 8-chloroadenosine. The inhibitory effect of 2 μM 8-chloroadenosine was seen at 24 h (19 ± 10% of control, $P < 0.05$) and this effect was maintained for 48–120 h (39 ± 8%, 48 h; 27 ± 5%, 72 h; 12 ± 4%, 96 h; 15 ± 5%, 120 h; Fig. 3A, top panel). The inhibition of cyclin D1 expression was enhanced when the 8-chloroadenosine concentrations were increased from 2 to 16 μM at 24 h (Fig. 3A, bottom panel, 16 ± 5% for 2 μM 8-chloroadenosine and were not detectable at 4–16 μM). Conversely, TPA (100 ng/ml), a chemical inducer of HL-60 cell maturation along monocytic lineage, induced cyclin D1 expression at 24 h (305 ± 39% of control, $P < 0.05$). The expression of two cyclin-dependent kinases, cdk2 and cdk4 (both closely associated with cyclin D1), was only slightly reduced following 8-chloroadenosine treatment (2 μM, 24–120 h) and reduced in TPA-induced differentiation cells (100 ng/ml, 24 h) as compared with the vehicle controls (Fig. 3B, top two panels). The TPA-induced increases in cyclin D1 and decreases in cdk2/cdk4 are consistent with previous reports [Akiyama et al., 1993; Burger et al., 1994; Horiguchi-Yamada et al., 1994].

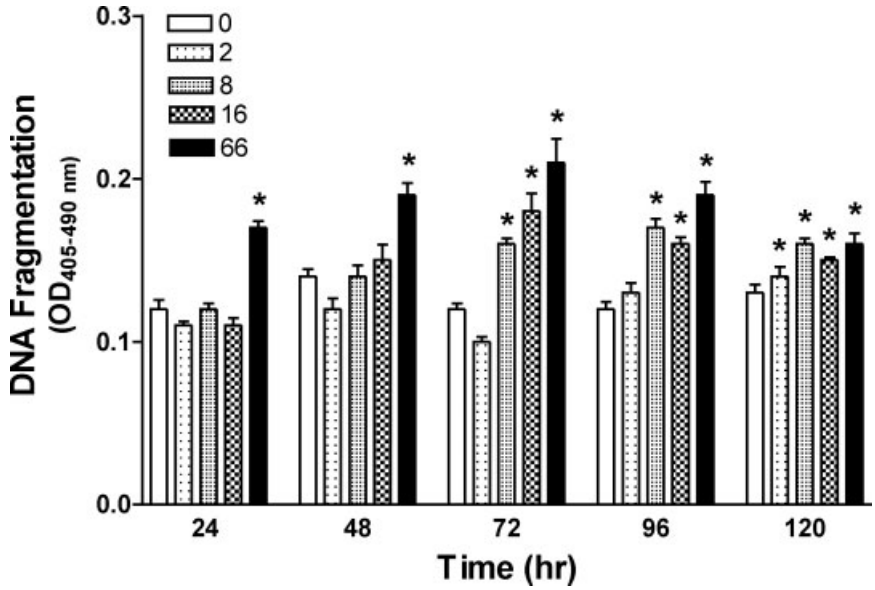

Fig. 2. 8-Chloroadenosine-induced apoptosis in HL-60 cells determined with DNA fragmentation for endonuclease-cleaved mono- and oligo-nucleosomes ($OD_{405-490\ nm}$). For comparative purposes, same amounts of cells (1,000 cell/20 μl cell lysis buffer) were used in the ELISA assay at the indicated time of cell culture and between the controls (open bars) and various concentrations of 8-chloroadenosine (solid bars, 2, 8, 16, and 66 μM from left to the right). Data represent data from one of three repeated experiments, mean ± SEM (n = 4).

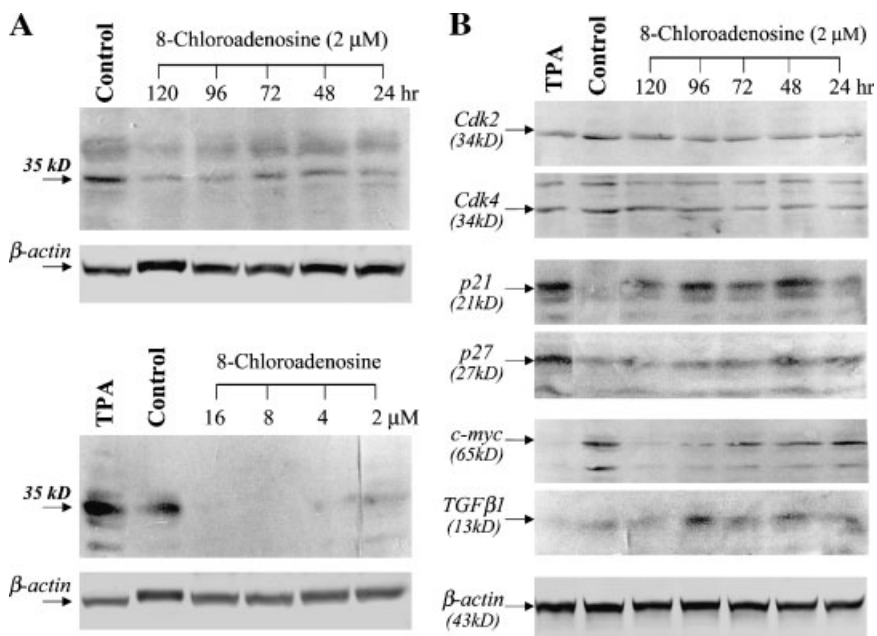

Fig. 3. 8-Chloroadenosine reduced cyclin D1 and altered associated proteins expressions in HL-60 cell. **A**: Decreased expression of cyclin D1 expression was found in 8-chloroadenosine treated HL-60 cells. Time course of cyclin D1 in HL-60 cells treated by 2 μM 8-chloroadenosine for 24–120 h (**top panel**), and by different doses of 8-chloroadenosine treated at 24 h (**bottom panel**). **B**: Increase in $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and transforming growth factor β1 (TGFβ1), decrease in *c-myc*, and smaller decrease in cdk2/cdk4 expressions were found in 8-chloroadenosine (2 μM) treated HL-60 cells for 24–120 h. Whole cell extractions (100 μg/lane) were applied on 12.5% SDS–PAGE gel, and Western blots analysis using different antibodies were carried out as described in ''Materials and Methods.'' Mixture of cell extracts from vehicle control (Control) at 0–120 h was used for most of blots except the one of showing cyclin D1 with various concentrations of 8-chloroadenosine was prepared at 24 h. Cell extracts for TPA-treatment (TPA, 100 ng/ml) were prepared at 24 h. Blots of β-actin (43 kDa) were used as internal control for protein loading. Data represent data from one of three repeated experiments and are quantified by densitometry.

Induced $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ Expressions in 8-Chloroadenosine Treated HL-60 Cells

HL-60 cells express endogenous trace levels of $p21^{WAF1/CIP1}$ and basal levels of $p27^{KIP1}$ (Fig. 3B, middle two panels). The expression of $p21^{WAF1/CIP1}$ was induced by 8-chloroadenosine (2 μM) after 24 h treatment ($136 \pm 12\%$ of control, $P < 0.05$), reached the maximum at 48–96 h ($296 \pm 46\%$, 48 h; $204 \pm 52\%$, 72 h; $271 \pm 55\%$, 96 h) and was maintained until 120 h ($150 \pm 24\%$). There was also a smaller induction of $p27^{KIP1}$ by 8-chloroadenosine, with a shorter duration of the effect ($147 \pm 28\%$, 24 h; $153 \pm 30\%$, 48 h; $120 \pm 22\%$, 72 h; $112 \pm 15\%$, 96 h; $109 \pm 10\%$, 120 h). The TPA-differentiated HL-60 cells (24 h) showed potent inductions of both $p21^{WAF1/CIP1}$ ($355 \pm 84\%$ of control, $P < 0.05$) and $p27^{KIP1}$ ($267 \pm 71\%$ of control, $P < 0.05$), results that are consistent with previous reports in literature [Jiang et al., 1994; Schwaller et al., 1995; Millard et al., 1997].

We also tested alterations of transcriptional factor *c-myc* [Philipp et al., 1994; Alexandrow and Moses, 1995] and transforming growth factor TGFβ1 [Silberstein and Daniel, 1987; Polyak et al., 1994; Alexandrow and Moses, 1995; Datto et al., 1995] in 8-chloroadenosine treated HL-60 cells. Our data showed that HL-60 cells had a high basal expression of *c-myc* and with trace levels of TGFβ1 expression. Following treatment of HL-60 cells with 8-chloroadenosine (2 μM) for 24–120 h, *c-myc* expression was decreased ($95 \pm 13\%$, 24 h; $64 \pm 17\%$, 48 h; $59 \pm 13\%$, 72 h; $10 \pm 4\%$ 96 h) and in a measurably undetected level at 120 h. In contrast, TGFβ1 expression was increased by 24 h exposure to 8-chloroadenosine treatment ($112 \pm 8\%$, $P > 0.05$), increased slightly more at 48 h ($137 \pm 15\%$, $P < 0.05$) and 72 h ($121 \pm 16\%$), andreached a maximum effect at 96 h ($159 \pm 23\%$, Fig. 3B, bottom two panels). TPA also inhibited *c-myc* expression (measurably undetected) but had no effect on the level of TGFβ1 expression ($89 \pm 25\%$ of control, $P > 0.05$) at the 24 h treatment point.

8-Chloroadenosine Attenuated Telomerase Activity in Treated HL-60 Cells but did not Directly Influence Enzyme Activity

Telomerase activity is important to maintain immortality and continuous replication of HL-60 cell [Bestilny et al., 1996]. Following treatment of HL-60 cell with various concentrations of 8-chloroadenosine (2–16 μM) for 24–120 h, telomerase activity in cells extract was decreased in a dose-dependent and time-dependent manner (Fig. 4A,B). 8-Chloroadenosine, however, showed no direct effect on the catalytic activity of telomerase when tested in vitro. The values of telomerase activity measured in the presence and absence of various concentrations of 8-chloroadenosine were: $97.1 \pm 8.1\%$ (0.1 μM), $83.7 \pm 8.7\%$ (1 μM), $91.6 \pm 4.9\%$ (4 μM), $82.4 \pm 6.2\%$ (8 μM), and $80.7 \pm 9.6\%$ (16 μM) as compared to control activity of $100 \pm 11.2\%$ (by four different exp). These results indicate that 8-chloroadenosine suppress the expression of telomerase in HL-60 cell without directly impairing the catalytic activity to any significant degree.

DISCUSSION

A human leukemia cell line (HL-60) has been used as a model system to compare the ability of certain chemicals to inhibit carcinoma cell growth and induce a more differentiated phenotype. The exact mechanism by which these agents induce these effects is not fully understood though a number of target enzymes and regulatory proteins have been proposed to play a critical role(s) in the processes. Table III lists several known chemical inducers of HL-60 cells and some of the proposed mechanisms involved. The inducers include TPA, 1,25D3, DMSO, RA, and 8-Cl-cAMP and its derivative 8-chloroadenosine. In the present study, we tested the dose- and time-dependent effects exhibited by 8-chloroadenosine on cell growth and differentiation in the model system of human leukemia and compared its effects to that reported for other inducers. We found that 8-chloroadenosine exhibits a similar potency of growth inhibition ($IC_{50} = 1.35$ μM) and differentiation (NBT reduction, $EC_{50} = 5.75$ μM) in HL-60 cell. The time course of G_1/G_0 phase arrest (24 h) in 8-chloroadenosine-treated HL-60 cell is earlier than the appearance of granulocytic markers (>72 h). These effects of 8-chloroadenosine are similar to those findings observed in granulocytic inducers like DMSO and RA [Bestilny et al., 1996], but differed from those monocytic inducers like TPA and 1,25D3 [Burger et al., 1994; Zhang et al., 1994; Bestilny et al., 1996; Wang et al., 1996].

A number of significant effects of 8-chloroadenosine were observed: these included a

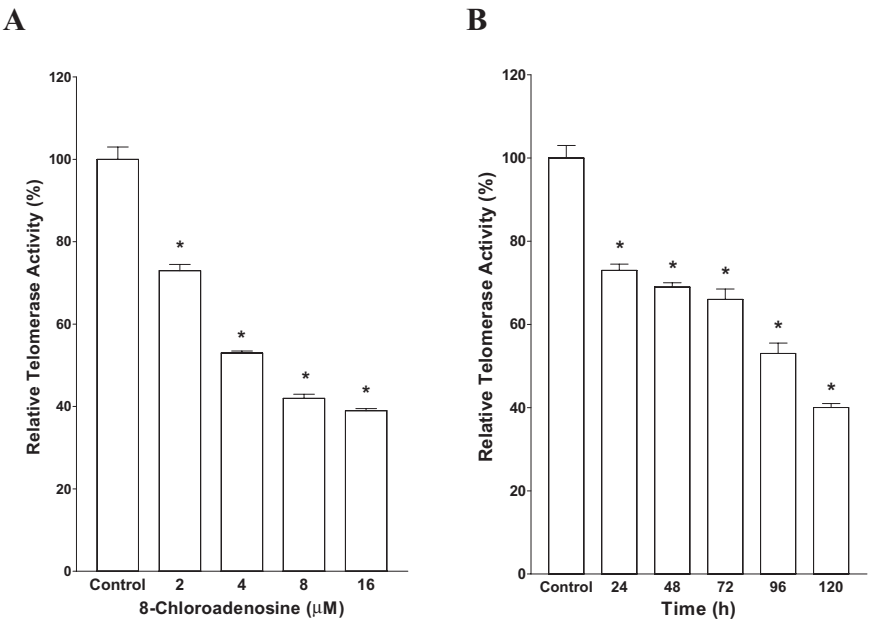

Fig. 4. 8-Chloroadenosine reduced telomerase expression in HL-60 cells. **A**: Dose-related reduction of telomerase activity in HL-60 cell treated with different concentrations of 8-chloroadenosine for 24 h. **B**: Time course of telomerase activity in HL-60 cells treated by 2 μM 8-chloroadenosine for 24–120 h. The telemorase activity in each group of cellular preparation was assayed with the telomere repeat amplification protocol (TRAP)-ELISA protocol as described in ''Materials and Methods.'' The relative telomerase activity per microgram total cellular protein is expressed as a percentage of the telomerase activity detected in vehicle control cells. Data represent the mean ± SEM from five separate experiments performed in triplicate. *, $P < 0.05$ as compared with control.

reduction in cyclin D1 expression and telomerase activity. Conversely, an up-regulation of $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $TGF\beta1$ genes thought to be important in differentiation, development and transformation, respectively, were detected. All these genes changed by 8-chloroadenosine with the cell-cycle kinetics in HL-60 cell are not parallel to its prodrug, 8-Cl-cAMP [Pepe et al., 1991; Park et al., 2002]. 8-Chloroadenosine also reduced the expression of *c-myc* in HL-60 cell, the transcriptional factor believed to be required for cell growth and progression of the cell cycle from G_1 phase to S phase involving the G_1-phase related cyclins including cyclin D1 [Philipp et al., 1994; Alexandrow and Moses, 1995]. A reduction of *c-myc* was also detected in 8-Cl-cAMP treated K-562 leukemia cell [Tortora et al., 1989].

Cyclin D1 is down-regulated when using granulocytic inducers like DMSO and 8-chloroadenosine but except RA [Burger et al., 1994]. However, an up-regulation of cyclin D1 has been found in monocytic inducers like TPA and 1,25D3. [Akiyama et al., 1993; Burger et al., 1994; Horiguchi-Yamada et al., 1994; Wang et al., 1996]. So, the earlier decrease in cyclin D1 at 12–24 h is a factor for "phenotype selection" to granulocytes, and it is accompanied with a G_1/G_0 arrest and a slow process of maturation. The decreased cyclin D1 may directly block cell-cycle progression through G_1 phase to S phase via the reduction of the activated complex of cyclin D1 with cdk2/cdk4 [Prall et al., 1997]. But if there is no initial decrease in cyclin D1, cells quickly maturate to monocytes, and then those cells maturated along lineage of monocytes. So, the alteration of cyclin D1 is a checkpoint marker for the selection of promyelotic HL-60 cell towards different lineages of maturation rather than the specific marker for G_1/G_0 arrest.

In certain instances, changes have been shown to occur in HL-60 cells treated with the various agents but not with 8-chloroadenosine. For example, G_1/G_0 phase-related cdk2/cdk4 are reduced in these cells following treatment with TPA, RA, or DMSO [Burger et al., 1994; Horiguchi-Yamada et al., 1994], but are not changed by 8-chloroadenosine. It is also true of the G_2/M/S cyclins and associated proteins, for example, cyclin A, cyclin B, cdc2, and cdc25, where the changes observed in differentiated HL-60 cells do not correlate with either

TABLE III. Comparison of 8-Chloroadenosine Affecting Cell-Cycle Proteins in HL-60 Cell With Known Differentiation Inducers[a]

Reagent[b]	Phenotype of differentiation	Markers (time)	Cell-cycle arrest (time)	Changes of G_1-cyclins	Other cyclins, cdks[c]	Changes of CKIs[d]	Apoptosis (%)[e]
TPA[(a1–a10)]	Monocyte/marcophage	CD11 (size ↑, pseudopodia) (24 h <)	G_1/G_0 (24–36 h)[(a5, a8)]	cyclin D1 ↑[(a1, a4, a5)] cyclin E ↓[(a1, a5)]	cyclin A,B ↓[(a1, a5, a8, a9)] cdk2, cdk4, cdc2 ↓[(a1, a5, a8, a9)]	p21 ↑[(a2, a6)] p27 ↑[(a7)] p15 ↑, p18/19 ↓[(a10)]	<10
1,25D3[(a2, a6, a11, a12)]	Monocyte/marcophage	CD11 (24 h <)	G_1/G_0[(a11, a12)] (>24 h)	cyclin D1 ↑[(a11)] cyclin E ↑[(a11)]	?	p21 ↑[(a2, a6, a11)] p27 ↑[(a11)]	<10
DMSO[(a1–a3, a9, a10)]	Granulocytes	NBT reduction (size ↓, segment/ lobulated nuclei (>48 h)	G_1/G_0 (24 h)	Cyclin D1/D2 ↓[(a1)] cylcin E ± [(a1)]	cyclcin A, B ± [(a1, a9)] cdc2, cdc25 ± [(a9)] cdk2, cdk4 ↓[(a1)]	p21 ↑[(a2)] p18/19 ↑[(a10)]	<5
RA[(a1–a3)]	Granulocytes	NBT reduction (>48 h)	G_1/G_0 (24 h)	Cyclin D1 ± [(a1)]	cyclin E ↓[(a1)] cdk2 ↓ cdk4 ± [(a1)]	p21 ↑[(a2)]	<15
8-ClA (8-Cl-cAMP)[f]	Granulocytes*	NBT reduction* (>48 h)	G_1/G_0 (24 h)	cyclin D1 ↓	cdk2,cdk4 ±	p21 ↑ p27 ↑	<15

8-Cl-cAMP, 8-chloroadenosine 3′,5′-monophosphate; NBT, nitro blue tetrazolium.

[a]Data are obtained from references indicated as: Burger et al. [1994] (a1); Jiang et al. [1994] (a2); Bestilny et al. [1996] (a3); Akiyama et al. [1993] (a4); Horiguchi-Yamada et al. [1994] (a5); Schwaller et al. [1995] (a6); Millard et al. [1997] (a7); Hass et al. [1992] (a8); Horiguchi-Yamada and Yamada [1993] (a9); Schwaller et al. [1997] (a10); Wang et al. [1996] (a11); Zhang et al. [1994] (a12); Pepe et al. [1991] (a13). The marker used as: "↑" increase, "↓" decrease, "±" no change, "?" not known.

[b]Abbreviations for HL-60 chemical inducers are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 1,25D3, 1,25-dihydroxyvitamin D3; DMSO, dimethyl sulfoxide; RA, all-trans retinoic acid; 8-CA, 8-chloroadenosine.

[c]Cyclin A ($S + G_2$ phase), cyclin B (G_2 phase), cyclin E (late G_1/S phase), and cyclin D (non-periodic but affecting G_1 phase).

[d]Cyclin kinase inhibitors (CKIs): p15–p19: $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$; p21: $p21^{WAF1/CIP1}$; p27: $p27^{KIP1}$.

[e]The apoptosis rate is indicated by the concentrations for each reagent induced a significant rate of growth inhibition and differentiation.

[f]Consistent effects for 8-Cl-cAMP on HL-60 differentiation with 8-chloroadenosine (8-ClA) are indicated by "*" (from reference a13 and therein).

phenotype selection or G_1/G_0 growth arrest [Hass et al., 1992; Horiguchi-Yamada and Yamada, 1993; Burger et al., 1994; Horiguchi-Yamada et al., 1994]. Even cyclin E, a G_1-phase cyclin, is increased in 1,25D3-treatment, decreased in TPA- and RA-treatments, but not changed in DMSO-treatment [Burger et al., 1994; Horiguchi-Yamada et al., 1994; Wang et al., 1996].

In contrast, the expression of $p21^{WAF1/CIP1}$ is up-regulated with five known inducers, and accompanied with an up-regulation of $p27^{KIP1}$ in three agents including TPA, 1,25D3, and 8-chloroadenosine. $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ are known for their inhibition in cell-cycle progression and their regulatory effects on cell differentiation. Even regarded as a universal cdk-inhibitor [Xiong et al., 1993], $p21^{WAF1/CIP1}$ is obviously characterized by its G_1 effects [Gartel et al., 1996]. This suggests that the induction of $p21^{WAF1/CIP1}$ in HL-60 cell by 8-chloroadenosine is closely related to the role of G_1/G_0 arrest and it may play a role in HL-60 differentiation while cell maturation is selected [Freemerman et al., 1997; Li et al., 1998]. Further, the increased $p21^{WAF1/CIP1}$ in 8-chloroadenosine treated HL-60 cell leads to a low rate in apoptosis, consistent with other inducers [Jiang et al., 1994; Schwaller et al., 1995; Bestilny et al., 1996; Wang and Studzinski, 1997; Rahmani et al., 2003]. $p27^{KIP1}$ plays key roles in HL-60 cell arrested in G_1 phase by TPA and 1,25D3, but it is not correlated with HL-60 cell differentiation [Wang et al., 1996, 1998; Millard et al., 1997]. In addition, transforming factor TGFβ1 activates $p27^{KIP1}$ and/or $p21^{WAF1/CIP1}$ but inhibits *c-myc* that regulates cell-cycle progression [Silberstein and Daniel, 1987; Polyak et al., 1994; Alexandrow and Moses, 1995; Datto et al., 1995]. So, we attribute 8-chloroadenosine-indued growth inhibition, G_1/G_0 arrest and differentiation in HL-60 cells to a correlation of up-regulation $p21^{WAF1/CIP1}$ and/or $p27^{KIP1}$ with the induced TGFβ1 but reduced *c-myc*.

8-Chloroadenosine reduces telemorase activity, a critically important enzyme responsible for the proliferation and immortality of HL-60 cells [Bestilny et al., 1996; Janknecht, 2004]. Other chemical differentiation inducers, for example, TPA, DMSO, RA, and 1,25D3, also inhibit the expression of telomerase in these cells [Bestilny et al., 1996; Reichman et al., 1997]. This suggests that the telomerase activity may serve as a cellular marker for the differentiation process involved in HL-60 cells. However, the exact mechanism by which 8-chloroadenosine reduces telomerase activity at transcriptional and/or translational levels warrants further investigation. In this regard, recently a TGFβ1–$p21^{WAF1/CIP1}$ pathway has been reported to negatively regulate human telomerase reverse transcriptase (hTERT) and an up-regulation of telomerase associate proteins (TP1) as shown by several investigators [Reichman et al., 1997; Kagawa et al., 1999; Harada et al., 2000; Henderson et al., 2000; Rama et al., 2001, 2003; Newbold, 2002; Zeng and Tu, 2003]. The telomerase-specific inhibitors, Telomestatin (SOT-095) and polycyclic acridines, were also shown to enhance $p21^{WAF1/CIP1}$ expression in human leukemia cells [Missailidis et al., 2002; Tauchi et al., 2003], highlighting the importance of this molecule in the process of mediating the effects of 8-chloroadenosine on growth inhibition, G_1/G_0 arrest and differentiation.

In summary, we have shown that 8-chloroadenosine inhibits cell growth, alters differentiation, and arrests the cell cycle at G_1/G_0 phase, and that these effects correlated with reduced levels of cyclin D1 and telomerase and increased levels of $p21^{WAF1/CIP1}$ in a model system of human leukemia.

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