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8-CL-CAMP INDUCES CELL CYCLE-SPECIFIC APOPTOSIS IN HUMAN CANCER CELLS

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8-Cl-cyclic adenosine monophosphate (8-Cl-cAMP) has been known to induce growth inhibition and differentiation in a variety of cancer cells by differential modulation of protein kinase A isozymes. To understand the anticancer activity of 8-Cl-cAMP further, we investigated the effect of 8-Cl-cAMP on apoptosis in human cancer cells. Most of the tested human cancer cells exhibited apoptosis upon treatment with 8-Cl-cAMP, albeit with different sensitivity. Among them, SH-SY5Y neuroblastoma cells and HL60 leukemic cells showed the most extensive apoptosis. The effect of 8-Cl-cAMP was not reproduced by other cAMP analogues or cAMP-elevating agents, showing that the effect of 8-Cl-cAMP was not caused by simple activation of protein kinase A (PKA). However, competition experiments showed that the binding of 8-Cl-cAMP to the cAMP receptor was essential for the induction of apoptosis. After the treatment of 8-Cl-cAMP, cells initially accumulated at the S and G2/M phases of the cell cycle and then apoptosis began to occur among the population of cells at the S/G2/M cell cycle phases, indicating that the 8-Cl-cAMP-induced apoptosis is closely related to cell cycle control. In support of this assumption, 8-Cl-cAMP-induced apoptosis was blocked by concomitant treatment with mimosine, which blocks the cell cycle at early S phase. Interestingly, 8-Cl-cAMP did not induce apoptosis in primary cultured normal cells and non-transformed cell lines, showing that 8-Cl-cAMP-induced apoptosis is specific to transformed cells. Taken together, our results show that the induction of apoptosis is one of the mechanisms through which 8-Cl-cAMP exerts anticancer activity.

Key words: *8-Cl-cAMP; apoptosis; protein kinase A; cell cycle; human cancer cells*

Signals mediated by cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) have been implicated in the control of cell proliferation.[1,2] PKA consists of a regulatory (R) subunit dimer and two catalytic (C) subunits. Two isozymes of PKA, type I and type II, are distinguished by the association of type I R subunit (RI) and type II R subunit (RII) with the common C subunits, respectively. Four isoforms of the R subunit (RIα, RIβ, RIIα and RIIβ) and three isoforms of C subunit (Cα, Cβ and Cγ have been identified.[3]

Distinct roles of PKA isozymes in the control of cell growth and differentiation have been suggested. A high level of RI or type I PKA activity correlates with active cell growth and transformation, and a high level of RII or type II PKA activity is related to growth arrest and differentiation.[1] Indeed, introduction of RIα into normal epithelial cells conferred transformed phenotypes, *i.e.*, active cell proliferation and serum-independent growth,[4] whereas overexpression of RIIβ in cancer cells suppressed cell proliferation and transformed cell phenotypes.[5–7] It has been reported that selective modulation of PKA isozymes using site-selective cAMP analogues induces growth inhibition in a variety of cancer cells.[8] Among the tested cAMP analogues, 8-Cl-cAMP was most effective in the growth inhibition of cancer cells. In addition, 8-Cl-cAMP was shown to induce differentiation of human leukemic cells.[9,10] Through the studies with the antisense oligonucleotide against RIIβ mRNA and the mutant RIIβ, it was found that the RIIβ protein is essential in the 8-Cl-cAMP-induced growth inhibition and differentiation of cancer cells.[5,7,11] These reports suggested that 8-Cl-cAMP reverses the transformed phenotype of cancer cells through RIIβ/type II PKA.

The differentiation process both *in vivo* and *in vitro* often accompanies apoptotic cell death, suggesting that cell differentiation and apoptotic cell death are closely linked. Previously, we and others reported that 8-Cl-cAMP, alone or in combination with other drugs, induced apoptotic cell death in some cell lines,[12–15] suggesting that the induction of apoptotic cell death may be involved in the anticancer activity of 8-Cl-cAMP. Although the differential role of PKA and the potency of 8-Cl-cAMP in the control of cancer cell growth and differentiation have been well documented, the signaling pathway initiated by 8-Cl-cAMP leading to apoptotic cell death is still unclear. Given that growth inhibition and differentiation by 8-Cl-cAMP are caused by the selective modulation of PKA isozymes, it is likely that apoptotic cell death could also be explained by differential modulation of PKA isozymes.

In this study, we employed a variety of human cancer cell lines, non-transformed normal cell lines and primary culture of normal cells to examine the effect of 8-Cl-cAMP on cell growth and apoptotic cell death. We found that the induction of apoptotic cell death as well as growth inhibition was induced in most of the human cancer cell lines. None of the non-transformed cell lines and primary culture cells showed apoptotic cell death in response to 8-Cl-cAMP treatment. In addition, we demonstrate that 8-Cl-cAMP-induced apoptosis is mediated by the cAMP signal transduction pathway and is specific to the S/G2/M cell cycle phase, showing that cell cycle control is involved in the cAMP-mediated growth inhibition and induction of apoptosis in cancer cells.

Abbreviations: cAMP, cyclic AMP; PKA, protein kinase A; R and C, regulatory and catalytic subunits of protein kinase A, respectively; RI and RII, type I and type II regulatory subunits of protein kinase A, respectively; PARP, poly (ADP-ribose) polymerase; TUNEL, terminal deoxythymidine transferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacryl amide gel electrophoresis; FITC, fluorescein-5-isothiocyanate; HPLC, high performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; dbcAMP, N^6, 2′-*O*-dibutyryl cyclic AMP; H89, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cyclic AMP; cdk, cyclin-dependent kinase; PMA, phorbol 12-myristate 13-acetate.

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MATERIAL AND METHODS

Cell culture

SH-SY5Y, BE(2)-c, SK-N-SH, LS-174T, HeLa, MCF7 and CHO-K1 cells were maintained in Eagle's minimum essential medium (EMEM). SK-OV-3, OVCAR-3, DT and NIH/3T3 cells were grown in DMEM. The 2774, H460, H1299, HL60 and K562 cells were maintained in RPMI-1640 medium. L-132 cells were grown in RPMI-1640 medium containing 100 mM sodium pyruvate. Culture media were supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Gaithersburg, MD), 100 units/ml penicillin G and 100 μg/ml streptomycin. MCF-10A cells were maintained in DMEM/F-12 (1:1) supplemented with 5% heat-inactivated horse serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 0.5 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF) and 10 μg/ml insulin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. For the monolayer growth assay, cell number was determined with a Coulter's counter at the indicated time points.

Primary mouse cortical neuron cultures were prepared from fetal mice at 14 to 16 days of gestation. Dissociated cortical cells from 2.5 hemispheres of embryo brain were plated on a 24-well plate pre-coated with poly-D-lysine (50 μg/ml) and laminin (1 μg/ml). Cells were maintained in DMEM supplemented with 20 mM glucose, 2 mM glutamine, 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum (GIBCO-BRL) in a humidified atmosphere of 5% CO_2 at 37°C. Ten micromolar cytosine arabinoside was added 4 days after the plating and maintained for 2 days to halt the growth of non-neuronal cells. 8-Cl-cAMP was treated after 7 to 10 days of culture. For primary mouse splenocyte culture, single-cell suspensions were prepared by triturating the spleen with a syringe needle. Cells were grown at an initial density of 10^6/ml in Iscove's modified Dulbecco's medium containing 0.6% β-mercaptoethanol, 100 units/ml penicillin G, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum.

Acridine orange staining

Monolayer cells on culture dishes were stained *in situ* with acridine orange (4 μg/ml in culture medium). Suspension cells were harvested and resuspended in culture medium containing 4 μg/ml acridine orange. Cell suspensions were placed on a microscope slide for observation. Nuclear morphology was examined under a 10× objective with epifluorescence microscope.

Chromosomal DNA isolation and ladder formation assay

Harvested cells were suspended in lysis buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 0.5% laurylsarcosine) containing 50 μg/ml RNase A. After incubation at 37°C for 1 hr, proteinase K was added to the cell lysate at a concentration of 50 μg/ml and samples were incubated at 55°C for 2 hr. Chromosomal DNA was purified by phenol/chloroform extraction and precipitated with ethanol. Five micrograms of chromosomal DNA were electrophoresed in 2% agarose gel and visualized by staining with ethidium bromide.

Cell cycle analysis and evaluation of apoptosis by flow cytometry

For adherent cells, cells were harvested by gentle trypsinization and then suspended in PBS before fixation. Suspended cells were fixed with ice-cold 70% ethanol for at least 3 hr. Fixed cells were stained with 50 μg/ml propidium iodide containing 50 μg/ml RNase A at 37°C for 30 min. DNA contents of cells (10,000 cells/experimental group) were analyzed by a FACStar^{PLUS} flow cytometer (B&D, Mountain View, CA) using Lysis II and CELLFIT programs (B&D).

Protein kinase A activity assay

Preparation of cell extract and protein kinase A activity assay were performed as described.[16]

Western blot analysis

Preparation of cell extracts and Western blot analysis were performed as previously described.[6,16] Polyclonal antibodies against PKA subunits were also previously described.[16,17] Antibody for poly (ADP-ribose) polymerase was purchased from PharMingen (San Diego, CA). Antibody for CPP32 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The specific band for each protein was quantified by densitometry.

Cyclin B/cdk1 activity assay

Harvested cells were washed with PBS, suspended in extraction buffer (50 mM Tris-Cl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 50 mM NaF, 0.1 mM $NaVO_4$, 100 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 mM leupeptin, 0.5 μg/ml aprotinin), and incubated on ice for 20 min. After centrifugation at 10,000*g* at 4°C for 15 min, supernatant was collected as cell extract. One microgram of cyclin B1 monoclonal antibody (Santa Cruz Biotechnology) was added to 300 μg of cell extract in 500 μl of extraction buffer and incubated for 4 hr with continuous agitation. Immune complex was collected with protein A-sepharose and washed three times with extraction buffer and twice with kinase buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT]) consecutively. Immunoprecipitates were suspended in 50 μl of kinase reaction mixture (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 μM [γ-^{32}P]ATP, 5 μg histone H1) and incubated at 37°C for 30 min. Reaction was stopped by the addition of 50 μl of 2× SDS-PAGE loading buffer. ^{32}P-labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

TUNEL analysis combined with cell cycle analysis

Terminal deoxythymidine transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using an *in situ* cell death detection kit (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. In brief, harvested cells were fixed in 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100. Fixed cells were labeled with fluorescein isothiocyanate (FITC)-dUTP using terminal deoxythymidine transferase and stained with 5 μg/ml propidium iodide. DNA content and FITC labels of cells (10,000 cells/experimental group) were analyzed by a FACStar^{PLUS} flow cytometer (B&D) using Lysis II program (B&D).

RESULTS

Induction of apoptosis in human cancer cells by 8-Cl-cAMP

Previous reports showed that 8-Cl-cAMP induces growth inhibition and differentiation in a variety of cancer cells by differential regulation of PKA isozymes.[1] Since apoptosis is closely related to cell proliferation and differentiation, we tested whether 8-Cl-cAMP could induce apoptosis as well as growth inhibition and differentiation in cancer cells. Growth of the most human cancer cells was inhibited by more than 50% after 4 days of 10 μM 8-Cl-cAMP treatment (Fig. 1*a*). However, non-transformed cell lines showed only a marginal growth inhibition in response to 8-Cl-cAMP treatment. This tendency was evident when the effects on cancer cells were compared with those on their normal counterpart (H460 and H1299 *vs.* L-132; MCF7 *vs.* MCF-10A; DT *vs.* NIH/3T3).

To test whether apoptosis also occurs in 8-Cl-cAMP-treated cancer cells, nuclear morphology was examined by staining with acridine orange. Although the sensitivities were different, most of the tested cells exhibited typical nuclear morphology of apoptotic cells, *i.e.*, condensed or fragmented nuclei (Fig. 1*a*,*b*). In contrast, none of the non-transformed cell lines showed any sign of apoptotic cell death (Fig. 1*a*). Among the human cancer cells, SH-SY5Y neuroblastoma cells and HL60 leukemic cells showed the most evident sign of apoptosis. These two cell lines also exhibited a chromosomal DNA ladder in agarose gel electrophoresis (Fig. 1*c*).

A

Cell Lines	Growth inhibition	Apoptosis
LS-174T	+++	+
HeLa	+++	++
SK-N-SH	+++	+
BE(2)-c	+++	++
SH-SY5Y	+++	++
HL60	++++	++++
K562	+++	-
2774	++++	+
SK-OV-3	+++	++
OVCAR-3	++	++
CHO-K1	+	-
H460	+++	+
H1299	+++	+
L-132	-	-
MCF7	++++	++
MCF-10A	+	-
DT	++++	-
NIH/3T3	++	-

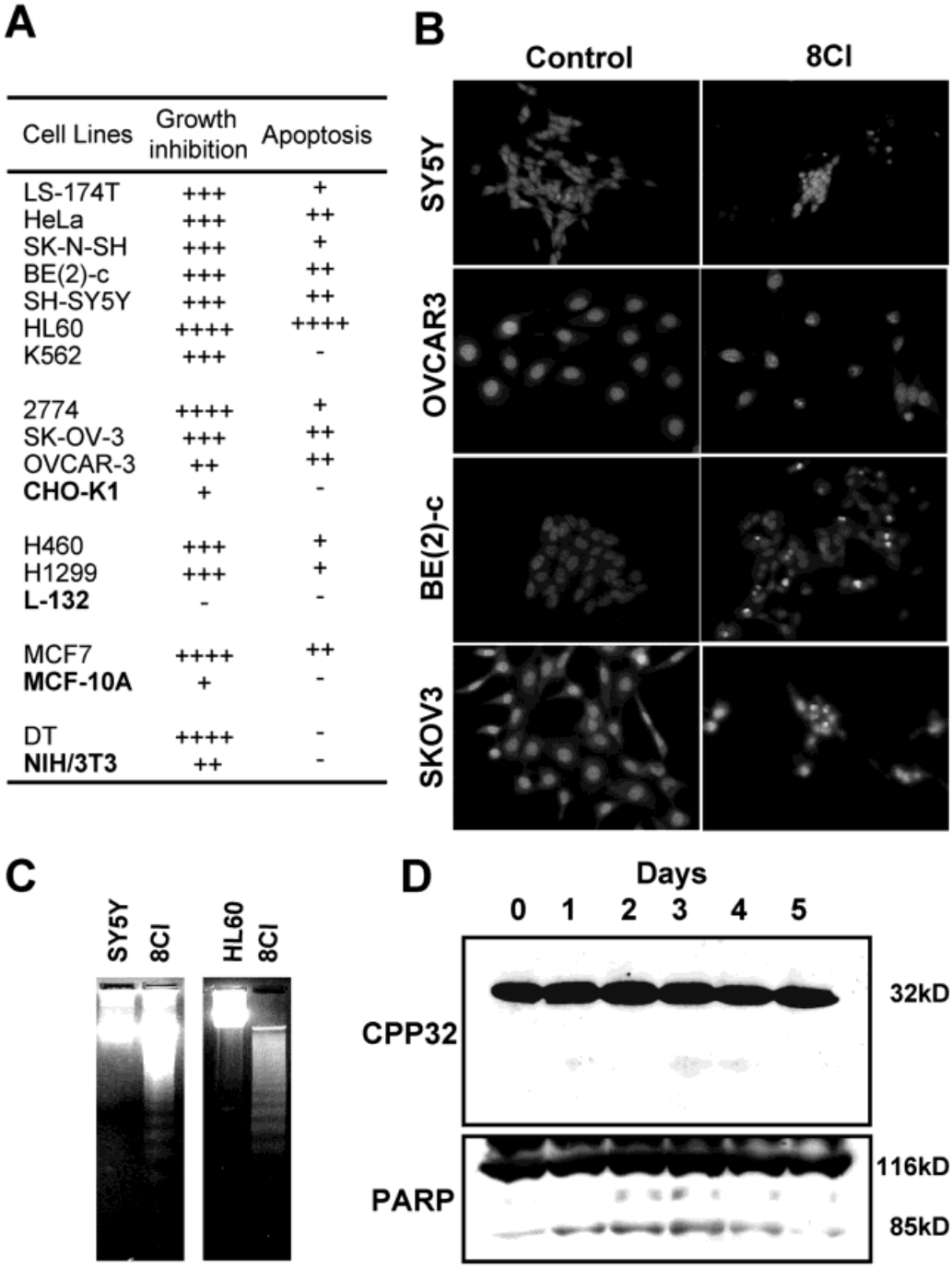

FIGURE 1 – Growth inhibition and induction of apoptosis by 8-Cl-cAMP in human cancer cells. (*a*) Growth inhibition by 8-Cl-cAMP. Cells were seeded at a density of 2×10^4 to 2×10^5 cells/35 mm dish, and 10 μM 8-Cl-cAMP was treated after 16 hr. Cell numbers were determined at day 4. For HL60 cells, 5 μM 8-Cl-cAMP was treated. Non-transformed cells are in boldface and are positioned below the cancer cells of the same tissue origin. Apoptotic cells were scored by counting the cells with condensed or fragmented nuclei when stained with acridine orange. The symbols for growth inhibition and apoptosis are as follows: −, less than 5%; +, 5% to 25%; ++, 25% to 50%; +++, 50% to 75%; ++++, 75% to 100%. (*b*) Nuclear morphology of 8-Cl-cAMP-treated cells. Cells treated with 10 μM 8-Cl-cAMP for 4 days were stained with acridine orange to visualize nuclear morphology. Apoptotic cells with condensed or fragmented nuclei are visible in 8-Cl-cAMP treated cells. Magnification ×417. (*c*) Chromosomal DNA ladder formation. Chromosomal DNA from the cells treated with 10 μM 8-Cl-cAMP for 4 days (SH-SY5Y cells) and with 5 μM 8-Cl-cAMP for 2 days (HL60 cells) was electrophoresed in 2% agarose gel. (*d*) Western blot analyses on the CPP32 and PARP. The fragmentations of CPP32 and PARP were examined in SH-SY5Y cells treated with 10 μM 8-Cl-cAMP. The durations of 8-Cl-cAMP treatment are indicated.

To test whether the activation of caspase is involved in the 8-Cl-cAMP-induced apoptosis, we examined the cleavage of CPP32 and poly (ADP-ribose) polymerase (PARP) by Western blot analyses. Up to day 5, the cleaved fragment of CPP32 was not detected in 8-Cl-cAMP-treated SH-SY5Y cells (Fig. 1*d*). However, the cleaved form of PARP was increased up to day 3 and then

decreased thereafter (Fig. 1*d*). The increase in PARP cleavage was followed by an increase in apoptotic cells containing sub-2N DNA content after a 1-day time interval (see Fig. 5*a*). These results suggest that the activation of caspase, other than CPP32, is involved in the 8-Cl-cAMP-induced apoptosis.

Down-regulation of type I regulatory subunit of protein kinase A in 8-Cl-cAMP-treated cells

The selective activation and down-regulation of type I PKA by 8-Cl-cAMP has been extensively studied in HL60 cells.[18] Since type I PKA has been linked to active cell growth and transformation, down-regulation of type I PKA has been considered an important event in the 8-Cl-cAMP-induced growth inhibition. To test whether 8-Cl-cAMP suppresses type I PKA in SH-SY5Y cells as in HL60 cells, the effects of 8-Cl-cAMP on the PKA activity and isozyme pattern were examined. In cells treated with 10 μM 8-Cl-cAMP, the basal PKA activity (measured in the absence of cAMP) increased and the total PKA activity (assessed in the presence of 5 μM cAMP) decreased compared with non-treated control (Fig. 2*a*). These changes in the PKA activity were consistent in the repetitive experiments, even though they were not so remarkable. These results indicate that 8-Cl-cAMP does activate intracellular PKA and down-regulate PKA holoenzyme.

The effect of 8-Cl-cAMP on the PKA isozyme pattern was also examined by Western blot analyses of the PKA subunits (Fig. 2*b*). In the 8-Cl-cAMP-treated cells, the RIα subunit level decreased by 50% at day 1 and further decreased up to day 5. Levels of RIIα and RIIβ subunits did not change under the same conditions. The Cα subunit level decreased by 50% at day 3 and the decreased level was maintained thereafter. To confirm that the down-regulation of RIα is specific to 8-Cl-cAMP, we examined the changes in the PKA subunit level in cells treated with other cAMP analogues for 4 days. None of the cAMP analogues, N^6,2′-O-dibutyryl-cAMP (dbcAMP), 8-Br-cAMP and 8-(4-chlorophenylthio)-cyclic AMP (8-CPT-cAMP), caused a decrease in RIα. dbcAMP-treated cells showed even higher levels of RIα protein. These results show that only 8-Cl-cAMP selectively down-regulates type I PKA in SH-SY5Y cells as in HL60 cells.

Effects of non-selective cAMP analogues or cAMP-elevating agents

Since 8-Cl-cAMP is a cAMP analogue that activates PKA (Fig. 2*a*), we decided to determine whether the 8-Cl-cAMP-induced apoptosis is caused by a simple increase in PKA catalytic activity or by the isozyme-selective action of 8-Cl-cAMP. We therefore tested whether non-selective activation of PKA is enough to in-

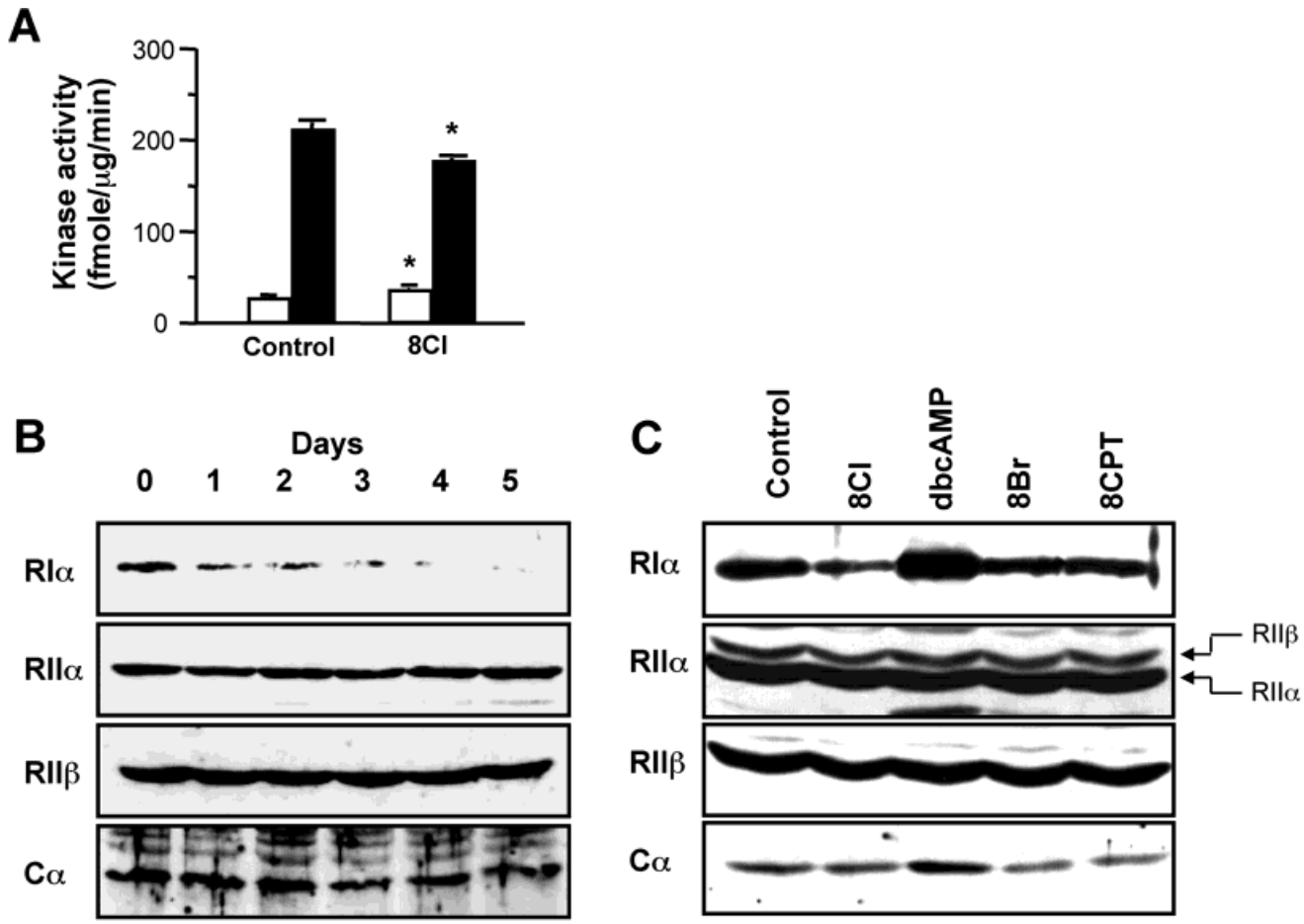

Figure 2 – Effect of 8-Cl-cAMP on the catalytic activity and the subunit levels of PKA in SH-SY5Y cells. (*a*) Catalytic activity of PKA. SH-SY5Y cells were mock-treated (Control) or treated with 10 μM 8-Cl-cAMP (8Cl) for 3 days and the catalytic activity of PKA was analyzed. PKA activity was determined in the absence (open bar) or presence (closed bar) of 5 μM cAMP. Data show mean ± SD of three independent experiments of duplicate determinations. Asterisks show significant difference of PKA activity between control and 8-Cl-cAMP-treated cells (*, $p < 0.05$, two-sided *t*-test). (*b*) Western blot analysis on PKA subunits. SH-SY5Y cells were treated with 10 μM 8-Cl-cAMP and changes in the levels of PKA subunits were examined. Durations of 8-Cl-cAMP treatment are indicated. (*c*) Effects of various cAMP analogues on the levels of PKA subunits. SY-SY5Y cells were treated with 10 μM 8-Cl-cAMP, 300 μM dbcAMP, 10 μM 8-Br-cAMP and 10 μM 8-CPT-cAMP for 4 days and the levels of PKA subunits were examined. An RIIβ band was also detected with the RIIα antibody because of the cross-reactivity.

duce growth inhibition and apoptosis. The PKA activators forskolin (1 to 20 μM), 3-isobutyl-1-methylxanthine (IBMX; 50 to 500 μM) and dbcAMP (100 to 500 μM) effectively increased PKA catalytic activity in SH-SY5Y and HL60 cells (data not shown). Although the effects of these PKA activators on cell proliferation were varied (Fig. 3*a*,*c*), none of them induced apoptosis in both cell lines (Fig. 3*b*,*d*). 8-CPT-cAMP, 8-Br-cAMP and N^6-monobutyryl cAMP did not induce apoptosis either (data not shown). These results indicate that a simple increase in PKA catalytic activity does not cause apoptosis in cancer cells.

Mediation of 8-Cl-cAMP-induced apoptosis by cAMP signal

Since 8-Cl-cAMP-induced growth inhibition and apoptosis could not be reproduced by other cAMP analogues or cAMP-elevating agents, we checked whether the action of 8-Cl-cAMP is mediated by cAMP signal. To this end, we tested whether blocking the binding of 8-Cl-cAMP to a cAMP receptor could abrogate 8-Cl-cAMP-induced apoptosis, using cAMP or dbcAMP as a competitor for binding to the cAMP receptor. 8-Cl-cAMP-induced apoptosis was blocked by co-treatment with IBMX, which increases intracellular cAMP concentration through the inhibition of phosphodiesterase, or dbcAMP (Fig. 4*a* [8Cl/dbcAMP], *c*). Although we have used IBMX and dbcAMP to inhibit the binding of 8-Cl-cAMP to its receptor, it is also possible that the increase in PKA catalytic activity and the resulting changes in the sensitivity toward apoptosis signal caused the suppression of 8-Cl-cAMP-induced apoptosis. To clarify this point, we tested the effect of dbcAMP when PKA catalytic activity was suppressed. Using H89, a specific inhibitor of PKA catalytic activity, we tested the effect of dbcAMP without an increase of PKA catalytic activity. The apoptosis-inhibiting effect of dbcAMP was not abrogated by concomitant treatment of H89 (Fig. 4*a* [8Cl/dbcAMP/H89]). Under this condition, 8-Cl-cAMP single-treated cells and 8Cl/dbcAMP/H89 triple-treated cells showed comparable intracellular PKA activity (Fig. 4*b*, open bar).

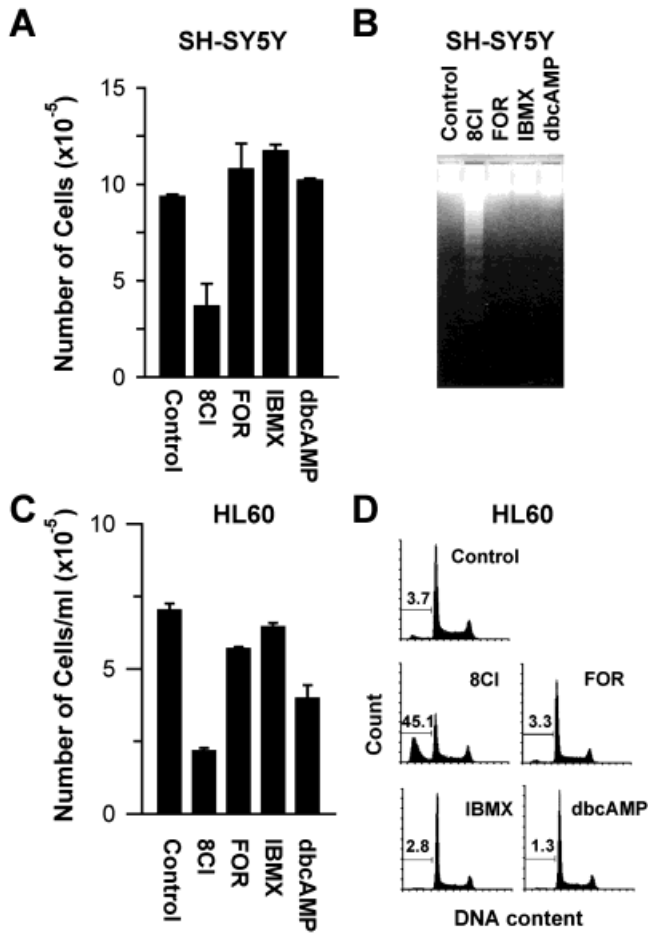

FIGURE 3 – Effects of dbcAMP and cAMP-elevating agents on cell growth and apoptosis. (*a*) Effects on monolayer growth in SH-SY5Y cells. Cells were seeded at a density of 2×10^5 cells/35 mm dish on day 0 and mock-treated (Control) or treated with 10 μM 8-Cl-cAMP (8Cl), 10 μM forskolin (FOR), 100 μM IBMX (IBMX) or 300 μM dbcAMP (dbcAMP) on day 1. Cell numbers were determined on day 5. (*b*) Effect on apoptosis in SH-SY5Y cells. SH-SY5Y cells treated as in (*a*) were subjected to chromosomal DNA ladder formation assay. Lane designations are the same as in (*a*).(*c*) Effects on cell growth in HL60 cells. Cells (initial density of 10^5 cells/ml) were treated at the same concentrations as in (*a*) except that of 8-Cl-cAMP (5 μM) for 3 days. (*d*) Effects on apoptosis in HL60 cells. Cells were mock-treated (Control), treated with 5 μM 8-Cl-cAMP (8Cl), 10 μM forskolin (FOR), 100 μM IBMX or 300 μM dbcAMP (dbcAMP) for 48 hr and then analyzed by flow cytometry. In (*a*) and (*c*), data show mean ± SD of three independent experiments of triplicate determinations. (*b*) and (*d*) show representatives from three separate experiments that gave similar results.

These results show that a simple increase in PKA catalytic activity is not responsible for the inhibition of 8-Cl-cAMP-induced apoptosis. We also found that dbcAMP is not a general inhibitor of apoptosis in that the apoptosis induced by staurosporine or carboplatin was not affected by co-treatment with dbcAMP (Fig. 4*d*). Therefore, the apoptosis-inhibiting effect of dbcAMP is caused by the competitive inhibition of 8-Cl-cAMP to the cAMP receptor. These results, taken together, indicate that the action of 8-Cl-cAMP is mediated by the cAMP signaling pathway, in which the binding of cAMP to its receptor is essential.

Cell cycle-specific occurrence of 8-Cl-cAMP-induced apoptosis

Since cell proliferation is closely related to cell cycle progression, it was expected that the treatment of 8-Cl-cAMP would change the cell cycle distribution. In both SH-SY5Y cells and HL60 cells, the population of the G1 cell cycle phase decreased upon treatment with 8-Cl-cAMP (Fig. 5*a*,*b*). During the same period, cells in the S/G2/M cell cycle phase increased and then started to decrease with the appearance of apoptotic cells (day 3 in Fig. 5*a* and 24 hr in Fig. 5*b*). The effect of 8-Cl-cAMP on cell cycle distribution was also confirmed by examining the cell cycle-specific kinase activity. The cyclin B/cdk1 activity, which peaks at mitosis, increased more than 3-fold in 8-Cl-cAMP-treated SH-SY5Y cells, indicating that 8-Cl-cAMP indeed increases the M phase cells or promotes the activation of cyclin B/cdk1 (Fig. 5*c*).

Because the decrease in S/G2/M cells and the increase in apoptotic cells occurred simultaneously, we hypothesized that the cells were arrested during or after DNA synthesis and then driven to apoptosis specifically at these cell cycle phases. To test this hypothesis, the cell cycle and the occurrence of apoptosis in the respective cells were examined using flow cytometry (Fig. 5*d*). Since the cell cycle phase of dying cells could not be identified after severe DNA fragmentation, the initial time point of apoptosis occurrence was chosen for the analysis. SH-SY5Y cells were treated with 8-Cl-cAMP for 2.5 days and then double-stained with terminal deoxythymidine transferase-mediated dUTP nick end labeling (TUNEL) and propidium iodide. In 8-Cl-cAMP-treated cells, most of the TUNEL-positive (apoptotic) cells showed >2N DNA content. Thus, it is likely that 8-Cl-cAMP-induced apoptosis occurs specifically in the population of cells at the S/G2/M cell cycle phases.

Mimosine is an amino acid derivative that effectively inhibits DNA replication in mammalian cells and blocks cell cycle at early S phase.[19] Since 8-Cl-cAMP-induced apoptosis occurs specifically at the S/G2/M phase, inhibition of S phase progression by mimosine treatment would block 8-Cl-cAMP-induced apoptosis. Mimosine alone (200 μM to 1 mM) induced apoptosis in SH-SY5Y cells. When 8-Cl-cAMP and mimosine were treated simultaneously, however, the effect on apoptosis was not so much differ-

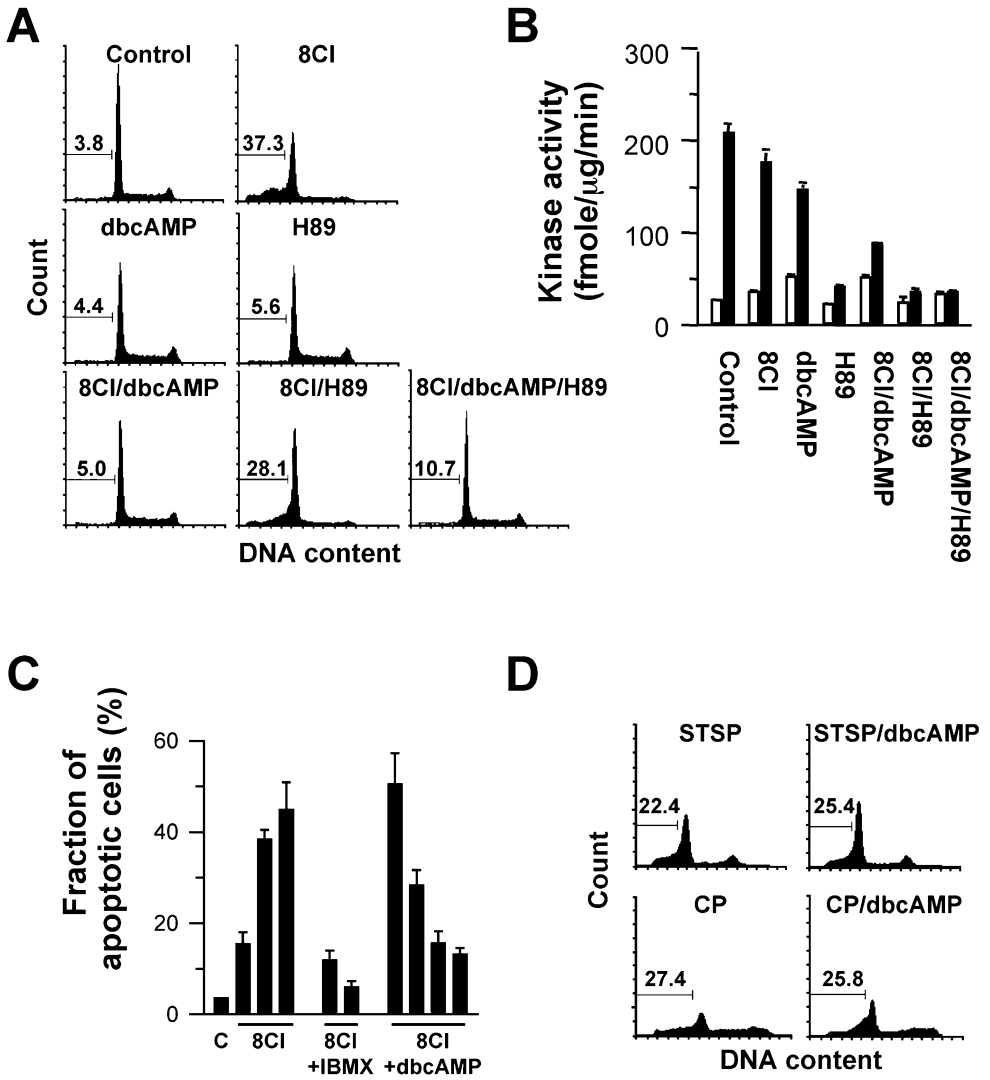

FIGURE 4 – Blocking of 8-Cl-cAMP-induced apoptosis by competitive inhibition of 8-Cl-cAMP binding to cAMP receptor. (*a*) Blocking of 8-Cl-cAMP-induced apoptosis by dbcAMP in SH-SY5Y cells. Cells were mock-treated (Control) or treated with 10 μM 8-Cl-cAMP (8Cl), 300 μM dbcAMP (dbcAMP), 5 μM H89 (H89) or their combinations. After 4 days of treatment, the induction of apoptosis was measured by propidium iodide staining followed by flow cytometry. Three separate experiments gave similar results. (*b*) Protein kinase A activity. SH-SY5Y cells were treated as in (*a*) for 3 days and the catalytic activity of PKA was measured in the absence (open bar) and the presence (closed bar) of 5 μM cAMP. Data show mean ± SD of three independent experiments of duplicate determinations. (*c*) Dose-dependent blocking of 8-Cl-cAMP-induced apoptosis by IBMX and dbcAMP in HL60 cells. Cells were mock-treated (C), treated with 8-Cl-cAMP alone (1, 3 and 5 μM), 8-Cl-cAMP (5 μM) plus IBMX (100 and 300 μM), or 8-Cl-cAMP (5 μM) plus dbcAMP (10, 50, 100 and 300 μM) for 48 hr and the apoptosis was measured by propidium iodide staining followed by flow cytometry. Data show mean ± SD of three separate experiments. (*d*) Effects of dbcAMP on staurosporine- and carboplatin-induced apoptosis. SH-SY5Y cells were treated with staurosporine (STSP) alone (30 μM), staurosporine plus dbcAMP (30 and 300 μM, respectively), carboplatin (CP) alone (1.5 μg/ml) or carboplatin plus dbcAMP (1.5 μg/ml and 300 μM, respectively) for 4 days before flow cytometry analysis.

ent from that of mimosine single treatment (Fig. 5*e*). These data also confirm that 8-Cl-cAMP-induced apoptosis is restricted at the S/G2/M cell cycle phase.

Effect of 8-Cl-cAMP in non-transformed cells

Because 8-Cl-cAMP restrains cancer cell growth by reversing transformed phenotype, it could be expected that the apoptosis-inducing effect of 8-Cl-cAMP is specific to transformed cells. It has already been reported that the growth-inhibiting effect of 8-Cl-cAMP is confined to transformed cells.[1] Specificity for transformed cells in the apoptosis-inducing effect is a preferable characteristic of therapeutic cancer drugs. To examine the effect of 8-Cl-cAMP in non-transformed cells *in vitro*, we employed primary culture of mouse embryonic brain cortical neurons. Because the apoptosis-inducing effect of 8-Cl-cAMP was evident in neuroblastoma cells like SH-SY5Y and BE(2)-c, a primary neuron culture would serve as a proper non-transformed cell control. This culture shows fully differentiated features of neurons, such as extended neurites and expression of several neuron-specific marker proteins including neuron-specific enolase and microtubule-associated protein II (data not shown). Interestingly, 8-Cl-cAMP-treated neurons did not show any sign of cell death after examination by cellular morphology and chromosomal DNA ladder formation assay (Fig. 6*a*).

To examine the effect of 8-Cl-cAMP on lymphocytes that play important roles in the host defense against diseases, we also set up a primary mouse spleen cell culture. *In vitro* activation of lym-

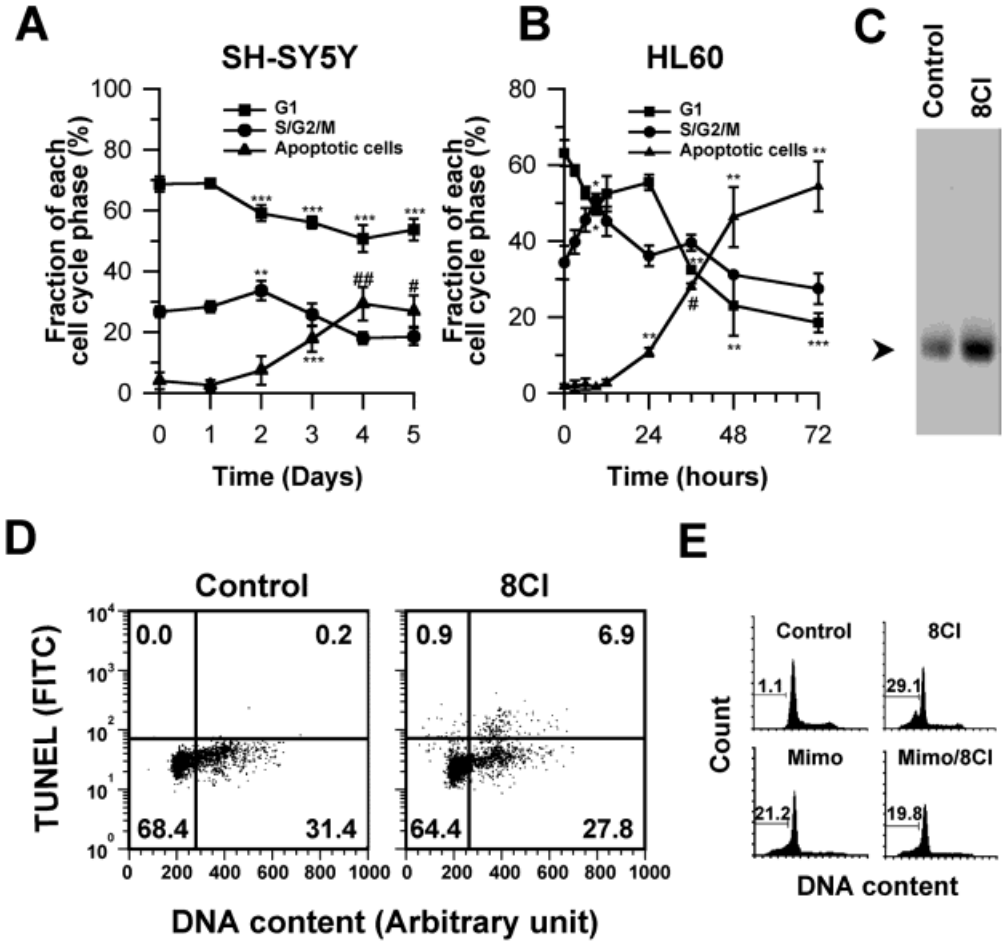

FIGURE 5 – Cell cycle-specific induction of apoptosis by 8-Cl-cAMP. (*a, b*) Changes in the cell cycle distribution in 8-Cl-cAMP-treated SH-SY5Y cells (*a*) and HL60 cells (*b*). Cells were treated with 10 μM (SH-SY5Y) or 5 μM (HL60) 8-Cl-cAMP on day 0 and the cell cycle was analyzed using propidium-iodide staining followed by flow cytometry. Cells with less than 2N of DNA content were scored as apoptotic cells. Graphs show mean ± SD of four (*a*) and three (*b*) separate experiments. Asterisks and sharps show significant differences of the percentage in each cell cycle phase between day 0 and each time point (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; #, $p < 0.001$; ##, $p < 0.0005$, two-sided *t*-test). (*c*) Change in cyclin B/cdk1 activity in 8-Cl-cAMP treated SH-SY5Y cells. Cyclin B-associated histone H1 kinase activity was examined in mock-treated cells (Control) or cells treated with 10 μM 8-Cl-cAMP (8Cl) on day 3 of treatment. The histone H1 band is marked with arrowhead. (*d*) Induction of apoptosis at S/G2/M phases. SH-SY5Y cells were mock-treated (Control) or treated with 10 μM 8-Cl-cAMP (8Cl) for 2.5 days. Cells were stained with TUNEL using FITC-labeled dUTP and then with propidium iodide. Staining with both fluorescent dyes was analyzed by flow cytometry. The percentage of cells in each window is indicated. Four separate experiments gave similar results. (*e*) Effect of mimosine on the 8-Cl-cAMP-induced apoptosis. SH-SY5Y cells were treated individually or in combination as indicated and apoptosis was analyzed on day 4. Designations are as follows: Control, mock treatment; 8Cl, 10 μM 8-Cl-cAMP alone; Mimo, 800 μM mimosine.

phocytes by combined treatment with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore caused an increase in apoptotic cell death, which is a well-known effect of lymphocyte activation. However, apoptotic cell death in either non-activated or activated lymphocytes was not affected by the 8-Cl-cAMP treatment (Fig. 6*b*). We also tested the effect of 8-Cl-cAMP on established non-transformed cell lines. In all the tested non-transformed cell lines, NIH/3T3, CHO-K1, MCF-10A and L-132, no sign of apoptosis was seen, after examination by nuclear morphology (Fig. 6*c*) and any other methods described above (data not shown). Taken together, these results indicate that the apoptosis-inducing effect of 8-Cl-cAMP is restricted to transformed cells.

DISCUSSION

It is known that differential regulation of PKA isozymes by cAMP analogues or suppression of type I PKA by antisense oligonucleotide can reverse the transformed phenotype of cancer cells *in vitro* and can inhibit tumor growth *in vivo*.[20,21] Since these effects were thought to be due to the restoration of normal cAMP signaling and to be specific to transformed cells,[1] we considered cAMP signaling as a target for the selective induction of apoptosis as well as growth inhibition and differentiation in cancer cells. We examined the effect of 8-Cl-cAMP on cell growth and apoptosis in a group of human cancer cells originating from various tissues including colon (LS-174T), uterus (HeLa), ovary (2774, SK-OV-3 and OVCAR-3), lung (H460 and H1299), breast (MCF7), brain (SK-N-SH, BE(2)-c and SH-SY5Y) and hematopoietic cells (HL60 and K562).

To compare the effect of 8-Cl-cAMP in non-transformed cells, several non-transformed cell lines were also tested (L-132, lung; MCF-10A, breast; NIH/3T3, fibroblast; CHO-K1, ovary). Most of the tested human cancer cells showed significant growth inhibition on day 4 of 10 μM 8-Cl-cAMP treatment and the typical nuclear morphology of apoptotic cells (Fig. 1*a*,*b*). Among the human cancer cells, SH-SY5Y neuroblastoma cells and HL60 leukemic cells exhibited the most evident signs of apoptosis including chro-

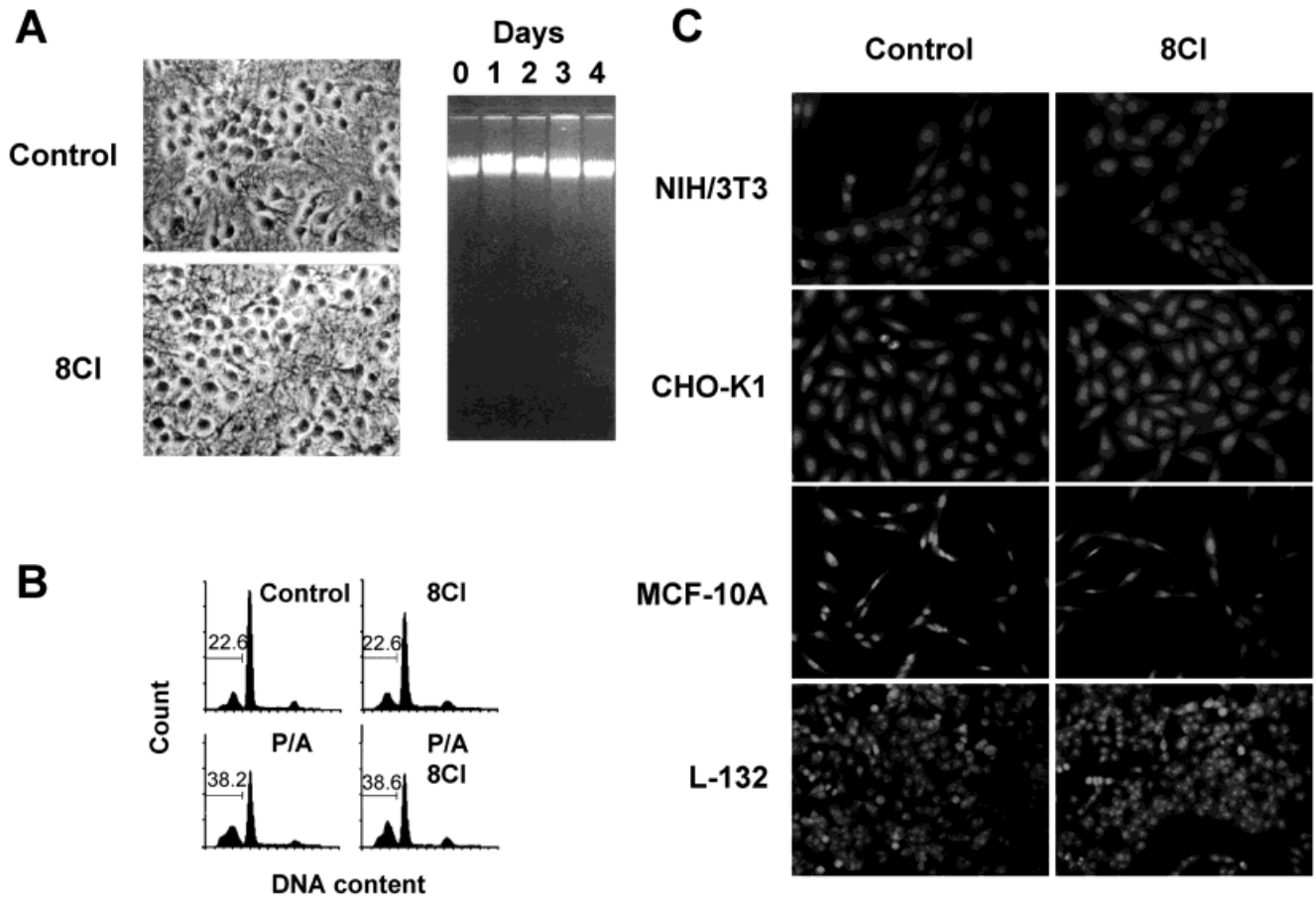

FIGURE 6 – Effect of 8-Cl-cAMP in non-transformed cells. (*a*) Effect in mouse cortical neuron culture. Mouse cortical neuron cultures were treated with 8-Cl-cAMP for 4 days and apoptosis was examined by cellular morphology and chromosomal DNA ladder formation. Control, mock-treated cells; 8Cl, 10 μM 8-Cl-cAMP-treated cells. Durations of 8-Cl-cAMP treatment for chromosomal DNA ladder formation assay are indicated. (*b*) Effect on splenocyte culture. Mouse splenocyte cultures were treated with 10 μM 8-Cl-cAMP (8Cl), 12 nM PMA plus 1 mM A23187 (P/A) alone or in combination as indicated. Apoptotic cell death was examined on day 2 of treatment. (*c*) Effect in non-transformed cell lines. NIH/3T3, CHO-K1, MCF-10A and L-132 cells were treated with 10 μM 8-Cl-cAMP for 4 days. Nuclear morphology was examined by acridine orange staining. Original magnification ×417.

mosomal DNA ladder formation (Fig. 1*c*) and typical apoptotic morphology by electron microscopy (data not shown). Although we could not detect activation of CPP32, the most common apoptosis-causing caspase, caspase action seems to be necessary for 8-Cl-cAMP-induced apoptosis. Indeed, PARP was cleaved to an 85 kDa fragment during 8-Cl-cAMP-induced apoptosis (Fig. 1*d*), and zVAD-fmk, a broad-range caspase inhibitor, efficiently blocked 8-Cl-cAMP-induced apoptosis.[14]

The most interesting finding in the present study is that the apoptosis-inducing effect of 8-Cl-cAMP is restricted to cancer cells. Primary cortical neuron culture and four non-transformed cell lines showed no signs of apoptosis in response to 8-Cl-cAMP treatment (Fig. 6). Apoptosis in activated or non-activated primary splenocyte (peripheral lymphocyte) cultures was also not affected by 8-Cl-cAMP treatment. The resistance of primary normal cells and non-transformed cell lines to apoptosis induction is not general to other apoptosis-inducing drugs in that other drugs, such as staurosporine and ceramide, efficiently induced apoptosis in these cells as in cancer cells (data not shown). Thus, it is likely that the apoptosis-inducing effect of 8-Cl-cAMP is specific to transformed or cancer cells. Currently we do not know why the apoptosis-inducing effect of 8-Cl-cAMP is specific to cancer cells. One possible explanation is that 8-Cl-cAMP reverses the abnormal cAMP signaling in cancer cells and activates the apoptosis pathway that can prevent abnormal cell proliferation. It is also possible that the conflict between differentiation/growth inhibition stimulus provided by 8-Cl-cAMP and the tendency of cancer cells to proliferate continuously caused apoptotic cell death. In this light, it would be interesting to test the effect of 8-Cl-cAMP on non-transformed cells of various origins and in various growth conditions that can affect cell proliferation rate.

Our results also showed that 8-Cl-cAMP-induced apoptosis is related to cell cycle control (Fig. 5). Cells accumulated at the S/G2/M cell cycle phases during the early period of 8-Cl-cAMP treatment, cyclin B/cdk1 activity was increased and the induction of apoptosis was specific to the S/G2/M cell cycle phase. The cell cycle specificity of apoptosis was verified by the observation that the cells arrested at early S phase by mimosine treatment were not sensitive to 8-Cl-cAMP-induced apoptosis. This point is quite interesting because only proliferating cells are targeted for 8-Cl-cAMP-induced apoptosis. Although the proliferating non-transformed cells that we have tested in this study are resistant to 8-Cl-cAMP-induced apoptosis, the cell cycle specificity may provide an additional advantage for the use of 8-Cl-cAMP as a therapeutic cancer drug.

Up to the present, two families of cAMP receptor proteins have been identified. One of them is the regulatory subunit of PKA, which was considered as the sole effector molecule of cAMP signaling in mammalian cells. Another family of cAMP receptor

proteins, named cAMP-GEF or Epac, was also identified.[22,23] These proteins are Rap-1 guanine nucleotide exchange factors whose activities are regulated by cAMP. Through these proteins, cAMP can activate the Rap-1-dependent signal transduction pathway. A well-known downstream effector of Rap1 is ERK-type MAP kinase.[24] Since cAMP may change MAP kinase activity through cAMP-GEFs and MAP kinase activity is known to suppress apoptosis,[25] changes in MAP kinase activity might be involved in 8-Cl-cAMP-induced growth inhibition and apoptosis. However, we found that 8-Cl-cAMP did not influence MAP kinase activity significantly and an inhibitor of MEK (MAP kinase kinase; PD98059, 1 to 10 μM) did not affect 8-Cl-cAMP-induced apoptosis of SH-SY5Y cells (data not shown). Considering that the antiproliferative and differentiation-inducing effect of 8-Cl-cAMP is mediated by type II PKA,[5,11] we reason that the major effector of 8-Cl-cAMP-induced apoptosis is PKA.

In the present report, we provide experimental evidence that 8-Cl-cAMP induces apoptosis specifically in cancer cells but not in primary cultured normal cells or non-transformed cell lines. 8-Cl-cAMP-induced apoptosis is also related to cell cycle control. We propose that the cAMP signal transduction pathway can be a therapeutic target for cancer treatment through modulation of the cell cycle and the induction of apoptosis. In addition, we present the positive aspects of 8-Cl-cAMP as a therapeutic cancer drug.

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