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Dual Anticancer Activity of 8-Cl-cAMP: Inhibition of Cell Proliferation and Induction of Apoptotic Cell Death

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8-Cl-cAMP induces apoptotic cell death in human cancer cells. To look at this more closely, we examined the changes in the levels of Bcl-2 family proteins during 8-Cl-cAMP-induced apoptosis of SH-SY5Y human neuroblastoma cells. Following the treatment with 8-Cl-cAMP, Bcl-2 was transiently down-regulated and Bad was increased continuously up to day 5. In addition, overexpression of Bcl-2 efficiently blocked the 8-Cl-cAMP-induced apoptosis, suggesting Bcl-2 family proteins may be involved in the 8-Cl-cAMP-induced apoptosis. The contribution of the apoptotic cell death and the inhibition of cell proliferation in the 8-Cl-cAMP-induced growth inhibition was closely monitored in the Bcl-2-overexpressing cells. Though the apoptosis was reduced significantly, no significant difference was observed in the inhibition of cell proliferation up to day 2 of 8-Cl-cAMP treatment. These results suggest that 8-Cl-cAMP exerts anticancer activity by two distinct mechanisms, i.e., through the inhibition of cell proliferation as well as the induction of apoptosis. Supporting this notion was the observations that (1) suppression of apoptosis by zVAD did not abrogate 8-Cl-cAMP-induced inhibition of cell proliferation, and (2) 8-Cl-cAMP did not show additive inhibition of cell proliferation in RIIβ-overexpressing cells.

Key Words: **8-Cl-cAMP; inhibition of cell proliferation; apoptosis; anticancer activity.**

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Protein kinase A (PKA) is a serine/threonine protein kinase that is activated by cAMP. It 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 common C subunits, respectively. Four isoforms of R subunits (RIα, RIβ, RIIα, and RIIβ) and three isoforms of C subunits (Cα, Cβ, and Cγ) have been identified (1). A high level of RI or type I PKA correlates with active cell growth and transformation, and a high level of RII or type II PKA is related to the growth arrest and differentiation (2).

Based on the different roles of PKA isozymes in the control of cell growth and differentiation, several lines of attempts were made to suppress transformed phenotype of cancer cells by the differential modulation of PKA isozymes. Suppression of type I PKA using antisense oligonucleotide against RIα mRNA successfully induced growth inhibition of cancer cells *in vitro* and *in vivo* (3, 4). A group of site-selective cAMP analogues inhibited cell growth in a variety of cancer cells and induced differentiation in leukemic cells (5, 6). In addition, introduction of RIIβ subunit of PKA, which resulted in the increase of type II PKA and the down-regulation of type I PKA, suppressed growth and transformed phenotype of transformed or cancer cells (7–9).

8-Cl-cAMP is a cAMP analogue that showed most potent growth-inhibiting effect in a variety of human cancer cells (10). 8-Cl-cAMP-treated cancer cells showed a decrease of RIα and type I PKA, an increase of RIIβ and type II PKA, and inhibition of cell growth (11–13). Especially, *ras*-transformed mouse fibroblast (DT) cells showed morphological differentiation and human leukemic cells showed differentiated characteristics upon the treatment with 8-Cl-cAMP (6, 14). We also found that 8-Cl-cAMP induces apoptotic cell death in human cancer cells including SH-SY5Y human neu-

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roblastoma cells and HL60 human leukemic cells (unpublished results).

To further understand the anticancer activity of 8-Cl-cAMP, we evaluated the contribution of the inhibition of cell proliferation and the apoptotic cell death separately in the 8-Cl-cAMP-induced growth inhibition. In this report, we present the evidence supporting that 8-Cl-cAMP exerts dual anticancer activity through the inhibition of cell proliferation and the induction of apoptotic cell death.

MATERIALS AND METHODS

Cell culture. SH-SY5Y human neuroblastoma cells were maintained in Eagle's minimum essential medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL) in a humidified atmosphere of 5% CO_2 at 37°C. In all experiments, cells were seeded at the same density of 2×10^4 cells/cm^2, and 8-Cl-cAMP was treated on the day after seeding. For the monolayer growth assay, 2×10^5 cells were seeded on 35 mm culture dish at day −1. 8-Cl-cAMP was treated at day 0 and the cell number was determined with Coulter's counter at the indicated time points.

Generation of stable cell lines. Expression construct for RIIβ was generated by inserting human RIIβ cDNA (16) into *Eco*RI cloning site of pLXSN (17). Bcl-2 overexpression construct was generated by inserting Bcl-2 cDNA into *Hpa*I cloning site of pLXSN. SH-SY5Y cells were transfected with the expression construct by calcium phosphate/DNA co-precipitation method. After selection against 500 μg/ml G418, high-expressor clonal cells were obtained by dilution cloning and screening with Western blot analysis.

Chromosomal DNA 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 h, proteinase K was added to the cell lysate at a concentration of 50 μg/ml and samples were incubated at 55°C for 2 h. 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.

Western blot analysis. At appropriate time points after treatment with 10 μM 8-Cl-cAMP, monolayer cells were washed with PBS and then harvested by scraping and centrifugation. Cell pellet was suspended in an extraction buffer (20 mM Tris–Cl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.5 μg/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, 1 mM benzamidine), and incubated on ice for 15 min. After the centrifugation at 10,000*g* for 15 min at 4°C, supernatant was taken as cell extract. Western blot analysis was performed as described previously (8). Polyclonal antibodies against PKA subunits were generated in rabbit using recombinant proteins (18, 19). Monoclonal antibody against Bcl-2 was purchased from DAKO. Polyclonal antibodies against Bcl-X_L, Bax, and Bad were obtained from Santa Cruz Biotechnology.

Flow cytometric analysis of apoptotic cell death. Cells were harvested by gentle trypsinization, washed with PBS, and then fixed with ice-cold 70% ethanol for 1 h. Fixed cells were washed with PBS, and 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) using Lysis II and CELL-FIT programs (B&D). Cells with less than 2N of DNA content were scored as apoptotic cells.

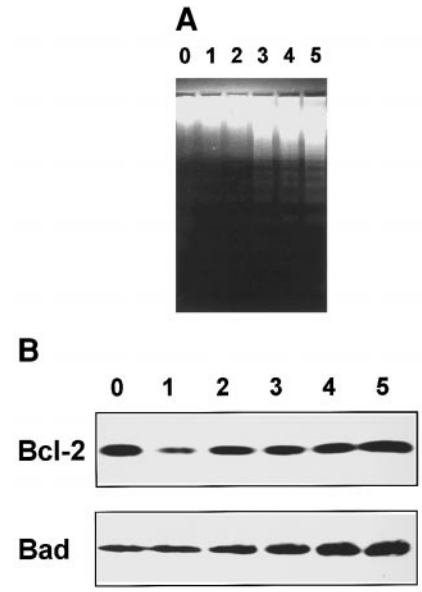

FIG. 1. Changes in Bcl-2 family proteins during 8-Cl-cAMP-induced apoptosis. (A) Assessment of 8-Cl-cAMP-induced apoptosis by chromosomal DNA ladder formation assay. SH-SY5Y cells were treated with 10 μM 8-Cl-cAMP and the chromosomal DNA was analyzed. The durations of 8-Cl-cAMP-treatment were indicated as days. (B) Western blot analyses of the Bcl-2 family proteins. The changes in the level of Bcl-2 and Bad proteins during 8-Cl-cAMP-induced apoptosis were examined by Western blot analyses. The durations of 8-Cl-cAMP-treatment were indicated as days. The approximate size of each protein is 27 kDa for Bcl-2 and 24 kDa for Bad.

RESULTS

Changes of Bcl-2 family proteins during 8-Cl-cAMP-induced apoptosis. Bcl-2 family proteins are evolutionarily well-conserved apoptosis-regulating proteins. Bcl-2, a prototype of Bcl-2 family proteins, is known to suppress apoptotic cell death induced by a variety of apoptosis-inducing signals. Tolerance of neoplastic cells to anticancer drugs is also related in part to the increased level of Bcl-2 (20). SH-SY5Y cells underwent apoptotic cell death in response to 8-Cl-cAMP treatment in a time dependent manner (Fig. 1A). In an attempt to test the possibility that the changes in the levels of Bcl-2 family proteins are involved in the 8-Cl-cAMP-induced apoptosis of SH-SY5Y cells, the levels of Bcl-2 family proteins were examined by Western blot analyses. The level of Bcl-2 transiently decreased at day 1 of 8-Cl-cAMP-treatment and then increased even above the original level. Continuous increase of Bad was observed in 8-Cl-cAMP-treated cells and the level of Bad increased up to 3-fold at day 5 (Fig. 1B). Bcl-X_L exhibited invariably low level, and Bax was not detected in this cell line (data not shown). These results suggest that the modulation of Bcl-2 family proteins may be involved in the induction of apoptosis by 8-Cl-cAMP.

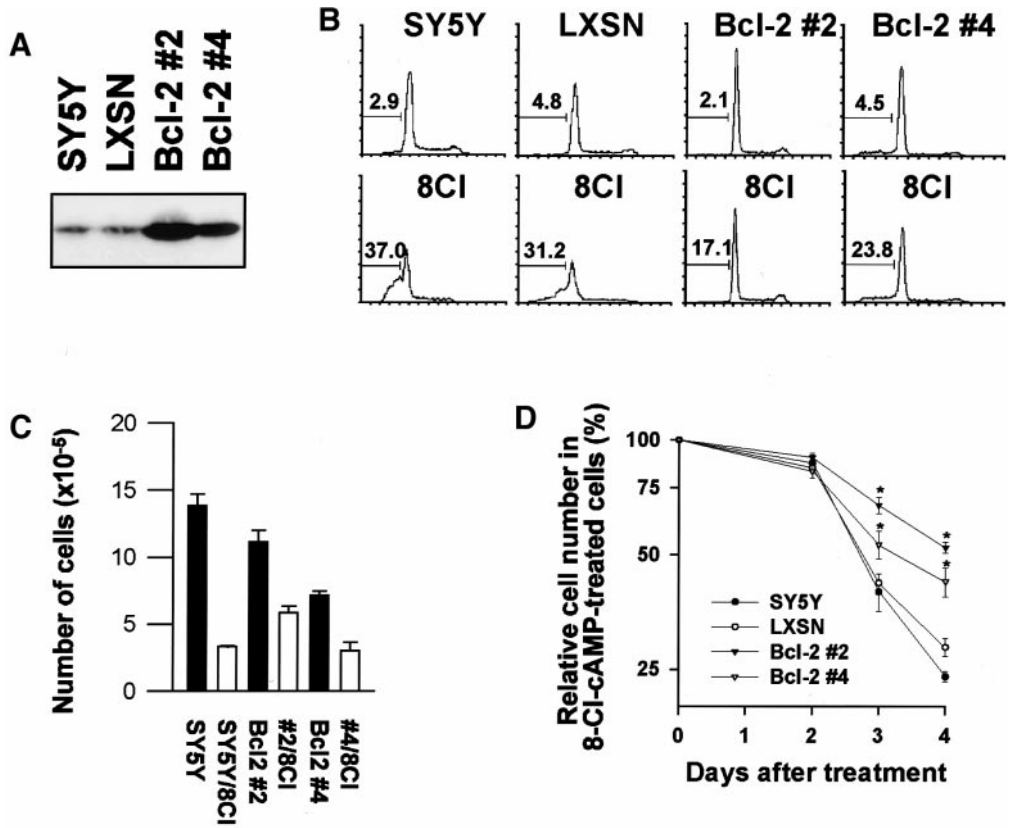

FIG. 2. Effects of Bcl-2 overexpression on 8-Cl-cAMP-induced apoptosis and growth inhibition. (A) Western blot analysis of Bcl-2 protein. The levels of Bcl-2 protein in Bcl-2 high-expressor cells were examined by Western blot analysis. Designations are: SY5Y, parental SH-SY5Y cells; LXSN, vector control cells; Bcl-2 #2 and Bcl-2 #4, Bcl-2 high-expressor clones. (B) Quantification of apoptosis by propidium iodide staining and flow cytometry. Cells were treated with 10 μM 8-Cl-cAMP for 4 days before analysis. Cells with less than 2N of DNA content were considered as apoptotic cells. Percentages of apoptotic cells are indicated in the histogram. (C) Growth inhibition by 8-Cl-cAMP in Bcl-2 overexpressing cells. Cells were treated with 10 μM 8-Cl-cAMP for 4 days and cell numbers were determined. (D) Time course of 8-Cl-cAMP-induced growth inhibition. Cells were treated with 10 μM 8-Cl-cAMP at day 0 and cell numbers were determined at days 2, 3, and 4. Asterisks show the significant difference compared to parental or vector control cells (*, $P < 0.05$, $n = 3$, two-sided t-test).

Suppression of 8-Cl-cAMP-induced apoptosis by overexpressed Bcl-2. To test whether the Bcl-2 or its family proteins affect 8-Cl-cAMP-induced apoptosis, Bcl-2 was overexpressed in SH-SY5Y cells. Two high-expressor clonal lines which showed 7- and 4-fold higher levels of Bcl-2 protein than that of parental cells were isolated (Fig. 2A). The effect of the Bcl-2 overexpression on the 8-Cl-cAMP-induced apoptosis was checked by flow cytometric analysis. When 8-Cl-cAMP was treated, both Bcl-2 overexpressing cell lines showed reduced apoptotic cell death compared to the parental cells. The clone with higher Bcl-2 level (#2) showed greater inhibition of apoptotic cell death (Fig. 2B). These results show that 8-Cl-cAMP-induced apoptotic cell death is blocked by high level of Bcl-2 and the apoptosis inducing signal executes at the upstream of the regulatory level of Bcl-2.

Effect of Bcl-2 overexpression on the 8-Cl-cAMP-induced growth inhibition. We also examined the effect of the overexpression of Bcl-2 on the 8-Cl-cAMP-induced inhibition of cell proliferation. For the convenience of description, we define the growth inhibition as a reduction of cell number in an experimental group compared to a control group. Hence, growth inhibition may be caused not only by the inhibition of cell proliferation but also by the apoptotic as well as any other type of cell death. We hypothesized that 8-Cl-cAMP-induced growth inhibition would also be blocked by the overexpression of Bcl-2 if the growth inhibition is caused only by the apoptotic cell death. If the induction of apoptotic cell death is a separate event from the inhibition of cell proliferation, the growth of Bcl-2 overexpressing cells will also be inhibited by 8-Cl-cAMP. To clear this point, we examined the proliferation of Bcl-2 overexpressing cells in the presence and absence of 8-Cl-cAMP. Since 8-Cl-cAMP-induced apoptotic cell death occurs from day 3 of treatment (Fig. 1A), we checked the cell number at days 2, 3, and 4 to examine the effect of 8-Cl-cAMP before and after the induction of apoptosis. The proliferations of Bcl-2 overexpressing

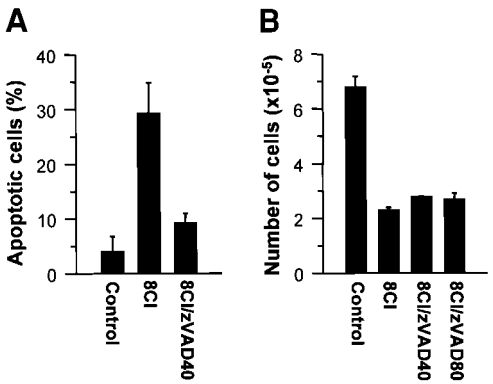

FIG. 3. Effects of zVAD on 8-Cl-cAMP-induced apoptosis and growth inhibition. (A) Suppression of 8-Cl-cAMP-induced apoptosis by zVAD. Cells were mock-treated (control), treated with 10 μM 8-Cl-cAMP (8-Cl), or with 10 μM 8-Cl-cAMP plus 40 μM zVAD (zVAD40). Apoptosis at day 4 of treatment was quantified by propidium iodide staining followed by flow cytometric analysis. Cells with less than 2N of DNA content were scored as apoptotic cells. (B) Effect of zVAD on 8-Cl-cAMP-induced growth inhibition. Cells were treated as indicated and cell number was determined at day 4 of 8-Cl-cAMP-treatment. zVAD40 and zVAD80 indicates cotreatment with 40 μM and 80 μM zVAD, respectively.

cells were slower than that of parental cells (Fig. 2C), which is in agreement with previous reports (21, 22). However, the inhibition of cell proliferation by 8-Cl-cAMP in parental and Bcl-2 overexpressing cells were not significantly different up to day 2 of 8-Cl-cAMP-treatment (Fig. 2D). The relative cell number in 8-Cl-cAMP-treated cells compared to non-treated cells at day 2 were 87.1 $\pm$ 4.3% for SH-SY5Y cells, 89.9 $\pm$ 2.2% for Bcl-2 #2, and 82.7 $\pm$ 3.2% for Bcl-2 #4 ($n = 3$). These results suggest that 8-Cl-cAMP inhibits cell proliferation without the induction of apoptotic cell death until day 2. From day 3 of 8-Cl-cAMP treatment, Bcl-2 overexpressing cells showed reduced growth inhibition as compared to parental cells, showing that apoptotic cell death plays a major role in the late period of 8-Cl-cAMP-induced growth inhibition.

Effect of zVAD on the apoptosis and the growth inhibition by 8-Cl-cAMP. To confirm that 8-Cl-cAMP inhibits cell proliferation in addition to the induction of apoptotic cell death, we examined the effect of zVAD, a broad range-caspase inhibitor, on the apoptosis and growth inhibition induced by 8-Cl-cAMP. 8-Cl-cAMP-induced apoptosis was suppressed by the co-treatment with 40 μM zVAD (Fig. 3A). However, 8-Cl-cAMP-induced growth inhibition was not significantly affected by the cotreatment with zVAD even at a higher concentration (Fig. 3B). These results also indicate that 8-Cl-cAMP inhibits cell proliferation, which is independent of apoptotic cell death.

Effect of 8-Cl-cAMP in RIIβ-overexpressing cells. The growth of cancer cells are also inhibited by the overexpression of RIIβ (7–9). To test whether overexpression of RIIβ also induces apoptotic cell death as well as inhibition of cell proliferation, we overexpressed RIIβ in SH-SY5Y cells. RIIβ-overexpressing cells showed twofold higher RIIβ level and reduced RIα level than those of parental cells (Fig. 4A). As reported in other cells, RIIβ-overexpressing cells showed retarded growth compared to parental cells. Interestingly, when treated with 8-Cl-cAMP, SH-SY5Y cells and RIIβ-overexpressing cells showed similar cell number (Fig. 4B), showing that overexpressed RIIβ and 8-Cl-cAMP did not show additive growth inhibition. To examine the effect of the inhibition of cell proliferation and the effect of apoptosis separately, cell numbers were counted at days 2, 3, and 4 of treatment. RIIβ-overexpressing cells showed no additional inhibition of cell proliferation by 8-Cl-cAMP until day 2 (Fig. 4C), showing again the inhibition of cell proliferation by 8-Cl-cAMP and overexpressed RIIβ share common signal transduction pathway. However, 8-Cl-cAMP-induced apoptosis, which is evident from day 3 of treatment, was not different between parental and RIIβ-overexpressing cells (Fig. 4D). These results indicate that 8-Cl-cAMP-induced growth inhibition can be divided into two independent events: one is the inhibition of cell proliferation and the other is the induction of apoptotic cell death.

DISCUSSION

8-Cl-cAMP was primarily selected as a cancer therapeutic drug which reverses the transformed phenotype of cancer cells (10). Recently, we found that 8-Cl-cAMP induces apoptotic cell death in human cancer cells (unpublished results). In addition, the 8-Cl-cAMP-induced apoptosis was specific to transformed or cancer cells. The results of phase I clinical trial suggested that effective plasma level of 8-Cl-cAMP could be maintained below the maximum tolerated dose (23), implicating that 8-Cl-cAMP can be used for the treatment of human cancer.

In the present study, our primary interest was to understand the nature of anticancer activity of 8-Cl-cAMP. Initially, 8-Cl-cAMP was reported to inhibit cancer cell proliferation without inducing cell death (10). However, recent reports showed that 8-Cl-cAMP induces apoptotic cell death as well in cancer cells (24, 25). Therefore, we assumed that both the inhibition of cell proliferation and the apoptotic cell death may constitute 8-Cl-cAMP-induced growth inhibition. To clear this point, we employed two independent approaches. First, we overexpressed Bcl-2 or treated zVAD to specifically block apoptotic cell death without affecting the inhibition of cell proliferation. Second, we examined the effect of 8-Cl-cAMP in RIIβ-overexpressing cells.

Since 8-Cl-cAMP-induced apoptosis occurs after 3 days of treatment, we examined the cell numbers at day 2 to examine the inhibition of cell proliferation and

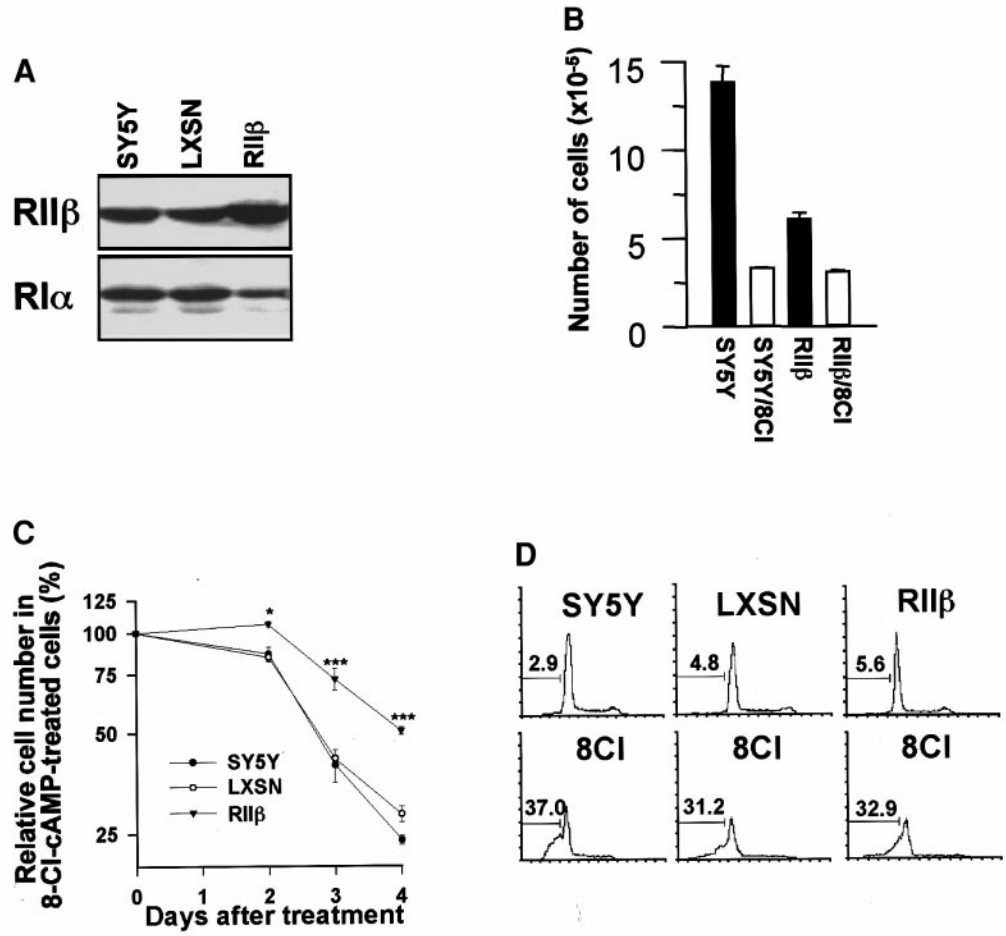

FIG. 4. Effects of 8-Cl-cAMP in RIIβ-overexpressing cells. (A) Western blot analysis of RIIβ and RIα. The levels of RIIβ and RIα proteins in RIIβ-overexpressing cells were examined by Western blot analyses. Designations are: SY5Y, parental SH-SY5Y cells; LXSN, vector control cells; RIIβ, RIIβ overexpressing cells. (B) Growth inhibition by 8-Cl-cAMP in RIIβ-overexpressing cells. Cells were treated with 10 μM 8-Cl-cAMP for 4 days and cell numbers were determined. (C) Time course of 8-Cl-cAMP-induced growth inhibition. Cells were treated with 10 μM 8-Cl-cAMP at day 0 and cell numbers were determined at days 2, 3, and 4. Asterisks show the significant difference compared to parental or vector control cells (*, $P < 0.05$; ***, $P < 0.005$, $n = 3$, two-sided t-test). (D) Quantification of apoptosis by propidium iodide staining and flow cytometry. Cells were treated with 10 μM 8-Cl-cAMP for 4 days before analysis. Cells with less than 2N of DNA content were considered as apoptotic cells. Percentages of apoptotic cells are indicated in the histogram.

days 3 and 4 to examine the growth inhibition including the effect of apoptosis. Apoptosis was analyzed at day 4. As expected, Bcl-2 overexpressing cells showed reduced apoptotic cell death (Fig. 2B). Interestingly, the inhibition of cell proliferation, which was measured before cells undergo apoptosis (day 2), was not different between parental and Bcl-2-overexpressing cells (Fig. 2D). Thus, 8-Cl-cAMP inhibits cell proliferation even without inducing apoptotic cell death. This notion was confirmed by the experiment using zVAD, a broad range-caspase inhibitor. Even when apoptosis was almost blocked by the co-treatment with zVAD, 8-Cl-cAMP-induced growth inhibition was not significantly affected (Fig. 3). Therefore, the anticancer activity of 8-Cl-cAMP was twofold: the inhibition of cell proliferation and the induction of apoptotic cell death.

RIIβ-overexpressing SH-SY5Y cells also showed reduced cell growth (Fig. 4B), as reported in other cancer cell lines (7–9). However, RIIβ-overexpressing cells did not show any spontaneous apoptosis, and underwent apoptosis with the same degree to the parental cells upon treatment with 8-Cl-cAMP (Fig. 4D). Thus, it was a suitable system to analyze the inhibition of cell proliferation and the apoptosis separately. RIIβ-overexpressing cells did not show further inhibition of cell proliferation up to day 2 of 8-Cl-cAMP treatment (Fig. 4C), indicating that the inhibition of cell proliferation by overexpressed RIIβ and 8-Cl-cAMP share common signal transduction pathway. These results show again that anticancer activity of 8-Cl-cAMP can be separated into two activities. One is the inhibition of cell proliferation which shares common signal trans-

duction pathway with RIIβ-induced inhibition of cell proliferation, and the other is the induction of apoptotic cell death that is unique effect of 8-Cl-cAMP.

We also found that Bcl-2 family proteins are modulated during the process of 8-Cl-cAMP-induced apoptosis. Bcl-2 was transiently down-regulated at day 1 and Bad was steadily increased up to day 5 of treatment (Fig. 1B). However, the transient down-regulation of Bcl-2 was not considered to play significant role, since Bcl-2 level was rebounded before the induction of apoptosis. Recently, regulations of Bcl-2 family proteins by cAMP in the apoptotic cell death were reported. cAMP decreases Bcl-2 in SH-SY5Y cells and enhances apoptotic cell death induced by serum deprivation (26). Contrary to this report, cAMP increases *bcl-2* promoter activity in other system through cAMP-response element (27). Bcl-2 protein is also phosphorylated and inactivated by PKA during the apoptosis induced by microtubule-damaging agents (28). In human B-precursor cells, cAMP specifically down-regulates Mcl-1 and induces apoptosis (29). Bad was reported to be phosphorylated and inactivated by mitochondrial type II PKA (30). These reports suggest that cAMP and PKA may play roles in the regulation of apoptosis through modulating the levels or activities of Bcl-2 family proteins. Our result that overexpression of Bcl-2 inhibit 8-Cl-cAMP-induced apoptosis suggests that the increased Bad may take part in the 8-Cl-cAMP-induced apoptosis. The elucidation of the role of increased Bad and possible modifications of Bcl-2 family proteins awaits further investigation.

Resistance of cancer cells toward anticancer drugs is one of the major clinical problems of chemotherapeutic treatment of human cancer. Alteration in drug accumulation and metabolism, amplification of drug target, and changes in molecules involved in DNA repair are well-known mechanisms of drug resistance (31). In addition, inability of cancer cells to activate the apoptotic response has also been proposed (32). Previous reports showed that differentiated neuroblastoma cells express high level of Bcl-2 and show resistance to chemotherapeutic drugs (33, 34), which may cause drug resistance in the chemotherapy. Our data showed that 8-Cl-cAMP induces both the inhibition of cell proliferation and the apoptotic cell death. Through these mechanisms, 8-Cl-cAMP brought about marked growth inhibition even in cells expressing much higher level of Bcl-2. Since Bcl-2 family proteins are modulated by PKA, through phosphorylation and the regulation of expression, the combination of conventional anticancer drugs and cAMP analogues including 8-Cl-cAMP may enhance the performance of anticancer therapy. Indeed, it was already reported that 8-Cl-cAMP and paclitaxel or cisplatin synergistically induce apoptotic cell death in human cancer cells (35), supporting the possibility that 8-Cl-cAMP may increase the sensitivity to anticancer drugs. In this sense, our present report may provide the rationale for the use of 8-Cl-cAMP as a cancer chemotherapeutic agent.

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