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8-Cl-cAMP and its Metabolite, 8-Cl-Adenosine Induce Growth Inhibition in Mouse Fibroblast DT Cells Through the Same Pathways: Protein Kinase C Activation and Cyclin B Down-Regulation

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8-Chloro-cyclic AMP (8-Cl-cAMP) is known to be most effective in inducing growth inhibition and differentiation of a number of cancer cells. Also, its cellular metabolite, 8-Cl-adenosine was shown to induce growth inhibition in a variety of cell lines. However, the signaling mechanism that governs the effects of 8-Cl-cAMP and/or 8-Cl-adenosine is still uncertain and it is not even sure which of the two is the key molecule that induces growth inhibition. In this study using mouse fibroblast DT cells, it was found that adenosine kinase inhibitor and adenosine deaminase could reverse cellular growth inhibition induced by 8-Cl-cAMP and 8-Cl-adenosine. And 8-Cl-cAMP could not induce growth inhibition in the presence of phosphodiesterase (PDE) inhibitor, but 8-Cl-adenosine could. We also found that protein kinase C (PKC) inhibitor could restore this growth inhibition, and both the 8-Cl-cAMP and 8-Cl-adenosine could activate the enzymatic activity of PKC. Besides, after 8-Cl-cAMP and 8-Cl-adenosine treatment, cyclin B was down-regulated and a CDK inhibitor, p27 was up-regulated in a time-dependent manner. These results suggest that it is not 8-Cl-cAMP but 8-Cl-adenosine which induces growth inhibition, and 8-Cl-cAMP must be metabolized to exert this effect. Furthermore, there might exist signaling cascade such as PKC activation and cyclin B down-regulation after 8-Cl-cAMP and 8-Cl-adenosine treatment. J. Cell. Physiol. 201: 277–285, 2004. © 2004 Wiley-Liss, Inc.

cAMP has been known as a secondary messenger that is closely related to the regulation of a variety of cellular functions, such as metabolism (Krebs and Beavo, 1979), secretion (Litvin et al., 1984), cell proliferation (Stork and Schmitt, 2002), ion channel regulation (Ewald et al., 1985), and gene induction (Maurer, 1981; Jungmann et al., 1983). cAMP can stimulate cell growth in many cell types such as PC12 cells, pre-adipocytes and Sertoli cells (Vossler et al., 1997; Yarwood et al., 1998; Boulogne et al., 2003), whereas, it inhibits cell growth in NIH/3T3 cells and adipocytes (Sevetson et al., 1993; Chen and Iyengar, 1994).

It has been known that 8-chloro-cyclic AMP (8-Cl-cAMP), one of the cAMP analogues, initiates growth inhibition and apoptosis in a broad spectrum of cancer cell lines by differential modulation of PKA (cAMP-dependent protein kinase) isozymes, PKA type I and II. These two isozymes are distinguished by the association of a different regulatory (R) subunit, RI or RII to an identical catalytic (C) subunit (Kim et al., 2001). An increased level of intracellular RI or type I PKA activity enhances cell growth, and an increased level of RII or type II PKA activity provokes growth arrest and differentiation (Beebe et al., 1989; Cho-Chung et al., 1989). That is, anticancer activity of 8-Cl-cAMP in cancer cells may be mediated through PKA pathway, which selectively down-regulates the level of RI or type I PKA activity. Although this anti-proliferation effect of 8-Cl-cAMP has been widely reported and 8-Cl-cAMP is anticipated to be an effective anticancer drug (Cho-Chung, 1992; Cummings et al., 1994), the mechanisms involved in anticancer activity of 8-Cl-cAMP are not fully understood. And the relevance of differential

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modulation of PKA-R subunits during 8-Cl-cAMP-induced growth inhibition and cytotoxicity is still under debates (Lamb and Steinberg, 2002). Moreover, many researchers reported that growth inhibition and cytotoxicity induced by 8-Cl-cAMP are mediated by its cellular metabolite, 8-Cl-adenosine (Halgren et al., 1998; Gandhi et al., 2001; Lamb and Steinberg, 2002). Adenosine deaminase that converts 8-Cl-adenosine into 8-Cl-inosine abrogated the cytotoxic effects of not only 8-Cl-adenosine but also 8-Cl-cAMP in multiple myeloma cell lines (Halgren et al., 1998). Furthermore, adenosine kinase-deficient L1210 mouse leukemic cells were resistant to both agents (Gandhi et al., 2001). In an attempt to elucidate the signaling pathways involved in the 8-Cl-cAMP/8-Cl-adenosine-induced growth inhibition, and to manifest which of the two is the key molecule that induces growth inhibition, we employed DT cells, a K-ras-transformed mouse fibroblast, as a model system. In DT cells, 8-Cl-cAMP does not induce cell death (apoptosis) but only inhibits proliferation (Kim et al., 2001), so we can limit our focus solely into the growth inhibition mechanism by 8-Cl-cAMP excluding the apoptotic aspect.

Protein kinase C (PKC), a family of phospholipid-dependent Ser/Thr kinases, regulates many cellular events, such as cell growth, cell cycle progression and differentiation (Nishizuka, 1992; Dekker and Parker, 1994), and it might be another mediator of cAMP signaling. PKC can exert positive or negative regulation depending on when PKC is activated or which isozymes are involved in relevant event (Black, 2000). Because PKC isozymes were shown to be involved in the cellular proliferation and anti-tumor drug resistance, it has long been tried to exploit PKC as a major target for anti-tumor treatment. Subsequent works provided the evidences that it is essential to activate PKC for anti-tumor action in certain cell types (Yoshida et al., 1998). There may exist the mutual interactions between cAMP and PKC, because phorbol esters such as TPA, which are potent activators of PKC pathways, inhibit TSH (cAMP)-mediated human thyroid cell differentiation, whereas TSH attenuates PKC-mediated thyroid cell mitogenesis and anti-mitogenesis (Kraiem et al., 1995). In PC12 cells, cAMP induces differentiation and inhibits cell growth, and PKC activation acts synergistically with cAMP on these effects (Kvanta and Fredholm, 1993). Although there are many evidences confirming the cross-talk between cAMP and PKC, few experiments has been undertaken concerning the effects of PKC on the growth inhibition induced by 8-Cl-cAMP.

Evidently, cell cycle regulation is the crucial point of growth inhibition. Previously, we reported that 8-Cl-cAMP induced cell death (apoptosis) in a cell cycle-dependent manner (Kim et al., 2001). In SH-SY5Y human neuroblastoma cells and HL60 leukemic cells, after the treatment of 8-Cl-cAMP, cells initially accumulated at the S and G_2/M phases of the cell cycle and then apoptosis began to occur among the population of cells at the S/G_2/M cell cycle phases, indicating that the 8-Cl-cAMP-induced apoptosis is closely related to cell cycle control. And time-dependent suppression of cyclin B1 was detected in human glioma cell lines, which might be responsible for the observed G_2 delay (Grbovic et al., 2002). Also, 8-Cl-adenosine inhibited growth in primary mouse epidermal keratinocytes through an elevation in the protein level of $p21^{WAF1/Cip1}$, a cyclin-dependent kinase (CDK) inhibitor (Dransfield et al., 2001).

In this study, we carried out a series of experiments to clarify which is the real effector during 8-Cl-cAMP-induced growth inhibition, between 8-Cl-cAMP itself and its metabolite 8-Cl-adenosine. Furthermore, we tried to elucidate the mechanism(s) through which 8-Cl-cAMP and/or 8-Cl-adenosine induce growth inhibition in regard to the regulation of PKC activity and modulation of the level of cyclin proteins.

MATERIALS AND METHODS

Chemicals and drugs

8-Cl-cAMP, 8-Cl-adenosine, and 8-Cl-AMP were purchased from Biolog (Bremen, Germany). [γ-^{32}P]-ATP (6,000 Ci/mmol) was purchased from Dupont NEN (Boston, MA). Selectide^{TM} (a PKC-specific substrate) and kemptide (a PKA-specific substrate) were from Calbiochem (San Diego, CA). GF-109203x (a PKC inhibitor) was obtained from A. G. Scientific (San Diego, CA). A-134974 (an adenosine kinase inhibitor) and adenosine deaminase were purchased from Sigma-Aldrich (St. Louis, MO). All the other chemicals used in this research were obtained from Sigma-Aldrich.

Cell culture

DT (K-ras-transformed NIH/3T3) cells were grown in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT). 2774, H1299 and HL60 cells were maintained in RPMI 1640 medium (HyClone). SH-SY5Y and LS-174T cells were maintained in minimum essential medium with Earle's salt (EMEM, HyClone). Culture media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. For the monolayer growth assay, cell number was determined with Coulter^{TM} counter (Beckman Coulter, Fullerton, CA) at the indicated time points. For cell growth test, MTT assay using 3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) was carried out. Five thousand cells were seeded in flat-bottomed 24-well-plates and at an appropriate time interval, MTT (2 mg/ml in phosphate-buffered saline (PBS)) was added to the cultures. After incubation for 4 h, the resulting color reaction product, MTT formazan, was dissolved with dimethyl sulfoxide, and the absorbance was measured at 570 nm using microplate reader (Bio-Rad, Hercules, CA). For soft agar colony formation assay, top agar (0.3% Difco Noble agar) medium including 5×10^4 cells was layered over 1 ml of 0.8% bottom agar medium in 35 mm culture dish. After 7 days, colonies were stained with nitro blue tetrazolium for overnight, and then photographed under the microscope.

PKC activity assay

Cells were washed twice with PBS and harvested by scraping and centrifugation, and then homogenized in an extraction buffer (25 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 mM PMSF, 1 μg/ml each of aprotinin, pepstatin A, antipain, and leupeptin, 1 mM Na_3VO_4, 50 mM NaF, and 10 mM pyrophosphate) on ice for

15 min. The supernatant was obtained by centrifugation at 10,000*g* for 10 min. Ten micrograms of total cell extract was mixed with kinase assay buffer (200 mM Tris pH 7.5, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 1 μg/ml each of aprotinin, pepstatin A, antipain and leupeptin, 15 μM ATP, 5 μM [γ-^{32}P]-ATP, and 25 μM of PKC-specific substrate, Selectide^{TM} [Calbiochem, H-Ala-Ala-Lys-Ile-Gln-Ala-Ser-Phe-Arg-Gly-His-Met-Ala-Arg-Lys-Lys-OH]), and incubated at 30°C for 10 min. Ten microliters of ice-cold 5% acetic acid was added to the mixtures to stop the reaction and 30 μl of the reaction mixtures were spotted onto phosphocellulose filters (GIBCO-Invitrogen, Carlsbad, CA), and then washed three times with 1% phosphoric acid. Filters were air-dried and radioactivities were measured with liquid scintillation counter (Wallac, Turku, Finland).

PKA activity assay

For the determination of total cellular PKA activity, cell extract was prepared as in PKC activity assay. Ten micrograms total cell extract in 10 μl were mixed with 50 μl of kinase assay buffer (50 mM Tris pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 30 μM Kemptide [H-Leu-Arg-Arg-Ala-Ser-Leu-Gly-OH], and 5 μM [γ-^{32}P]-ATP) in the presence or absence of 5 μM cAMP, and incubated at 37°C for 5 min. After the reaction, radioactivity was measured as in PKC activity assay.

Western blot analysis

At appropriate time points after treatment of 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 $MgCl_2$, 1 mM PMSF, 1 μg/ml each of aprotinin, pepstatin A, antipain, and leupeptin), 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. The extracts were electrophoresed on a 10% SDS/polyacrylamide gel and transferred onto PVDF membrane. Protein-bound membrane was incubated with appropriate antibodies, followed by horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad). The relevant protein bands were then visualized using Enhanced Chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ).

Polyclonal antibodies against PKA subunits were previously described (Lee et al., 1999). Antibodies for cyclin B, A, D, and E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for $p27^{Kip}$ was obtained from BD Transduction Laboratories (San Diego, CA).

RESULTS

8-Cl-cAMP metabolism is essential for growth inhibition

We first checked the growth of DT cells by MTT assay after treating the cells with 8-Cl-cAMP and 8-Cl-adenosine. Both 8-Cl-cAMP and 8-Cl-adenosine induced growth inhibition in a dose-dependent manner (Fig. 1a,b; IC_{50} for 8-Cl-cAMP is about 2 μM and for 8-Cl-adenosine is about 0.5 μM). 8-Cl-cAMP is first converted to 8-Cl-AMP by phosphodiesterase (PDE), and then further metabolized to 8-Cl-adenosine by the action of nucleotide phosphatase. To determine whether 8-Cl-cAMP metabolism toward 8-Cl-adenosine is essential for growth inhibition, we co-treated PDE inhibitor, IBMX (3-isobutyl-1-methyl-xanthine, 100 μM) with both drugs. IBMX could reverse the 8-Cl-cAMP-induced growth inhibition (Fig. 1a), but had no effect on 8-Cl-adenosine (Fig. 1b). However, IBMX could not attenuate the PKA activity increased by the treatment with 8-Cl-cAMP (Fig. 1c). This result suggests that the growth inhibition might occur through another mechanism(s) rather than conventional cAMP-PKA signaling pathway. IBMX itself can activate PKA because it increases intracellular cAMP by blockage of PDE action, which changes cAMP to AMP. Furthermore, both 8-Cl-cAMP and 8-Cl-adenosine down-regulated the PKA RIα subunit level and IBMX had no effect on this RIα down-regulation (Fig. 1d). When the cells were co-treated with adenosine deaminase (AD, 1.6 U/ml) that converts 8-Cl-adenosine to 8-Cl-inosine, both 8-Cl-cAMP and 8-Cl-adenosine did not show any growth inhibitory effects (Fig. 1a,b). In addition, adenosine kinase inhibitor (A-134974, 1 μM) almost completely blocked the growth inhibition induced by 8-Cl-cAMP and 8-Cl-adenosine (Fig. 1e). A-134974 showed the identical reversal of growth inhibition when treated in the cultures of various human cancer cell lines, even in HL60 and SH-SY5Y that undergo apoptosis as well as growth inhibition after 8-Cl-cAMP treatment (Fig. 2). Taken together, these results suggest that 8-Cl-adenosine is the key molecule and the metabolism of 8-Cl-cAMP is prerequired for inducing the growth inhibition in a variety of cancer cell lines.

8-Cl-cAMP and 8-Cl-adenosine induce growth inhibition through PKC activation

Next, we tried to identify the signaling pathway that governs the 8-Cl-cAMP-induced growth inhibition. Unexpectedly, it turned out that inhibition of PKA catalytic activity did not attenuate the growth inhibition (data not shown). We accordingly sought after another pathway(s) known to be involved in the regulation of cell growth. Among them, PKC turned out to be the most plausible candidate because a PKC inhibitor, GF-109203x could restore the 8-Cl-cAMP- and 8-Cl-adenosine-induced growth inhibition. 8-Cl-cAMP (10 μM) suppressed the DT cell growth down to 10% of control. However, when cells were co-treated with GF-109203x (3 μM), the cell number increased up to 50% (Fig. 3a). Even if GF-109203x itself had some growth inhibitory effect, it significantly attenuated 8-Cl-cAMP-induced growth inhibition. To verify the relationship between PKC and 8-Cl-cAMP, we carried out PKC activity assay using PKC-specific peptide substrate, Selectide^{TM} (Fig. 3b). The enzymatic activity of PKC subtly increased after 12 h and greatly augmented at 72 h after the treatment with 8-Cl-cAMP. This enhanced activity was sustained and further increased until 5 days after treatment. To investigate whether the anti-tumor activity of 8-Cl-cAMP was also affected by PKC inhibition, we performed soft agar culture assay, which can be used to test the anchorage-independent growth activity (Fig. 3c). Control DT cells showed anchorage-independent growth that is a major phenotype of

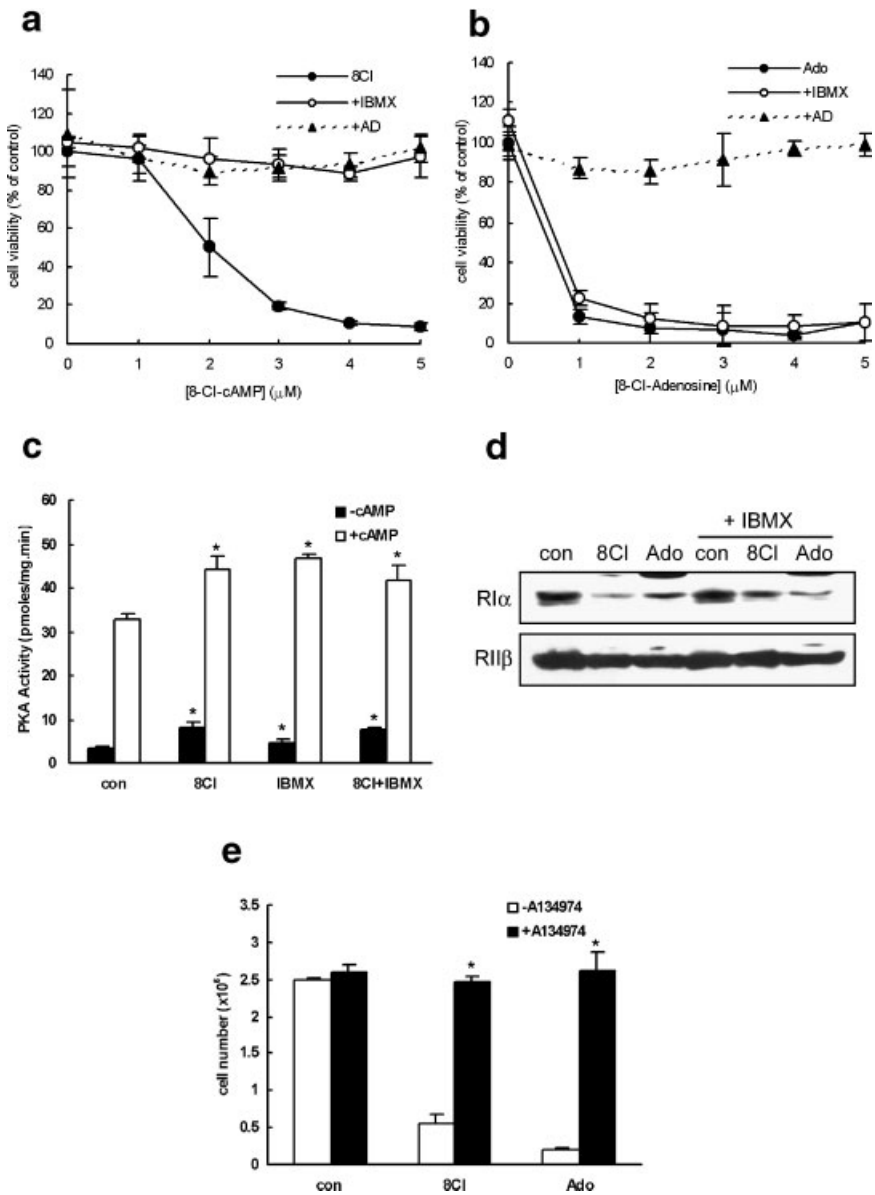

Fig. 1. 8-Chloro-cyclic AMP (8-Cl-cAMP)-induced growth inhibition is mediated by its metabolite, 8-Cl-adenosine (Ado). **a**: Cell growth assay after 8-Cl-cAMP treatment. Mouse fibroblast DT cells were incubated with 8-Cl-cAMP for 3 days. 8-Cl-cAMP (8Cl) induced growth inhibition in a dose-dependent manner (IC_{50} is about 2 μM). This growth inhibition was reverted to normal level by the co-treatment of phosphodiesterase (PDE) inhibitor (+IBMX, 100 μM) or adenosine deaminase (+AD, 1.6 U/ml). The graph denotes cellular growth (mean ± SD, n = 4) measured using MTT assay as described in "Materials and Methods." **b**: Cell growth assay after Ado treatment. After 3 day-treatment of Ado, cellular growth was measured as in part (a). Ado-induced growth inhibition (IC_{50} is about 0.5 μM) was blocked by adenosine deaminase, but IBMX had no effect. **c**: The effect of IBMX on PKA activity increased by 8-Cl-cAMP. Bars denote PKA activities (mean + SD, n = 3) measured as in "Materials and Methods." 8-Cl-cAMP (10 μM for 3 days) increased basal (−cAMP) and total (+cAMP) PKA activities, where co-treatment with IBMX (100 μM) had no effect. IBMX alone could evoke PKA activation. Asterisks (*) denote differences from mock-treated control group ($P < 0.05$, two-tailed t-test). **d**: The effect of IBMX on RIα down-regulation. Both 8-Cl-cAMP (8Cl, 10 μM) and Ado (2 μM) down-regulated protein level of PKA RIα subunit, which was not affected by IBMX co-treatment. RIIβ protein levels showed less variation between the treated groups compared with RIα. The intensity of each band was quantitated with densitometer. In RIα blot, the relative values are 1, 0.12, 0.37, 0.94, 0.41, and 0.17, however, in RIIβ blot, these values are 1, 0.76, 0.74, 0.84, 0.86, and 0.68. These are representatives from three separate experiments with similar results. **e**: The effect of adenosine kinase inhibitor on growth inhibition induced by 8-Cl-cAMP and Ado. Bars denote cell numbers (mean + SD, n = 4) counted at 3 days after treatments of indicated drugs. Adenosine kinase inhibitor (A134974, 1 μM) completely blocked growth inhibition induced by both 8-Cl-cAMP (8Cl) and Ado. Asterisks (*) denote differences from 8Cl and Ado group, respectively ($P < 0.01$, two-tailed t-test).

transformed cell lines. However, 8-Cl-cAMP-treated cells could not grow well on soft agar medium. In parallel with the data in growth rate experiments, inhibition of PKC activity by GF-109203x also reversed the reduced anchorage independent growth of DT cells caused by 8-Cl-cAMP treatment.

8-Cl-adenosine also influenced cell growth in a PKC-dependent manner. 8-Cl-adenosine (1 μM) induced

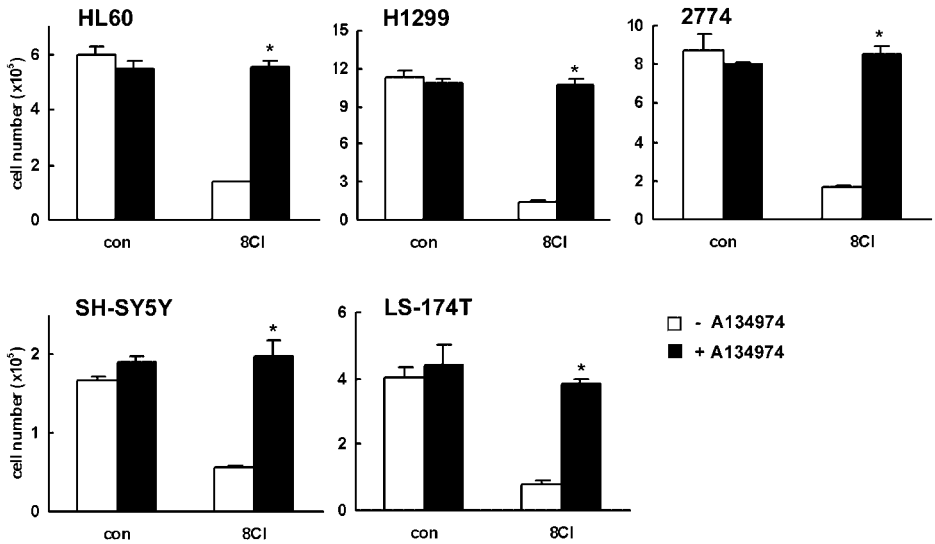

Fig. 2. Adenosine kinase inhibitor (A134974) attenuated 8-Cl-cAMP-induced growth inhibition in various human cancer cell lines (HL60, leukemia cells; H1299, lung carcinoma cells; 2774, ovarian carcinoma cells; SH-SY5Y, neuroblastoma cells; LS-174T, colon carcinoma cells). Each graph represents cell number (mean + SD, n = 3) counted after 8-Cl-cAMP treatment (10 μM for 3 days) in the presence or absence of 1 μM A-134974. Asterisks (*) denote differences from only 8-Cl-cAMP-treated group ($P < 0.01$, two-tailed t-test).

about 50% growth inhibition compared with mock-treated control, and this inhibition was almost completely blocked by treatment of GF-109203x, in parallel with the 8-Cl-cAMP (Fig. 3d). Likewise, the activity of PKC was enhanced by 8-Cl-adenosine, and the enhanced activity of PKC returned to normal level by co-treatment of adenosine kinase inhibitor (A-134974) in both the 8-Cl-cAMP- and 8-Cl-adenosine-treated samples (Fig. 3e). IBMX also could diminish the 8-Cl-cAMP-induced PKC activation, but IBMX alone could not influence the PKC activity (Fig. 3f). These suggest that 8-Cl-cAMP inhibits cell growth via PKC activation, and it must be metabolized to 8-Cl-adenosine to activate PKC.

8-Cl-cAMP and 8-Cl-adenosine down-regulate cyclin B and up-regulate $p27^{Kip1}$

Previously, we verified that 8-Cl-cAMP induces cell cycle-specific apoptosis in human cancer cells (Kim et al., 2001). In SH-SY5Y and HL60 cells, after 8-Cl-cAMP-treatment, cells were accumulated in $S/G_2/M$ phase and then underwent cell death. To prove whether this cell cycle specific effect is also related with growth inhibition in DT cells, we investigated the protein expression levels of cyclins and $p27^{Kip1}$ CDK inhibitor using Western blotting. Among the four cyclin proteins tested, only cyclin B that is important for G_2-M transition showed a decreasing trend from 36 h after 8-Cl-cAMP-treatment (Fig. 4a). Expression levels of other cyclins such as cyclin A, D, and E did not change during 8-Cl-cAMP-incubation. Furthermore, 8-Cl-cAMP increased the $p27^{Kip1}$ protein level, which belongs to the Cip/Kip CDK inhibitor family (Fig. 4a). 8-Cl-adenosine also decreased the cyclin B (Fig. 4c) and increased $p27^{Kip1}$ protein level (Fig. 4b). In addition, adenosine kinase inhibitor and PKC inhibitor, just like the effects mentioned above, restored these distorted level of cell cycle proteins to normal conditions (Fig. 4c,d). IBMX, a PDE inhibitor, blocked the effect of 8-Cl-cAMP on cyclin B down-regulation. However, it could not recover the decreased level of cyclin B caused by 8-Cl-adenosine or 8-Cl-AMP (Fig. 4e). 8-Cl-cAMP is converted to 8-Cl-AMP by PDE and further metabolized to 8-Cl-adenosine by nucleotide phosphatase. Hence, the cell cycle protein regulation of 8-Cl-cAMP is also dependent upon the activity of PKC and for this process, it is still necessary for 8-Cl-cAMP to be metabolized into 8-Cl-adenosine or further metabolites.

DISCUSSION

It has been known that 8-Cl-cAMP, a site selective analogue of cAMP, exerted growth inhibition in numerous cancer cells (Cho-Chung et al., 1989) and, moreover, induced apoptosis even more dominantly in cancer cells compared to the non-transformed counterparts (Kim et al., 2001). Based on these findings, many researchers have been trying to adopt 8-Cl-cAMP as a new therapeutic agent against cancer and phase I clinical test has been accomplished (Cummings et al., 1996; Propper et al., 1999). However, the mechanisms through which 8-Cl-cAMP induces growth inhibition and cell death are still unclear and under debate. Cho-Chung et al. proved that 8-Cl-cAMP differentially regulates the regulatory subunits of PKA, i.e., it preferentially decreases RI subunit level so that the RI/RII ratio went down. They found that these decreased RI/RII ratio caused growth retardation because RIα antisense oligonucleotides that block the expression of RIα led to the growth inhibition and RIIβ antisense oligonucleotides attenuated 8-Cl-cAMP-induced growth inhibition (Tortora et al., 1990; Yokozaki et al., 1993).

However, lots of articles describe the effects of 8-Cl-cAMP on cellular growth in somewhat different ways (Langeveld et al., 1997; Halgren et al., 1998; Gandhi et al., 2001; Lamb and Steinberg, 2002). They showed that 8-Cl-adenosine, a metabolite of 8-Cl-cAMP, can induce growth inhibition, and moreover, the growth inhibitory effect of 8-Cl-cAMP is mediated by

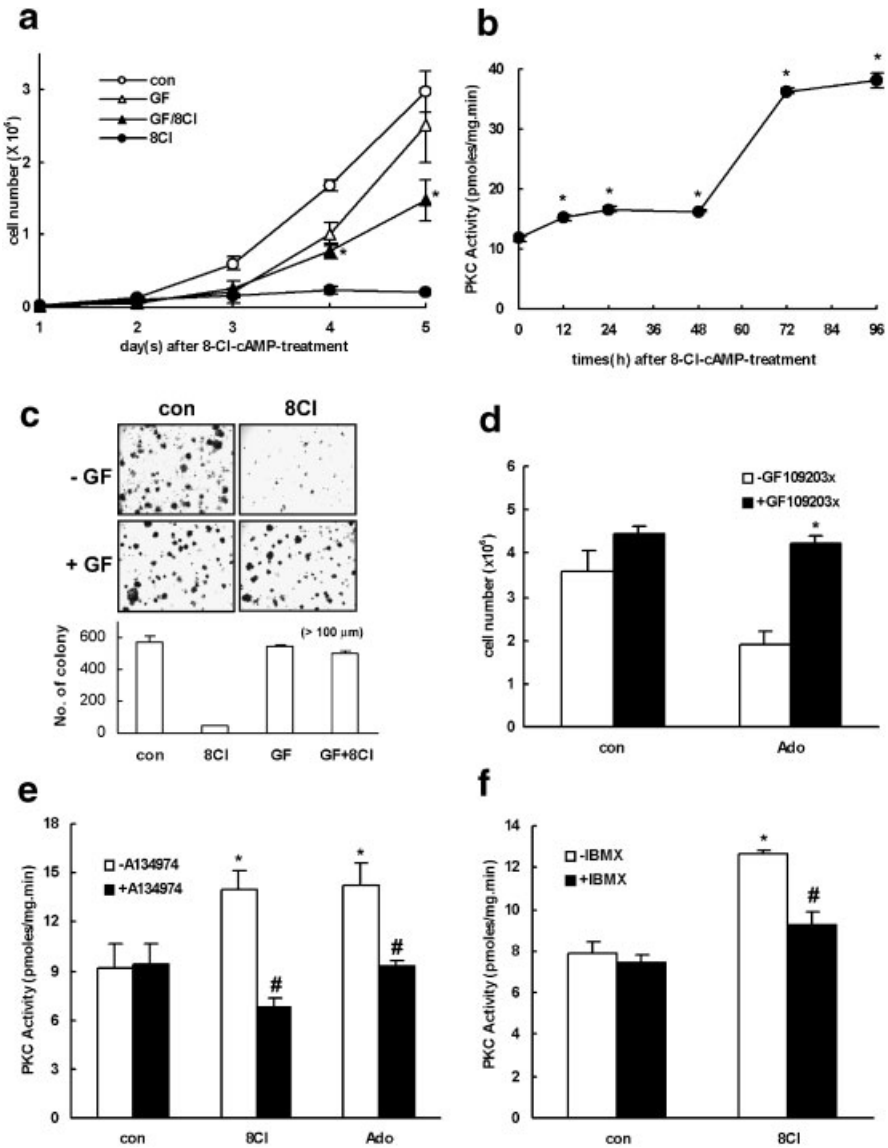

Fig. 3. 8-Cl-cAMP and Ado induce growth inhibition through PKC activation. **a**: The effect of PKC inhibitor on 8-Cl-cAMP-induced growth inhibition. DT cells were treated with 8-Cl-cAMP (8Cl, 10 μM) for the indicated times with or without PKC inhibitor, GF-109203x (GF, 3 μM). The graph shows cell numbers (mean ± SD, n = 3) counted after the treatments. GF-109203x itself had some growth inhibitory effect, but it significantly attenuated 8-Cl-cAMP-induced growth inhibition. Asterisks (*) denote differences from only 8-Cl-cAMP-treated group ($P < 0.01$, two-tailed t-test). **b**: PKC activity assay after 8-Cl-cAMP treatment. Graph represents PKC activity (mean ± SD, n = 3) measured after 10 μM 8-Cl-cAMP-treatment for indicated times according to the protocol described in "Materials and Methods." 8-Cl-cAMP started to induce PKC activation after 12 h of incubation and further increased the PKC activity at 72 h after the treatment. Asterisks (*) denote differences from 0 h-treated group ($P < 0.01$, two-tailed t-test). **c**: The effect of PKC inhibitor on 8-Cl-cAMP-induced anti-tumor activity. Soft agar colony formation assay was carried out as described in "Materials and Methods." PKC inhibition also reversed the reduced anchorage independent growth of DT cells caused by 8-Cl-cAMP treatment. The graph represents the colony numbers (larger than 100 μm in diameter; mean + SD, n = 3) counted at three independent microscopic fields (×40). **d**: The effect of PKC inhibitor on Ado induced growth inhibition. After Ado (1 μM) treatment for 3 days in the presence or absence of GF-109203x (3 μM), cell numbers (mean + SD, n = 3) were counted. GF-109203x attenuated Ado-induced growth inhibition, too. Asterisk (*) denotes difference from Ado group ($P < 0.01$, two-tailed t-test). **e**: The effect of adenosine kinase inhibitor on PKC activation by 8-Cl-cAMP and Ado. As in part (b), PKC activities were measured after the treatment of 8-Cl-cAMP (8Cl, 10 μM) and Ado (2 μM) for 3 days with or without adenosine kinase inhibitor, A-134974 (1 μM). A-134974 suppressed PKC enzymatic activity induced by both 8-Cl-cAMP and Ado. Asterisks (*) denote differences from mock-treated control group ($P < 0.05$) and sharps (#) represent difference from 8Cl and Ado group, respectively ($P < 0.01$, two-tailed t-test). **f**: The effect of IBMX on PKC activation by 8-Cl-cAMP. 8-Cl-cAMP was treated for 3 days in combinations with IBMX (100 μM) and then PKC activities were measured. IBMX also attenuated 8-Cl-cAMP-induced PKC activation. Asterisk (*) denotes difference from mock-treated control group ($P < 0.01$) and sharp (#) means difference from 8Cl group ($P < 0.01$, two-tailed t-test).

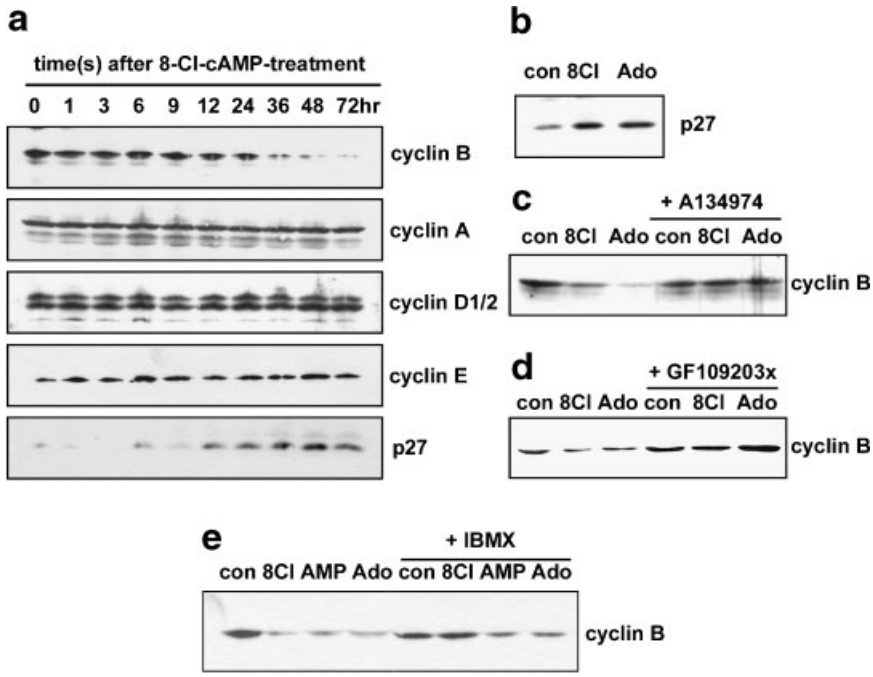

Fig. 4. 8-Cl-cAMP- and Ado-induced growth inhibitions are mediated by cyclin B down-regulation. **a**: The effect of 8-Cl-cAMP on protein expressions of cyclins and $p27^{Kip1}$. After the treatment of 8-Cl-cAMP for the indicated times, cyclins and $p27^{Kip1}$ protein levels were determined by Western blotting as described in "Materials and Methods." Among the tested cyclins, only cyclin B protein started to decrease at 36 h after the treatment and nearly disappeared after 72 h. On the contrary, a CDK inhibitor $p27^{Kip1}$ was increased after 12 h of the treatment. **b**: $p27^{Kip1}$ expression after Ado treatment. After the treatment of 8-Cl-cAMP (8Cl, 10 μM) and Ado (2 μM) for 3 days, $p27^{Kip1}$ protein level was measured as in part (a). Ado also increased $p27^{Kip1}$ protein expression level. **c**: The effect of adenosine kinase inhibitor on cyclin B down-regulation by 8-Cl-cAMP and Ado. After 8-Cl-cAMP- or Ado-treatment for 72 h in the presence or absence of A-134974, cyclin B protein levels were measured. A-134974 restored cyclin B protein level down-regulated by 8-Cl-cAMP and Ado. **d**: The effect of PKC inhibitor on cyclin B down-regulation. As in part (**c**), cyclin B Western blotting was performed but A-134974 was substituted by GF-109203x. GF-109203x also restored cyclin B protein level to normal. **e**: The effect of IBMX on cyclin B down-regulation. Cyclin B protein levels were decreased by the treatment of 8-Cl-cAMP (8Cl, 10 μM), 8-Cl-AMP (AMP, 10 μM), and Ado (2 μM) for 72 h. Cyclin B down-regulation by 8-Cl-cAMP was recovered by IMBX. In the Ado- and 8-Cl-AMP-treated groups, IBMX showed no effect.

8-Cl-adenosine. 8-Cl-cAMP is converted to 8-Cl-AMP by the action of PDEs and further metabolized into 8-Cl-adenosine by nucleotide phosphatase. This 8-Cl-adenosine is then modified to 8-Cl-inosine by adenosine deaminase or to 8-Cl-ATP by adenosine kinase. The chemicals that inhibit the metabolic pathway of 8-Cl-cAMP can offset the growth inhibitory effect of 8-Cl-cAMP (Lamb and Steinberg, 2002). The action mechanism through which 8-Cl-adenosine exerts its effect on cell growth is also uncertain. However, it was reported that 8-Cl-adenosine might disturb the intracellular ATP pool and further inhibit nucleic acid synthesis, especially RNA synthesis (Gandhi et al., 2001).

We showed that 8-Cl-cAMP-induced growth inhibition was blocked by the chemicals or enzyme that prohibit the progression of 8-Cl-cAMP metabolism, which suggests that 8-Cl-adenosine or its further metabolites are the key molecule(s) in cellular responses to 8-Cl-cAMP. 8-Cl-ATP into which 8-Cl-adenosine is converted by adenosine kinase may play an important role in this process. Treatment of 8-Cl-adenosine increased the intracellular concentration of 8-Cl-ATP, whereas that of ATP inside the cell was decreased (Gandhi et al., 2001). It is possible that this newly produced 8-Cl-ATP may substitute for ATP in various cellular actions such as cofactor of many enzymes and precursor of nucleotide synthesis.

As described above, 8-Cl-cAMP can differentially modulate regulatory subunits of PKA, that is, increases type II regulatory subunit but decreases type I, and this unbalanced RI/RII ratio causes growth inhibition and cell death (Cho-Chung, 1992). However, IBMX that completely blocked the growth inhibitory effect of 8-Cl-cAMP could not restore RIα protein level decreased by 8-Cl-cAMP-treatement. Furthermore, 8-Cl-adenosine, which is not a cAMP derivative, also down-regulated RIα protein, just like 8-Cl-cAMP did. These results suggest that RIα down-regulation is not the major cause of growth inhibition and there might exist another growth inhibitory mechanisms rather than RIα down-regulation. In this study, we proposed that PKC activation by 8-Cl-cAMP and 8-Cl-adenosine takes an important role during progression of growth inhibition in mouse fibroblasts.

There have been many evidences showing that the cross-talk might exist between cAMP and PKC signaling pathways (Cambier et al., 1987; Liedtke and Cole, 1998; Roberson et al., 1999). For example, in mouse B-lymphocytes, cAMP-generating signal can activate nuclear translocation of PKC and this nuclear PKC may function in the regulation of gene expression (Cambier et al., 1987). 8-Cl-cAMP might also have some correlation with PKC. In human mammary xenografts, 8-Cl-cAMP-treatment induced growth stasis and

rebound in tumor growth occurred when the treatment was ceased. During this process, PKC activity was also modulated (Parandoosh et al., 1990). Nevertheless, there has been no evidence representing 8-Cl-cAMP-induced growth inhibition is directly related with PKC activation. In our experiments, it was found that PKC inhibitor could revert 8-Cl-cAMP- and 8-Cl-adenosine-induced growth inhibition and PKC activity was elevated by these treatments. Furthermore, PKC inhibitor also recovered cyclin B expression down-regulated by 8-Cl-cAMP. However, 8-Cl-cAMP-induced cell death was not influenced by PKC inhibition. In SH-SY5Y and HL60 cell lines, where 8-Cl-cAMP can induce not only inhibition of proliferation but also cell death, PKC inhibitor could not increase the cell growth suppressed by 8-Cl-cAMP (data not shown). It is suggested that 8-Cl-cAMP-induced growth inhibition is dependent on the activity of PKC but cell death is not.

As mentioned above, 8-Cl-cAMP and 8-Cl-adenosine are undergoing phase I clinical test against human cancers (Cummings et al., 1996; Propper et al., 1999). However, the debate is still going on as to whether 8-Cl-cAMP is a prodrug for its metabolite, 8-Cl-adenosine. The data presented here support the hypothesis that the metabolism of 8-Cl-cAMP is necessary for its growth inhibitory action. Nonetheless, it is still tenable that RIα down-regulation by 8-Cl-cAMP is responsible for growth inhibitory effect, because 8-Cl-adenosine can also modulate the RIα protein level. Further researches must be carried out to elucidate the exact mechanisms of 8-Cl-cAMP-induced growth inhibition. But based on our experiments performed here, there might exist at least two pathways that transduce 8-Cl-cAMP signal; one is through the modulation of PKA regulatory subunits and the other is through PKC activation and cyclin B down-regulation.

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