8-Chloro-Cyclic AMP–Induced Growth Inhibition and Apoptosis Is Mediated by p38 Mitogen-Activated Protein Kinase Activation in HL60 Cells

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Abstract

8-Chloro-cyclic AMP (8-Cl-cAMP), which is known to induce growth inhibition, apoptosis, and differentiation in various cancer cell lines, has been studied as a putative anticancer drug. However, the mechanism of anticancer activities of 8-Cl-cAMP has not been fully understood. Previously, we reported that the 8-Cl-cAMP-induced growth inhibition is mediated by protein kinase C (PKC) activation. In this study, we found that p38 mitogen-activated protein kinase (MAPK) also plays important roles during the 8-Cl-cAMP-induced growth inhibition and apoptosis. SB203580 (a p38-specific inhibitor) recovered the 8-Cl-cAMP-induced growth inhibition and apoptosis, whereas other MAPK inhibitors, such as PD98059 (an extracellular signal-regulated kinase–specific inhibitor) and SP600125 (a c-Jun NH_2-terminal kinase–specific inhibitor), had no effect. The phosphorylation (activation) of p38 MAPK was increased in a time-dependent manner after 8-Cl-cAMP treatment. Furthermore, SB203580 was able to block PKC activation induced by 8-Cl-cAMP. However, PKC inhibitor (GF109203x) could not attenuate p38 activation, indicating that p38 MAPK activation is upstream of PKC activation during the 8-Cl-cAMP-induced growth inhibition. 8-Chloro-adenosine, a metabolite of 8-Cl-cAMP, also activated p38 MAPK and this activation was blocked by adenosine kinase inhibitor. These results suggest that 8-Cl-cAMP exerts its anticancer activity through p38 MAPK activation and the metabolite(s) of 8-Cl-cAMP mediates this process. (Cancer Res 2005; 65(11): 4896-901)

Introduction

Cyclic AMP (cAMP) is a well-known secondary messenger that triggers various cellular physiologic responses, such as cell growth and proliferation (1), regulation of metabolism (2), gene induction (3, 4), and ion channel regulation (5). These biological actions of cAMP seem to be cell type specific. For example, cAMP can stimulate cell growth in many cell types, such as PC12 cells, preadipocytes, and Sertoli cells (6–8), whereas it inhibits cell growth in other cells, such as NIH3T3 and adipocytes (9, 10). Classically, cAMP has been known to provoke its cellular responses through protein kinase A (PKA) activation. After binding of cAMP to the regulatory (R) subunits of PKA, R subunits are dissociated from the holoenzyme and catalytic (C) subunits are activated. Although this cAMP-PKA signaling is a well-established pathway, another cAMP signal transducer has been recently identified, the exchange protein directly activated by cAMP-Rap1 pathway (11, 12). This exchange protein directly activated by cAMP-Rap1 signal can regulate cellular growth by modulating the activities of mitogen-activated protein kinases (MAPK; ref. 13).

8-Chloro-cAMP (8-Cl-cAMP), one of the cAMP analogues, induces inhibition of cellular proliferation and apoptosis when treated to various cancer cell lines (14). Due to its antitransforming activities, 8-Cl-cAMP has been tested as a cancer therapeutic drug (15, 16). However, the signaling mechanisms that govern the 8-Cl-cAMP-induced growth inhibition are still uncertain. Cho-Chung et al. suggested that differential regulation of PKA isozymes, PKA type I and II, is the major cause of the 8-Cl-cAMP-induced anticancer activity (17). When treated to cancer cells, 8-Cl-cAMP selectively down-regulates type I regulatory (RI) subunit; then, the decreased ratio of RI/RII provokes growth inhibition. However, the relevance of differential modulation of PKA-R subunits during the 8-Cl-cAMP-induced growth inhibition and cytotoxicity is still under debate (18–20). Furthermore, we recently showed that activation of protein kinase C (PKC), rather than PKA, is involved during the 8-Cl-cAMP-induced growth inhibition in mouse fibroblast DT cells (21).

MAPKs are other important candidates that might be involved in the 8-Cl-cAMP-induced growth inhibition and apoptosis. It has long been reported that cAMP and MAPKs are closely related in the regulation of cell proliferation (reviewed in ref. 1). There are distinct subfamilies in MAPKs that have evolved for transmitting different types of signals, such as the extracellular signal-regulated kinase (ERK) for mitogenic stimuli and differentiation and the p38 MAPK and c-Jun NH_2-terminal kinase (JNK) for stress factors (22). p38 MAPK and JNK are usually related to cell growth inhibition or apoptosis, whereas ERK promotes cell proliferation (23), and cAMP signal can intervene during these processes. In many instances, cAMP can inhibit cellular proliferation by blocking ERK signaling stimulated by growth factors (13, 24–26). The activity of p38 MAPK can also be regulated by cAMP signals; for example, cAMP-increasing signals induce p38 MAPK activation and this activation is blocked by PKA catalytic inhibitor in NG108-15 neuroblastoma × glioma hybrid cells (27), SK-N-MC human neuroblastoma cells (28), and Chinese hamster ovary cells stably transfected with the human thyroid-stimulating hormone (TSH) receptor (29). In this study, we tested whether MAPK pathway is involved in the 8-Cl-cAMP-induced growth inhibition and apoptosis and found that p38 MAPK is indispensable in evoking these events.

Materials and Methods

Chemicals and drugs. 8-Cl-cAMP and 8-chloro-adenosine (8-Cl-adenosine) were purchased from Biolog (Bremen, Germany). [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from Dupont NEN (Boston, MA). Selectide

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(a PKC-specific substrate) was from Calbiochem (San Diego, CA). GF109203x (a PKC inhibitor), SB203580 (a p38 MAPK inhibitor), PD98059 (an ERK inhibitor), and SP600125 (a JNK inhibitor) were obtained from A.G. Scientific (San Diego, CA). H89 (a PKA inhibitor) and A134974 (an adenosine kinase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). All the other chemicals used in this research were obtained from Sigma-Aldrich.

Cell culture. HL60 and K562 cells were maintained in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone), 100 units/mL penicillin G, and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. For the growth assay, cell number was determined with Coulter counter (Beckman Coulter, Fullerton, CA) at the indicated time points. To investigate cell morphology, cells were stained with acridine orange (10 μg/mL) and then observed under Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan).

Protein kinase C activity assay. Suspended cells were harvested by centrifugation, washed with PBS, and then incubated with lysis buffer [25 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L β-mercaptoethanol, 0.05% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL each of aprotinin, pepstatin A, antipain, and leupeptin, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 10 mmol/L pyrophosphate] on ice for 15 minutes. The supernatant was obtained by centrifugation at 14,000 × *g* for 10 minutes. Total cell extract (10 μg) was mixed with kinase assay buffer [200 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $CaCl_2$, 100 mmol/L $MgCl_2$, 1 μg/mL each of aprotinin, pepstatin A, antipain, and leupeptin, 15 μmol/L ATP, 5 μmol/L [γ-^{32}P]ATP, 25 μmol/L PKC-specific substrate, Selectide (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 minutes. Ice-cold 5% acetic acid (10 μL) was added to the mixtures to stop the reaction and the reaction mixtures (30 μL) were spotted onto phosphocellulose filters (Life Technologies-Invitrogen, Carlsbad, CA), and then washed thrice with 1% phosphoric acid. Filters were air dried and radioactivities were measured with liquid scintillation counter (Wallac, Turku, Finland).

Western blot analysis. Cells were harvested by centrifugation and washed with PBS. Cell pellet was suspended in an extraction buffer [20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL each of aprotinin, pepstatin A, antipain, and leupeptin] and incubated on ice for 15 minutes. After centrifugation at 14,000 × *g* for 15 minutes at 4°C, the supernatant was taken as cell extract. The extracts were separated on a 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Protein-bound membrane was incubated with appropriate antibodies followed by horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Bio-Rad, Hercules, CA). The relevant protein bands were then visualized using enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ). Polyclonal antibody against PKA RIα subunits was described previously (30). Antibody for phospho-p38 was obtained from BD Transduction Laboratories (San Diego, CA). Antibodies for p38 and phospho–MAPK kinase (MKK) 3/6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA ladder formation assay. Harvested cells were suspended in lysis buffer [5 mmol/L Tris-HCl (pH 7.4), 20 mmol/L EDTA, 0.5% Triton X-100] and incubated on ice for 30 minutes. After centrifugation at 14,000 × *g* for 30 minutes at 4°C, DNA in the supernatant was purified by phenol/chloroform extraction and precipitated with ethanol. The DNA was then electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide.

Fluorescence-activated cell sorting analysis. Harvested cells were suspended in PBS and then fixed with ice-cold 70% ethanol for at least 3 hours. Fixed cells were stained with 50 μg/mL propidium iodide containing 50 μg/mL RNase A at 37°C for 30 minutes. DNA contents of cells (10,000 cells per experimental group) were analyzed by FACStar^{PLUS} flow cytometer (B&D, Mountain View, CA) using Lysis II and CELL-FIT programs (B&D).

Results

8-Chloro-cyclic AMP–induced growth inhibition is blocked by p38 mitogen-activated protein kinase inhibitor in HL60 cells. Previously, we reported that 8-Cl-cAMP induces inhibition of proliferation and apoptosis in various human cancer cell lines, including SH-SY5Y and HL60 (14). In HL60 (acute promyelocytic leukemia) cells, growth inhibition was induced after the treatment of 8-Cl-cAMP in time- and dose-dependent manners (Fig. 1*A* and *B*). However, K562 (chronic myelogenous leukemia) was more resistant to the growth inhibitory effect of 8-Cl-cAMP than HL60. It has been reported that K562 cells express the BCR-ABL fusion protein due to genetic rearrangement that maintains resistance to apoptosis induced under various conditions (31, 32).

To find out the signaling mechanisms involved in this growth inhibition, we first carried out kinase-inhibitor study (Fig. 1*C*). In parallel with the growth inhibition study in DT cells (21), PKA inhibitor (H89, 10 μmol/L) did not show any recovery effect on the 8-Cl-cAMP-induced growth inhibition, which means that this process is independent of PKA catalytic activity. GF109203x (3 μmol/L), a potent PKC inhibitor, could not reverse the 8-Cl-cAMP-induced growth inhibition, which seems to be incompatible to our previous experiments using DT cells (21). However, unlike the case in DT cells, 8-Cl-cAMP induces not only inhibition of proliferation but also apoptosis in HL60 cells. Therefore, this result can be interpreted that 8-Cl-cAMP may evoke apoptosis via another signaling pathway(s) rather than PKC activation through which inhibition of proliferation is induced.

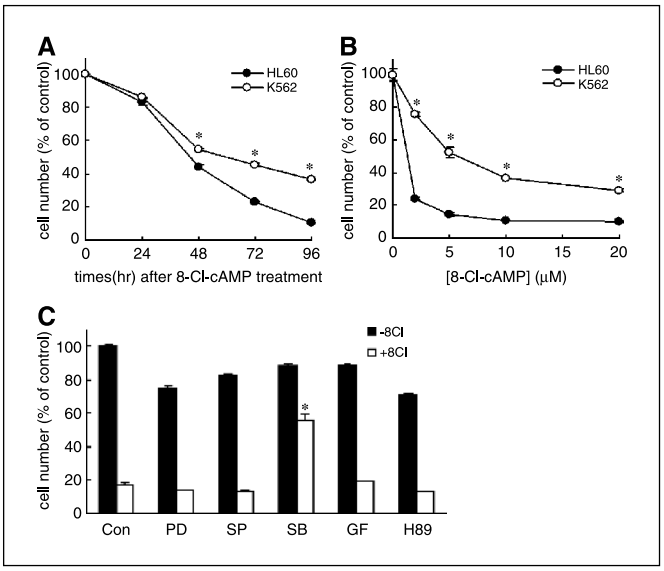

Figure 1. 8-Cl-cAMP-induced growth inhibition is blocked by p38 MAPK inhibitor. *A* and *B,* cell growth test after 8-Cl-cAMP treatment. HL60 and K562 cells were seeded at the concentration of 1 × 10^5 per well on 12-well plates. *A,* HL60 and K562 cells were treated with 8-Cl-cAMP (10 μmol/L) for the indicated times. *B,* HL60 and K562 cells were treated with the indicated concentrations of 8-Cl-cAMP for 3 days. After the treatment, cell numbers were measured with Coulter counter and normalized to control groups (100%). K562 cells were more resistant to the growth inhibitory effect of 8-Cl-cAMP than HL60 cells. *Asterisks,* differences between two groups ($P < 0.01$, two-tailed *t* test). *C,* effects of kinase inhibitors on the 8-Cl-cAMP-induced growth inhibition. HL60 cells were seeded at the concentration of 1 × 10^5 per well on 12-well plates and incubated with 8-Cl-cAMP (*8Cl*, 10 μmol/L) + various kinase inhibitors for 3 days. Only SB203580 (*SB*) could attenuate the 8-Cl-cAMP-induced growth inhibition. *Asterisk,* difference from 8-Cl-cAMP alone–treated group ($P < 0.01$, two-tailed *t* test). The inhibitors used in this experiment were PD98059 (*PD*; 10 μmol/L), SP600125 (*SP*; 10 μmol/L), SB203580 (10 μmol/L), GF109203x (*GF*; 3 μmol/L), and H89 (10 μmol/L).

Neither PD98059 (10 μmol/L; ERK inhibitor) nor SP600125 (10 μmol/L; JNK inhibitor) could reverse the 8-Cl-cAMP-induced apoptosis. However, SB203580 (10 μmol/L; a p38 MAPK inhibitor) attenuated the growth inhibitory effect when cotreated with 8-Cl-cAMP (Fig. 1*C*). In this respect, p38 MAPK can be regarded as a signaling mediator of the 8-Cl-cAMP-induced growth inhibition and/or apoptosis in HL60 cells.

8-Chloro-cyclic AMP–induced apoptosis is mediated by p38 mitogen-activated protein kinase activation. 8-Cl-cAMP-treated HL60 cells showed cell body shrinkage, nuclear condensation, and nuclear fragmentation (Fig. 2*A*, *C*, and *D*), which are the typical characteristics of apoptosis (33). As might be expected, this apoptotic morphology disappeared by the inhibition of p38 MAPK activity (Fig. 2*A*). Moreover, in DNA fragmentation assay and fluorescence-activated cell sorting analysis, p38 MAPK inhibitor completely restored the 8-Cl-cAMP-induced apoptosis (Fig. 2*C* and *D*). However, 8-Cl-cAMP-treated K562 cells did not show any morphologic differences from mock-treated control cells (Fig. 2*B*).

To investigate whether p38 MAPK activation is actually involved in the 8-Cl-cAMP-induced apoptosis, we measured the phosphorylated (activated) form of p38 MAPK using a phospho-specific p38 MAPK antibody. In HL60 cells, phosphorylated p38 MAPK started to increase at 24 hours after the treatment of 8-Cl-cAMP (Fig. 3*A*), and this phosphorylation exhibited dosage dependency (Fig. 3*C*). However, K562 cells, which are more tolerant to 8-Cl-cAMP, showed constant level of phospho–p38 MAPK (Fig. 3*B*). Furthermore, MKK3/6, which are known as upstream kinases of p38 MAPK, were also activated by the treatment of 8-Cl-cAMP in HL60 cells (Fig. 3*D*). All these data suggest that the 8-Cl-cAMP-induced apoptosis is mediated by MKK3/6-p38 MAPK signaling pathway.

p38 mitogen-activated protein kinase activation is upstream of protein kinase C activation during the 8-chloro-cyclic AMP–induced apoptosis. Although PKC inhibitor could not attenuate growth inhibition by 8-Cl-cAMP in HL60 cells, it is necessary to clarify the hierarchical relationship between p38 MAPK and PKC during the 8-Cl-cAMP-induced growth inhibition. To investigate whether PKC activity is also increased by 8-Cl-cAMP in HL60 cells, we carried out PKC activity assay using a PKC-specific peptide substrate, Selectide. Although the 8-Cl-cAMP-induced apoptosis was not attenuated by the cotreatment of PKC inhibitor (Fig. 1*C*), 8-Cl-cAMP was still able to stimulate the PKC activity in HL60 cells (Fig. 4*A*). It can be argued that induction of apoptosis, which is independent of PKC activation, might be more predominant than inhibition of proliferation in HL60 cells that total cell number decreased by 8-Cl-cAMP was hardly affected by PKC inhibition.

After the treatment of 8-Cl-cAMP, both p38 MAPK and PKC were activated as shown previously. Irrespective of the presence of PKC inhibitor (GF109203x), 8-Cl-cAMP still increased the phosphorylation of p38 MAPK (Fig. 4*B*). On the contrary, PKC activity was reduced to the normal level by the cotreatment of p38 MAPK inhibitor (SB203580; Fig. 4*C*). Practically, both SB203580 and GF109203x could recover DT cell growth suppressed by 8-Cl-cAMP (Fig. 4*D*). These results suggest that after 8-Cl-cAMP treatment PKC activation also occurs in HL60 cells, and this PKC activation is regulated by p38 MAPK activation (i.e., PKC resides or acts downstream of p38 MAPK).

p38 mitogen-activated protein kinase activation by 8-chloro-cyclic AMP is independent of PKA catalytic activity. It has been reported that p38 MAPK activation is controlled by PKA under certain conditions (27–29). For example, in Chinese hamster ovary cells transfected with TSH receptor, TSH and forskolin induced MKK3/6 and p38 MAPK activation, and this activation was blocked by PKA inhibitor, H89 (29). To verify whether p38 MAPK activation by 8-Cl-cAMP is also dependent on PKA activity, we did phospho–p38 MAPK Western blotting after cotreatment of 8-Cl-cAMP and H89 (10 μmol/L). H89 has been known to completely block PKA enzymatic activity even at 5 μmol/L (14). Nevertheless, it had no effect on p38 MAPK phosphorylation induced by 8-Cl-cAMP (Fig. 4*E*). The type I PKA regulatory subunit α (RIα) down-regulation by 8-Cl-cAMP, which has been regarded as an important cause of the 8-Cl-cAMP-induced growth inhibition (17), was also returned to normal levels by blocking the p38 MAPK activation (Fig. 4*F*). Therefore, p38 MAPK activation is not regulated by PKA at least in our experimental system. On the contrary, it suggests that RIα down-regulation signal by 8-Cl-cAMP may be transduced through the p38 MAPK pathway.

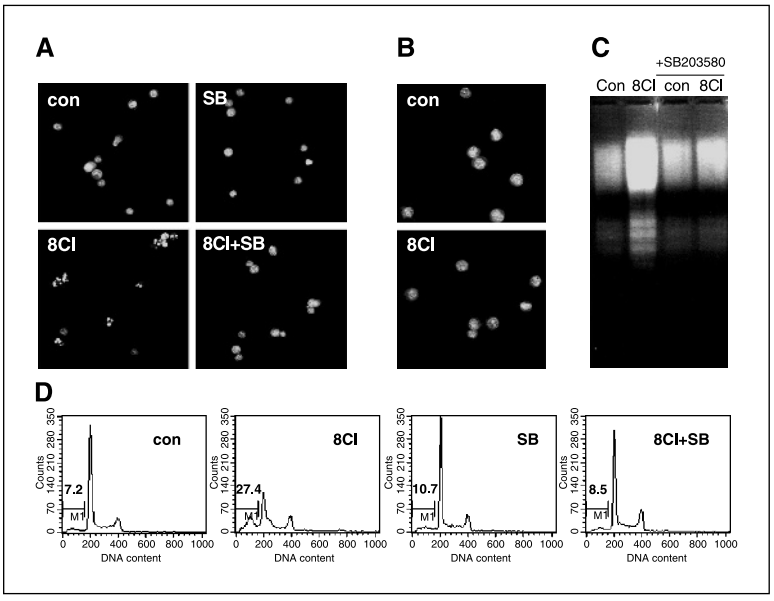

Figure 2. 8-Cl-cAMP-induced apoptosis is blocked by p38 MAPK inhibition. *A,* acridine orange staining in HL60 cells. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) in the presence or absence of SB203580 (10 μmol/L) for 3 days. To show the nuclear morphology, HL60 cells were stained with acridine orange (10 μg/mL) and then observed under a fluorescence microscope. 8-Cl-cAMP-treated cells showed nuclear fragmentation and condensation (*arrows*), which was blocked by the cotreatment of SB203580 (10 μmol/L). *B,* acridine orange staining in K562 cells. K562 cells were also treated as in (*A*), and acridine orange staining was done. 8-Cl-cAMP could not affect the cell morphology. *C,* DNA fragmentation assay. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) in the presence or absence of SB203580 (10 μmol/L) for 3 days. Then, chromosomal DNA was prepared and electrophoresed in 2% agarose gel. Chromosomal DNA obtained from 8-Cl-cAMP-treated cells showed fragmented pattern, which was disappeared by p38 MAPK inhibition. *D,* fluorescence-activated cell sorting analysis of cell cycle. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) in the presence or absence of SB203580 (10 μmol/L) for 3 days and then stained with 50 μg/mL propidium iodide. After propidium iodide staining, the cell cycle profile was measured using flow cytometry. *Numbers,* percentage of sub-G_1 phase (i.e., apoptotic population). 8-Cl-cAMP increased sub-G_1 population (27.4%), which was diminished by the treatment with 10 μmol/L SB203580 (8.5%).

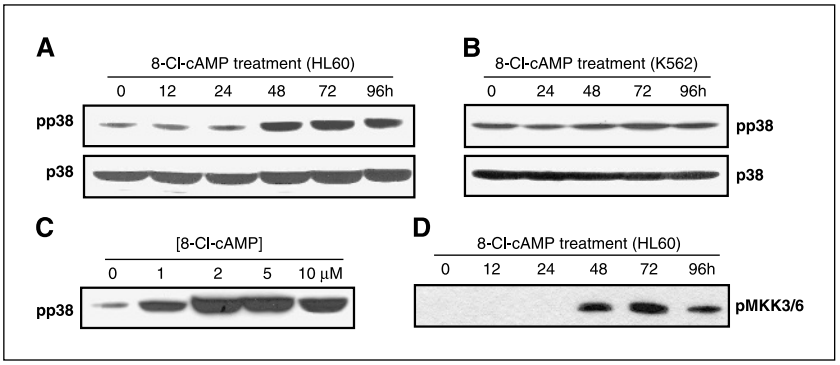

Figure 3. p38 MAPK is activated after 8-Cl-cAMP-treatment. *A* and *B,* phopho-p38 Western blotting after 8-Cl-cAMP treatment. HL60 (*A*) and K562 (*B*) cells were treated with 8-Cl-cAMP (10 μmol/L) for the indicated times; then, Western blotting was carried out using anti-phospho-p38 (*pp38*) or anti-total p38 (*p38*) antibody. Phospho-p38 increased time dependently in HL60 after 8-Cl-cAMP treatment, whereas it was not changed in K562 cells. Total p38 showed constant level in both cell lines. *C,* phopho-p38 Western blotting in HL60 cells. After treatment of indicated concentrations of 8-Cl-cAMP for 3 days, phospho-p38 Western blotting was carried out. Even at 2 μmol/L 8-Cl-cAMP, p38 MAPK was phosphorylated eminently. *D,* phospho-MKK3/6 Western blotting. After 8-Cl-cAMP was treated as in (*A*), phosphorylation (activation) of MKK3/6 was determined by Western blotting using anti-phospho-MKK3/6 (pMKK3/6) antibody in HL60 cells. pMKK3/6 was also increased after 8-Cl-cAMP treatment.

p38 mitogen-activated protein kinase activation by 8-chloro-cyclic AMP is also mediated by its metabolite, 8-chloro-adenosine. As we have shown previously (21), metabolism of 8-Cl-cAMP into 8-Cl-adenosine or further metabolites is indispensable for the induction of growth inhibition and cell death in various cancer cell lines. In HL60 cells, the 8-Cl-cAMP-induced or 8-Cl-adenosine-induced growth inhibition is also blocked by the cotreatment of an adenosine kinase inhibitor, A134974 (10 μmol/L; Fig. 5*A*). Furthermore, A134974 down-regulated the 8-Cl-cAMP-induced or 8-Cl-adenosine-inducd apoptosis, which was assessed by poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 5*B*). To clarify whether p38 MAPK activation is also dependent on the 8-Cl-cAMP metabolism, we checked cell growth after 8-Cl-adenosine treatment in the presence or absence of 10 μmol/L SB203580. Just as with 8-Cl-cAMP, 8-Cl-adenosine did not induce growth inhibition in the presence of SB203580 (Fig. 5*C*). We also looked at the p38 MAPK activation by Western blotting after 8-Cl-adenosine treatment (Fig. 5*D*). Both 8-Cl-cAMP and 8-Cl-adenosine increased the p38 MAPK phosphorylation, and it returned to normal levels by the cotreatment of A134974. We can surmise from these results that the 8-Cl-cAMP-induced p38 MAPK activation requires the metabolic modification to 8-Cl-adenosine by adenosine kinase.

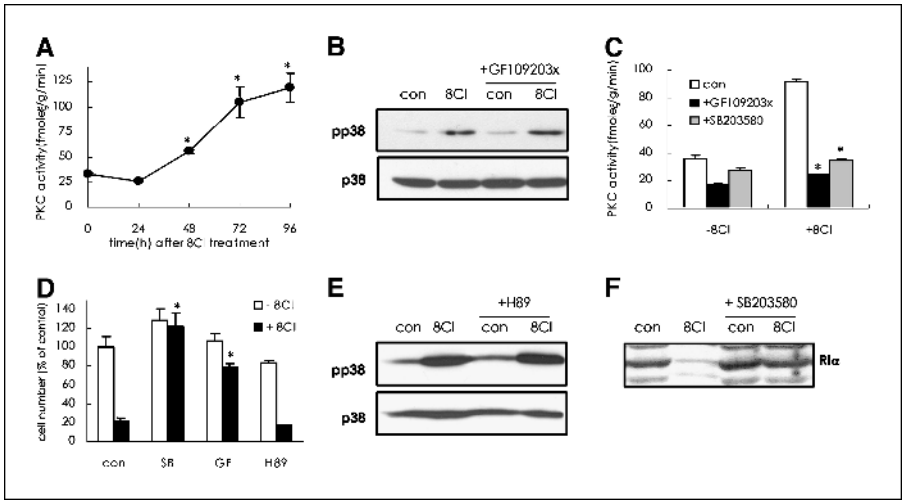

Figure 4. p38 activation is upstream of PKC activation and is independent of PKA activation. *A,* PKC activity assay. After 8-Cl-cAMP treatment to HL60 cells for indicated times, PKC activity was evaluated using a PKC-specific substrate, Selectide, as mentioned in Materials and Methods. After 48 hours, PKC enzymatic activity started to increase. *Asterisks,* differences from 0-hour group ($P < 0.01$, two-tailed *t* test). *B,* effect of PKC inhibitor on p38 phosphorylation by 8-Cl-cAMP. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) in the presence or absence of GF109203x (3 μmol/L) for 3 days; then, phospho-p38 Western blotting was carried out. GF109203x could not attenuate p38 phosphorylation induced by 8-Cl-cAMP. *C,* effect of p38 inhibitor on PKC activation by 8-Cl-cAMP. HL60 cells were treated with 8-Cl-cAMP + GF109203x (3 μmol/L) or SB203580 (10 μmol/L) for 3 days; then, PKC activity was measured using Selectide peptide as a substrate. SB203580 completely reduced PKC activity increased by 8-Cl-cAMP. *Asterisks,* differences from 8-Cl-cAMP group ($P < 0.01$, two-tailed *t* test). *D,* effect of p38 and PKC inhibitors on the 8-Cl-cAMP-induced growth inhibition in DT cells. 8-Cl-cAMP (10 μmol/L) was treated to mouse fibroblast DT cells with p38 or PKC inhibitors for 3 days; then, cell numbers were counted. Both SB203580 (10 μmol/L) and GF109203x (3 μmol/L) could recover cell growth inhibited by 8-Cl-cAMP. *Asterisks,* differences from 8-Cl-cAMP group ($P < 0.01$, two-tailed *t* test). *E,* effect of PKA inhibitor on p38 phosphorylation by 8-Cl-cAMP. HL60 cells were treated with 8-Cl-cAMP in the presence or absence of H89 (10 μmol/L) for 3 days; then, phospho-p38 level was measured by Western blot analysis. H89 could not attenuate p38 phosphorylation induced by 8-Cl-cAMP. *F,* effect of p38 inhibitor on RIα down-regulation induced by 8-Cl-cAMP. HL60 cells were treated with 8-Cl-cAMP in the presence or absence of SB203580 (10 μmol/L); then, RIα level was measured. SB203580 could reverse the RIα protein level decreased by 8-Cl-cAMP.

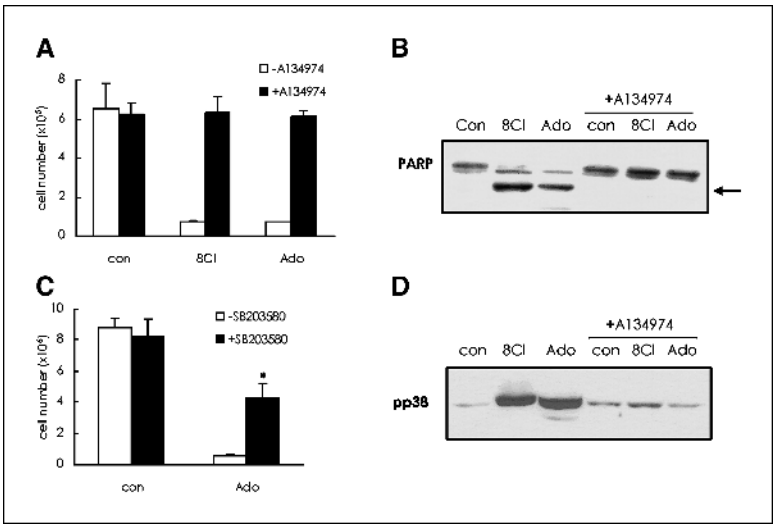

Figure 5. p38 MAPK activation by 8-Cl-cAMP is mediated by its metabolite, 8-Cl-adenosine. *A,* effect of adenosine kinase inhibitor on the 8-Cl-cAMP-induced or 8-Cl-adenosine-induced growth inhibition. HL60 cells were seeded at the concentration of 1×10^5 per well on 12-well plates and incubated with 8-Cl-cAMP (10 μmol/L) or 8-Cl-adenosine (*Ado*, 2 μmol/L) in the presence or absence of A134974 (10 μmol/L) for 3 days; then, cell numbers were counted with Coulter counter. A134974 completely blocked the growth inhibition induced by 8-Cl-cAMP and 8-Cl-adenosine. *B,* PARP cleavage after 8-Cl-cAMP or 8-Cl-adenosine treatment. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) or 8-Cl-adenosine (2 μmol/L) in the presence or absence of A134974 (10 μmol/L) for 3 days; then, Western blotting was carried out using anti-PARP antibody. Cleaved forms of PAPR (*arrow*) appeared after 8-Cl-cAMP or 8-Cl-adenosine treatment, which was blocked by the addition of A134974. *C,* effect of p38 inhibitor on the 8-Cl-adenosine-induced growth inhibition. HL60 cells were seeded at the concentration of 1×10^5 per well on 12-well plates and incubated with 8-Cl-adenosine (2 μmol/L) in the presence or absence of SB203580 (10 μmol/L) for 3 days; then, cell numbers were counted. SB203580 could also attenuate the growth inhibition by 8-Cl-adenosine. *D,* effect of adenosine kinase inhibitor on the 8-Cl-cAMP-induced or 8-Cl-adenosine-induced p38 phosphorylation. HL60 cells were treated with 8-Cl-cAMP (10 μmol/L) or 8-Cl-adenosine (2 μmol/L) in the presence or absence of A134974 (10 μmol/L) for 3 days; then, Western blotting was done using anti-phospho-p38 antibody. 8-Cl-adenosine as well as 8-Cl-cAMP increased p38 phosphorylation, which was reduced by adenosine kinase inhibitor A134974.

Discussion

Due to its growth inhibitory effect, 8-Cl-cAMP has been studied as one of the candidates for anticancer therapeutic agent for many years (15, 16). 8-Cl-cAMP inhibits cell proliferation and induces cell death (apoptosis) in various cell lines. In general, this growth inhibition occurs more evidently in transformed cell lines than in normal cell lines. For example, 8-Cl-cAMP induced growth inhibition and apoptosis in MCF-7 human breast cancer cells, but it hardly affected the growth of MCF-10A human mammary epithelial cells (14). However, the mechanisms of its anticancer activity have not been fully understood and are still under debates. Cho-Chung et al. have done considerable amount of studies that suggest 8-Cl-cAMP induces growth inhibition by the differential regulation of PKA regulatory subunits, RI and RII (15, 17, 34, 35); that is, 8-Cl-cAMP decreases the level of RI protein that distorts the intracellular RI/RII ratio, which is important for the maintenance of cellular physiology and the effects of 8-Cl-cAMP can be mimicked by the addition of RIα antisense oligonucleotide.

On the contrary, several lines of evidence indicate that the regulation of PKA-R subunits by 8-Cl-cAMP is not the major cause of growth inhibition (18–20, 36). 8-Cl-cAMP could not induce growth inhibition in the presence of IBMX (a phosphodiesterase inhibitor) and A134974 (an adenosine kinase inhibitor), which lead to the blockage of 8-Cl-cAMP metabolic pathway (20). In addition, not 8-Cl-cAMP, but its metabolite, 8-Cl-adenosine, is the key molecule that is responsible for the growth inhibitory effect. Furthermore, during this growth inhibitory process, PKA catalytic activity is not required. Instead, as in our previous report, it was found that PKC activation was involved in the 8-Cl-cAMP-induced or 8-Cl-adenosine-induced growth inhibition in DT cells (21).

In HL60 cells, PKC activity was also increased by 8-Cl-cAMP treatment, but this activation was not essential in inducing the growth inhibition or apoptosis. If 8-Cl-cAMP induces proliferation inhibition and apoptosis through different signaling pathways, in HL60 cells where the 8-Cl-cAMP-induced apoptosis occurs more dominantly, the contribution of PKC activation to the total cell growth may not be so critical. In this report, we found that 8-Cl-cAMP induces apoptosis through another signaling pathway, p38 MAPK activation.

p38 MAPK activation is well known to mediate apoptotic signal transduction. Inhibition of p38 MAPK pathway blocked apoptosis induced by various stimuli, such as trophic factor deprivation (37–39), oxidative stresses (40, 41), ceramides (42, 43), and UV radiation (44–46). In addition, there are many experimental evidence that imply cross-talk between p38 MAPK and cAMP signaling. For example, TSH and forskolin induced p38 MAPK activation in thyroid cells or Chinese hamster ovary cells stably transfected with TSH receptor (29). According to the results presented here, the 8-Cl-cAMP-induced apoptosis was also mediated by p38 MAPK activation in HL60 cells. However, in K562 cells, which are tolerable to many apoptosis-inducing agents, p38 MAPK was not activated and apoptotic cell death was not observed after 8-Cl-cAMP treatment. The mechanisms by which the BCR-ABL-positive leukemia cells resist the apoptotic signals are not fully understood, although delayed caspase activation or increased Bcl-X_L level in K562 cells has been reported (32). We showed here that p38 MAPK activation can be regarded as another determinant to execute apoptosis in leukemia cells.

In this study, it was also found that the 8-Cl-cAMP-induced apoptosis is dependent on its metabolic pathway because it is entirely blocked by an adenosine kinase inhibitor. 8-Cl-cAMP can be converted to 8-Cl-adenosine by the actions of phosphodiesterase and nucleotide phosphatase, and 8-Cl-adenosine is further metabolized by adenosine kinase into 8-Cl-AMP or 8-Cl-ATP. In

many experimental systems, 8-Cl-cAMP must be metabolized to induce growth inhibition; nevertheless, it is still debatable (18–20, 36). It cannot be overlooked that down-regulation of PKA-RI subunit by 8-Cl-cAMP is a critical aspect during the induction of growth inhibition. However, 8-Cl-adenosine can also decrease the RIα subunit, which is blocked by adenosine kinase inhibitor (data not shown). Additionally, p38 MAPK pathway may be involved in this RIα down-regulation process because cotreatment of the p38 MAPK inhibitor, SB203580, reinstated the RIα protein level decreased by 8-Cl-cAMP and 8-Cl-adenosine. Therefore, two hypotheses explain the growth inhibition mechanism of 8-Cl-cAMP; that is, RIα down-regulation by 8-Cl-cAMP itself or the importance of its metabolic pathway do not seem to be so incompatible. To elucidate the exact mechanism of the 8-Cl-cAMP-induced growth inhibition or apoptosis, further researches must be carried out more delicately.

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References

1. Stork PJ, Schmitt JM. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 2002;12:258–66.

2. Krebs EG, Beavo JA. Phosphorylation-dephosphorylation of enzymes. Annu Rev Biochem 1979;48:923–59.

3. Maurer RA. Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. Nature 1981;294:94–7.

4. Jungmann RA, Kelley DC, Miles MF, et al. Cyclic AMP regulation of lactate dehydrogenase. Isoproterenol and N^6,O_2-dibutyryl cyclic amp increase the rate of transcription and change the stability of lactate dehydrogenase a subunit messenger RNA in rat C6 glioma cells. J Biol Chem 1983;258:5312–8.

5. Ewald DA, Williams A, Levitan IB. Modulation of single Ca^{2+}-dependent K^+-channel activity by protein phosphorylation. Nature 1985;315:503–6.

6. Vossler MR, Yao H, York RD, et al. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 1997;89:73–82.

7. Yarwood SJ, Kilgour E, Anderson NG. Cyclic AMP potentiates growth hormone-dependent differentiation of 3T3-F442A preadipocytes: possible involvement of the transcription factor CREB. Mol Cell Endocrinol 1998;138:41–50.

8. Boulogne B, Habert R, Levacher C. Regulation of the proliferation of cocultured gonocytes and Sertoli cells by retinoids, triiodothyronine, and intracellular signaling factors: differences between fetal and neonatal cells. Mol Reprod Dev 2003;65:194–203.

9. Sevetson BR, Kong X, Lawrence JC Jr. Increasing cAMP attenuates activation of mitogen-activated protein kinase. Proc Natl Acad Sci U S A 1993;90:10305–9.

10. Chen J, Iyengar R. Suppression of Ras-induced transformation of NIH 3T3 cells by activated Gαs. Science 1994;263:1278–81.

11. Altschuler DL, Peterson SN, Ostrowski MC, et al. Cyclic AMP-dependent activation of Rap1b. J Biol Chem 1995;270:10373–6.

12. de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 1998;396:474–7.

13. Schmitt JM, Stork PJ. Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1. Mol Cell Biol 2001;21:3671–83.

14. Kim SN, Ahn YH, Kim SG, et al. 8-Cl-cAMP induces cell cycle-specific apoptosis in human cancer cells. Int J Cancer 2001;93:33–41.

15. Cho-Chung YS. Modulation of adenylate cyclase signalling. Semin Cancer Biol 1992;3:361–7.

16. Cummings J, Fielding H, Miller WR. Pharmaceutical analysis of 8-chloroadenosine 3′, 5′-monophosphate. J Pharm Biomed Anal 1994;12:1289–94.

17. Cho-Chung YS, Clair T, Tagliaferri P, et al. Site-selective cyclic AMP analogs as new biological tools in growth control, differentiation, and proto-oncogene regulation. Cancer Invest 1989;7:161–77.

18. Halgren RG, Traynor AE, Pillay S, et al. 8Cl-cAMP cytotoxicity in both steroid sensitive and insensitive multiple myeloma cell lines is mediated by 8Cl-adenosine. Blood 1998;92:2893–8.

19. Gandhi V, Ayres M, Halgren RG, et al. 8-Chloro-cAMP and 8-chloro-adenosine act by the same mechanism in multiple myeloma cells. Cancer Res 2001;61:5474–9.

20. Lamb D, Steinberg RA. Anti-proliferative effects of 8-chloro-cAMP and other cAMP analogs are unrelated to their effects on protein kinase A regulatory subunit expression. J Cell Physiol 2002;192:216–24.

21. Ahn YH, Jung JM, Hong SH. 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. J Cell Physiol 2004;201:277–85.

22. Efimova T, LaCelle P, Welter JF, et al. Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. J Biol Chem 1998;273:24387–95.

23. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 2002;12:9–18.

24. Wang L, Liu F, Adamo ML. Cyclic AMP inhibits extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways by inhibiting Rap1. J Biol Chem 2001;276:37242–9.

25. Fujita T, Meguro T, Fukuyama R, et al. New signaling pathway for parathyroid hormone and cyclic AMP action on extracellular-regulated kinase and cell proliferation in bone cells. Checkpoint of modulation by cyclic AMP. J Biol Chem 2002;277:22191–200.

26. Hecquet C, Lefevre G, Valtink M, et al. cAMP inhibits the proliferation of retinal pigmented epithelial cells through the inhibition of ERK1/2 in a PKA-independent manner. Oncogene 2002;21:6101–12.

27. Zhang Z, Xin SM, Wu GX, et al. Endogenous δ-opioid and ORL1 receptors couple to phosphorylation and activation of p38 MAPK in NG108-15 cells and this is regulated by protein kinase A and protein kinase C. J Neurochem 1999;73:1502–9.

28. Zhen X, Uryu K, Wang HY, et al. D1 dopamine receptor agonists mediate activation of p38 mitogen-activated protein kinase and c-Jun amino-terminal kinase by a protein kinase A-dependent mechanism in SK-N-MC human neuroblastoma cells. Mol Pharmacol 1998;54:453–8.

29. Pomerance M, Abdullah HB, Kamerji S, et al. Thyroid-stimulating hormone and cyclic AMP activate p38 mitogen-activated protein kinase cascade. Involvement of protein kinase A, rac1, and reactive oxygen species. J Biol Chem 2000;275:40539–46.

30. Lee GR, Kim SN, Noguchi K, et al. Ala99ser mutation in RIα regulatory subunit of protein kinase A causes reduced kinase activation by cAMP and arrest of hormone-dependent breast cancer cell growth. Mol Cell Biochem 1999;195:77–86.

31. McGahon A, Bissonnette R, Schmitt M, et al. BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. Blood 1994;83:1179–87.

32. Gangemi RM, Santamaria B, Bargellesi A, et al. Late apoptotic effects of taxanes on K562 erythroleukemia cells: apoptosis is delayed upstream of caspase-3 activation. Int J Cancer 2000;85:527–33.

33. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–57.

34. Tortora G, Clair T, Cho-Chung YS. An antisense oligodeoxynucleotide targeted against the type II β regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects. Proc Natl Acad Sci U S A 1990;87:705–8.

35. Yokozaki H, Budillon A, Tortora G, et al. An antisense oligodeoxynucleotide that depletes RIα subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. Cancer Res 1993;53:868–72.

36. Langeveld CH, Jongenelen CA, Theeuwes JW, et al. The antiproliferative effect of 8-chloro-adenosine, an active metabolite of 8-chloro-cyclic adenosine monophosphate, and disturbances in nucleic acid synthesis and cell cycle kinetics. Biochem Pharmacol 1997;53:141–8.

37. Xia Z, Dickens M, Raingeaud J, et al. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.

38. Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. J Biol Chem 1997;272:20490–4.

39. Rosini P, De Chiara G, Lucibello M, et al. NGF withdrawal induces apoptosis in CESS B cell line through p38 MAPK activation and Bcl-2 phosphorylation. Biochem Biophys Res Commun 2000;278:753–9.

40. Huot J, Houle F, Rousseau S, et al. SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. J Cell Biol 1998;143:1361–73.

41. Zhuang S, Demirs JT, Kochevar IE. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. J Biol Chem 2000;275:25939–48.

42. Brenner B, Koppenhoefer U, Weinstock C, et al. Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. J Biol Chem 1997;272:22173–81.

43. Willaime S, Vanhoutte P, Caboche J, et al. Ceramide-induced apoptosis in cortical neurons is mediated by an increase in p38 phosphorylation and not by the decrease in ERK phosphorylation. Eur J Neurosci 2001;13:2037–46.

44. Bulavin DV, Saito S, Hollander MC, et al. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 1999;18:6845–54.

45. Shimizu H, Banno Y, Sumi N, et al. Activation of p38 mitogen-activated protein kinase and caspases in UVB-induced apoptosis of human keratinocyte HaCaT cells. J Invest Dermatol 1999;112:769–74.

46. Assefa Z, Vantieghem A, Garmyn M, et al. p38 mitogen-activated protein kinase regulates a novel, caspase-independent pathway for the mitochondrial cytochrome *c* release in ultraviolet B radiation-induced apoptosis. J Biol Chem 2000;275:21416–21.