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Inhibition of pRB Phosphorylation and Induction of p21WAF1/CIP1 Occur During cAMP-induced Growth Arrest in Human Neuroblastoma Cells

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Abstract

To develop a new approach to the treatment of neuroblastoma cells we evaluated the effect of cAMP on the Ewing's sarcoma cell line CHP-100. We observed that the proliferation-inhibitory effect of cAMP analogs was due to cell cycle arrest and induction of apoptosis, which was confirmed by observing the morphological changes and DNA fragmentation. DNA flow cytometric analysis revealed that cAMP arrested the cell cycle progression at the G1 phase, which effects were associated with inhibition of phosphorylation of retinoblastoma protein (pRB) and enhanced binding of pRB and the transcription factor E2F-1. cAMP also suppressed the cyclin-dependent kinase (Cdk) 2 and cyclin E-associated kinase activity without changes of their expressions. Furthermore, cAMP induced the levels of Cdk inhibitor $p21^{WAF1/CIP1}$ expression and p21 proteins induced by cAMP were associated with Cdk2. Overall, our results identify a combined mechanism involving the inhibition of pRB phosphorylation and induction of p21 as targets for cAMP, and this may explain some of its anti-cancer effects.

Key words – cAMP, Apoptosis, G1 arrest, pRB, p21

Introduction

Regulation of cell proliferation is a complex process involving the regulated expression and/or modification of discrete gene products, which control transition between different stages of the cell cycle[24]. Cyclins have appeared as major positive regulators in this network, because their association to the cyclin-dependent kinases (Cdks) allows the subsequent activation of the Cdk/cyclin complexes and their catalatic activity. On the other hand, the Cdk inhibitors negatively control the activity of Cdk/cyclin complex by coordinating internal and/or external signals and impending proliferation at several key checkpoints[20]. Among them, $p21^{WAF1/CIP1}$ is required for p53-induced growth arrest, which can inhibit kinase activity of Cdks during the G1/S cell cycle checkpoint[5,32]. Cyclic adenosine monophosphate (cAMP) has been described as a mediator of numerous physiological processes and it also plays a critical role in the regulation of cell growth and differentiation through modulation of cAMP-dependent protein kinase signaling pathway[23,26]. Previous studies have shown that cAMP analogues exhibit growth inhibitory activity *in vitro* and *in vivo* in a broad spectrum of human carcinoma, fibrosarcoma and leukaemia cell lines without causing cytotoxicity[13,14,21,30]. However, the molecular mechanisms of its anti-proliferative action on cell growth are not

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known completely.

Ewings sarcoma, the second most frequent bone tumor in children and adolescents, is charecterazed by a highly specific rearrangement between the EWS gene and one of five ETS-related transcription factors, resulting in the formation of a chimeric transcription factor[15,31]. The predominant fusion protein, EWS-FLI1, has been shown to function as an aberrant transcription factor, and both EWS-FLI1 and the variant chimeric protein EWS-ETV1 activate common signaling pathways and promote oncogenesis[1,27,28]. Although the histogenesis of Ewings sarcoma has not been firmly established, this tumor is generally thought to be derived from primitive neuronal progenitor cells[29], and retain the potential for neural differentiation *in vitro* and *in vivo*[16,29,]. The progenosis of patients with localized disease has been improved dramatically by multimodality therapy[10,19]. In contrast, the prognosis is still poor for patients with locally recurrent or metastatic disease[11].

The present study was carried out to characterize the probable mechanisms involved in cAMP-mediated growth inhibitory effect in Ewing's sarcoma CHP-100 cell line. We demonstrated that cAMP induced cell cycle arrest at G1 phase and apoptosis through a combined mechanism involving the inhibition of phosphorylation of pRB and the induction of Cdk inhibitor p21.

Materials and Methods

Cell culture

Human neuroblastoma CHP-100 cells were grown at 37°C in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 IU/mL of penicillin, and 100 μg/ml of streptomycin (Biofluids, Gaithersburg, MD, USA), in a humidified atmosphere with 5% CO_2.

cAMP analogs and antibodies

Dibutyryl-cAMP (db-cAMP, N^6,2′-*O*-dibutyryl-adenosine-3′:5′-monophosphate, cyclic monosodium salt) and 8-bromo-cAMP (8-br-cAMP, 8-bromo-adenosine-3′:5′-monophosphate, cyclic monosodium salt) were obtained from Boehringer Mannheim (Indianapolis, IN, USA). Monoclonal anti-pRB antibody, polyclonal anti-E2F1, -cdk2, -cdk4, -cdk6, -cyclin D1, and -cyclin E antibodies were purchased from Santa Cruz Biotechnology (dilution, 1:500-1,000, Santa Cruz, CA, USA). Monoclonal anti-p21 antibody was purchased from Upstate Biotechnology (dilution, 1:1,000, Lake Placid, NY, USA). Monoclonal anti-p53 antibody was purchased from Calbiochem (dilution, 1:1,000, Cambridge, MA, USA). Peroxidase-labeled secondary antibodies were purchased from Amersham Corp. (dilution, 1:1,000, Arlington Height, IL, USA).

Cell cycle analysis

Exponentially growing cells were compared to cells treated with db-cAMP and 8-br-cAMP and cells were harvested, fixed in 50% ethanol and incubated with RNase A and the DNA intercalating dye propidium iodide (Sigma Chemical Co., St. Louis, MO, USA). Cell cycle phase analysis was performed by flow cytometry using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA)[2,3].

Nuclear staining with DAPI

Cells were washed two times with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature [2]. The cells were washed two more times with PBS and analyzed via a fluorescence microscope.

Assessment of DNA degradation

Cells were washed with PBS and resuspended in lysis buffer [1 mM EDTA, 10 mM Tris (pH 8.0), 1% SDS, 1 μg /ml proteinase K]. After 1 h incubation at 37°C, RNase

A was added and incubation continued for a further 1 h. Crude DNA preparations were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). DNA samples were subsequently run at 120 V on a 1.0% agarose gel containing ethidium bromide (EtBr, Sigma). Gel was examined on an ultraviolet light source and photographed.

Immunoprecipitation and Western bolt analysis

Total cell lysates were lysed in extraction buffer as previously described[2]. For immunoprecipitation, cell extracts were incubated with immunoprecipitating antibody or normal rabbit serum in extraction buffer for 1 h at 4°C. The immuno-complex was collected on protein A-Sepharose beads (Sigma) and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Western blot analysis was performed as described[2] using enhanced chemiluminescence (ECL) detection system (Amersham).

In vitro immuno-complex kinase assay

Whole cell lysates were incubated with primary antibody, precipitated and resuspended in kinase assay mixture buffer[3] containing [γ-^{32}P] ATP (ICN, Irvine, CA, USA) and histone H1 (Sigma) as substrate. After incubation at 37°C for 30 min, the reaction was stopped by addition of the same amount of 2×SDS sample buffer. After boiling and spinning, the samples were separated on 10% SDS-polyacrylamide gels, dried and bands were detected by autoradiography.

Results

Growth inhibition and G1 arrest by cAMP

To evaluate the effect of cell-permeant cAMP analogs, db-cAMP and 8-br-cAMP, on cell proliferation, we initially determined the effects of cAMP on the growth of human neuroblastoma CHP-100 cells. Either db-cAMP or 8-br-cAMP had a strong inhibitory effect on cell proliferation in a time-dependent manner (about 45% decrease from baseline in the presence of 1 mM cAMP for 72 h, data not shown). As shown in Table 1, the cell cycle phase distribution of exponentially growing CHP-100 cells was compared to cells treated with cAMP analogs. Twenty-four hours after cAMP addition there was a marked increase in the percent of cells in G1. By 72 h following db-cAMP and 8-br-cAMP addition, 81.4 and 91.9% of the CHP-100 cells were arrested in G1, respectively, which was accompanied with a significant decrease in their G2/M phase as compared with untreated control cells, suggesting that the growth inhibitory effect of cAMP was the result of a block during this G1 phase and that such cells did not enter S phase.

Apoptotic cell death induced by cAMP

To investigate whether the anti-proliferative effects of cAMP were due to apoptosis in CHP-100 cells, we assessed the morphological changes with DAPI staining. Fig. 1A shows the morphologic changes of CHP-100 cells incubated with or without cAMP. The control cells displayed intact nuclear structure, while cells treated

Table 1. Effect of cAMP on cell cycle phase distribution in human neuroblastoma CHP-100 cells. Exponentially growing cells and cells treated with 1 mM db-cAMP and 1 mM 8-br-cAMP for 24 and and 48 h were harvested and the cell cycle phase distribution was determined by flow cytometric analysis of DNA content

cAMP	Time (h)	% of cells		
		G1	S	G2/M
	0	48.13	45.65	6.22
db-cAMP	24	61.60	29.29	9.11
	48	81.40	18.50	0.10
8-br-cAMP	24	69.11	27.30	3.59
	48	91.92	7.25	0.83

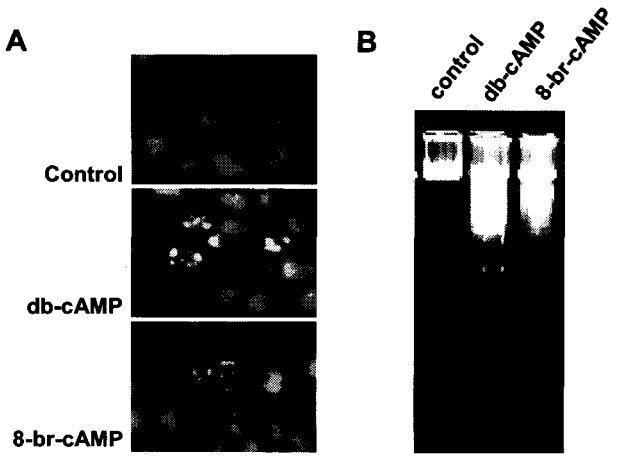

Fig. 1. cAMP analogs induce apoptosis in human neuroblastoma CHP-100 cells. Cells were cultured in the absence (control) or presence of either 1 mM 8-br-cAMP or 1 mM db-cAMP for 72 h. (A) The cells were fixed with formaldehyde, stained with the nuclear stain DAPI, and examined by fluorescence microscopy (×400). (B) Following incubation of cells in the presence of cAMP, genomic DNA was extracted and resolved in an 1% agarose gel and visualized using EtBr.

with either db-cAMP or 8-br-cAMP displayed chromosomal condensation and formation of apoptotic bodies, indicating that exposure to cAMP induced programmed cell death. Moreover, after 72 h treatment of cells with cAMP, typical genomic DNA laddering was clearly visible in EtBr-stained gel (Fig. 1B) again indicating the occurrence of apoptosis. Taken together, these results indicate that the growth inhibition observed in response to cAMP is due to either a growth arrest of the cells or apoptotic cell death.

Inhibition of pRB phosphorylation and increased binding of pRB and E2F-1 by cAMP

Since cAMP analogs arrest cells in G1 phase as assessed by DNA flow cytometry, and then the *RB* gene product pRB is an important checkpoint protein in G1 phase of the cell cycle. We determined the kinetics between phosphorylation of pRB and the transcription factor E2F-1. The total levels of pRB expression were decreased remarkably, and changed from hyperphosphorylated form to hypophosphorylated form by cAMP treatment (Fig. 2A). Association of pRB and E2F-1 was almost undetectable by co-immunoprecipitation analysis of the untreated control cells. However, there was a strong increase in the association of pRB and E2F-1 in cAMP-treated cells (Fig. 2B) suggesting that cAMP inhibits the releasing of E2F-1 protein from pRB.

cAMP down-regulates Cdk2 and cyclin E-dependent kinase activity

We next tested whether cAMP analogs altered the expression levels of G1 regulatory proteins, and as demonstrated in Fig. 3A, they did not affect the protein

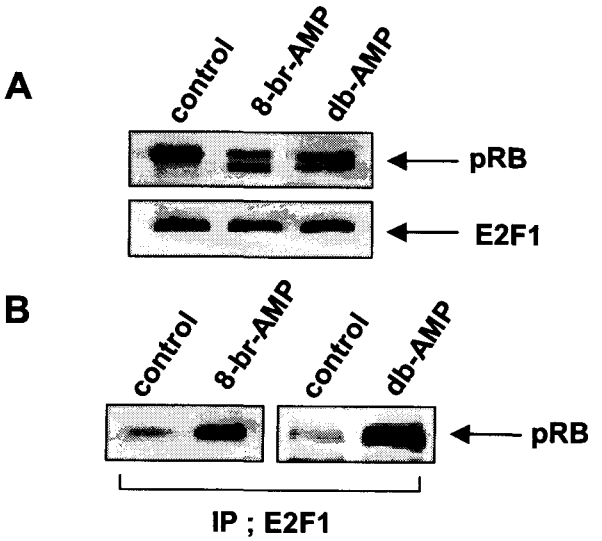

Fig. 2. Hypophosphorylation of pRB and enhanced association of pRB and E2F-1 in CHP-100 cells after exposure to cAMP. (A) Total cell lysates were prepared and separated by 8% SDS-polyacrylamide gel. Western blotting was performed using anti-pRB and ECL detection. (B) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with cAMP for 72 h were immunoprecipitated with anti-E2F-1 antibody. Immuno-complexes were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-pRB antibody. Proteins were detected by ECL detection.

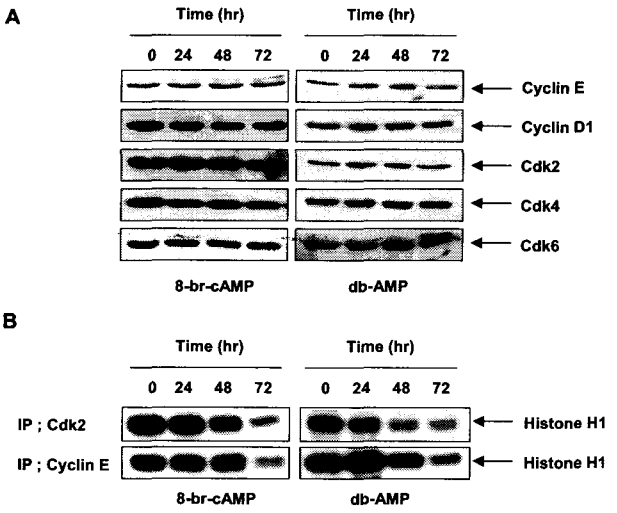

Fig. 3. Effects of cAMP on the protein levels of G1 cyclins and Cdks, and their kinase activities in CHP-100 cells. (A) Total cell lysates were prepared and subjected to 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Western blots were detected with indicated antibodies, and ECL detection. (B) Total cell lysates (0.5 mg protein) from control cells and cells treated with cAMP were immunoprecipitated with anti-Cdk2 or anti-cyclin E antibody. Kinase activity was assayed using histone H1 as substrates. Phosphorylated histone H1 was analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

levels of cyclin E, cyclin D1, Cdk2, Cdk4 and Cdk6. However, as shown in Fig. 3B, the kinase activity of either cyclin E or Cdk2 was significantly inhibited in response to cAMP in a time-dependent manner. These results suggest that the suppressive effect of cAMP on cell growth of CHP-100 cells was partly caused by down-regulating the activity of Cdks and cyclin E-associated kinase without altering the expression of their proteins.

cAMP induces Cdk inhibitor p21 protein and enhanced association of p21 with Cdk2

To understand the molecular mechanism by which cAMP down-regulates Cdk2 and cyclin E-dependent kinase activity, we investigated whether Cdk inhibitors were involved in the cAMP-induced growth arrest in CHP-100 cells (Fig. 4). In the untreated control cells, the expression of p21 protein was undetectable. However, incubation of cells with cAMP analogs caused a striking time-dependent increase (Fig. 4A). In addition, association of p21 with Cdk2 was not detected in the untreated control cells, whereas, treatment of cells with cAMP resulted in a significant association between p21 and Cdk2 (Fig. 4B), suggesting that the down regulation of Cdks kinase activity by cAMP is mainly caused by highly induced p21 expression.

Discussion

Cell cycle progression is regulated by the orchestrated activation and inactivation of a family of Cdks[24]. Derangements in the cell cycle machinery are pivotal to the

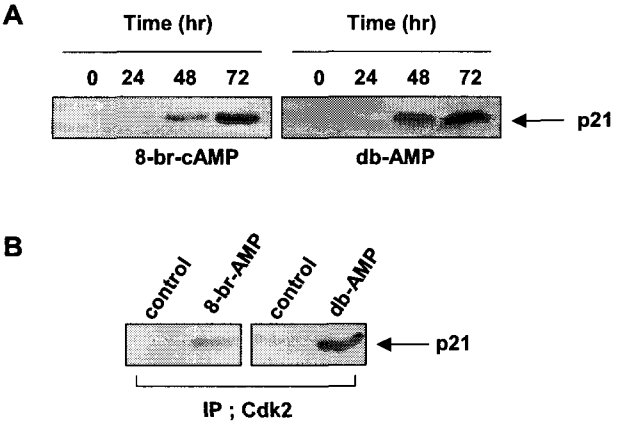

Fig. 4. Induction of Cdk inhibitor p21, and enhanced association of p21 with Cdk2 in CHP-100 cells after exposure to cAMP. (A) Cells were treated with 1 mM cAMP for the time indicated. (B) Cells were treated with 1 mM cAMP for 72 h. Cell lysates were immunoprecipitated with anti-Cdk2 antibody and immunoprecipitates were collected. Equal amounts of whole proteins or immunoprecipitated proteins were separated by 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-p21 antibody. Proteins were detected by ECL detection.

uncontrolled cell growth characteristic of malignant neoplasms[7]. In mammalian cells, early to mid G1 progression and late G1 progression leading to S phase entry are directed by D-type cyclins-Cdk4, 6 and cyclin E-Cdk2 both of which can phosphorylate pRB[17,25]. pRB, is a transcriptional repressor which, in its unphosphorylated state, binds to members of the E2F transcription factor family and blocks E2F-dependent transcription of genes controlling the G1 to S phase transition and subsequent DNA synthesis[4,25]. Cyclin A is produced in late G1 and expressed during S and G2 phase, and expression of B-type cyclins is typically maximal during the G2 to M phase transition and it controls the passage through M phase. They primarily associate with and activate Cdk2, and Cdc2, respectively[9,22]. On the other hand, the Cdk inhibitors negatively control the activity of Cdk/cyclin complex by coordinating internal and/or external signals and impending proliferation at several key checkpoints[2,6]. Among them, p21 is required for p53-induced growth arrest, which can inhibit kinase activity of Cdks during the G1/S cell cycle checkpoint[5,32].

In the data presented here, elevation of intracellular cAMP with hydrolysis-resistant cAMP analogs, db-cAMP and 8-br-cAMP, resulted in a time-dependent inhibition of cell viability in human neuroblastoma cells, which was associated with gross morphological changes (data not shown). Subsequent experiments addressed the issue of whether these compounds induce apoptosis and perturb the cell cycle. Both cAMP analogs induced chromatin condensation and DNA fragmentation indicating that their anti-proliferative effects were associated with apoptotic cell death (Fig. 1). Furthermore, flow cytometric data of cAMP-treated cells clearly revealed a cell-cycle block at G1 to S phase transition (Table 1) and an accumulation of cells at sub-G1 apoptotic region (data not shown), which contained less DNA than G1 cells [8]. These results suggested that cAMP interferes with proliferation and induces apoptosis in close association with the G1 arrest by modulation of cell cycle-regulatory proteins and associated kinase activities as a possible molecular mechanism of the effect of cAMP. Thus, we investigated the effects of cAMP on the expression of G1/S transition regulatory proteins to analyze the mechanism of G1 arrest.

As shown in Fig. 2A, we found that there was a loss of phosphorylatd pRB protein by treatment with cAMP and an enhanced binding of pRB and E2F-1 in response to cAMP treatment (Fig. 2B) indicating that cAMP analogs indirectly inhibited the transcriptional activity of E2F family for S phase entry [4,25]. The results from the *in vitro* immuno-complex kinase assay using antibodies to Cdk2 and cyclin E, and histone H1 as substrates demonstrated that cAMP inhibits Cdk2 and cyclin E-dependent activity, rather than altering those protein levels (Fig. 4), which plays an essential role in cell cycle progression at the G1 to S phase transition stage[12,18]. These results were consistent with the effective time of cAMP treatment that could significantly induce the growth inhibition, cell cycle arrest and apoptosis. Taken together, these results in part indicated that cAMP suppressed cell proliferation by down-regulating the activity of G1 regulatory proteins such as Cdk2 and cyclin E.

Furthermore, we supposed the down-regulation of Cdk2 and cyclin E-dependent kinase activity and the cell cycle arrest by cAMP may be also involved by an additional inhibitory mechanism (s). To elucidate this hypothesis we next investigated whether Cdk inhibitors such as p21, p27 and p16 are involved in the cAMP-induced growth arrest using Western blotting and co-immunoprecipitation analysis. This hypothesis was clearly supported by the facts that cAMP selectively induced the expression of Cdk inhibitor p21 protein (Fig. 4A), which was blocked by transcription inhibitor actinomycin D (data not shown), and the increased p21 proteins by cAMP treatment were tightly associated with Cdk2 (Fig. 4B). These results demonstrate that the down-regulations of Cdks and cyclin E-dependent kinase

activities are mainly caused by selective induction of p21 expression.

In summary, we have addressed here that cAMP strongly inhibits cell proliferation and induces apoptosis in the neuroblastoma CHP-100 cells. The results obtained provide convincing evidence that cAMP analogs exert their effect on cell cycle progression of CHP-100 cells by two pathways. First, by a significant decrease in the phosphorylated forms of tumor suppressor pRB that lead to its inhibitory effect of the E2F-dependent transcriptional activity of genes controlling the transition from G1 to S phase and subsequent DNA synthesis, and second, by an increase in p21 expression that leads to its increased binding with cyclin/Cdk complexes, resulting in a marked decrease in their kinase activities. These data suggest that the cAMP/PKA pathway may be a useful target in the development of new signal transduction-based approaches to neuroblastoma chemoprevention and therapy.

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초록 : 인체 신경아세포종에서 cAMP 처리에 의한 pRB의 인산화 억제 및 p21WAF1/CIP1의 유도

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인체 신경아세포종의 성장에 미치는 cAMP의 영향을 조사하기 위하여 Ewing's sarcoma 세포주인 CHP-100 세포에 dibutyryl-cAMP 및 8-bromo-cAMP를 처리하였다. 두 종류의 cAMP analog처리 시간 증가에 따라 CHP-100 세포의 증식이 처리 시간 의존적으로 억제되었으며, 이는 핵의 형태변화 및 DNA 단편화 현상을 수반한 apoptosis 유발과 연관성이 있었다. 또한 DNA flow cytometry 분석결과 cAMP는 세포주기 G1기 특이적 arrest를 유발하였다. cAMP 처리에 의하여 retinoblastoma 단백질(pRB)의 인산화가 억제되었으며, 전사조절인자 E2F-1과의 결합이 증대되었다. cAMP는 cyclin-dependent kinase (Cdk) 2 및 cyclin E 단백질의 발현변화에는 영향을 미치지 않았으나, 그들의 kinase 활성은 처리시간 의존적으로 매우 감소되었다. 또한 cAMP 처리에 의하여 Cdk inhibitor인 p21*WAF1/CIP1*의 발현이 증가되었으며, 증가된 p21 단백질은 Cdk2와 강한 결합을 형성하고 있었다. 이상의 결과에서 cAMP의 암세포 성장억제 효과에 pRB 및 p21이 매우 중요한 역할을 함을 알 수 있었다.