Inhibitory Effect of 8-Chloro-cyclic Adenosine 3′,5′-Monophosphate on Cell Growth of Gastric Carcinoma Cell Lines

Atsushi Takanashi,[1] Wataru Yasui,[1] Kazuhiro Yoshida,[1] Hiroshi Yokozaki,[1,2] Daizo Saito,[3] Kaoru Abe,[4] Kenichi Urakami,[5] Keizaburo Miki[5] and Eiichi Tahara[1,6]

[1]First Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, [2]Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA, [3]Department of Internal Medicine, National Cancer Center Hospital, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, [4]National Matsudo Hospital, 123-1 Takatsuka Shinden, Matsudo, Chiba 271 and [5]Corporate Research and Development Laboratory, Fundamental Research Laboratories, Tonen Corporation, 3-1, Nishitsurugaoka 1-chome, Oi-cho, Iruma-gun, Saitama 354

A cAMP analogue, 8-chloro-cAMP (8-Cl-cAMP), selectively binds to site 1 receptor of type II regulatory subunit (RII) of cAMP-dependent protein kinase. The effects of 8-Cl-cAMP on human gastric carcinoma cell lines were studied. Twenty μM 8-Cl-cAMP clearly inhibited cell growth in six cell lines (TMK-1, KATO-III, MKN-7, -28, -45, and -74) but not in MKN-1. Cell population in the G_1 phase was increased in KATO III cells, which were most responsive to 8-Cl-cAMP, while cell cycle progression in TMK-1 and MKN-1 cells was apparently not influenced by 8-Cl-cAMP. The various changes induced by 8-Cl-cAMP were further analyzed in TMK-1 cells. Decrease of type I regulatory subunit (RI) of cAMP-dependent protein kinase and translocation of RII from cytosol to nucleus were induced by 8-Cl-cAMP treatment. 8-Cl-cAMP increased the level of cAMP-response element (CRE) binding protein in addition to inducing FOS mRNA, whose promoter contains CRE. 8-Cl-cAMP decreased the expression of mRNA for transforming growth factor-α (TGF-α), while the expression of epidermal growth factor receptor was not changed. Expression of HRAS and MYC mRNAs was slightly increased, whereas the amounts of HRAS and MYC proteins remained unchanged. Our results overall suggest that 8-Cl-cAMP might be a useful tool for antitumor therapy of gastric cancers and that cell growth inhibition by 8-Cl-cAMP might account for the decrease of TGF-α expression by tumor cells.

Key words: Gastric carcinoma — cAMP-dependent protein kinase — 8-Cl-cAMP — TGF-α

Cyclic adenosine 3′:5′-monophosphate (cAMP) is a second messenger in a variety of extracellular signals. It binds to the regulatory subunit of cAMP-dependent protein kinase, which consists of two catalytic subunits and two regulatory subunits. Based on the difference in regulatory subunits, this protein kinase is classified into type I and type II. The differential effect of cAMP on cell growth may be brought about through the difference in regulatory subunits of cAMP-dependent protein kinase.[1] We have previously reported that type I isoenzyme is higher in gastric cancer tissue than in non-neoplastic mucosa, suggesting that type I isoenzyme may contribute to abnormal proliferation of cancer cells.[2]

On the other hand, Cho-Chung et al. have recently reported that the synthetic analogue 8-chloro-cAMP (8-Cl-cAMP), which selectively binds to site 1 receptor of type II regulatory subunit (RII),[3] suppresses the cell growth of breast cancer, colon cancer[4] and leukemia cell lines.[5]

However, there has been no report concerning the inhibitory effect of 8-Cl-cAMP on cell growth of human gastric carcinoma. This report describes the effect of 8-Cl-cAMP on human gastric carcinoma cell lines from various viewpoints, including regulatory subunits, cAMP-response element (CRE)-binding protein, oncogene products, transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) receptor.

MATERIALS AND METHODS

Chemicals and oligonucleotides 8-Cl-cAMP was kindly provided by Dr. Y. S. Cho-Chung (Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, NIH, USA) and the Research and Development Laboratory, Tonen Co. (Tokyo). 8-Azidoadenosine-3′,5′-cyclic [^{32}P]monophosphate (8-N$_3$-[^{32}P]cAMP) was purchased from ICN Radiochemicals (Irvine, CA). The CRE-containing oligonucleotide used in this study contains TGAC as the first 5′ to 3′ downstream sequence and GTCA as the next sequence. Oligonucleotide consisting of a 3-fold repeat of TGACGTCA with a complementary

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second strand and a 5'-dinucleotide overhang was synthesized and was kindly provided by Tonen Co.

Cell culture Seven human gastric carcinoma cell lines were used. TMK-1, poorly differentiated adenocarcinoma, was established in our laboratory.[6] KATO-III (signet-ring-cell carcinoma) was kindly provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo). The other five cell lines (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical University, Fukushima). Cells were cultured in RPMI-1640 (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) (M.A. Bioproducts, Walkersville, MD).

Cell growth Cells (2×10^5) were seeded on 60 mm dishes and cultured in the presence of various concentrations of 8-Cl-cAMP. The medium was changed every 48 h. Cell number was counted in triplicate.

Cell cycle analysis The cell cycle was analyzed by the method of Braylan et al. using the DNA-intercalating dye propidium iodide.[7] An argon laser operating at 488 nm was used for fluorescence excitation. Flow cytometry was performed using a FACSCAN (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

Photoaffinity labeling of cAMP regulatory subunits The cell pellets were lysed in buffer TEN (0.1 M NaCl, 5 mM MgCl$_2$, 1% Nonidet P-40, 0.5% Na deoxycholate, 20 mM Tris-HCl, pH 7.4) for 30 min and centrifuged at 3,000g for 5 min according to Katsaros et al.[4] The supernatant was used for photoaffinity labeling. Photoactivated incorporation of 8-N$_3$-[^{32}P]cAMP was performed as described.[3, 8, 9] Thirty μg of cell lysate was mixed with 1 mM 8-N$_3$-[^{32}P]cAMP in 30 μl of buffer TEN, then left at 4°C for 30 min in the dark, and irradiated with UV light (254 nm) for 5 min. After addition of 10 μl of ×4 SDS-sample buffer, the mixture was boiled and subjected to electrophoresis in SDS-polyacrylamide gradient gel (PAG PLATE 10/20, Daiichi Pure Chemicals Co., Tokyo). The separated proteins were transferred to nitrocellulose sheets, followed by autoradiography.

Gel retardation assay Nuclear extracts were prepared as described.[3, 10] The cell pellets were lysed with a glass homogenizer in 10 mM Tris-HCl (pH 7.9) containing 10 mM KCl and 1.5 mM MgCl$_2$, and centrifuged at 3,000g for 8 min. The pellet was stirred in 50 mM Tris HCl (pH 7.5) containing 10% sucrose, 0.42 M KCl, 5 mM MgCl$_2$, 0.1 mM EDTA and 20% glycerol for 30 min and centrifuged at 25,000g for 1 h. The resulting pellet was dialyzed for 3 h against 50 mM Tris HCl (pH 7.9) containing 0.1 M KCl, 12.5 mM MgCl$_2$, 1 mM EDTA, 20% glycerol and 0.1% NP40. DNA-binding assay was performed as described.[11, 12] An aliquot (10 μg) of nuclear extract was incubated with 5 ng of labeled oligonucleotide and 0.2–2 μg of poly (dI·dC) for 30 min at 30°C. Each sample was applied to a 4% polyacrylamide gel and then the gel was dried, and autoradiographed.

Northern blot analysis Total RNAs were isolated by homogenization in guanidine isothiocyanate and pelleted through cesium chloride gradients.[13] Poly(A)$^+$-selected RNAs (10 μg) were electrophoresed on 1% agarose gel containing 6% formaldehyde and blotted onto a nitrocellulose filter or zeta-probe nylon filter membrane (BioRad, Richmond, CA). The ^{32}P-labeled DNA probes were hybridized according to the standard procedure.[14] The filters were washed with standard saline citrate containing 0.1% sodium dodecyl sulfate (SDS) and autoradiographed. The 2.1-kb human FOS cDNA and the 0.4-kb PstI-PstI human MYC DNA including the second exon were kindly provided by the Japanese Cancer Research Resources Bank. The 0.8-kb human HRAS cDNA was purchased from Oncogene Science, Inc. (Mineola, NY). The 1.4-kb human TGF-α cDNA was kindly provided by Dr. R. Derynck (Genentech, Inc. South San Francisco, CA).[15]

RESULTS

Inhibitory effect of 8-Cl-cAMP on cell growth The inhibitory effect of 8-Cl-cAMP on cell growth was examined in seven human gastric carcinoma cell lines. As shown in Table I, the growth of six carcinoma cell lines (TMK-1, KATO-III, MKN-7, -28, -45, -74) was significantly inhibited by 8-Cl-cAMP treatment at a concentration of 20 μM, whereas that of MKN-1 was not. Percentage growth inhibition on the 4th day was as high as 80% in the six cell lines. At a concentration of 10 μM, the growth inhibition of 8-Cl-cAMP was submaximal and a little weaker than the inhibition at 100 μM (Fig. 1).

Table I. Growth Inhibition of Gastric Carcinoma Cell Lines by 8-Cl-cAMP

Cell line	% Growth inhibition[a]
TMK-1	84.4
KATO-III	101.2
MKN-1	29.3
MKN-7	79.0
MKN-28	87.6
MKN-45	80.1
MKN-74	93.4

a) Data are expressed as percentage growth inhibition with respect to the growth of untreated control cells on the 4th day and represent average values of three separate experiments.
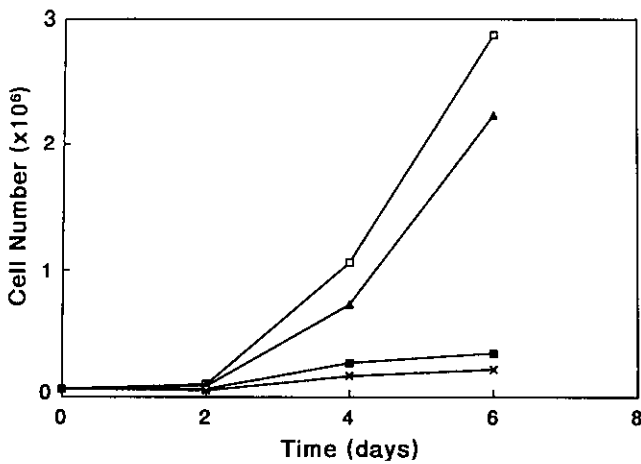
Fig. 1. Dose-dependent inhibition by 8-Cl-cAMP of the cell growth of TMK-1 cells. 8-Cl-cAMP (1 μM (▲), 10 μM (■), 100 μM (×)) was added to about 2×10^5 cells in the medium containing 10% FBS. Cell number was counted in triplicate and average values were calculated. Control (□).
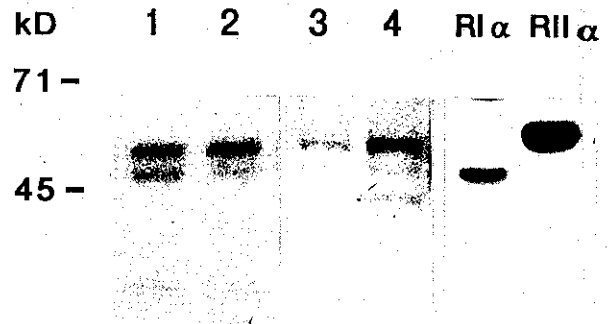

Fig. 2. Photoaffinity labeling of RI and RII in TMK-1 cells. Lanes 1 and 2, whole cell lysates; lanes 3 and 4, nuclear proteins. Lanes 2 and 4, cells treated for 3 days with 10 μM 8-Cl-cAMP; lanes 1 and 3, untreated control cells. RI$_\alpha$, rabbit RI (Sigma, St. Louis, MO); RII$_\alpha$, bovine RII (Sigma). Cell lysate was prepared and labeled with 8-N$_3$-[^{32}P]cAMP as described in "Materials and Methods."

Table II. Effect of 8-Cl-cAMP on Cell Cycle Progression

Cell line		% Cell population in		
		G$_1$	S	G$_2$M
TMK-1	Control	45	31	24
	8-Cl-cAMP	45	33	22
KATO III	Control	45	40	15
	8-Cl-cAMP	61	25	14
MKN-1	Control	60	29	11
	8-Cl-cAMP	60	30	10

Growth inhibition by 8-Cl-cAMP was much stronger than that by dibutyryl cAMP (dbcAMP). That is, even when 1 mM dbcAMP was added to the culture medium, growth inhibition was weaker than that by 10 μM 8-Cl-cAMP (data not shown).

Effect of 8-Cl-cAMP on cell cycle progression To determine whether the suppressed cell proliferation is due to a specific block in one phase of the cell cycle, the cells were treated with 10 μM 8-Cl-cAMP for 3 days and the cell cycle was analyzed by flow cytometry. As shown in Table II, the fractions of TMK-1 and MKN-1 cells in G$_1$, S and G$_2$/M phases were not significantly different between the control cells (untreated) and 8-Cl-cAMP-treated cells. However, in KATO-III cells, 8-Cl-cAMP induced an appreciable increase of cell population in the G$_1$ phase, associated with a marked reduction in S-phase.

The effect of 8-Cl-cAMP on the levels of RI and RII cAMP regulatory subunits The effect of 8-Cl-cAMP on the levels of RI and RII cAMP regulatory subunits was analyzed using the photoaffinity ligand 8-N$_3$-[^{32}P]cAMP. As shown in Fig. 2, TMK-1 cells contained two cAMP receptor proteins. The 48,000 Mr protein appears to be RI$_\alpha$, because it comigrated in SDS-PAGE with the purified preparation of the 48,000 Mr RI$_\alpha$ from rabbit skeletal muscle.[16] The other 52,000 Mr protein appears to be RII$_\beta$, because its molecular weight is smaller than that of the 56,000 Mr RII$_\alpha$ from the bovine heart.[16, 17] The other gastric carcinoma cell lines contained the same two cAMP receptor proteins as TMK-1 (data not shown). Treatment with 10 μM 8-Cl-cAMP decreased RI$_\alpha$ (lanes 1, 2) in TMK-1 cells. RII$_\beta$ was translocated from cytosol to nucleus (lanes 3, 4), though the total amount of RII$_\beta$ did not change significantly (lanes 1, 2). In the unresponsive cell line MKN-1, no change in RI or RII was observed (data not shown). No labeled protein was detected till 6 h after 8-Cl-cAMP treatment (data not shown).

Effect of 8-Cl-cAMP on FOS gene expression and the levels of CRE-binding protein cAMP-inducible genes such as FOS, somatostatin gene and vasoactive intestinal peptide gene have been shown to contain CRE, which is essential for transcription of these genes.[18] A CRE-binding protein (CREB) has been identified.[19] 8-Cl-cAMP evidently increased expression of FOS mRNA (Fig. 3). We then examined the effect of 8-Cl-cAMP on the level of CREB in TMK-1 and MKN-1 cells. In TMK-1 cells, whose growth was inhibited by 8-Cl-cAMP, treatment of 8-Cl-cAMP for 3 days increased CREB in nuclear fraction. On the contrary, in the unresponsive cell line MKN-
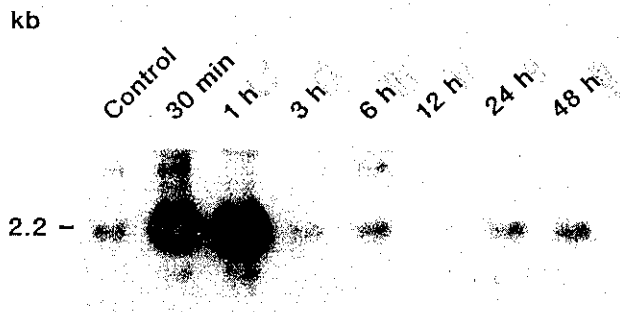
Fig. 3. Northern blot analysis of FOS mRNA in TMK-1 cells. TMK-1 cells were cultured in medium containing 10% FBS and treated with 10 μM 8-Cl-cAMP for the periods indicated. Northern blot analysis was carried out as described in "Materials and Methods."

Fig. 5. Northern blot analysis of HRAS and MYC mRNA in TMK-1 cells. Cells were cultured in medium containing 10% fetal bovine serum and treated with 10 μM 8-Cl-cAMP for the periods indicated.
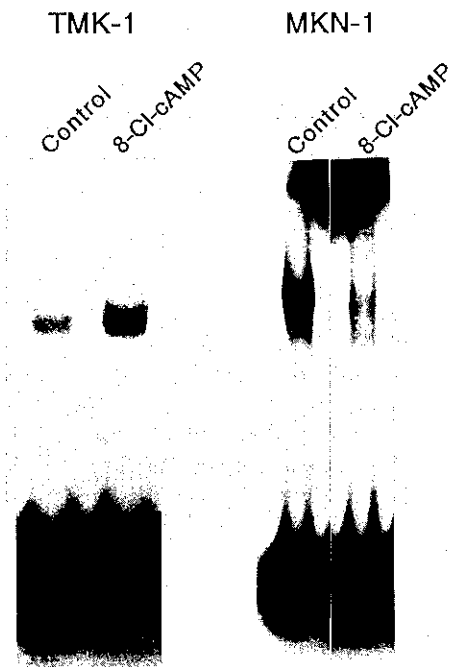

Fig. 4. Gel retardation assay of CRE-binding activity in TMK-1 and MKN-1 cells. Cells were cultured in medium containing 10% FBS in the presence or absence of 10 μM 8-Cl-cAMP for 3 days. Nuclear protein was incubated with labeled oligonucleotide containing CRE and subjected to 4% polyacrylamide gel electrophoresis as described in "Materials and Methods."

Fig. 6. Northern blot analysis of TGF-α and EGF receptor (EGFR) mRNA in TMK-1 cells. Cells were cultured in medium containing 10% fetal bovine serum and treated with 10 μM 8-Cl-cAMP for the periods indicated.

1, CREB was decreased (Fig. 4). After 8-Cl-cAMP treatment from 15 min to 1 day in both tumor cell lines, there was no remarkable change of CREB (data not shown).
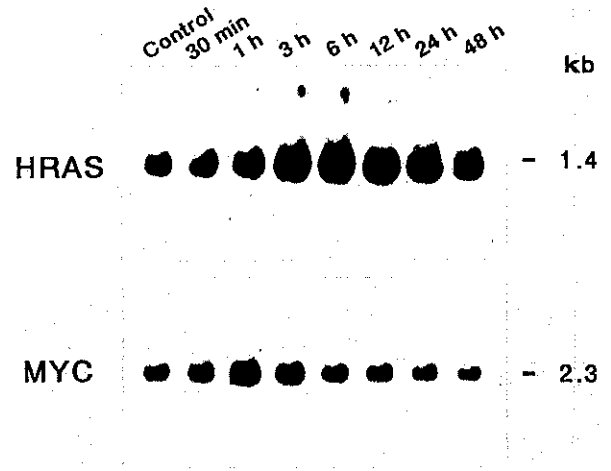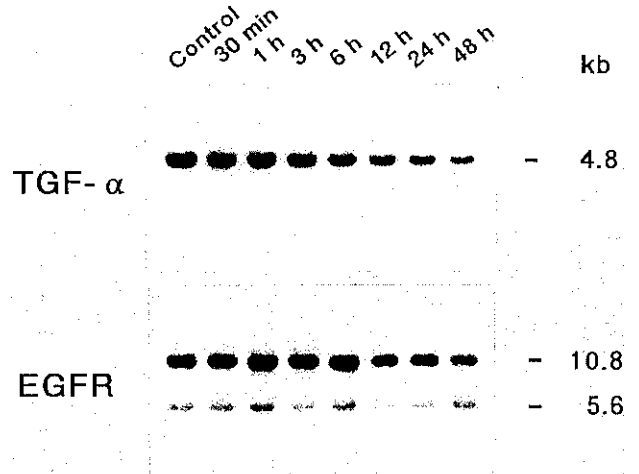
Effect of 8-Cl-cAMP on the expression of HRAS and MYC genes In TMK-1 cells, HRAS mRNA accumulation increased up to 2-fold at 6 h after 8-Cl-cAMP treatment and thereafter decreased gradually, while MYC mRNA accumulation also increased slightly at 1 h after treatment (Fig. 5). Western blot analysis showed no remarkable change in the amounts of HRAS p21 protein and MYC p62 protein during treatment for 7 days (data not shown).

Effects of 8-Cl-cAMP on the expression of TGF-α and EGF receptor genes The effect of 8-Cl-cAMP on the expression of TGF-α and EGF receptor genes was examined. 8-Cl-cAMP decreased expression of TGF-α mRNA by TMK-1 cells to about 35% of the control level as determined by densitometric tracing (Fig. 6). However, the expression of EGF receptor gene was not altered by 8-Cl-cAMP treatment.

DISCUSSION

In the present study, we found that 8-Cl-cAMP inhibited the growth of gastric carcinoma cell lines, as reported for other carcinoma cell lines. Apparent cell death was not observed during treatment of TMK-1 cells with 8-Cl-cAMP for 2 weeks. 8-Cl-cAMP did not block any specific phase of the cell cycle in TMK-1 and MKN-1 cells, although the cell growth in TMK-1 cells but not in MKN-1 cells was inhibited by 8-Cl-cAMP. Similar findings had been observed in colon cancer cell lines,[20] though we do not know the reason at present. The change of cell cycle observed in KATO III cells, however, might account for the high sensitivity to 8-Cl-cAMP.

As to the effect of 8-Cl-cAMP on RI and RII, the amount of RI decreased and RII was translocated from cytosol to nucleus by 8-Cl-cAMP treatment in TMK-1 cells. In the unresponsive cell line MKN-1, regulatory subunits were not altered. These observations suggest that RI and RII may have an essential role in growth control of cancer cells. These findings are consistent with our previous data that type I cAMP-dependent protein kinase increased but type II decreased in human gastric carcinoma tissues.[2] There are two species of RII, 54-kD and 52-kD form[21] or 56-kD and 52-kD form.[3] The proteins of higher and lower molecular weight are defined as $RII_α$ and $RII_β$, respectively.[21-23] In gastric carcinoma cell lines, $RII_β$ was dominant while $RII_α$ was absent or at a very low level, if present. When human colon cancer cells or Friend erythroleukemia cells are treated with Me_2SO, 8-Br-cAMP or 8-Cl-cAMP, the amount of $RII_β$ increases but that of $RII_α$ does not.[3, 21] In addition, the 52-kD RII, which translocates from cytosol to nucleus in gastric and colon cancer cell lines, has been identified as $RII_β$.[3] $RII_β$ has the specific amino acid sequence KKRK for nuclear location that is not present in RI.[24] Yokozaki et al. found that the nuclear trans-

location of $RII_β$ induced by 8-Cl-cAMP is 5- to 10-fold greater than that induced by dibutyryl cAMP, indicating that the amount of $RII_β$ translocation parallels the growth-inhibitory effect of 8-Cl-cAMP.[25] It is quite likely that translocation of $RII_β$ from cytosol to nucleus may play an important role in the inhibitory effect of 8-Cl-cAMP on cell growth. In fact, Cho-Chung reported that $RII_β$ antisense oligomer specifically blocked the growth inhibition or differentiation induced by 8-Cl-cAMP.[26]

Catalytic subunit of cAMP-dependent protein kinase, which is activated by binding of cAMP with regulatory subunits, phosphorylates CREB, leading to enhanced transcription of genes containing CRE.[27] 8-Cl-cAMP induced rapid expression of FOS gene containing CRE. Furthermore, an increase in CREB was observed in the 8-Cl-cAMP-responsive TMK-1 cells but not in the 8-Cl-cAMP-unresponsive MKN-1 cells. These results clearly indicate that the site-selective cAMP analogue increases transcription factors that bind to CRE.

Although 8-Cl-cAMP induced decreases of HRAS and MYC gene expression by breast carcinoma cells and leukemia cells,[5, 28] we did not observe any significant change of these genes in gastric carcinoma cell lines. The growth suppression signal of tumor cells by 8-Cl-cAMP might be different between gastric carcinoma cells and other cancer cell lines. What is more interesting in the present study is that TGF-α transcription was significantly suppressed by 8-Cl-cAMP treatment in TMK-1 cells even though expression of EGF receptor gene was not changed. Recently, we have demonstrated that TGF-α expressed by MKN-28 cells may act as an autocrine growth factor.[29] Therefore, it is likely that 8-Cl-cAMP suppresses the expression of TGF-α by tumor cells, resulting in inhibition of cancer cell growth. As regards the mechanism of the growth inhibition by 8-Cl-cAMP, two possibilities can be considered. First, DNA-binding $RII_β$ cAMP receptor protein may repress transcription of the TGF-α promoter directly. Secondly, this protein may induce transcriptional repressor for TGF-α gene expression.

In conclusion, 8-Cl-cAMP permits us to investigate the role of cAMP-dependent protein kinase in cell growth and differentiation of cancer cells, although the mechanism of growth inhibition by 8-Cl-cAMP still remains speculative. Furthermore, 8-Cl-cAMP is expected to be an effective tool for therapy of human gastric cancer.

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