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Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor α production in Ki-*ras*-transformed rat fibroblasts

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A site-selective cAMP analog, 8-chloroadenosine 3',5'-cyclic monophosphate (8-Cl-cAMP), was demonstrated to be a potent inhibitor of both the monolayer and soft agar growth of normal rat kidney (NRK) fibroblasts that had been transformed with the v-Ki-*ras* oncogene or treated with transforming growth factor α (TGFα). The growth inhibition was dose dependent and reversible and was accompanied by reversion of the transformed phenotype, suppression of TGFα production, and a decrease in p21ras protein levels. These effects of 8-Cl-cAMP were linked to the cAMP analog's selective modulation of the type I and type II cAMP-dependent protein kinase regulatory subunits, RI and RII, present in Ki-*ras*-transformed and TGFα-treated NRK cells.

cyclic AMP receptor protein; Nuclear translocation; Reverse transformation; Protein, p21ras

1. INTRODUCTION

We previously reported that new site-selective cAMP analogs produce potent growth inhibition and differentiation in a variety of cancer cell lines [1-5]. These cAMP analogs exert their biological effects by provoking a differential regulation of type I versus type II cAMP-dependent protein kinase isozymes [1-5]. We have also demonstrated that cAMP-induced reversion of the transformed phenotype and growth inhibition leads to a suppression in p21*ras* expression [1,4,5]. TGFα is a potent mitogen for fibroblasts and epithelial cells [6] that has been circumstantially implicated in the autocrine growth of a variety of transformed cells. Enhanced synthesis and secretion of TGFα have been demonstrated in a number of rodent and human tumor cell lines and in oncogene-transformed rodent fibroblasts and epithelial cells [7-12]. In the present work, we investigated the ability of 8-Cl-cAMP (which has been selected by the National Cancer Institute as preclinical phase I antineoplastic drug) to antagonize the effects of exogenous TGFα in NRK fibroblasts and to inhibit the production of TGFα by Ki-*ras*-transformed NRK (K-NRK) cells.

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Abbreviations: cAMP, cyclic adenosine 3',5'-monophosphate; 8-Cl-cAMP, 8-chloroadenosine 3',5'-cyclic monophosphate; TGFα, transforming growth factor α; NRK, normal rat kidney; K-NRK, Ki-*ras*-transformed NRK; DMEM, Dulbecco's modified Eaglemedium; FBS, fetal bovine serum; RIA, radioimmunoassay; RRA, radioreceptor assay; EGF, epidermal growth factor

2. MATERIALS AND METHODS

8-Cl-cAMP was synthesized at the Nucleic Acid Research Institute (Costa Mesa, CA) [13]. Synthetic TGFα was purchased from Bachem (Torrance, CA). A clone, 49F, of NRK cells was obtained from Dr J. DeLarco (NCI, Bethesda, MD) and K-

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NRK cells (NRK cells transformed by the viral Kirsten *ras* oncogene) were a gift from Dr R.H. Bassin (NCI, Bethesda). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY) in a 5% humidified atmosphere at 37°C.

For cell growth experiments in monolayer culture, 1×10^5 cells/60 mm dish were seeded in DMEM containing 10% FBS, and 24 h later (day 0) medium was changed and TGFα (5 ng/ml) was added. Fresh medium was added every 48 h thereafter. 8-Cl-cAMP was added at the time of medium replenishment starting day zero. At the indicated times, cells were harvested and cell counts were performed in duplicate on a ZBI Coulter counter (Coulter Electronics, Hialeah, FL).

For experiments in soft agar, 2×10^4 cells/35 mm dish were seeded into 0.3% Difco Noble agar supplemented with DMEM containing 10% FBS, layered over a base layer of 0.8% agar medium, and treated with a variety of concentrations of 8-Cl-cAMP. After 12 days, cells were stained with Nitro blue tetrazolium, and colonies larger than 50 μm were counted with an Artek 880 colony counter (Artek Systems, Inc., Farmingdale, NY).

Preparation of conditioned media from cultured cells and determination of TGFα by RIA and by RRA were performed as previously described [11–14].

Western blot analysis of p21*ras* was performed using Y13-259, a rat monoclonal anti-p21 antibody, as previously described [15,16].

The levels of RI and RII regulatory subunits of cAMP-dependent protein kinase were determined by applying the photoaffinity labeling technique to the nuclear and cytosolic fractions of cell extracts as previously described [17].

3. RESULTS AND DISCUSSION

8-Cl-cAMP, the most potent site-selective cAMP analog [1], was tested for its effect on the growth of Ki-*ras*-transformed K-NRK cells, and the results are shown in fig.1A. At a concentration of 10 μM, the cAMP analog inhibited growth by approx. 20% at day 2 and by 40% at day 7 when compared to the untreated control cells. At a higher concentration (50 μM), 8-Cl-cAMP inhibited growth by 50% as early as day 3, and no cell growth was observed thereafter (fig.1A). In agreement with our previously reported results [4], the effect of 8-Cl-cAMP was cytostatic and reversible, as shown by the resumption in cell growth after analog removal (fig.1A).

It has been shown [18] that TGFα can induce phenotypic transformation of NRK fibroblasts grown in the presence of serum containing TGFβ and other growth factors. Addition of TGFα (5 ng/ml) to NRK cells produces a dramatic increase in cell growth in monolayer culture and induces morphological alterations typical of transformed phenotype, as is observed in *ras*-transformed K-NRK cells [19,20]. In addition, NRK cells treated with TGFα exhibit anchorage-independent growth in semi-solid agar medium [20,21].

The addition of 50 μM 8-Cl-cAMP to NRK cells that were treated with TGFα in a monolayer culture resulted in a 60% inhibition of TGFα-stimulated growth by day 4 (fig.1B) and induced a reversion of the morphological effects produced by TGFα (fig.2, panel D). Interestingly, 8-Cl-cAMP did not inhibit the growth of NRK cells in the absence of TGFα; rather, it slightly stimulated growth. A greater degree of growth inhibition was observed, in a dose-dependent fashion, on the TGFα-induced soft agar growth of NRK cells (fig.1C). 8-Cl-cAMP at 50 μM resulted in 80% inhibition of TGFα-stimulated colony formation.

We next investigated whether the growth inhibitory effects of 8-Cl-cAMP on K-NRK cells are associated with a specific interference with the production of endogenous TGFα. Conditioned medium was collected from K-NRK and NRK cells, and the levels of immunorective and biologically active TGFα were measured by RIA and RRA, respectively. As shown in fig.3A, both immunoreactive and biologically active TGFα were reduced by approx. 50% within 2 days of 8-Cl-cAMP treatment. Thus, the decrease in TGFα production precedes or is at least concomitant with the inhibition of cell growth (fig. 1A) that follows 8-Cl-cAMP treatment. Transformation of mouse mammary epithelial cells or NIH3T3 cells by an activated *ras* cellular proto-oncogene or v-Ki-*ras* leads to a lack of response by these cells to exogenous TGFα or EGF as a consequence of the increased capacity of these cells to secrete high levels of endogenous TGFα into the culture medium [11,14,22].

In a previous report we noted that 8-Cl-cAMP leads to a suppression of p21*ras* expression in several cancer cell lines [1,4,5]. We therefore investigated by Western blotting analysis the effect of 8-Cl-cAMP on p21*ras* expression in K-NRK cells and in NRK cells stimulated with exogenous TGFα. As shown in fig.3B, treatment of K-NRK cells with 50 μM 8-Cl-cAMP resulted in a marked decrease in the levels of p21ras protein. Quantification by densitometry showed that the p21 levels decreased to 50% of untreated control cell level within 2 days of 8-Cl-cAMP treatment (not

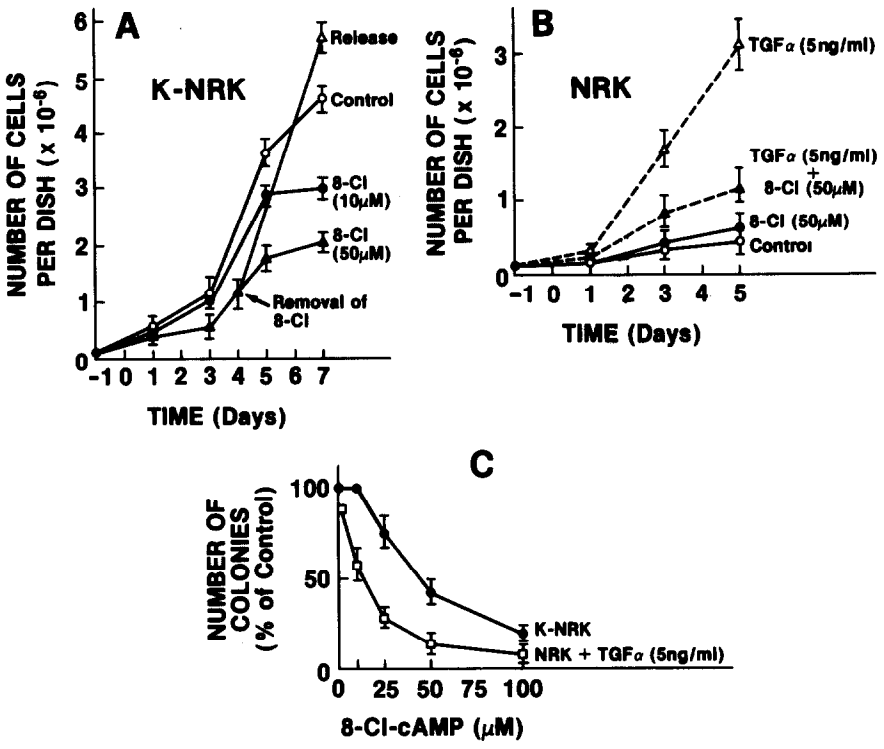
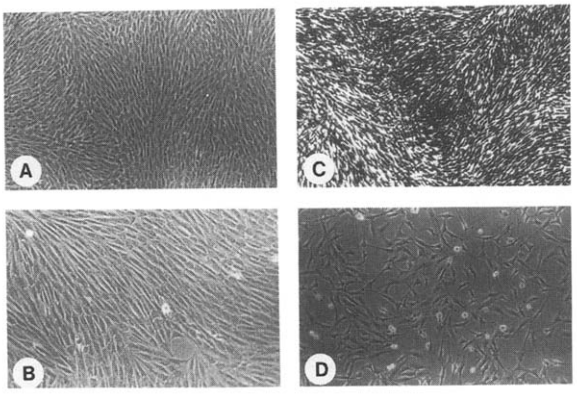


Fig.1. Effect of 8-Cl-cAMP on the growth of K-NRK cells, NRK cells, and NRK cells treated with TGFα. (A) Monolayer growth of K-NRK cells; (B) monolayer growth of NRK cells; (C) soft agar growth of K-NRK cells and NRK cells treated with TGFα. Data represent an average ± SD from four experiments. Colonies in the untreated controls were: K-NRK, 1280 (± 55) colonies/dish and NRK + TGFα (5 ng/ml), 1195 (± 70) colonies/dish.



A, Control; B, 8-Cl-cAMP (50μM); C, TGFα (5 ng/ml); D, TGFα (5 ng/ml) + 8-Cl-cAMP (50μM)

Fig.2. Effect of 8-Cl-cAMP and TGFα on the morphology of NRK cells. (A) Untreated control cells; (B) treatment of cells for 4 days with 8-Cl-cAMP (50 μM); (C) TGFα (5 ng/ml); (D) TGFα (5 ng/ml) + 8-Cl-cAMP (50 μM). × 125.

shown). Thus, the decreases in endogenous TGFα production and in p21ras protein levels occur simultaneously and precede the growth arrest and morphological changes in K-NRK cells following 8-Cl-cAMP treatment. Moreover, we found that the addition of TGFα (5 ng/ml) to NRK cells resulted in the appearance of endogenous p21ras, which was at an undetectable level in the untreated control cells, and the treatment with 8-Cl-cAMP sharply reduced the p21 level (not shown).

It has been shown that the expression of the type I isozyme of the cAMP-dependent protein kinase and its regulatory subunit, RI, is associated with an increase in cell proliferation and with neoplastic transformation, whereas an increase in the levels of the type II protein kinase and its regulatory subunit, RII, is linked to cellular growth inhibition and differentiation [15,23,25]. We recently demonstrated that the antineoplastic activity of 8-Cl-cAMP is associated with an early increase

A

Cells	Treatment	TGFα in the conditioned media (ng/10^8 cells/48 hr) RIA	RRA
NRK	none	20 ± 3.0	22 ± 1.0
K-NRK	none	264 ± 12 (100)	200 ± 20 (100)
K-NRK	8-Cl-cAMP (2 days)	128 ± 17 (49)	143 ± 5 (57)
K-NRK	8-Cl-cAMP (4 days)	124 ± 10 (47)	113 ± 11 (45)

B

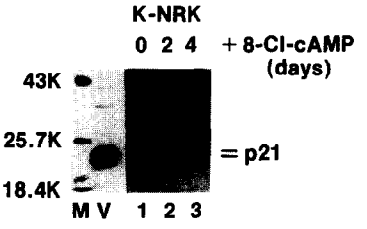


Fig.3. Effect of 8-Cl-cAMP on TGFα production and p21ras protein levels in K-NRK cells. (A) TGFα protein from concentrated conditioned medium was evaluated in a TGFα-specific RIA and in an EGF/TGFα RRA as previously described [11,14]. Values represent the mean ± SD from four experiments. Values in parentheses are the percentages. (B) Western blotting of p21 protein was performed as previously described [15,16]. M, marker proteins of known molecular mass (Bethesda Research Laboratories). V, cell lysate from Ha-MuSV-transformed NIH3T3 clone 13-3-B4 [2].

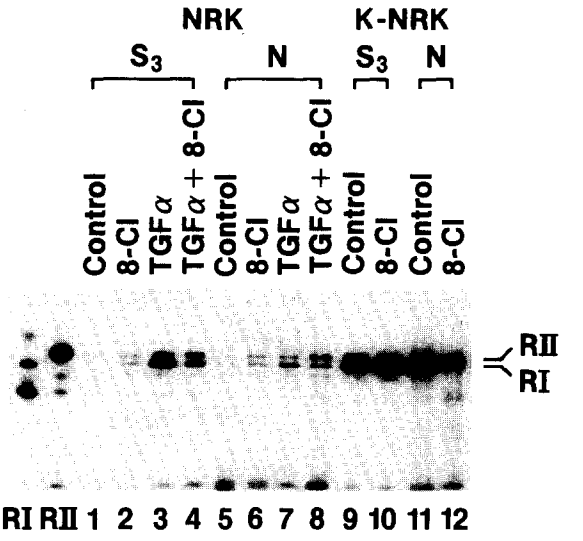


Fig.4. Effect of 8-Cl-cAMP and TGFα on the levels of RI and RII cAMP receptor proteins in the cytosol (S_3) and nuclei (N) of NRK cells and K-NRK cells. Photoactivated incorporation of 8-N_3-[^{32}P]cAMP on the nuclear and the cytosolic fractions was performed as previously described [17]. RI, the 48-kDa RI cAMP receptor protein; RII, the 56-kDa RII cAMP receptor protein (Sigma Chemical Co., St. Louis, MO).

(within 10 min) of RII in the nucleus of treated LS-174T human colon cancer cells [17]. We therefore measured the levels of the RI and RII regulatory subunits in the cytosolic and nuclear fractions of K-NRK and NRK cells, using a 8-N_3-[^{32}P]cAMP photoaffinity ligand. As shown in fig.4, in K-NRK cells treatment with 50 μM of 8-Cl-cAMP brought about a marked reduction of RI level in nuclei and an increase of RII levels in both the cytosol and nuclei (lanes 9–12). In NRK cells, the addition of TGFα resulted in a marked increase of RI levels in both the cytosol and nuclei (lanes 3 and 7), thereby mimicking the effect of *ras* transformation (lanes 9 and 11). The addition of 8-Cl-cAMP to TGFα-treated NRK cells markedly reduced the RI levels in both the cytosol and nuclei (lanes 4 and 8). Concomitantly, RII levels increased in the cytosol and particularly in the nucleus (lanes 4 and 8), where there was an inversion in the RI/RII ratio as compared with that in untreated cells. Interestingly, treatment with 8-Cl-cAMP alone, which did not inhibit growth in NRK cells (fig.1B), had no appreciable effect upon the levels of RI or RII in comparison with untreated cells (cf. lanes 2 and 6 with 1 and 5).

The results of this study represent, to our knowledge, the first evidence demonstrating a direct role for a cAMP analog, 8-Cl-cAMP, in the control of TGFα production and activity in transformed fibroblasts. We have shown that 8-Cl-cAMP inhibits the growth of *ras*-transformed K-NRK cells and of TGFα-treated NRK cells, both in monolayer and soft agar cultures. Growth inhibition was accompanied by a reduction in TGFα production and inhibition of the p21*ras* level in K-NRK cells. Furthermore, these effects of 8-Cl-cAMP appear to be related to a selective modulation of the levels of RI versus RII cAMP receptor, the regulatory subunits of protein kinase isozymes present in these cells (namely, a decrease in the RI level and a nuclear translocation of RII). These data strongly support the role of 8-Cl-cAMP as an antiproliferative and differentiating antineoplastic

drug and directly implicate it in the control of neoplastic transformation induced by certain growth factors and/or specific oncogenes.

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