8-Chloro-cyclic AMP Inhibits Autocrine and Angiogenic Growth Factor Production in Human Colorectal and Breast Cancer

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ABSTRACT

8-Chloro-cyclic AMP (8-Cl-cAMP) is a cAMP analogue that specifically down-regulates type I protein kinase A, a signaling protein directly involved in cell proliferation and neoplastic transformation, and that causes growth inhibition in a variety of human cancer cell types. In this report, we have investigated the effects of 8-Cl-cAMP on the expression of several growth factors in human colon (GE0 and LS174T) and breast (MDA-MB468) cancer cell lines. 8-Cl-cAMP treatment caused in the three cancer cell lines a significant dose- and time-dependent inhibition in the expression of various endogenous autocrine growth factors, such as transforming growth factor α, amphiregulin, and CRIPTO, and of two angiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor, at both the mRNA and protein levels. Furthermore, 8-Cl-cAMP treatment markedly inhibited the ability of all three cell lines to invade a basement membrane matrix in a chemoinvasion assay. Finally, 8-Cl-cAMP-induced inhibition of GEO tumor growth in nude mice was accompanied by a significant suppression of transforming growth factor α, amphiregulin, CRIPTO, basic fibroblast growth factor, and vascular endothelial growth factor production by the tumor cells, and of neoangiogenesis, as detected by factor VIII staining of host blood cells. These results demonstrate that 8-Cl-cAMP is a novel antitumor drug that inhibits the production of various autocrine and paracrine tumor growth factors that are important in sustaining autonomous local growth and facilitate invasion and metastasis.

INTRODUCTION

Growth factors are proteins that regulate normal cellular proliferation and differentiation and that are important in initiating and maintaining neoplastic transformation (1, 2). Cancer cells generally exhibit a decreased requirement for exogenous growth factors as compared to normal cells. The relaxation in growth factor dependency is due in part to the ability of tumor cells to synthesize growth factors that can regulate their proliferation through autocrine and paracrine mechanisms. Among these, the EGF receptor family of growth factors such as TGF-α, AR, and CRIPTO play an important role in the pathogenesis of human colon and breast cancers (3). TGF-α, AR, and CRIPTO are expressed by the majority of human primary and metastatic breast and colorectal cancers (4, 5). Inhibition of synthesis of these growth factors by a specific antisense RNA or DNA approach inhibits human colon and breast cancer cell growth (6–9). Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in tumor survival and metastatic spreading (10, 11). Several growth factors, such as bFGF, VEGF, TGF-α, and PIGF, have been identified as potential regulators of angiogenesis (12–14). These proteins are produced by the tumor cells and can stimulate normal endothelial cell growth through paracrine mechanisms (10).

cAMP acts in mammalian cells by binding to either of two distinct isoenzymes of PKA, termed PKAI and PKAII (15). PKA and PKAII have identical catalytic subunits but differ in the regulatory subunits (defined as RI in PKA and RII in PKAII, respectively; Ref. 15). Differential expression of PKAI and PKAII has been correlated with cell differentiation and neoplastic transformation. In fact, preferential expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected in tumor cells and in normal cells following exposure to mitogenic stimuli (16). Moreover, we have shown recently that PKAI plays a role in the transduction of mitogenic signals (17, 18). PKAI is generally overexpressed in human cancer cell lines and primary tumors (16). 8-Cl-cAMP is a site-selective cAMP analogue that specifically inhibits PKAI by facilitating the degradation of the RII regulatory subunit while up-regulating at the transcriptional level RII expression (19). Decreased expression of RII induced by 8-Cl-cAMP treatment is associated with growth inhibition in various human cancer cell lines in vitro and in vivo (20–24). We have demonstrated recently in a Phase 1 clinical trial that 8-Cl-
cAMP can be given to cancer patients at doses that allow patients to reach plasma concentrations within the potential therapeutic range for growth inhibition (25).

In this study, we have investigated the effects of 8-Cl-cAMP on the expression and production of several autocrine and angiogenic growth factors in human colon (GEO and LS174T) and breast (MDA-MB468) cancer cell lines. Treatment with 8-Cl-cAMP determines a dose- and time-dependent down-regulation in the production of TGF-α, AR, CRIP1TO, VEGF, and bFGF in all cell lines. Moreover, 8-Cl-cAMP treatment interferes with the ability of these cells to invade the basement membrane matrix in a chemoinvasion assay. Finally, 8-Cl-cAMP is effective in inhibiting the growth of well-established GEO tumor xenografts in nude mice with an almost complete suppression of cancer cell production of endogenous growth factors and angiogenic peptides.

MATERIALS AND METHODS

Cell Cultures. GEO and LS174T cells were maintained in McCoy’s medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. MDA-MB468 cells were grown in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine (ICN). Early-passage HUVE cells were kindly provided by Dr. G. Viglietto (Istituto Nazionale Tumori-Fondazione Pascale, Naples, Italy). HUVE cells were cultured in DMEM supplemented with 20% heat-inactivated FBS, 100 µg/ml ECGF (Sigma Chemical Co., Milan, Italy), 100 µg/ml heparin (Sigma), 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine.

Monolayer Growth. Cells (10⁵/well) were plated in six multiwell cluster dishes (Becton Dickinson, Milan, Italy) and treated every 48 h with different concentrations of 8-Cl-cAMP. After 4 and 6 days of treatment, the cells were trypsinized and counted with a hemocytometer.

Soft Agar Growth. Cells (5 × 10³/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) in complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-complete medium in 24 multiwell cluster dishes (Becton Dickinson) and treated with different concentrations of 8-Cl-cAMP. After 12 days, the cells were stained with nitroblue tetrazolium (Sigma), and colonies were counted as described previously (7).

Analysis of DNA Fragmentation. To evaluate the potential induction of programmed cell death by 8-Cl-cAMP, cells were treated every 48 h with various concentrations of 8-Cl-cAMP. After 1, 3, or 4 days, both adherent and detached cells were harvested and lysed, and DNA was extracted and electrophoresed as described (26).

RNA Isolation and Northern Blotting. Total cellular RNA was isolated as described previously (23). Twenty µg of total RNA were electrophoresed through a denaturating 1.2% agarose/2.2 M formaldehyde gel. Ethidium bromide staining of the gels showed that equivalent amounts of RNA were contained in each lane. The samples were transferred to Hybond-N membranes (Amersham Corp., Arlington Heights, IL) and hybridized with the following 32P-labeled human cDNA probes: TGF-α (27), CRIP1TO (7), VEGF (28), KDR (29), FLT-1 (30), PI GF (31), β-actin (27), and glyceraldehyde 3-phosphate dehydrogenase (32). The cDNA probes for human VEGF, KDR, FLT-1, and PI GF were kindly provided by Dr. G. Viglietto (Istituto Nazionale Tumori-Fondazione Pascale).

Western Blotting. Protein lysates or concentrated CM (50 µg of total protein/lane) were separated by SDS-PAGE on 10% precast gels (Bio-Rad Laboratories, Milan, Italy), transferred to nitrocellulose filters, and incubated with either a rabbit polyclonal anti-human VEGF antiserum (Preprotech, London, United Kingdom) at 1:500 dilution or a rabbit polyclonal anti-human CRIP1TO antiserum (kindly provided by Dr. R. Harkins; Berlex Biosciences, Richmond, CA) at 1:500 dilution, as described previously (7). Immunoepreactive proteins were visualized by a chemiluminescence ECL Western blotting kit (Amersham Corp.).

Cell Cycle Distribution of HUVE Cells. HUVE cells were plated in 35-mm dishes (Becton Dickinson) in complete medium. After 24 h, the cells were washed twice with PBS and incubated in DMEM containing 10% FBS without ECGF and heparin. Under these conditions, HUVE cells ceased to proliferate and underwent accumulation in the G0-G1 phases of the cell cycle within 48 h. HUVE cells were then washed twice with PBS and incubated for an additional 24 h, either with complete medium containing ECGF and heparin or with GEO cell serum-free CM. For preparation of GEO cell CM, GEO cells were grown in CM with or without different concentrations of 8-Cl-cAMP for 96 h, rinsed twice with PBS (Sigma Chemical Co.) and incubated for an additional 24 h in serum-free medium without 8-Cl-cAMP. CM were then collected and centrifuged at 3000 rpm for 10 min at 4°C to eliminate cell debris. CM were diluted in a 2:1 (v/v) aceton:serum-free medium and stored overnight at −20°C. The precipitates were resuspended in PBS and added to HUVE cells at a final concentration of 200 µg/ml total proteins. HUVE cells were trypsinized, washed twice with PBS, and fixed in 70% ethanol. Cells (10⁶) were incubated for 30 min at room temperature in 1 ml of propidium iodide staining solution (50 µg/ml propidium iodide in PBS). DNA and cell cycle analysis were performed in duplicate with a FACScan flow cytometer (Becton Dickinson) as reported previously (26).

Chemoinvasion Assay. The chemoinvasion assay was performed using Biocoat Matrigel 35-mm dish invasion chambers (Becton Dickinson), as described previously (33). GEO, LS174T, and MDA-MB468 cells (10⁶ cells/dish) were plated in 35-mm culture dishes (Becton Dickinson) and treated every 48 h with different concentrations of 8-Cl-cAMP. After 96 h, the cells were trypsinized, washed twice with PBS, resuspended in serum-free medium containing 0.1% BSA (Sigma Chemical Co.), and placed (5 × 10³ cells/dish) in the upper compartment of the invasion chamber. Concentrated serum-free CM obtained from NIH-3T3 mouse fibroblasts was used as chemoattractant in the chamber lower compartment (33). After incubation for 18 h in a humidified atmosphere of 95% air and 5% CO2 at 37°C, the cells on the upper surface of the filters were mechanically removed, and the filters were fixed and stained. Ten fields per
each experimental point were counted, as described previously (34).

**GEO Xenografts in Nude Mice.** Five- to 6-week-old female BALB/c athymic (nu+/nu+) mice were purchased from Charles River Laboratories, Milan, Italy. The research protocol was approved, and mice were maintained in accordance to institutional guidelines of the University of Naples Animal Care Committee. Animals were injected s.c. with $10^7$ GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products). After 7 days, when well-established xenografts were detectable with an approximate 0.30–0.35 cm$^3$ tumor size, mice were treated twice weekly with i.p. injections of different doses of 8-Cl-cAMP for the indicated period of time. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)$^2$ (35).

**Immunocytochemistry and Evaluation of Immunoperoxidase Staining.** Slides containing GEO, LS174T, and MDA-MB468 cells treated in vitro with different concentrations of 8-Cl-cAMP or GEO tumor sections from GEO tumor xenografts grown s.c. in nude mice treated i.p. with various doses of 8-Cl-cAMP were incubated for 30 min at 20°C with methanol containing 0.3% hydrogen peroxide to block any endogenous peroxidase activity. After several washes with PBS, the sections were blocked for 45 min with 10% goat serum, washed with PBS, and incubated overnight with the appropriate primary antibody at 4°C. The following antibodies were used: An anti-CRIPTO rabbit antiserum raised against a 17-mer peptide corresponding to amino acid residues 97–113 of the human CRIPTO protein was used at 1:400 dilution (5). An anti-AR rabbit antiserum generated against a 17-mer peptide corresponding to amino acid residues 159–175 of the rat AR protein was used at 1:200 dilution (5). Both the anti-CRIPTO and the anti-AR rabbit antiseras were kindly provided by Dr. W. J. Gullick (Imperial Cancer Research Fund, London, United Kingdom). An anti-human TGF-α mouse mAb (Ab-2; Oncogene Science, Manhasset, NY) was used at 1:100 dilution. Each antibody was specific for TGF-α, AR, or CRIPTO and did not cross-react with the other two EGF-related peptides (5). An anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:50 dilution. An anti-bFGF rabbit polyclonal antibody (Santa Cruz Biotechnology) was used at 1:200 dilution. For proliferation cell nuclear antigen staining, PC 10 mAb (Novocastra Laboratory, Newcastle, United Kingdom) was used at 1:200 dilution. To detect factor VIII, a rabbit polyclonal antibody (Dakopatts, Glostrup, Denmark) was used at 1:1000 dilution. Sections were then washed three times with PBS and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit; Vector Laboratory, Burlingame, CA) for 30 min. Following several washes with PBS, the slides were reacted for 30 min with avidin biotinylated horseradish peroxidase H complex, rinsed twice in PBS, and incubated for 2 min in 0.05% diaminobenzidine and in 0.01% hydrogen peroxide. The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. Non-specific staining was evaluated for each specimen using either a similar concentration of preimmune rabbit serum or IgG or by adsorbing the primary antibody with the appropriate immunogenic peptide. Both intensity of staining (− to ++++) and percentage of immunopositive cells were scored. Two slides for each sample were evaluated by an investigator (G. F.) blinded to the treatment code. At least 1000 cancer cells per slide were counted and scored. Specific staining was semi-quantitated by assigning a score based on color intensity of the brown diaminobenzidine precipitate: (+, light brown staining; ++, a moderately brown color; ++++, an intense brown color), as described previously (8).

**RESULTS**

We have shown previously that 8-Cl-cAMP determines growth inhibition and differentiation in a wide variety of human cancer cell lines in vitro and in vivo (20–24). Treatment with 8-Cl-cAMP for 4 or 6 days induced a dose-dependent inhibition of monolayer growth in human breast (MDA-MB468) and colon (LS174T and GEO) cancer cell lines, with an IC$_{50}$ of 10, 5, and 20 μM, respectively. A comparable dose-dependent inhibition of soft agar cloning efficiency was caused in all three cell lines by the treatment with 8-Cl-cAMP (data not shown). The growth-inhibitory effect induced by 8-Cl-cAMP was cytostatic and not cytotoxic, as assessed by trypan blue dye exclusion. Furthermore, no evidence of apoptotic cell death was detected in the three cancer cell lines treated with 8-Cl-cAMP at concentrations up to 25 μM for 6 days, as evaluated by gel electrophoresis analysis of chromatin fragmentation into nucleosome ladders (data not shown; Ref. 26).

We next investigated the effects of 8-Cl-cAMP treatment on the expression of several growth factors involved in tumor growth and progression. GEO, LS174T, and MDA-MB468 cells were treated with different concentrations of 8-Cl-cAMP for 6, 24, and 96 h, and then total RNA was isolated and analyzed by Northern blotting. Control nontreated GEO and MDA-MB468 cells expressed specific TGF-α mRNA transcripts of 4.8 kb and of 4.8 and 1.6 kb, respectively (Fig. 1). Treatment with 8-Cl-cAMP caused a marked reduction in TGF-α mRNA levels that was time- and dose-dependent. In fact, an approximately 40–60% inhibition in TGF-α mRNA expression was already detectable within 6 h of treatment with 10 μM 8-Cl-cAMP in both cancer cell lines (Fig. 1). An almost complete down-regulation in TGF-α mRNA expression was observed in GEO and MDA-MB468 cells treated with 25 μM 8-Cl-cAMP for 96 h. A similar effect on TGF-α mRNA levels was observed in LS174T cells treated with 8-Cl-cAMP (data not shown). The expression of CRIPTO mRNA was also significantly reduced in GEO and LS174T cells treated with the cAMP analogue (Fig. 2). In fact, a reduction of 60–90% in CRIPTO mRNA expression was detected after treatment of GEO and LS174T cells with 25 μM 8-Cl-cAMP for 4 days as compared to control nontreated cells. A 3.9-kb VEGF-specific mRNA was detectable in GEO, LS174T, and MDA-MB468 cells (Fig. 3). In all three cell lines, 8-Cl-cAMP treatment determined a rapid and marked time- and dose-dependent inhibition in VEGF mRNA levels as compared to nontreated cells. In fact, an almost complete suppression in VEGF mRNA expression was observed in all three human cancer cell lines within 24 h of treatment with 10 μM 8-Cl-cAMP (Fig. 3). VEGF interacts with two high affinity distinct cell membrane receptors, FLT-1 and KDR, which possess an intracellular tyrosine kinase activity (29–30). These receptors are specifically expressed on endothelial cells and on some...
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levels of VEGF in the CM obtained from GEO, LS174T, and MDA-MB468 cells. CM were collected from control nontreated cells and from cells treated for 4 or 6 days with 10 or 25 μM 8-Cl-cAMP. VEGF may exist as four different isoforms consisting of 121, 165, 189, and 201 amino acids, respectively (28). VEGF165 is the more frequent isoform secreted by a variety of normal and transformed cells, which has been implicated in inducing angiogenesis (28). A major VEGF immunoreactive species with an apparent molecular weight of 45,000, which is consistent with VEGF165, was detected in all three tumor cell lines (Fig. 5). An approximate 60–70% reduction in VEGF protein levels was observed in GEO, LS174T, and MDA-MB468 cells treated with 10 μM 8-Cl-cAMP for 4 or 6 days as compared to nontreated control cells. A more marked down-regulation in VEGF protein levels was found in cells treated with 25 μM 8-Cl-cAMP for 4 or 6 days (Fig. 5). 8-Cl-cAMP treatment determined also a significant inhibition in the expression of CRIPTO protein. In fact, as evaluated by Western blotting on cell lysates, CRIPTO protein levels were reduced by 50–80% after treatment with 25 μM 8-Cl-cAMP for 2 or 4 days in GEO cells (Fig. 6) in LS174T and in MDA-MB468 cells (data not shown). We next performed an immunocytochemical analysis for TGF-α, AR, CRIPTO, VEGF, and bFGF protein expression in all three cancer cell lines treated with 10 or 25 μM 8-Cl-cAMP for 4 days. A mostly cytoplasmic-specific immunostaining for all growth factors was observed in the majority of cancer cell lines (36). Recent studies have suggested that FLT-1 and KDR have different signal transduction properties and that KDR may be the receptor involved in the regulation of neangiogenesis (37). Both KDR and FLT-1 mRNAs were not detectable in the three cancer cell lines before or after treatment with 8-Cl-cAMP (data not shown). We next analyzed the mRNA expression of PI GF, another angiogenic factor that is closely related to VEGF (14). It has been suggested recently that the activity of VEGF can be potentiated by PI GF (38). However, no PI GF-specific mRNA transcript could be detected in the three cancer cell lines, regardless of 8-Cl-cAMP treatment (data not shown).

Finally, as control we performed Northern blotting analysis of G3PD mRNA expression in GEO, LS174T, and MDA-MB468 cells treated with 8-Cl-cAMP. No significant changes in G3PD mRNA (Fig. 4) or β-actin (data not shown) were observed in all three cell lines following 8-Cl-cAMP treatment.

To determine whether the significant reduction in mRNA expression of mitogenic and angiogenic factors induced by 8-Cl-cAMP treatment is also accompanied by a decrease in the synthesis of the corresponding proteins, Western blotting and/or immunocytochemistry were performed on all three cancer cell lines that were treated with different concentrations of the cAMP analogue. We first evaluated by immunoblotting the
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Fig. 3 Northern blot analysis of VEGF mRNA expression in GEO (A), LS174T (B), and MDA-MB468 (C) cells. 
A: Lane 1, control nontreated GEO cells; Lanes 2–4, GEO cells treated with 5 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, GEO cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 8–10, GEO cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively. 
B: Lane 1, control nontreated LS174T cells; Lanes 2–4, LS174T cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, LS174T cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively. 
C: Lane 1, control nontreated MDA-MB468 cells; Lanes 2–4, MDA-MB468 cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, MDA-MB468 cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively.

Fig. 4 Northern blot analysis of glyceraldehyde 3-phosphate dehydrogenase mRNA expression in GEO (A), LS174T (B), and MDA-MB468 (C) cells. 
A: Lane 1, control nontreated GEO cells; Lanes 2–4, GEO cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, GEO cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively. 
B: Lane 1, control nontreated LS174T cells; Lanes 2–4, LS174T cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, LS174T cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively. 
C: Lane 1, control nontreated MDA-MB468 cells; Lanes 2–4, MDA-MB468 cells treated with 10 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively; Lanes 5–7, MDA-MB468 cells treated with 25 μM 8-Cl-cAMP for 6, 24, and 96 h, respectively.

GEO, LS174T, and MDA-MB468 cells (Table 1 and data not shown). A substantial dose-dependent reduction in both the percentage of immunoreactive cells and the intensity of specific cell staining for all growth factors was found in the three cancer cell lines with a weak expression of each protein in only 5–25% tumor cells after treatment with 25 μM 8-Cl-cAMP for 4 days (Table 1).

Because 8-Cl-cAMP treatment was able to significantly inhibit the production of various growth factors with angiogenic activity, such as VEGF, bFGF, and TGF-α, we evaluated if this reduction is biologically relevant using an in vitro assay of endothelial cell proliferation. HUVE cells require a mixture of 20% FBS, heparin, and ECGF for optimal proliferation. HUVE cells cultured for 24 h in a medium containing 10% FBS without heparin and ECGF became quiescent and accumulated in the G0-G1 phases of the cell cycle (Table 2). Incubation of quiescent HUVE cells for 24 h with concentrated CM obtained from control nontreated GEO cells stimulated their progression through the cell cycle because the percentage of HUVE cells in S phase raised from 5 to 20%. In contrast, incubation with concentrated CM obtained from GEO cells treated for 4 days with 10 or 25 μM 8-Cl-cAMP determined only a moderate increase in the percentage of HUVE cells in S phase (11 and 8%, respectively). These results suggest that 8-Cl-cAMP treatment of GEO cells causes a significant inhibition in the secretion of biologically active angiogenic growth factors.

Tumor cell invasion of the basement membrane is a critical step in the multistage process that leads to metastatic spreading (39). The invasive behavior of GEO, LS174T, and MDA-MB468 cells was evaluated in a chemoinvasion assay using Boyden chambers coated with Matrigel, a mixture of basement membrane components (33). CM from mouse NIH-3T3 fibroblasts, which contains defined (fibronectin and collagen) as well as undefined chemotactic factors, was used as chemoattractant...
Both control untreated and 8-Cl-cAMP-treated GEO tumor groups grew as moderately differentiated human colon adenocarcinomas (data not shown). 8-Cl-cAMP determined a dose-dependent inhibition of GEO tumor growth with an almost complete suppression of tumor cell proliferation, as assessed by proliferation cell nuclear antigen staining at 2 mg/dose 8-Cl-cAMP (Fig. 8). The in vivo growth-inhibitory effect of 8-Cl-cAMP on GEO tumors was cytostatic rather than cytotoxic. In fact, GEO tumors resumed a growth rate comparable to controls within 10 to 14 days following termination of 8-Cl-cAMP treatment (data not shown). To ascertain whether treatment with 8-Cl-cAMP was able to interfere with the production of growth factors also in vivo, immunohistochemical evaluation of the expression of TGF-α, AR, CRIPTO, bFGF, and VEGF was performed on GEO tumors at the end of the 4 weeks of treatment. A marked reduction in both the percentage of positive GEO cells and the average intensity of staining for all five growth factors were observed (Fig. 8B). The growth inhibition induced by 8-Cl-cAMP treatment was paralleled by a substantial inhibition of angiogenesis, as detected by factor VIII staining of mouse blood vessels.

**DISCUSSION**

Growth factors can regulate cancer initiation and progression through several mechanisms. These include: autonomous uncontrolled growth as a result of the autocrine production of endogenous growth factors by tumor cells; loss of sensitivity to growth inhibitors; and stimulation of tumor vascularization due to paracrine stimulation of normal host endothelial and mesenchymal cells by angiogenic growth factors secreted by cancer cells (1, 2).

The EGF family of growth factors, such as EGF, TGF-α, AR, and CRIPTO, plays a significant role in the development of human breast and colorectal cancer (3). We have demonstrated previously that inhibition of TGF-α, AR, or CRIPTO by antisense oligonucleotide treatment or by infection with antisense retroviral expression vectors is able to significantly block the growth of human breast and colon carcinoma cell lines (6–9).
bFGF and VEGF are potent angiogenic peptides that are secreted in several types of human malignancies (12-13, 41). It has been suggested that tumor cells secreting these growth factors may have a growth advantage in vivo. In this regard, overexpression of VEGF confers the ability of Chinese hamster ovary cells to form vascularized tumors in nude mice, whereas it does not have growth-stimulatory activity in vitro (42). A more direct evidence of the VEGF role in tumor-induced angiogenesis has been obtained with the use of specific anti-VEGF neutralizing mAbs. In fact, treatment of cancer cells with anti-VEGF neutralizing antibodies does not affect their proliferation in vitro, whereas it exerts a potent inhibitory effect on their growth as xenografts in nude mice (43). Moreover, treatment of nude mice bearing human colon carcinoma xenografts with an anti-VEGF mAb results in a marked decrease in the number and size of liver metastases (44). Similarly, tumor cells overexpressing bFGF become more aggressive and highly metastatic in vivo (45). However, treatment with an anti-bFGF neutralizing antibody induces a significant inhibition of primary and metastatic tumor growth (45). Therefore, interference with the production of angiogenic factors or with the signaling pathways regulated by angiogenic factors has been proposed as a form of cancer therapy that can be combined with more conventional cytotoxic anticancer drugs (46, 47).

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Furthermore, 8-Cl-cAMP has shown that 8-Cl-cAMP can be administered in several protocols of continuous iv. infusion at doses that allow the drug to reach plasma concentrations within a range potentially effective to suppress tumor growth (26).

The results of the present study suggest that inhibition of PKAI expression and activity by 8-Cl-cAMP treatment blocks the autocrine mitogenic signaling sustained by EGF-related growth factors. This has potential clinical relevance because inhibition of TGF-α, AR, and CRIPITO, which are frequently overexpressed in human colorectal and breast cancer as compared to normal colorectal and breast epithelium, may represent an important goal of novel biological cancer therapies. In this respect, several studies have demonstrated that blocking the activation of EGF-R, that is the specific receptor for EGF, TGF-α and AR, by the use of anti-EGF-R mAbs inhibits the in vitro and in vivo growth of several human cancer cell lines (48, 49). We have demonstrated recently that 8-Cl-cAMP and an anti-EGFR blocking mAb can be used in combination and determine a synergistic growth-inhibitory effect on human colon and breast cancer cell lines (26).

Fig. 8 A, antitumor activity of 8-Cl-cAMP treatment on established GEO human colon carcinoma xenografts. The mice were injected s.c. into the dorsal flank with 10^7 GEO cells as described in “Materials and Methods.” After 7 days (average tumor size, 0.30–0.35 cm^3) the mice were treated i.p. twice weekly with 8-Cl-cAMP at the following doses: Δ, 0.25 mg/dose; ○, 0.5 mg/dose; ■, 1 mg/dose; and ○, 2 mg/dose for 4 weeks. □, control. Each group consisted of 15 mice. Bars, SD. B, immunohistochemical evaluation of growth factor expression and of neoangiogenesis in GEO xenografts. Two mice per group were killed, and tumors were excised on day 35 after GEO cell injection. The percentage of positive cancer cells and the average intensity of specific immunostaining were determined as described in “Materials and Methods.”

activity, have been widely demonstrated in a variety of human cancer cell types (20–24). Furthermore, 8-Cl-cAMP has recently entered clinical evaluation in cancer patients. In fact, a Phase I trial in cancer patients refractory to standard therapies has shown that 8-Cl-cAMP can be administered in several cycles of continuous i.v. infusion at doses that allow the drug to reach plasma concentrations within a range potentially effective for tumor growth inhibition (25).

The results of this study demonstrate for the first time that the antiproliferative effect of 8-Cl-cAMP is accompanied by a marked inhibition in the synthesis and secretion of several growth factors such as TGF-α, AR, CRIPITO, VEGF, and bFGF, which are important modulators through autocrine and paracrine pathways of human cancer cell growth and of tumor-induced neoangiogenesis. The 8-Cl-cAMP-induced inhibition of growth factors and angiogenic peptides in human colon and breast cancer cells is time and dose dependent and probably occurs at a transcriptional level. In fact, a significant reduction in mRNA levels for these growth factors is detected within 6 to 24 h of 8-Cl-cAMP treatment and precedes the reduction in protein levels. These results are in agreement and further extend our previous observation of a specific down-regulation of TGF-α mRNA and protein in mouse mammary epithelial cells overexpressing the TGF-α gene following treatment with 8-Cl-cAMP (23). The reduction in the production of various peptide growth factors induced by 8-Cl-cAMP treatment does not appear as a nonspecific phenomenon due to cell growth inhibition. In fact, a 2- to 4-fold increase that is time and dose dependent in the expression of the EGFR on the cell surface of GEO, LS174T, and MDA-MB468 cells could be observed following treatment with 8-Cl-cAMP (26).

The inhibition in the synthesis and secretion of angiogenic growth factors such as VEGF and bFGF obtained in 8-Cl-cAMP treated cancer cells is functionally and biologically relevant. In fact, analysis of the cell cycle distribution of growth factor-depleted quiescent HUVE cells exposed to GEO cell CM reveals that GEO cells secrete factors that are able to stimulate normal endothelial cell proliferation. In contrast, treatment of GEO cells with 8-Cl-cAMP significantly inhibits the secretion of HUVE cell growth-stimulating factors.

The decreased production of autocrine and paracrine growth factors induced by 8-Cl-cAMP treatment is paralleled by the reduced ability of the tumor cells to invade the basement membrane in a chemo-invasion assay. In fact, a dose-dependent 40–60% inhibition in the capacity of GEO, LS174T, and MDA-MB468 cells to penetrate the Matrigel-coated filters is observed after 8-Cl-cAMP treatment. These results suggest that 8-Cl-cAMP may also interfere with the invasive behavior of cancer cells, which is a critical event in the multistep process that leads to the formation of distant metastases. Further studies will be necessary to determine whether 8-Cl-cAMP may inhibit the tumor cell production of various proteolytic enzymes, such as type IV collagenase, that degrade basement membrane components and that are involved in conferring the invasive phenotype (39).

Finally, 8-Cl-cAMP is highly effective in inhibiting the growth of GEO colon carcinoma cells in vivo. 8-Cl-cAMP treatment for 4 weeks results in a dose-dependent suppres-
sion of GEO xenografts growth in the absence of toxicity in the nude mice. The in vivo suppression of tumor growth is accompanied by a significant reduction in the expression of TGF-α, AR, CRIPTO, VEGF, and bFGF in tumor cells and by a strong inhibition of host neoangiogenesis, as measured by factor VIII staining of endothelial cells.

In summary, the results of the present study demonstrate that 8-Cl-cAMP is a novel anticancer drug with effects on the production of several growth and angiogenic factors that have potential therapeutic relevance. In fact, inhibition of endogenous autocrine and paracrine growth factors that sustain autonomous local growth and facilitate metastatic spreading of tumor cells is obtained by treatment with an agent that has different intracellular targets and that does not antagonize the effect of cytotoxic antineoplastic drugs. In this respect, long-term treatment with 8-Cl-cAMP can be proposed in adjunct to conventional chemotherapy in cancer patients.

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