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Cyclic AMP induces transforming growth factor β2 gene expression and growth arrest in the human androgen-independent prostate carcinoma cell line PC-3

(negative growth factor/hormone-refractory/cyclic AMP response element)

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ABSTRACT **The standard therapy for advanced prostate cancer is androgen ablation. Despite transitory responses, hormonally treated patients ultimately relapse with androgen-independent disease that is resistant to further hormonal manipulation and cytotoxic chemotherapy. To develop an additional approach to the treatment of advanced prostate cancer, we have been studying the signal transductions controlling the growth of human androgen-independent prostate carcinoma cell lines. We report here that elevation of intracellular cAMP markedly inhibits the growth of the hormone-refractory cell line PC-3. To examine the mechanism of cAMP action in PC-3 cells, we tested the effect of the cAMP analog dibutyryl cAMP (Bt_2-cAMP) on the regulation of the potent negative growth factor transforming growth factor β (TGF-β). Bt_2-cAMP selectively induced the secretion of TGF-β2 and not TGF-β1 by PC-3 cells. This TGF-β2 was shown to be bioactive by using the CCL-64 mink lung cell assay. TGF-β1 was not activated despite being present at 3-fold higher concentrations than TGF-β2. Northern analysis showed that Bt_2-cAMP induced an increase in the five characteristic TGF-β2 transcripts and had no effect on the level of TGF-β1 or TGF-β3 transcripts. TGF-β2 induction was only weakly enhanced by cycloheximide and was completely inhibited by actinomycin D. These data show that Bt_2-cAMP induces the expression of active TGF-β2 by PC-3 prostate carcinoma cells, suggesting a new approach to the treatment of prostate cancer and a new molecular mechanism of cAMP action.**

cAMP is a pleiotropic regulator of cell metabolism, growth, and differentiation. In eukaryotic cells, the only established mechanism of action of cAMP is through the activation of cAMP-dependent protein kinase A (1). cAMP exerts effects at the posttranslational level, through the activation of protein kinase A-catalyzed phosphorylation of cellular substrates, and at the transcriptional level, through protein kinase A-catalyzed phosphorylation of transcription factors bound to cis-acting elements in the promoter–regulatory regions of cAMP-responsive genes (2). One of the most prominent effects of cAMP is growth regulation. Both growth stimulation and growth inhibition have been observed, depending on the cell of origin, the state of activation or transformation, the concentration of cAMP, and the cell environment (3). As part of a search for new approaches to the treatment of advanced hormone-refractory prostate cancer, we have examined the effect of cAMP on human androgen-independent prostate carcinoma cell lines. The data presented here show that elevation of intracellular cAMP is highly inhibitory to growth of the hormone-refractory cell line PC-3.

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While recent progress in understanding the mechanism of cAMP regulation of gene expression in eukaryotic cells has been considerable, less is known about the molecular mechanism of cAMP control of cell growth. Studies of the reversal of the transformed phenotype by cAMP have led to the proposal that cAMP can restore the integrity of normal negative growth regulatory pathways that have been disrupted in the process of malignant transformation (4, 5). Among the most potent physiologic negative regulators of epithelial cell growth are the members of the polypeptide transforming growth factor β (TGF-β) family (for review, see ref. 6). Five members of the TGF-β family have been cloned, and three—TGF-β1, -β2, and -β3—are known to be expressed in human cells. The TGF-βs are synthesized and secreted in a latent form in which the amino-terminal peptide, which is cleaved during protein processing, is noncovalently bound to the carboxyl-terminal peptide containing the active region (7, 8). This inactive dimer is secreted with an additional component known as the latent TGF-β binding protein. The secreted complex must be activated for TGF-β to bind to its plasma membrane receptor. The receptor for TGF-β is expressed by many cell types, as is TGF-β itself. Therefore, activation of the latent complex is an important point of regulation of TGF-β action.

In studying the mechanism of cAMP-mediated growth arrest of PC-3 cells, we have examined the effect of cAMP on TGF-β regulation. It has been shown previously that PC-3 cells contain TGF-β transcripts (9, 10), secrete TGF-β (11), express TGF-β receptors, and are growth-inhibited by activated TGF-β (10, 12). These data suggest that it may be possible to activate a TGF-β autocrine negative growth pathway in PC-3 prostate cancer cells in a parallel way to the reported ability of antiestrogens to induce secretion of active TGF-β by the estrogen-dependent MCF-7 breast cancer cell line (13). Our results show that cAMP induces TGF-β secretion by PC-3 cells and that this effect is selective for the TGF-β2 isoform. In addition, we show that TGF-β inhibits the growth of PC-3 cells in the concentration range induced by cAMP treatment. Furthermore, our results show that the TGF-β2 secreted after addition of cAMP is activated and that activation is selective for TGF-β2 and not for TGF-β1, which is present in higher quantities but remains in latent form. Finally, Northern blot analysis suggests that cAMP is also exerting positive control on the regulation of TGF-β2 gene expression.

Abbreviations: TGF, transforming growth factor; CRE, cAMP response element; Bt_2-cAMP, dibutyryl cAMP.

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MATERIALS AND METHODS

Cell Culture. The human androgen-independent prostate carcinoma cell line PC-3 was obtained from the American Type Culture Collection. Cells were grown in Ham's F-12K medium containing 7% (vol/vol) fetal bovine serum and antibiotics (50 units of penicillin and 50 μg of streptomycin per ml). For growth in serum-free conditions, Ham's F-12K medium was supplemented with selenium (5 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), Hepes (20 mM), bovine serum albumin (1 mg/ml), and antibiotics.

Cell Growth. Cell growth was assessed by thymidine incorporation and by determination of cell number in a Coulter Counter. Thymidine incorporation was used to test the effect of increasing intracellular cAMP under the same assay conditions used for measurement of secreted TGF-β. Measurement of secreted TGF-β was performed by using confluent, serum-free cultures to maximize sensitivity for the detection of low quantities of TGF-β isoforms. For thymidine incorporation studies, cells (8×10^4 cells per well) were plated in 24-well plates in growth medium and incubated for 5 days prior to the assay. Under these conditions, PC-3 cells reach confluence by day 4. The cells were then washed once with serum-free medium and incubated in serum-free medium for 24 hr at 37°C. The culture medium was then replaced with fresh serum-free medium with or without test substances. After 22 or 46 hr, 1 μCi (37 kBq) of [*methyl*-^{3}H]thymidine was added for 2 hr, and trichloroacetic acid-precipitable radioactivity was determined in a liquid scintillation counter. Cell viability at the end of this procedure as determined by trypan blue exclusion was typically >90% (data not shown). However, some nonviable cells may be lost during cell washes. To assess the effect of elevating intracellular cAMP on a population of cells growing logarithmically in serum-containing medium, cell counts were performed by plating the cells at 3×10^3 per well in 24-well plates, allowing the cells to adhere for 24 hr, adding test substances every 48 hr, harvesting the cells after 6 days, and determining the cell number in a model ZM Coulter Counter.

Studies designed to test the effect of TGF-β2 on cell growth were performed with purified porcine platelet TGF-β2 from R & D Systems, Minneapolis.

Preparation of Conditioned Medium. For the preparation of conditioned medium, cells were plated at 1×10^6 cells per 100-mm dish and grown to confluence in complete medium. After 5 days of culture, cells were washed three times at 2-hr intervals with serum-free medium and were incubated in serum-free medium for 24 hr. This medium was discarded, and cells were incubated in 10 ml of fresh serum-free medium with or without dibutyryl cAMP (Bt_2-cAMP) for the indicated times. Aprotinin (1 μg/ml), leupeptin (1 μg/ml), and 1 mM phenylmethylsulfonyl fluoride were added, cell debris was removed by centrifugation, and the medium was stored at −70°C until use.

Quantitation of Secreted TGF-β1 and TGF-β2. The concentrations of TGF-β1 and TGF-β2 in conditioned medium were determined by enzyme-linked immunosorbent assay with antibodies against native TGF-β1 or TGF-β2 as described (14). Briefly, conditioned medium was concentrated by precipitation with trichloroacetic acid, the resulting precipitates were washed with ether/ethanol, 1:1 (vol/vol), lyophilized, and solubilized with 4 mM HCl containing 1 mg of bovine serum albumin per ml and 150 mM NaCl. Serial dilutions of the resulting neutralized samples were added to microtiter plates (Nunc Maxisorb), which were coated with turkey anti-TGF-β1 or anti-TGF-β2 antibodies. After incubation for 1 hr, the plates were incubated with turkey anti-TGF-β1 or anti-TGF-β2 antibodies, and after 1 hr phosphatase-conjugated goat anti-turkey IgG was added and incubated for 1 hr. The assay was developed with the phosphatase substrate *p*-nitrophenyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 to 3 hr at room temperature. The difference in absorbance at 410 and 450 nm was measured with a Dynatech MR600 microtiter plate spectrophotometer (Dynatech).

CCL-64 Mink Lung Cell Assay. Bioassay for active TGF-β was performed by testing for inhibition of the growth of the CCL-64 mink lung cell line as described (15). Briefly, CCL-64 cells were harvested, washed with assay buffer (Dulbecco's modified Eagle's medium supplemented with 0.2% fetal bovine serum and 10 mM Hepes), resuspended in assay buffer, and seeded at 5×10^4 cells in 0.5 ml in 24-well plates. After 1 hr, conditioned medium and antibody were added, and 22 hr later cells were pulsed with [^{3}H]thymidine for 2 hr at 37°C. Trichloroacetic acid-precipitable radioactivity was measured in a liquid scintillation counter.

RNA Analysis by Northern Hybridization. Confluent PC-3 cells were treated with various agents in serum-free medium, and total RNA was isolated by using single-step acid guanidinium isothiocyanate/phenol/chloroform extraction (16). Poly(A)$^+$ RNA was prepared with oligo(dT) columns (Pharmacia). The amount of RNA in an aqueous solution was quantitated by absorbance at 260 nm. RNA was separated on a 1.2% agarose/formaldehyde gel, transferred to Nytran (Schleicher & Schuell), and baked at 80°C for 2 hr. Blots were stained in 0.02% methylene blue in 0.3 M sodium acetate to verify the accuracy of transfer. Blots were prehybridized and hybridized in 1% bovine serum albumin/7% SDS/0.5 M sodium phosphate/1 mM EDTA at 65°C. Hybridized blots were washed in 1% bovine serum albumin/40 mM sodium phosphate/2 mM EDTA twice at room temperature and twice for 15 min at 65°C before exposure to Kodak XAR-2 film. Blots were stripped in 0.1× SSPE (0.87 g of NaCl, 0.138 g of NaH_2PO_4, and 0.037 of EDTA per liter, pH 7.4) containing 0.2% SDS at 100°C before reprobing.

RNA blots were sequentially hybridized with a 1.2-kilobase (kb) simian TGF-β2 cDNA (17), and a 1.2-kb rat TGF-β1 cDNA (18), which contained their respective coding regions and a rat glyceraldehyde-3′-phosphate dehydrogenase cDNA (19). Blots were routinely probed with $3–5 \times 10^5$ cpm of probe per ml, prepared by random primer labeling (Bethesda Research Laboratories) to a specific activity of 2×10^9 cpm/μg.

RESULTS

Inhibition of the Growth of PC-3 Prostate Carcinoma Cells by cAMP. Fig. 1 *Upper* shows the concentration-dependent inhibition of thymidine incorporation in the human androgen-independent prostate carcinoma cell line PC-3 after 48 hr of incubation in serum-free medium containing various concentrations of Bt_2-cAMP. The IC_{50} for inhibition of DNA synthesis was ≈90 μM. Bt_2-cAMP treatment also markedly inhibited the growth of logarithmic-phase PC-3 cells growing in medium containing 7% fetal bovine serum (Fig. 1 *Lower*). The IC_{50} for growth inhibition under these conditions was 150 μM. In experiments not shown, growth of PC-3 cells was also inhibited by the cAMP derivatives 8-chloro-cAMP and phosphorothioate cAMP, and by the phosphodiesterase inhibitors isobutylmethylxanthine, theophylline, and pentoxiphylline. Thus, the effect of Bt_2-cAMP on growth of PC-3 is through elevation of cAMP, rather than through an effect of the butyrate moiety.

Induction of TGF-β2 by cAMP. PC-3 cells were treated with 1 mM Bt_2-cAMP in serum-free medium, and concentrations of secreted TGF-β1 and TGF-β2 were measured using enzyme-linked immunosorbent assay. Bt_2-cAMP did not increase the secretion of TGF-β1, whereas Bt_2-cAMP treatment caused a significant increase in TGF-β2 secretion to values 4-fold higher than control values on day 1 and 6-fold

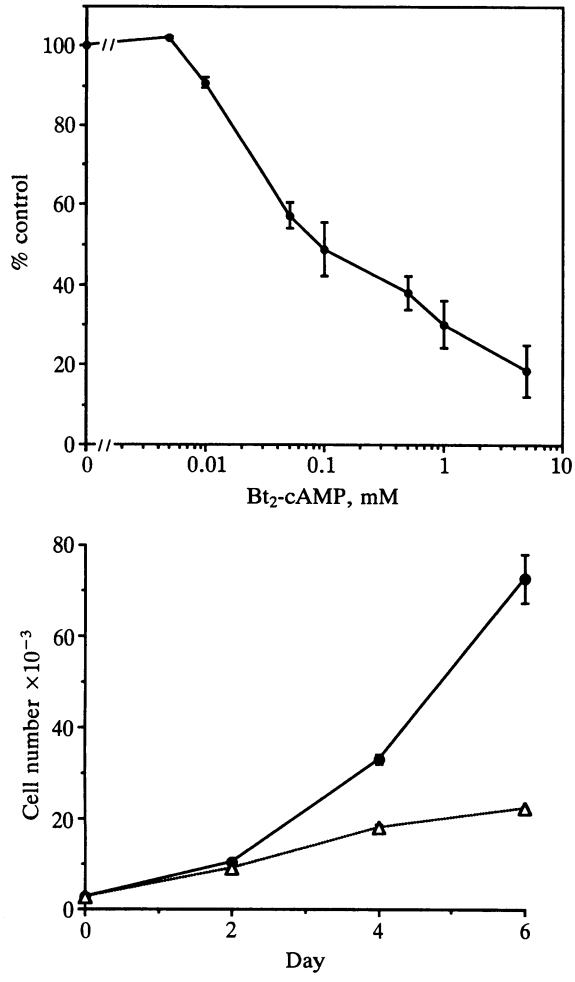

FIG. 1. (*Upper*) Concentration-dependent inhibition of thymidine incorporation in PC-3 cells by Bt_2-cAMP. Cells were plated in 24-well plates, and grown until confluent. Growth medium was replaced with serum-free medium and after 24 hr various concentrations of Bt_2-cAMP were added. After 46 hr, [^{3}H]thymidine was added for 2 hr, and the amount of [^{3}H]thymidine incorporation was measured by scintillation counting. The data are expressed as the percent of thymidine incorporation by cells in untreated control wells. The data are representative of three different experiments and the values are means ± SD (n = 3). (*Lower*) Inhibition of the growth of logarithmic-phase PC-3 cells by Bt_2-cAMP (△) as compared with controls (●). Cells were plated at low density, allowed to adhere for 24 hr (day 0) and Bt_2-cAMP (1 mM) was added on day 0 and every 48 hr subsequently. After 6 days the cells were harvested, and cell number was determined in a model ZM Coulter Counter.

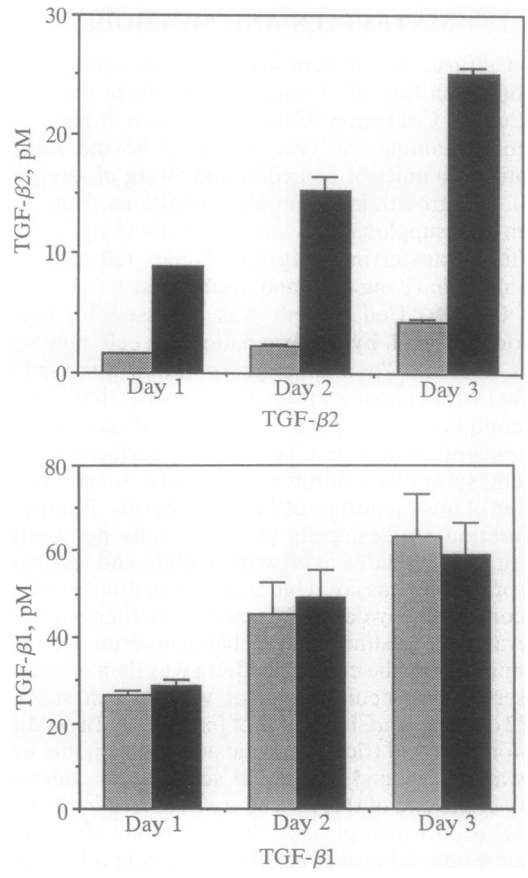

FIG. 2. Bt_2-cAMP induces secretion of TGF-β2 in PC-3 cells. PC-3 cells were plated at 1×10^6 cells per 100-mm dish, grown until confluent, washed with serum-free medium, and incubated in serum-free medium with vehicle (grey bars) or 1 mM Bt_2-cAMP (black bars) for the indicated times. Concentrations of TGF-β1 and TGF-β2 were determined by using enzyme-linked immunosorbent assay with specific antibodies against TGF-β1 and TGF-β2, respectively, as detailed in text. The data are representative of three different experiments, and the values are means ± SD of triplicate determinations.

higher than control values on days 2 and 3 (Fig. 2). Fig. 3 shows the concentration-dependent stimulation of TGF-β2 secretion by Bt_2-cAMP. The half maximal effect was seen at about 100 μM Bt_2-cAMP. In experiments not shown, TGF-β2 secretion was also stimulated by forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine.

TGF-β Inhibits the Growth of PC-3 Cells. The effect of TGF-β on the growth of PC-3 cells was tested by thymidine incorporation, and picomolar concentrations of TGF-β2 inhibited PC-3 cell thymidine incorporation by ≈50% (Fig. 4). These data were confirmed by determinations of cell number (data not shown).

cAMP Induces Secretion of Bioactive TGF-β2. As described above, TGF-β is secreted from cells as a latent complex of mature TGF-β in noncovalent association with its own amino-terminal precursor and in association with a TGF-β modulator peptide (7, 8). This latent form of TGF-β does not bind to the TGF-β receptor and is biologically inactive (20, 21). Originally described for TGF-β1, recently it has been shown that TGF-β2 and TGF-β3 are also synthesized as latent forms (22). The latent complex can be activated *in vitro* by transient acidification, alkalinization, exposure to urea, or heating (23). We used the CCL-64 growth inhibition assay to characterize the biologic activity of TGF-β in conditioned medium. These cells have been shown to be extremely sensitive to the growth-inhibitory action of TGF-β (24). Fig. 5 shows that conditioned medium from PC-3 cells treated with 1 mM Bt_2-cAMP for 3 days inhibited the growth of CCL-64 cells in a concentration-dependent manner. This inhibitory action was completely reversed by purified anti-TGF-β2 IgG (V6/30), whereas antibody to TGF-β1 or preimmune serum was without effect. The concentration of active TGF-β2 in the neutral conditioned medium was estimated to be ≈20 pM by comparison with a standard curve generated in the CCL-64 growth inhibition study. This concentration is almost identical to that determined by enzyme-linked immunosorbent assay in Fig. 2. In contrast, neutral conditioned medium from untreated PC-3 cells did not inhibit the growth of CCL-64 cells (data not shown). Also shown is the growth inhibitory effect of transiently acidified conditioned medium, which is a measure of total TGF-β present in the conditioned medium. The data in Fig. 5 show that conditioned medium from Bt_2-cAMP-treated PC-3 cells contains bioactive TGF-β2.

cAMP Effects on TGF-β2 Gene Expression. Poly(A)$^+$ RNA was prepared from PC-3 cells 24 hr after treatment with 1 mM Bt_2-cAMP. Fig. 6 shows that a very low level of the five

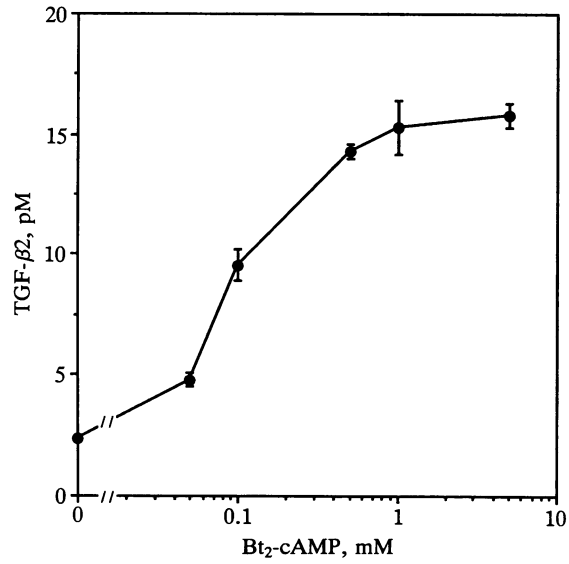

FIG. 3. Concentration dependence of TGF-β2 secretion in PC-3 cells by Bt_2-cAMP. PC-3 cells were cultured as described in Fig. 1 *Upper* legend and then incubated in serum-free medium for 2 days with various concentrations of Bt_2-cAMP. The data are representative of three different experiments and the values are means ± SD of triplicate determinations.

characteristic TGF-β2 transcripts was detected from untreated cells, while after treatment with Bt_2-cAMP there was a marked increase in the level of each transcript. This effect was specific for TGF-β2; there was no increase in the messenger RNA level for TGF-β1 after addition of cAMP, and TGF-β3 expression was not detected in PC-3 cells either before or after cAMP treatment (data not shown). Addition of cycloheximide (10 ng/ml) did not affect the induction of TGF-β2 mRNA, indicating that new protein synthesis is not required for TGF-β2 induction by Bt_2-cAMP. In contrast, addition of actinomycin D (3 μg/ml) completely blocked the induction of TGF-β2 mRNA, suggesting that, in addition to

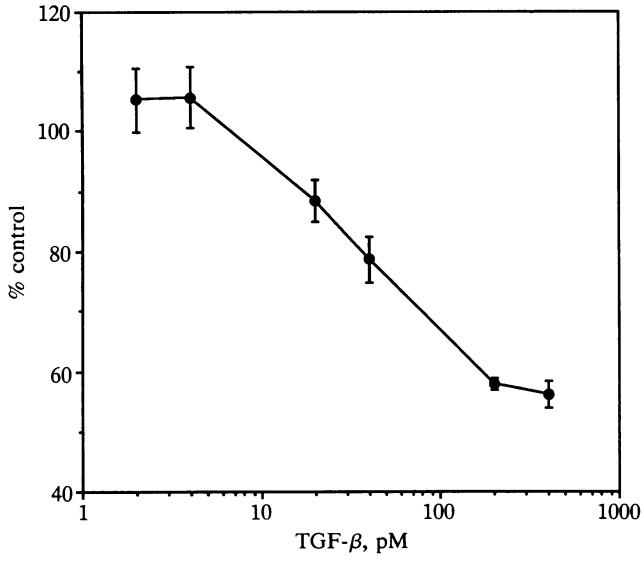

FIG. 4. Concentration-dependent inhibition of thymidine incorporation in PC-3 cells by TGF-β. Methods are as described in the legend to Fig. 1 except that cells were treated for 24 hr. The data are expressed as the percent of the thymidine incorporation by cells in untreated control wells. The data are representative of three different experiments, and the values are means ± SD (n = 3).

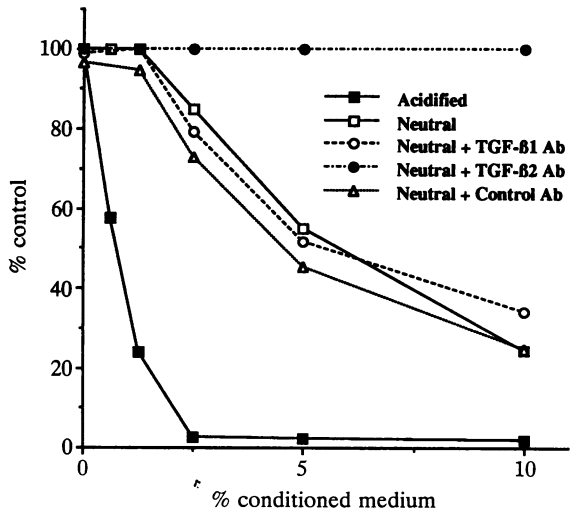

FIG. 5. Growth inhibition of CCL-64 mink lung cells by conditioned medium. CCL-64 cells were plated in 24-well plates. After 1 hr, conditioned medium (either neutral or transiently acidified) and antibodies were added, and 22 hr later cells were pulsed with [^{3}H]thymidine for 2 hr. The data are expressed as the percent of the thymidine incorporation in untreated control cells. The data are representative of three different experiments and the values are means of duplicate determinations.

posttranslational effects, cAMP is able to activate transcription of the TGF-β2 gene.

DISCUSSION

Prostate cancer is the second leading cause of cancer deaths in men in the United States (25). The standard treatment approach to advanced prostate cancer has been hormonal therapy, consisting of total androgen ablation through medical or surgical castration, with or without the addition of

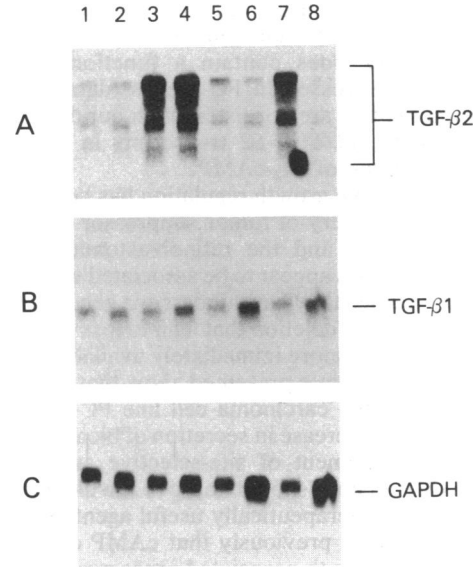

FIG. 6. Northern analysis of TGF-β1 and TGF-β2 mRNAs in PC-3 cells. After pretreatment with cycloheximide (10 μg/ml for 15 min), actinomycin D (3 μg/ml for 30 min), or vehicles only, cells were treated with 1 mM Bt_2-cAMP for 24 hr. Each lane contained 5 μg of poly(A)$^+$ RNA and was hybridized to a ^{32}P-labeled cDNA probe of TGF-β1, TGF-β2, or glyceraldehyde-3-phosphate dehydrogenase. Lanes: 1, control; 2, cycloheximide; 3, Bt_2-cAMP; 4, Bt_2-cAMP and cycloheximide; 5, control; 6, actinomycin D; 7, Bt_2-cAMP; 8, Bt_2-cAMP and actinomycin D.

antiandrogen (26). Hormonal therapy is not curative, and most hormonally treated patients relapse with androgen-independent disease (27). Because classical cytotoxic chemotherapy has not been effective in prostate cancer, we have been searching for a new approach to the treatment of advanced prostate cancer, targeted to the hormonally insensitive prostate cancer cell. The PC-3 cell line is a hormone-refractory human prostate carcinoma line that has lost androgen receptor expression. The data presented here demonstrate that this cell line is markedly growth inhibited by cAMP. We have also demonstrated that cAMP-induced growth arrest is associated with the selective production and activation of TGF-β2, a cytokine that is highly growth inhibitory to PC-3 cells.

The data presented here suggest that cAMP may be acting at more than one level to regulate TGF-β2 in PC-3 cells. We have shown that cAMP increases the level of extracellular activated TGF-β2. The physiologic mechanisms regulating the activation of the latent TGF-β complex have not been delineated, although some evidence suggests that proteases or glycosidases may play a role (6). The strikingly selective activation of TGF-β2 and not TGF-β1, despite higher constitutive levels of TGF-β1 in the culture supernatant, suggests that cAMP-treated PC-3 cells may offer a useful system in which to study the biochemical mechanisms of TGF-β activation.

In addition, the data in Fig. 6 suggest that cAMP may increase either the level of TGF-β2 transcription or message stability. The effects of cAMP on transcription are thought to be mediated through the activation of cis-acting promoter elements within the promoter–regulatory region of cAMP-sensitive genes. Two cAMP-responsive elements have been identified, the cAMP response element (CRE), which is also recognized by the activating transcription factor (ATF), and the AP-2 binding site, which is able to mediate both cAMP and phorbol ester induction of gene transcription. The TGF-β1, -β2, and -β3 5′ flanking regions have been characterized (28–30). The TGF-β2 promoter, which is currently least well characterized, appears to contain several CRE/ATF- and AP-2-like DNA consensus sequence elements (29). The TGF-β1 promoter does not contain a CRE site, and the TGF-β3 promoter does contain a functional CRE, but TGF-β3 is not expressed in PC-3 cells (data not shown). These structural data are consistent with our data showing a selective increase in TGF-β2 transcripts in PC-3 cells in response to addition of Bt_2-cAMP.

Interest in negative growth regulation has been intensified by the recent discovery of tumor suppressor genes such as *TP53* encoding p53 and the retinoblastoma-susceptibility gene *RB*. These genes appear to be associated with neoplastic transformation when they have undergone genetic alterations such as mutation and deletion that lead to loss of function. In contrast, TGF-β is a more immediately available therapeutic target. The data we have presented show that the androgen-independent prostate carcinoma cell line PC-3 responds to Bt_2-cAMP with an increase in secretion of bioactive TGF-β2. The recent development of site-selective and hydrolysis-resistant cAMP analogs should improve the likelihood of the development of a therapeutically useful agent (31).

It has been shown previously that cAMP can inhibit the expression of the growth-associated genes c-*myc* (32, 33) and the transferrin receptor gene (33). The data presented here demonstrate that cAMP can stimulate the expression of a negative growth regulator, suggesting a new molecular mechanism of cAMP action.

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