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Preclinical report

Expression and role of phosphodiesterase 3 in human squamous cell carcinoma KB cells

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Phosphodiesterase (PDE) 3s have been characterized in human squamous cell carcinoma KB cells. PDE3 activity was detected in homogenates of KB cells. PDE3A and 3B mRNAs were detected by RT-PCR in RNA from KB cells; the nucleotide sequences of the fragments were identical to those of human PDE3A and 3B. Immunoblotting with anti-PDE3 antibodies detected both PDE3A- and 3B-immunoreactive proteins in KB cells. The PDE3-specific inhibitor, cilostamide, inhibited the proliferation of KB cells. Our results indicate that PDE3s may be important regulators of the growth of KB cells. Therefore, PDE3 inhibitors may be potential new drugs for antiproliferative therapies in squamous cell carcinoma in the head and neck. [© 2002 Lippincott Williams & Wilkins.]

Key words: Phosphodiesterase, phosphodiesterase inhibitor, squamous cell carcinoma.

Introduction

Squamous cell carcinoma is the most common histologic type in head and neck cancer.[1–3] A number of drugs have been tested in patients. However, the response to these agents has not been complete.[4–6] The development of new drugs with activity against squamous cell carcinoma of the head and neck is critically important to increase the survival rates of patients with this disease.

Eleven different, but structurally related, PDE gene families (PDE1–11) have been identified in various tissues or cells.[7–18] PDE3 isoforms are found in a variety of tissues, including myocardium, platelets and adipose tissue.[7–9] They are characterized by high affinities for both cAMP and cGMP, with V_{max} for cAMP 4–10 times higher than that for cGMP and K_m values in the range of 0.1–0.8 μM, and competitive inhibition of its cAMP hydrolytic activity by cGMP, cilostamide and other drugs that increase myocardial contractility and inhibit platelet aggregation.[7–9] The two PDE3 isoforms, PDE3A and 3B, are products of distinct but related genes and differentially expressed in a variety of cells and tissues. Their deduced sequences indicate that the C-terminal regions of PDE3s contain the catalytic domain conserved among all mammalian PDEs, and that human and rat PDE3A (or 3B) are more similar than PDE3A and 3B from the same species.[7–12,19]

Recently, we first reported that human PDE3A and 3B mRNAs were expressed in human neoplastic submandibular gland intercalated duct HSG cells, and that the PDE3 inhibitor cilostamide inhibited the growth of these cells.[20] However, no data are available as to the expression of PDE3 isoforms in squamous cell carcinoma cells or the role of PDE3s in these cells. In this study, we examined both the expression and role of PDE3 isoforms in human squamous cell carcinoma KB cells.

Materials and methods

Cell line

Human squamous cell carcinoma KB cells[21] were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Life Technologies, Grand Island, NY). Media was changed 2 times per week.

PDE activity in cell extracts

KB cells were seeded at 1×10^6 cells/25 cm^2 flask (Nunc, Roskilde, Denmark). After 3 days, cells were

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washed twice with phosphate-buffered saline (PBS), harvested with a rubber policeman and homogenized in 2 ml of ice-cold homogenization buffer (100 mM TES, pH 7.4, 10 μg/ml each of pepstatin, leupeptin and aprotinin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, and 10% glycerol).

cAMP PDE assay

cAMP PDE activity was assayed by a modification of a previously described procedure.[22] Samples were incubated at 30°C for 10 min in a total volume (0.3 ml) containing 50 mM HEPES, pH 7.4, 0.1 mM EGTA, 8.3 mM $MgCl_2$ and 0.1 μM [^{3}H]cAMP (18 000 c.p.m.).

RT-PCR

Total RNA was isolated from KB cells (QuickPrep total RNA extraction kit; Amersham Pharmacia Biotech, Piscataway, NJ). First-strand cDNA was generated from total RNA using the Superscript preamplification system (Life Technologies). Two specific oligonucleotide primer sets were synthesized:[7,8,11,19] human PDE3A sense primer (5′-TCACCTCTCCAAGGGACTCCT), antisense primer (5′-CAGCATGTAAAACATCAGTGGC), human PDE3B sense primer (5′-AATTCTTCCAACCATCGACC), antisense primer (5′-GCTTGTAGCACATCTGTGGC). PCR amplification was carried out in a total volume of 50 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 U AmpliTag Gold (Perkin Elmer Applied Biosystems, Foster City, CA), and 0.8 μM sense and antisense primers. The PCR reaction was performed at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, for 35 cycles. Products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. No fragments were amplified in the absence of reverse transcriptase or RNA.

Expression of human PDE3A and 3B in Sf9 insect cells

Recombinant human PDE3A and 3B were expressed in Sf9 insect cells by a previously described procedure.[23,24] Sf9 cells (PharMingen, San Diego, CA) were maintained and propagated at 27°C in TNM-FH Insect Medium (PharMingen). Human PDE3A and 3B cDNA were inserted into pVL1393; transfer of human PDE3A and PDE3B from pVL1393 to *Autographa californica* nuclear polyhedrin virus was accomplished by homologous recombination using calcium phosphate transfection (Baculogold transfection kit; PharMingen). Supernatant containing recombinant virus was collected after 5 days and was amplified 2 times. A standard infection was carried out for 3 days with 3×10^6 Sf9 cells/25 cm^2 flask and 1 ml of supernatant that contained virus. Sf9 cells were collected and homogenized in 1 ml of homogenization buffer. Proteins in Sf9 cell lysates were used as positive control.

Immunoblotting

PDE3A. KB cells were homogenized in lysis buffer (100 mM TES, pH 7.4, 10 μg/ml each of pepstatin, leupeptin and aprotinin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, and 10% glycerol). Gel electrophoresis (8–25% polyacrylamide gels) and blotting were carried out in the PhastSystem (Amersham Pharmacia Biotech). Following electrophoresis, proteins were transferred to PVDF membranes and the membranes blocked by incubation with PBST (0.05% Tween 20 in PBS) supplemented with 10% powdered non-fat dry milk for 2 h. Blots were incubated with a primary antibody (polyclonal anti-PDE3A diluted 1:400 in PBST) overnight at 4°C and rinsed 3 times with PBST. Rinsed blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:6000 dilution) (Amersham Pharmacia Biotech) for 1 h, rinsed with PBST and immunoreactivity was detected by chemiluminescence using ECL Plus (Amersham Pharmacia Biotech) as per the manufacturer's recommendations.

PDE3B. KB cells were homogenized in buffer (100 mM TES, pH 7.4, 10 μg/ml each of pepstatin, leupeptin and aprotinin, 1 mM benzamidine, 0.5 mM. pebabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, and 10% glycerol) and centrifuged at 1000 g for 10 min.The supernatant was centrifuged at 100 000 g for 45 min at 4°C. Particulate fractions were suspended in lysis buffer. Gel electrophoresis and blotting were carried out as for PDE3A. Primary antibody (polyclonal anti-PDE3B) was diluted 1:200 in PBST.

Growth experiments. The cell survival was measured using the MTT assay, first described in 1983 by

Mosmann.[25] Briefly, KB cells were cultured with different concentrations of cilostamide in 96-well microplates for 7 days. Subsequently, the cells were incubated with 10 μl (5 mg/ml) of MTT for 6 h and after that with SDS for 6 h. Absorbance was measured at a wavelength of 540 nm.

Results

To test whether PDE3 is expressed in KB cells, we used the PDE3-specific inhibitor, cilostamide, and cGMP which is a competitive inhibitor of cGMP hydrolysis by PDE3. As shown in Figure 1, in extracts of KB cells, PDE activity was inhibited by cilostamide and cGMP, indicating the presence of PDE3 enzyme(s).

RT-PCR was performed on total RNA, using primer pairs specific for human PDE3A and 3B. PDE3A and 3B mRNAs were detected in RNA from KB cells (Figure 2); the nucleotide sequence of the amplified products was identical to that of PDE3A and 3B. No fragments were amplified in the absence of reverse transcriptase or RNA.

Several shorter types of PDE3A have been reported. Immunoblots of KB cell homogenates indicated the presence of 120-kDa PDE3A and 135-kDa PDE3B bands (Figure 3).

As there are no reports on PDE3s in KB cells, we examined function of PDE3s in KB cells. The PDE3-specific inhibitor, cilostamide, inhibited the growth of KB cells (Figure 4).

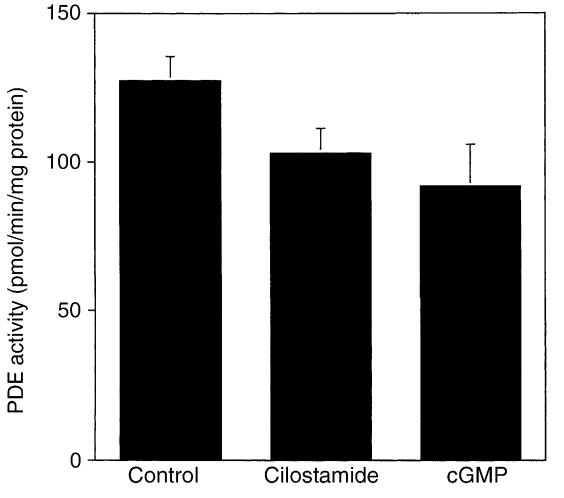

Figure 1. Effect of inhibitors on PDE activity in KB cells. Homogenates were prepared from the tumor cells and assayed for PDE activity with or without inhibitors as described in Materials and methods. Cilostamide (0.5 μM), cGMP (5 μM). Data are means $\pm$ SEM of three experiments.

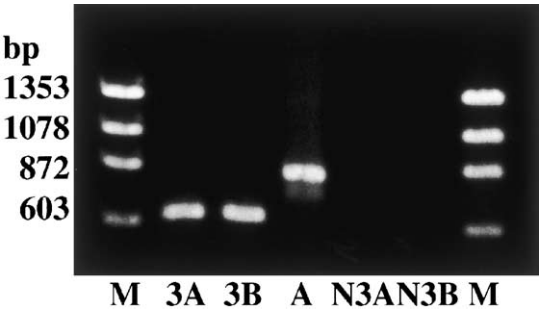

Figure 2. RT-PCR amplification of PDE3 mRNAs in KB cells. Total RNA was extracted as described in Materials and methods. cDNA was generated from 1 μg total RNA and amplified by PCR, using oligonucleotide primer sets based on sequences from PDE3A and 3B. The products were separated on agarose gels and photographed after ethidium bromide staining. PDE3A (3A), PDE3B (3B), β-actin (A), absence of RT: PDE3A (N3A), absence of RT: PDE3B (N3B), molecular markers (M).

Discussion

PDE3s are very sensitive to inhibition by certain drugs that augment myocardial contractility, and relax vascular smooth muscle such as cilostamide, enoximone and lixazinone.[7–9] cGMP is a competitive inhibitor of cAMP hydrolysis by PDE3. In extracts of KB cells, inhibition of PDE activity with cilostamide and cGMP suggested the presence of PDE3 enzyme(s).

Human PDE3A mRNAs are relatively highly expressed in heart, aorta and vascular smooth muscle; human PDE3B mRNAs, in rat adipose tissue[7–9] and 3T3-L1 adipocytes. However, there had been a few reports of expression of PDE3 mRNAs in tumor cells. Therefore, we were the first to report that human PDE3A and 3B were expressed in single-cell populations, human hepatocellular carcinoma Hep3B and HuH7 cells.[26] Furthermore, we demonstrated that human PDE3A and 3B were expressed and played an important role of the proliferation in human neoplastic submandibular gland intercalated duct HSG cells.[20] Using specific oligonucleotide primers, both PDE3A and 3B mRNAs were detected by RT-PCR in RNA from KB cells. These results were consistent with the inhibition of KB PDE activity by cilostamide and cGMP.

Two PDE3 subfamilies, PDE3A and 3B, had previously been cloned from human cardiac and human adipose tissue cDNA libraries.[11,19] PDE3A and 3B are products of distinct, but related genes. Human PDE3A cDNA encodes a protein of 1141 amino acids (around 125 kDa) and PDE3B cDNA encodes a protein of 1112 amino acids (around

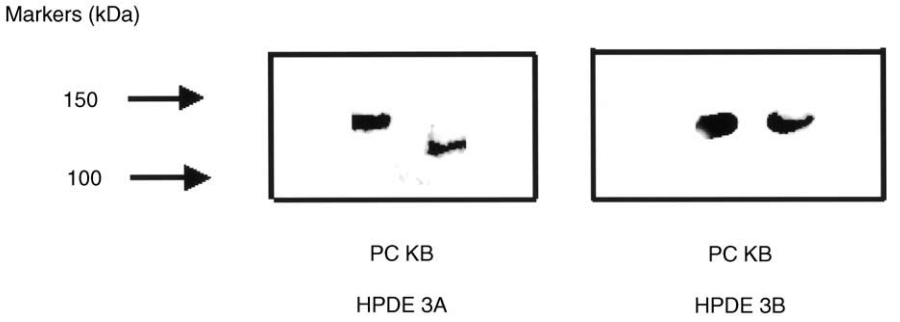

Figure 3. Immunoblot analysis of PDE3A (A) and 3B (B) in recombinant human PDE3 and KB cells. Samples were prepared as described in Materials and methods. Gel electrophoresis and blotting were carried out in the PhastSystem. Blots were incubated with an appropriate dilution of primary antibody (polyclonal anti-PDE3A 1:400 and 3B 1:200). Immunoreactivity was detected by chemiluminescence using ECL plus. The positions of molecular mass standards are indicated in kDa at the left.

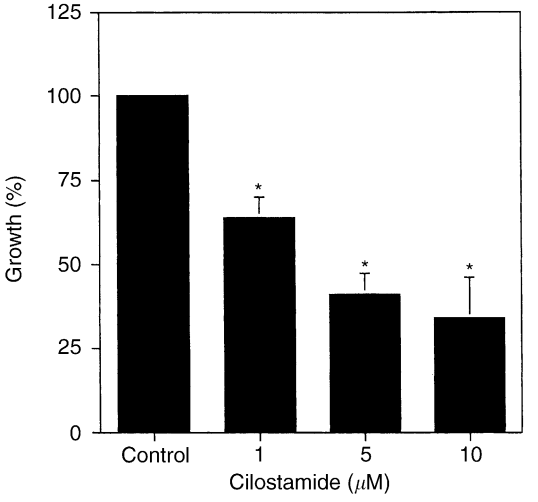

Figure 4. Effect of cilostamide on the growth of KB cells. Cells were plated in 96-well plates and cultured with the indicated concentrations of cilostamide. The cells were counted as described in Materials and methods. Data are means ± SEM of three different experiments. Significantly different from the control ($*p < 0.01$).

123 kDa).[11,19] Human PDE3A expression in *Spodoptera frugiperda* (Sf9) or NIH 3006 fibroblasts cells yields a protein that migrates with an apparent molecular mass of approximately 142 kDa on SDS–PAGE.[27] Analysis of the structure of human PDE3s indicated that a large N-terminal hydrophobic region containing six helical segments is thought to be involved in membrane association and a C-terminal catalytic region was highly conserved among phosphodiesterase families. Some shorter forms of PDE3A have also been identified. In mammalian myocardium, antibodies against PDE3A precipitate a 135-kDa phosphoprotein from microsomal fractions, a 116-kDa phosphoprotein from both microsomal and cytosolic fractions, and an 80-kDa phosphoprotein from cytosolic fractions.[27,28] PDE3A in human aortic myocytes is a 118-kDa protein that lacks N-terminal amino acids of the myocardial enzyme.[27] PDE3A in platelets is a 110-kDa cytosolic protein whose cDNA sequence is identical with that of the human myocardial PDE3A except for an apparent N-terminal deletion.[22,27,29] In this study, human PDE3A expressed in Sf9 cells was about 140 kDa and in KB cellswas about 120 kDa. PDE3A in KB perhaps lacks part of the N-terminal region as described for other PDE3A isoforms. However, very little is known about the characteristics and functional roles of the shorter (less than 135 kDa) forms of PDE3A found in KB cells. PDE3B in vascular smooth muscle might be the 125–135 kDa membrane-associated form.[27] Our results are consistent with previous data.

To establish whether PDE3 inhibitor might be useful as an antitumor drug in KB cells, we studied the effect of a PDE3-specific inhibitor, cilostamide, on the growth of KB cells. Cilostamide inhibited the growth of KB cells. Specific inhibitors of PDE3 inhibit proliferation of several types of cells.[30,31] Thus, inhibition of PDE3, which presumably results in an increase in cAMP (and/or cGMP), blocks proliferation of several types of cells. Many tumor cells have much lower basal levels of cAMP than normal cells and cAMP is a negative messenger for proliferation.[32] Increased intracellular cAMP also induces apoptosis in a subset of both normal and malignant lymphoid cells.[33]

Our results indicate that increased understanding of PDE3s in squamous cell carcinoma cells may be important for the development of therapeutic interventions involving disruption of cAMP signaling pathways.

Conclusion

PDE3s have been characterized in human squamous cell carcinoma KB cells. PDE3 activity was detected in homogenates of KB cells, and PDE3A and 3B mRNAs were detected by RT-PCR in RNA from KB cells. Immunoblotting experiments with anti-PDE3 antibodies allowed the detection of both PDE3A (120 kDa) and PDE3B (135 kDa) in KB cells. The PDE3-specific inhibitor, cilostamide, inhibited the growth of KB cells. Our data suggest that PDE3s may be important in the growth of KB cells, and PDE3 could be a potential target for therapy of human squamous cell carcinoma in head and neck.

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