Expression and Role of Phosphodiesterase 5 in Human Malignant Melanoma Cell Line

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Abstract. *Background: Eleven phosphodiesterase (PDE) gene families (PDE1-11) have been identified, and some PDE isoforms are selectively expressed in various cell types. Previously, we reported PDE1, PDE3 and PDE4 expressions in human malignant melanoma cells. However, the expression and role of PDE5 in malignant melanoma cells is not clear. Therefore, we characterized PDE5 in human malignant melanoma MAA cells. Materials and Methods: PDE5 activity and PDE5A mRNA expression were investigated in MAA cells. The full open reading frames for human PDE5A1 were sequenced. Effects of PDE5 inhibitors on cell growth were determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays. Results: PDE5 activity and PDE5A1 mRNA expression were detected in MAA cells. The nucleotide sequence of PDE5A1 was identical to that of human PDE5A1, previously published. Two PDE5 inhibitors inhibited the growth of cells. Conclusion: PDE5A1 mRNA is expressed and may play an important role in the growth of human malignant melanoma MAA cells.*

Eleven phosphodiesterase (PDE) gene families (PDE1-11) have been identified, and some PDE isoforms are selectively expressed in various tissues and cell types, but in different amounts, proportions and subcellular locations. All 11 PDE gene families encode proteins that exhibit a common structural organization, with a conserved catalytic domain in *C*-terminal portions and divergent regulatory modules and domains in *N*-terminal portions of the PDE molecules (1-4). By catalyzing the hydrolysis of cyclic nucleotides, PDEs regulate the intracellular concentrations and effects of these secondary messengers. Some PDE families are relatively specific for cAMP (PDEs 4, 7 and 8) or for cGMP (PDEs 5, 6 and 9); others hydrolyze both (PDEs 1-3, 10 and 11) (1, 2, 4).

PDE5 is relatively specific for cGMP and is expressed abundantly in vascular smooth muscle, including the pulmonary vasculature and corpus cavernosum of the penis. Three alternatively splicing variants of human *PDE5A* (*PDE5A1*, *PDE5A2* and *PDE5A3*) have been identified and their tissue distribution differs (2, 3, 5). PDE5 inhibitor sildenafil improves penile erection with a minimal risk of side-effects and adverse events in many men with erectile dysfunction (1-3, 5). However, the expression and role of PDE5 in human malignant melanoma cells is not clear. Therefore, we examined *PDE5* in human malignant melanoma MAA cells.

Materials and Methods

Cell culture. Human malignant melanoma MAA cells were established and maintained in RPMI 1640 containing 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA) at 37˚C in a humidified 5% CO_2 atmosphere in our laboratory (6).

cGMP PDE activity assay in cell extracts. The cells were seeded at 1×10^6 cells/25-cm^2 flask. After 3 days, the cells were washed twice with phosphate-buffered saline (PBS), harvested with a rubber policeman, and homogenized in ice-cold homogenization buffer (1 ml; 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). cGMP PDE activity was assayed by a modification of a previously described procedure (7). Samples were incubated at 30˚C for 10 min in a total volume of 0.3 ml containing 50 mM Hepes pH 7.5, 0.1 mM EGTA, 8.3 mM $MgCl_2$, and 0.5 μM [^{3}H] cGMP (18,000 cpm) with or without PDE5 inhibitor. PDE5 activity was measured as the cGMP PDE activity inhibited by 10 μM PDE5 inhibitor zaprinest (2, 5).

Reverse transcription polymerase chain reaction. The cells were seeded at 1×10^6 cells/25-cm^2 flask. After 3 days, total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany).

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Table I. *Primer sequences for PDE5A.*

Catalytic domain	
PDE5A-1	5'-ACTTGCATTGCTGATTGCTG-3'
PDE5A-2	5'-TTGAATAGGCCAGGGTTTTG-3'
PDE5A1	
HPDE5A-C	5'-GAGCACTGGTCCCCTTCAT-3'
HPDE5A1-2	5'-CGATCACTGGGACTTTACCT-3'
PDE5A2	
HPDE5A-C	5'-GAGCACTGGTCCCCTTCAT-3'
HPDE5A2-1	5'-TGCTATGTTGCCCTTTGGAG-3'
PDE5A3	
HPDE5A-C	5'-GAGCACTGGTCCCCTTCAT-3'
HPDE5A3-2	5'-AACATGACGGAACCTTGCCA-3'
Full length	
PDE5A1-F1	5'-AGGCCGAGTCCTGTTCTTCT-3'
PDE5A1-F2	5'-TGGATGTTGTTGATCCTTTCA-3'

First-strand cDNA was generated from total RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). PCR was performed with specific primer pairs for *PDE5A* (Table I). PCR amplification was carried out using HotStarTaq® Master Mix Kit (Qiagen) and 0.5 μM sense and antisense primers. HotStarTaq™ DNA Polymerase was activated by incubation at 95˚C for 15 min followed by 35 cycles of amplification (94˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min). Products were subjected to electrophoresis on 2% agarose gels and visualized by SYBR® Green I staining (Molecular Probes, Inc., Eugene, OR, USA).

Sequencing of PDE5A1. First-strand cDNA from MAA cells was used. PCR amplification was carried out using Easy-A® High-Fidelity PCR Cloning Enzyme (Agilent Technologies, Santa Clara, CA, USA) and 0.2 μM sense and antisense primers (*PDE5A1-F1* and *PDE5A1-F2*) for *PDE5A1* (Table I). The enzyme was activated by incubation at 95˚C for 2 min followed by 40 cycles of amplification (95˚C for 40 s, 60˚C for 30 s and 72˚C for 4 min). Products were subjected to electrophoresis on 1% agarose gels and visualized by SYBR® Green I staining (Molecular Probes). The PCR products were purified by GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd, Little Chalfont, UK) and verified by DNA sequencing.

Cell growth experiment. The cells were plated at 5×10^2 cells/well in a 96-well plate and allowed to adhere for 24 h. The cells were cultured in the presence or absence of different concentrations of PDE5 inhibitors (zaprinest (2, 5) or dipyridamole (2, 5)) for 5 days. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-^{2}H-tetrazolium, inner salt (MTS) assays were performed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), and the cell numbers were calculated. Data are evaluated using analysis of variance (ANOVA).

Results

PDE5 activity in MAA cells. To test whether PDE5 is expressed in human malignant melanoma MAA cells, we measured PDE5 activity. PDE5 activity is known to be inhibited by the PDE5 inhibitor zaprinest (2, 5). PDE5 activity was detected in MAA cells as being an average of 20.3±3.3 pmol/min/mg protein (±standard deviation).

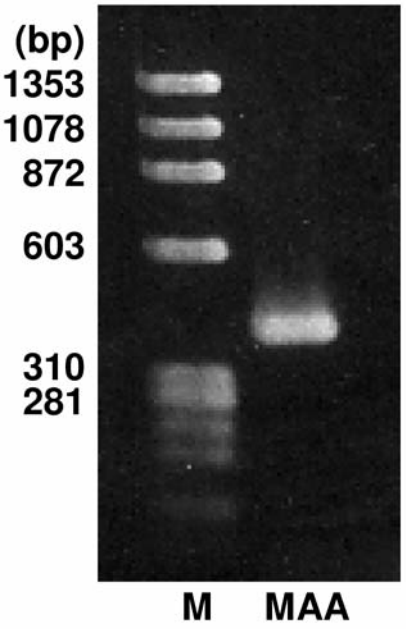

Figure 1. *Expression of PDE5A mRNA in human malignant melanoma MAA cells. Total RNA was extracted as described in the Materials and Methods. cDNA was generated from 1 μg total RNA and amplified by PCR, using oligonucleotide primer sets (catalytic domain) based on sequences from PDE5A. The products were separated on agarose gels and photographed after staining by SYBR® Green I staining. M, Molecular markers.*

PDE5A mRNA expression in MAA cells. Using specific oligonucleotide primers (Table I) based on published cDNA sequences, *PDE5A* mRNA was detected by RT-PCR (Figure 1). This findings are consistent with the detected *PDE5* activity. As three human *PDE5A* splicing variants (*PDE5A1*, *PDE5A2* and *PDE5A3*) have been reported (2, 3, 5), we investigated the expression of them in MAA cells using specific oligonucleotide primers (Table I). Only *PDE5A1* was detected (Figure 2).

Sequencing of PDE5A1. As only *PDE5A1* was detected, we examined the nucleotide sequences of *PDE5A1* from MAA cells. The nucleotide sequence of *PDE5A1* was identical to that of the previously published human *PDE5A1* (data not shown).

Cell growth experiments. As there have been no previous reports of PDE5A function in human malignant melanoma cells, we examined the effects of PDE5 inhibitors on the growth of MAA cells. The two PDE5 inhibitors used inhibited the cell growth of MAA cells in a dose-dependent manner (Figure 3).

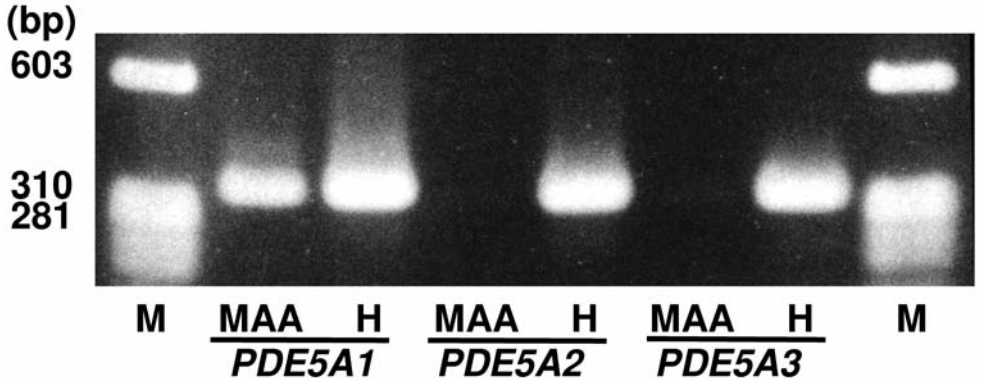

Figure 2. *Expression of PDE5A splice variants in human malignant melanoma MAA cells. Total RNA was extracted as described in the Materials and Methods. cDNA was generated from 1 μg total RNA and amplified by PCR, using oligonucleotide primer sets based on sequences from PDE5A1, PDE5A2 and PDE5A3. The products were separated on agarose gels and photographed after SYBR® Green I staining. M, molecular markers; H, human heart.*

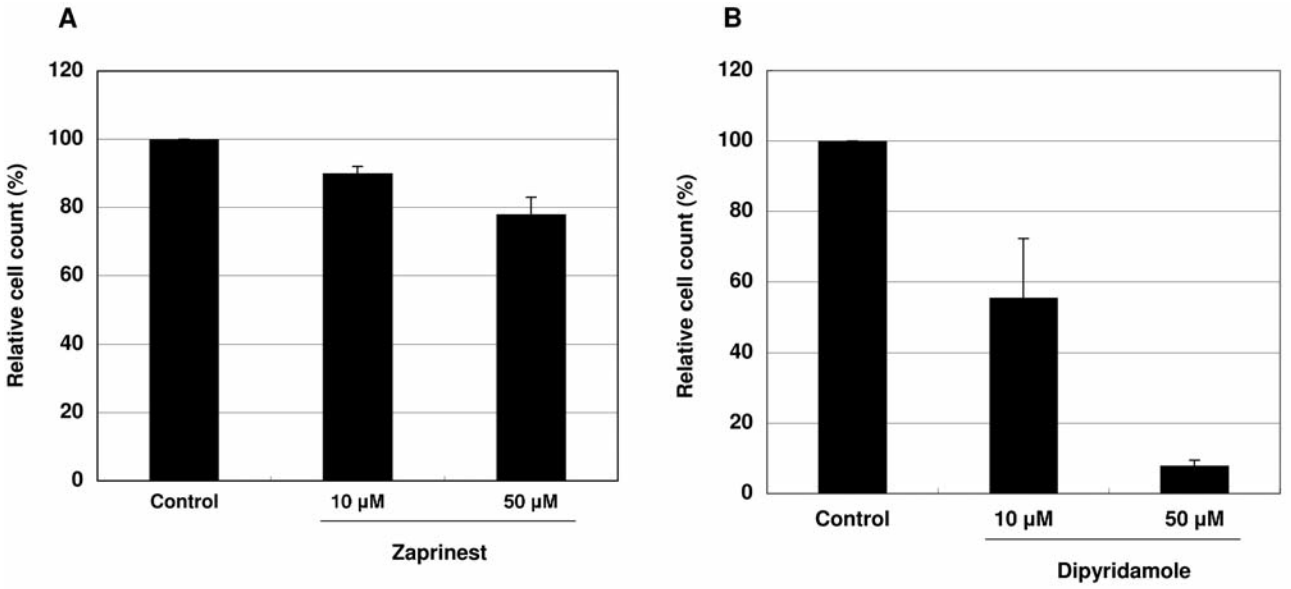

Figure 3. *Effect of the PDE5 inhibitors zaprinest (A) and dipyridamole (B) on the growth of human malignant melanoma MAA cells. Cells were plated in 96-well plates and cultured with different concentrations of inhibitor. The cells were counted as described in the Materials and Methods. Data are shown as the mean±S.D. of three different experiments. There was a significant ($p<0.001$) inhibition of the cell growth.*

Discussion

PDE5 activity was isolated from the supernatant fractions of mouse malignant melanoma B16 cells by Mono Q anion exchange column (8), human colon SW480 cells (9) and human bladder HT1376 cells (10) by DAEA Trisacryl M column. However, there has been no report of PDE5 expression in human malignant melanoma cells. Here, PDE5 activity was indeed detected in MAA cells, indicating the presence of PDE5 enzymes.

PDE5A mRNAs are expressed in malignant tumor cells: in human colon adenocarcinoma HT29 cells (11) and in mouse neuroblastoma N18TG2 cells (12). However, there was no data on *PDE5A* mRNA expression in human malignant melanoma cells. In this study, *PDE5A* mRNA was expressed in MAA cells (Figure 1). These data are consistent with the observed PDE5 activity. Three human *PDE5A* splice variants differ only in the 5' end of their respective mRNAs and the corresponding amino acid sequence at the extreme *N*-terminus in the protein products (5). In MAA cells, *PDE5A1*, but not *PDE5A2* or *PDE5A3*, was detected, and the nucleotide sequence of *PDE5A1* was identical to that of the previously published human *PDE5A1*. In human colonic T84 cells, *PDE5A1* and *PDE5A2* were cloned and a single base pair alteration (T152→C) was observed, which resulted in a change in the amino acid from valine to alanine at position 51 in the PDE5A2 protein sequence (13).

In human bladder HT1376 cells, the nonsteroidal anti-inflammatory drug, sulindac sulfone, induced apoptosis by inhibition of PDE5 (10). In addition, suppression of *PDE5* gene expression by antisense plasmid transfection inhibited growth and induced apoptosis in human colon tumor HT29 cells (11). However, the role of PDE5A in malignant melanoma cells is not known. As two PDE5 inhibitors inhibited growth of the MAA cells, PDE5A might regulate the growth of MAA cells.

In conclusion, these data suggest that *PDE5A1* is transcripted in human malignant melanoma MAA cells, and might have an important role in the growth of these cells.

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