Loss of 15-hydroxyprostaglandin dehydrogenase indicates a tumor suppressor role in pituitary adenomas

JI-WEI BAI[1*], ZHE WANG[1,2*], SONG-BAI GUI[3] and YA-ZHUO ZHANG[1]

[1]Beijing Neurosurgical Institute, Capital Medical University, Dongcheng District, Beijing 100050; [2]Department of Neurosurgery, Wei Fang People's Hospital, Weifang 261000; [3]Neurosurgical Department, Beijing Tiantan Hospital, Dongcheng District, Beijing 100050, P.R. China

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Abstract. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) may function as a tumor suppressor that antagonizes the action of the cyclooxygenase-2 (COX-2) oncogene in several types of tumors. However, it is unknown if it has a role in the pituitary. Recently, our group found that 15-PGDH expression was low in prolactin (PRL) secreting adenomas (prolactinomas) and growth hormone (GH) secreting adenomas (GHomas) using fiber-optic BeadArray technology. In this study, we examined the relative expression of 15-PGDH and COX-2 mRNA in clinical specimens and examined the effects of 15-PGDH on GH3 rat pituitary tumor cell proliferation, apoptosis and hormone secretion. 15-PGDH expression was lower and COX-2 expression was higher in prolactinomas and GHomas compared with normal controls. Overexpressed 15-PGDH inhibited tumor cell proliferation and induced apoptosis. It had a significant suppressive effect on mRNA levels and on the secretion of PRL and GH in GH3 cells. The inhibition of cell proliferation was accompanied by the decreased expression of COX-2, matrix metalloproteinase-9 (MMP-9) and B cell leukemia/lymphoma-2 (Bcl-2). These data are suggestive of a previously unrecognized pathway in pituitary tumorigenesis, and this novel observation may shed light on therapeutic strategies for pituitary tumors.

Introduction

Pituitary adenomas are the most common tumors that arise from anterior pituitary cells in the sella turcica, which represents up to 25% of brain tumors (1). Prolactin (PRL)secreting adenomas (prolactinomas) and growth hormone (GH) secreting adenomas (GHomas) are the most common types, accounting for 45% of all adenomas (2). During the past decade, significant advances have been made towards understanding the molecular mechanisms that regulate the development of pituitary adenomas and specific chromosomal alterations that lead to pituitary adenoma pathogenesis. However, the pathogenetic mechanisms that underlie pituitary adenomas are complex and remain enigmatic (1).

Recently, our group found several new candidate genes that suggested a role in the pathogenesis of prolactinomas and GHomas using a human genome-wide bead-based fiber-optic array (3,4). The HPGD is one of these genes, which is located on chromosome 4 and encodes a 29-kDa enzyme named 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The enzyme catalyzes the oxidation of the 15(S)-hydroxyl group of prostaglandins (PGs), resulting in the production of 15-keto-PGs and 15-keto-lipoxins, which have greatly reduced biological activities (5). Although previous studies on the distribution and activity of 15-PGDH have focused primarily on parturition and uterine biology, it is now recognized as a tumor suppressor and demonstrates antitumorigenic activity in various types of tumors including glioma, gastric, pancreatic, colon, breast, lung prostate and bladder tumors (6-13). COX-2, a rate-limiting enzyme in the arachidonic acid cascade, plays a key role in prostaglandin E_2 (PGE_2) biosynthesis. Overproduction of PGE_2 stimulates proliferation in various tumor cells, confers resistance to apoptosis in cancerous or transformed cells, and accelerates metastasis and angiogenesis. Excess PGE_2 undergoes metabolic inactivation catalyzed by 15-PGDH. 15-PGDH and COX-2 are reciprocally regulated in many tumor cells, although the molecular mechanism remains to be elucidated (13). Intense investigations of 15-PGDH have been conducted in many kinds of tumors and cell lines. However, its existence in the pituitary and its role in pituitary tumorigenesis have not been investigated.

In the present study, we examined the expression of 15-PGDH mRNA in clinical specimens and normal pituitaries and investigated the effects and possible mechanisms of 15-PGDH by transfecting GH3 cells with an expression plasmid encoding 15-PGDH in order to evaluate 15-PGDH effects on pituitary tumorigenesis.

Correspondence to: Professor Ya-Zhuo Zhang, Beijing Neurosurgical Institute, Capital Medical University, 6 Tiantan Xili, Dongcheng District, Beijing 100050, P.R. China
E-mail: zyz2004520@163.com

*Contributed equally

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Materials and methods

Clinical tissue specimens and normal pituitaries. Thirteen GHomas and eleven prolactinoma specimens were obtained from transsphenoidal surgery at Tiantan Hospital. Each tumor was classified according to the 2004 World Health Organization Classification of Endocrine Tumors guidelines (14). Due to the lack of a better control tissue available for human semi-quantitative real-time PCR (qPCR) study, normal pituitaries were chosen as control group despite the fact that normal pituitaries are not patient-matched. Five normal pituitaries were taken from autopsy cases that showed no evidence of endocrine diseases at 8-12 h after sudden death. All tissues were collected after written consent was obtained. The gender and age of the control group and of the patient groups are presented in Table I.

Cell culture, plasmid construction and transfection. The rat pituitary tumor-derived cell line GH3 is capable of secreting both PRL and GH. 15-PGDH expression levels were low in GH3 cells (data not shown). Therefore, GH3 cells were employed in this study and obtained from the Cell Center of the School of Basic Medicine, Peking Union Medical College. The cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Grand Island, NY, USA), and incubated at 37˚C in a humidified atmosphere of 5% CO_2.

The recombinant flag-tagged pcDNA3-PGDH encoding rat 15-PGDH cDNA was constructed in our laboratory. Briefly, a wild-type 15-PGDH cDNA was amplified from normal rat lung cDNA (amplification primer sequences were forward 5'-CGGGATCCATGCACGTGAACGGCAAAGTGGC-3', and reverse 5'-CCGCTCGAGTCATGGGGCTTTCGAAAAGG ATG-3'). The amplified 15-PGDH cDNA fragment and plasmid were digested by *Bam*HI and *Xho*I, and then ligated using T4-ligase into the HI-*Xho*I site of flag-tagged pcDNA 3.0 (kindly donated by Dr Xiang He). The recombinant vector named flag-tagged pcDNA3-PGDH was confirmed by restriction enzyme digestion and sequencing.

Lipofectamine 2000 transfection reagent was supplied by Invitrogen Life Technologies. Transfection of the recombinant vector or control empty vector was performed in accordance with the manufacturer's protocol. Briefly, cells were transferred to complete medium before transfection, and then a mixture of vector and Lipofectamine was added to the 25-cm^2 plates. After incubation for 4 h, the cells were incubated with fresh medium for the desired time period.

Plotting of cell growth curve. To obtain flag-tagged pcDNA3-PGDH transfected cells and the control group (cells transfected with empty flag-tagged pcDNA3.0 and cells without transfection), cells were seeded onto 24-well culture plates ($1x10^4$ cells/well). After transfection, three wells of each group were trypsinized and harvested every 12 h, and the viability and cell number of the samples were quantified under a light microscope after staining with trypan blue.

Cell inhibitory rate assays using WST-8. GH3 cells in log phase growth were washed twice with PBS, and plated on 96-well plates (5,000 cells/well) in 100 μl phenol red-free DMEM/F12 medium containing 10% FBS. Forty-eight hours after transfection, cell proliferation was assessed with the WST-8 cell staining kit (Neuronbc, Beijing, China). Briefly, 10 μl WST-8 solution was added to each well, followed by incubation for 4 h. The absorbance of each well was measured at 450 nm in a SpectraMax M5 multi-detection microplate reader (Molecular Devices, Sunnyvale, CA, USA). The cell inhibitory rate was calculated using the following equation: Inhibition rate (%) = $(OD_{control\ group} - OD_{drug\ group})/OD_{control\ group}$ x 100% (OD, optical density).

PGE_2, PRL and GH enzyme-linked immunosorbent assay (ELISA). Cells treated with empty vector, flag-tagged pcDNA3-PGDH plasmid or control solvent were plated onto 24-well plates ($3x10^4$ cell/well), and then cultured for 48 h in phenol red-free medium. The culture medium was collected from each well and centrifuged at 120 x g for 5 min at 4˚C. The supernatants were frozen at -80˚C until assay.

Secreted PGE_2, PRL and GH levels in the supernatants were measured using the ELISA kit (RapidBio, West Hills, CA, USA). The concentrations were calculated using a straight line regression equation of the standard curve with the standard density and the OD value. The concentrations were then normalized to viable cell numbers and expressed as % of control.

Measurement of apoptosis using propidium iodide (PI) and Annexin V double-staining flow cytometry. After transfection with flag-tagged pcDNA3-PGDH for 48 h, the cells were digested with trypsin to make a single-cell suspension, rinsed 3 times with PBS, resuspended in buffer, and adjusted to a density of $1x10^6$ cells/ml. PI and Annexin V were purchased from BD Pharmingen (San Jose, CA, USA). To 100 μl of cell suspension in each tube was added 15 μl of fluorescein-isothiocyanate-conjugated Annexin V and 10 μl of PI (20 μg/ml). The samples were stained at 4˚C for 20 min, and flow cytometry was used to examine cell apoptosis. A total of 10^4 cells were assayed for each sample.

SYBR-Green based qPCR analysis. The relative expression levels of several relevant mRNAs were tested in frozen clinical specimens and GH3 cells transfected for 48 h. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol and reverse-transcribed into cDNA using 1.5 μg of total RNA according to Quantscript RT Kit protocol (Tiangen Biotech, Beijing, China). The primers are listed in Table II. The qPCR reaction was performed using the SYBR-Green PCR kit on a Bio-Rad iCycler IQ Real-Time PCR Detection System according to the manufacturer's instructions in a 20 μl reaction containing the following: Master Mix (2X) 10 μl, ROXII (50X) 0.4 μl, primer F/R (0.4 μl each, 10 μmol/l), sample cDNA (1 μl), and MilliQ H_2O (7.8 μl). The amplification conditions were 94˚C for 15 min, followed by 40 cycles of 94˚C for 15 sec, 60 or 62˚C for 30 sec, and 72˚C for 30 sec. The relative expression levels of the analyzed genes were normalized to β-actin. The calculations were based on the cycle threshold (CT) value using the $2^{-\Delta\Delta CT}$ method for quantification (15).

Western blot analysis. The GH3 cells were transfected for 48 h, and the protein extracted from the cells was assayed

Table I. Characteristics of the three groups.

	Control group	GHomas group	Prolactinomas group
No. of patients	5	13	11
Gender (male/female)	3/2	6/7	6/5
Age (years)	21-43	26-59	24-56
Average age (mean ± SE)	31.2±9.86	44.23±9.68	41.8±10.08

No significant difference in gender and age among three groups was found (P>0.05). GHomas, growth hormone (GH) secreting adenomas.

Table II. Primers used in SYBR-Green-based semi-quantitative real-time PCR (qPCR) analysis.

Gene	Size (bp)	Forward sequence (5'→3')	Reverse sequence (5'→3')
Human PGDH	228	TCTGTTCATCCAGTGCGATGT	ATAATGATGCCGCCTTCACCT
Human COX-2	193	TAAACAGACATTTATTTCCAGAC	GAAAGAAATAGTCAATATGCTTG
Human β-actin	188	AGAAAATCTGGCACCACACC	AGAGGCGTACAGGGATAGCA
Rat COX-2	233	CCGGGTTGCTGGGGGAAGGA	CCACCAGCAGGGCGGGATACAG
Rat MMP-9	114	AAACATGCTGAAACCGGACC	GATCATCTCGGCTACCCTACCT
Rat Bcl-2	261	CACTGGCTTGACTGGCTGAA	CACAGACCTGGTTCGTGCTC
Rat β-actin	104	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACACA
Rat PRL	238	AGCCAAGTGTCAGCCCGGAAAG	TGGCCTTGGCAATAAACTCACGA
Rat GH	251	TATTGGGCAGATCCTCAAGC	CAAAGTGTAGGGGTGGCAGT

using western blot analysis. Bcl-2 and β-actin polyclonal antibodies were purchased from Abcam (Cambridge, UK). COX-2 and MMP-9 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The flag-tagged PGDH protein in GH3 cells exhibited a molecular weight of about 29 kDa. Monoclonal anti-flag antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used to detect flag-tagged PGDH. Horseradish peroxidase-labeled secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Briefly, experimental cells were lysed with cell lysis buffer, and their protein concentrations were determined using the BCA protein assay before separation by gel electrophoresis. Equal amounts of proteins were subjected to electrophoresis and then transferred onto polyvinylidene fluoride membranes. After transfer, the membranes were rinsed and incubated with blocking buffer (5% non-fat milk in TBST) for 1-2 h at room temperature. Membranes were then incubated overnight with primary antibodies at 4˚C, followed by three 10-min washes with TBST, and then incubation with secondary antibodies at room temperature for 1 h. After three 10-min washes with TBST, the antibody-antigen complex was detected with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis. All data are presented as mean ± standard error of the mean (SEM). All experiments were performed at least three times. Statistical analyses were performed using one-way ANOVA, the Student's t-test or the Kruskal-Wallis test (SPSS 13.0) and factorial design ANOVA (SAS 9.0). P<0.05 was assumed to be significant.

Results

Expression of 15-PGDH and COX-2 mRNA in clinical specimens. For further verification of our previous microarray results (3,4), 24 clinical specimens were analyzed with qPCR. There were no statistical differences in the age and gender distribution among the three groups (Table I, P>0.05). Possible effects of age and gender were ruled out. 15-PGDH mRNA was detected in all the normal controls, while it was detected in 5 of 13 GHomas, and 5 of 11 prolactinomas according to the melting curve. The 15-PGDH expression level was higher in normal control than in both the GHoma and prolactinoma groups (P<0.05, Fig. 1A) and there was no significant difference in 15-PGDH levels between the GHomas group and the prolactinomas group (P>0.05). COX-2 mRNA was detected in all specimens. All pituitary adenoma groups expressed higher COX-2 mRNA levels than the normal control group (P<0.05), and the subgroups of 15-PGDH-positive prolactinomas expressed higher COX-2 mRNA levels than the corresponding 15-PGDH-negative subgroups (P<0.05); although no significant difference was identified between 15-PGDH-positive GHomas and 15-PGDH-negative GHomas (P>0.05, Fig. 1B), COX-2 mRNA level were higher in 15-PGDH-positive GHomas.

Inhibitory effects of 15-PGDH on GH3 cell growth and proliferation. To observe the biological activity of 15-PGDH in GH3 cells, the flag-tagged pcDNA3-PGDH vector was transfected into GH3 cells. Forty-eight hours after transfection, 15-PGDH proteins were detected with anti-flag antibody

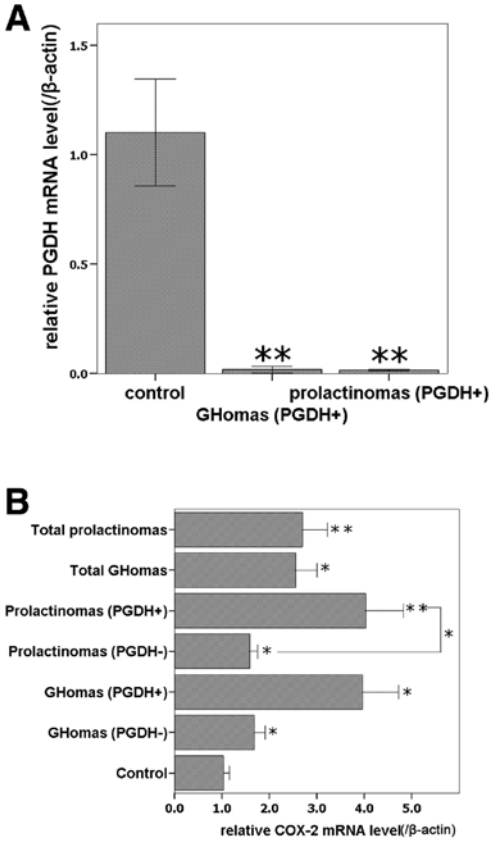

Figure 1. Relative (A) 15-PGDH and (B) COX-2 mRNA levels (normalized to β-actin) in clinical specimens and normal control. Control, normal pituitary glands; GHomas (PGDH$^+$), GHomas that expressed 15-PGDH mRNA; GHomas (PGDH$^-$), GHomas that were negative for 15-PGDH mRNA expression; prolactinomas (PGDH$^+$), prolactinomas that expressed 15-PGDH mRNA; prolactinomas (PGDH$^-$), prolactinomas that were negative for 15-PGDH mRNA expression; columns, mean from three experiments; bars, SEM; *P<0.05, **P<0.01 vs. the control group.

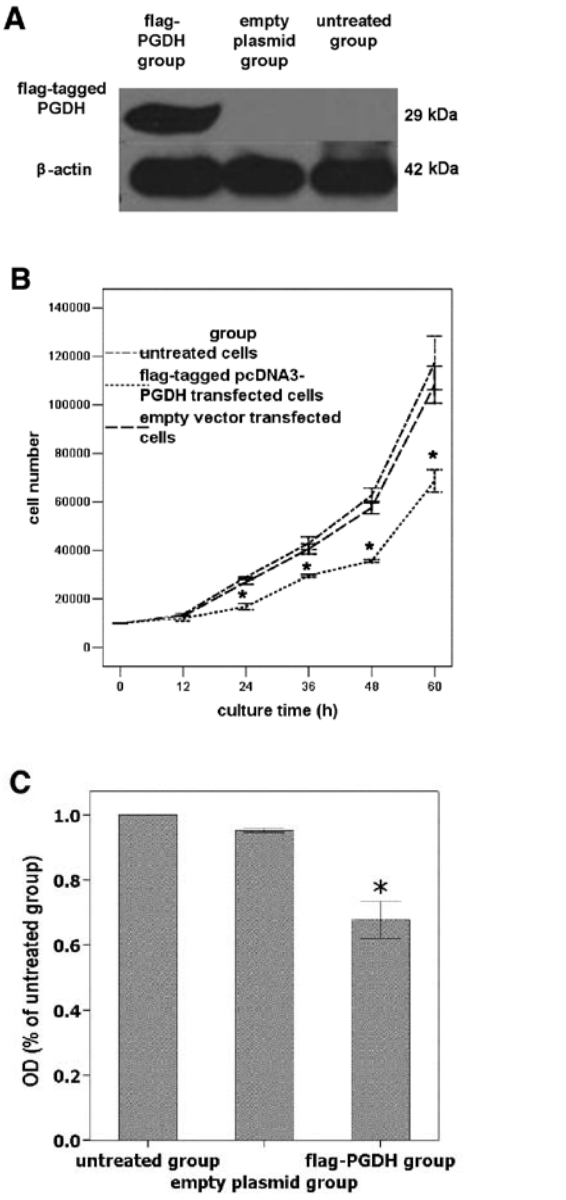

Figure 2. (A) Flag-tagged PGDH protein was detected with anti-flag antibody in rat flag-tagged pcDNA3-PGDH plasmid-transfected cells 48 h after transient transfection, while empty flag-pcDNA3 plasmid transfected cells and untreated cells were negative for flag-tagged PGDH protein. (B) Growth of GH3 cells were suppressed when 15-PGDH was overexpressed. (C) Effect of 15-PGDH on proliferation of GH3 cells using the WST-8 assay 48 h after transfection with rat flag-tagged pcDNA3-PGDH. Data are shown as percentages of the untreated control. *Statistical significance (P<0.05) vs. the corresponding untreated cells.

in the cells (Fig. 2A). The GH3 cell growth curve plotted for different treatment methods and different time points is shown in Fig. 2B. It indicates that forced overexpression of 15-PGDH suppressed cell growth 24 h after transfection (P<0.05).

As shown by the WST-8 assay results (Fig. 2C), transfection of GH3 cells with the 15-PGDH expression vector inhibited cell proliferation by 32.29±9.71% compared with untreated cells 48 h after transfection. Transfection with the empty expression vector generated a similar proliferation profile as the untreated cells, indicating that the suppression of tumor cell growth is specifically caused by 15-PGDH.

Overexpressed 15-PGDH promote apoptosis of GH3 cells. The flow cytometry results used to examine apoptosis are shown in Fig. 3. Compared with the control group (untreated cells), the overexpression of 15-PGDH resulted in increased numbers of both early-stage (8.4±0.38 vs. 22.15±4.67%, P<0.01) and late-stage apoptotic cells (0.12±0.08 vs. 0.68±0.19%, P<0.05).

Levels of PGE_2, PRL and GH are reduced by overexpressing 15-PGDH. To further investigate the activity of overexpressed 15-PGDH, PGE_2 levels in the culture medium were measured using an ELISA assay. As the expression of 15-PGDH protein increased, PGE_2 levels significantly decreased (Fig. 4A). The secreted PGE_2 levels of transfected cells were 60.1±23.91% of the untreated control group (P<0.05). PRL and GH expression were downregulated by 15-PGDH at both the secreted protein (Fig. 4B and C) and mRNA levels (Fig. 4D and E). Taken together, the data indicate that 15-PGDH protein was expressed and had a biological activity in GH3 cells after transfection.

Expressions of COX-2, MMP-9 and Bcl-2 were downregulated by overexpression of 15-PGDH in GH3 cells. To analyze the possible molecular mechanism of 15-PGDH, the relevant

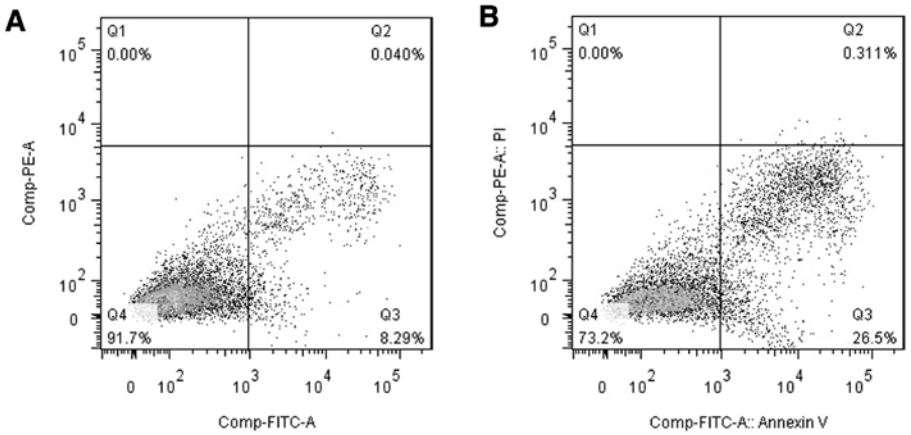

Figure 3. Flow cytometry analysis showing increased apoptosis after overexpressing 15-PGDH by transfection. (A) Untreated group (B) flag-tagged pcDNA3-PGDH plasmid transfected group. P<0.05 vs. the control group.

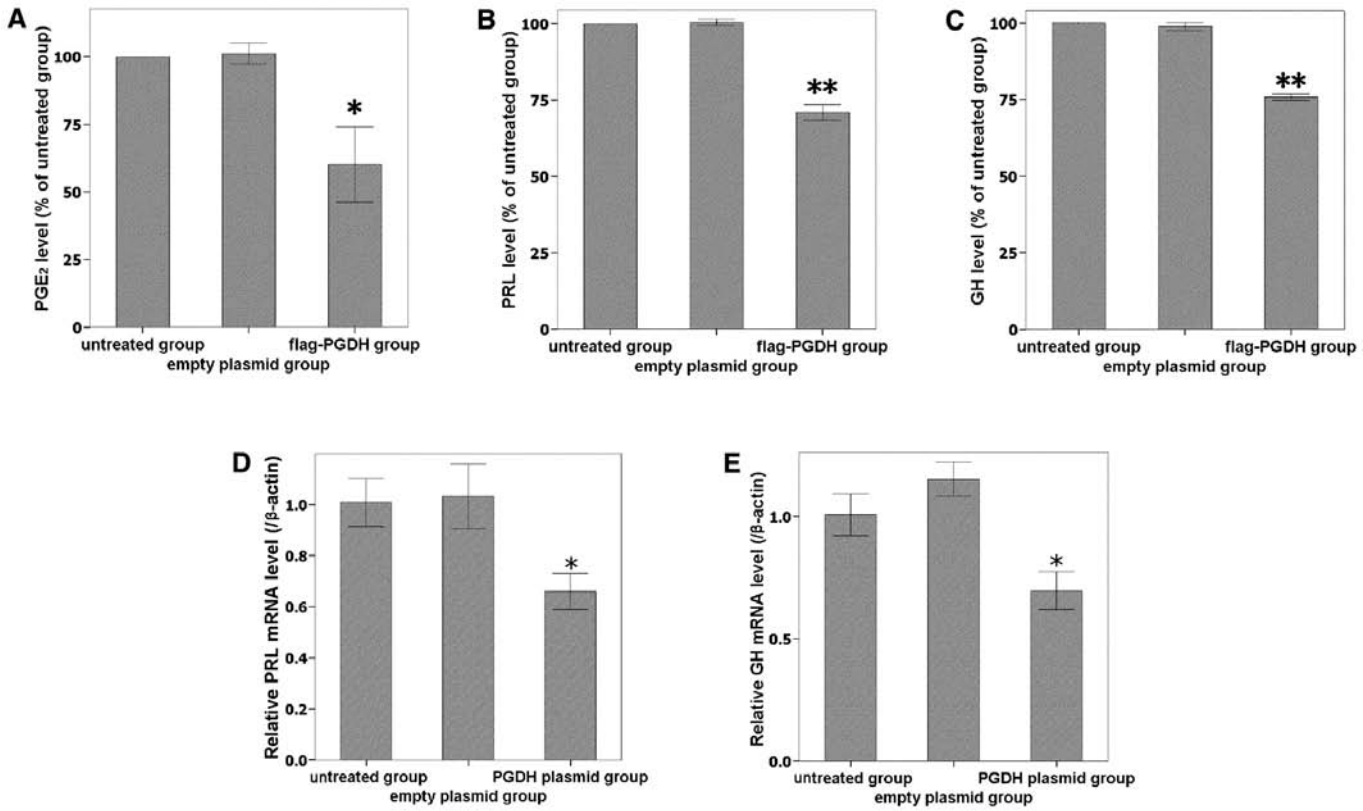

Figure 4. (A) PGE_2, (B) PRL and (C) GH secretion into the culture medium significantly decreased by 15-PGDH overexpression as measured by ELISA. (D) (D) PRL and (E) GH mRNA were downregulated after overexpressing 15-PGDH in GH3 cells as detected by semi-quantitative real-time PCR. Bars, mean of three experiments; error bars, SEM; *P<0.05, **P<0.01, vs. the untreated group.

genes including COX-2, MMP-9 and Bcl-2 were analyzed using qPCR and western blot analysis. COX-2, MMP-9 and Bcl-2 were all downregulated at both the mRNA (Fig. 5A-C) and protein (Fig. 5D) levels by the forced overexpression of 15-PGDH compared with untreated cells (P<0.05).

Discussion

15-PGDH is a member of the short chain dehydrogenase/reductase family which includes more than 60 different enzymes. The fact that 15-PGDH may function as a tumor suppressor has been exploited to examine the therapeutic efficacy *in vivo* of 15-PGDH-mediated cancer therapy (16). Although 15-PGDH appears to be widespread in mammalian tissues with relatively high activity in lung, kidney and placenta, there is no other research in relation to 15-PGDH and pituitary tumors until now. Low expression of 15-PGDH in pituitary tumors was reported by our group using fiber-optic BeadArray (3,4). In this study, we increased the number of specimens and tested the relative expression of 15-PGDH using qPCR. We

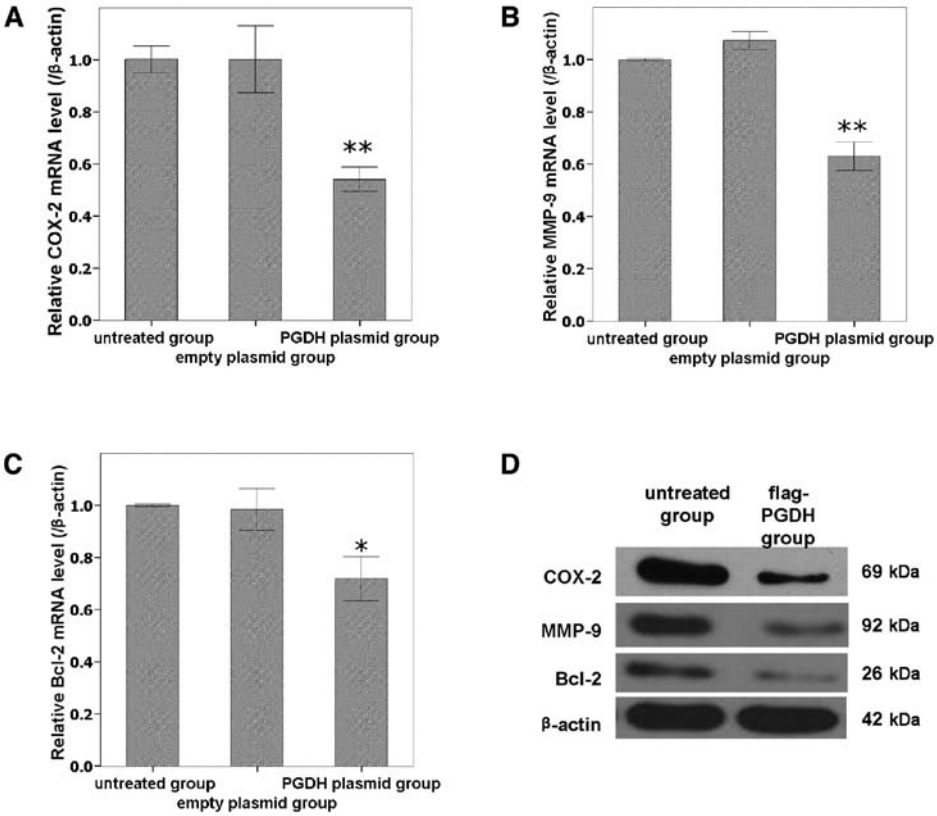

Figure 5. Expression levels of (A) COX-2, (B) MMP-9 and (C) Bcl-2 mRNA were downregulated after overexpressing 15-PGDH in GH3 cells as detected by semi-quantitative real-time PCR. $^{*}P<0.05$, $^{**}P<0.01$ vs. the untreated group. (D) COX-2, MMP-9 and Bcl-2 protein expression were downregulated by exogenous 15-PGDH after transfection as assessed by western blot analysis.

confirmed that 15-PGDH expression is low in both prolactinomas and GHomas. In addition, 15-PGDH was completely lost in some pituitary adenomas. As an inverse regulating enzyme of 15-PGDH, COX-2 was highly expressed in all pituitary adenomas, especially in 15-PGDH-negative specimens. Forced overexpression of 15-PGDH in GH3 cells resulted in significant growth inhibition and promotion of apoptosis and necrosis. These results indicate the importance of 15-PGDH in controlling the growth of pituitary tumor cells and pituitary adenoma pathogenesis. Accumulating evidence indicates that the downregulation of 15-PGDH in tumors is due to transcriptional repression and epigenetic silencing (10,17,18). Studying epigenetic mechanisms in clinical specimens and tumor cells may shed new light on therapeutic strategies for pituitary tumors.

COX-2 has been implicated in regulating tumorigenesis because it is overexpressed in many tumors, including pituitary adenomas (19). Expression of COX-2 has a wide range of biological activities, including increased angiogenesis, cellular proliferation and apoptosis inhibition. A strong correlation has been found between COX-2 expression and vascularization in pituitary tumors (19). Recently, R-flurbiprofen, a novel non-steroidal anti-inflammatory drug, was shown to decrease cell proliferation and to induce apoptosis in pituitary adenoma cells *in vitro*, which may function via COX-2 downregulation through inhibition of the upstream target, nuclear factor-κB (NF-κB) (20). COX-2 was downregulated by 15-PGDH in GH3 cells in the present study, which suggests that 15-PGDH functions via the COX-2 pathway in pituitary tumor cells. It strengthens the rationale for application of 15-PGDH to control pituitary tumors.

It was reported that PGE_2 enhanced PRL mRNA expression and PRL secretion in human endometrial stromal cells (21) and that PRL has a stimulatory effect on PGs in hamster Leydig cells (22). Therefore, PGE_2 and PRL may be reciprocally regulated in tumor cells. Furthermore, PGE_2 stimulates the release of GH and PRL from cultured bovine anterior pituitary cells (23). In the present study, PGE_2 secretion was reduced in the culture medium. Both mRNA expression and secretion of PRL and GH were also reduced after 15-PGDH overexpression. Taken together, it is possible that 15-PGDH may reduce PRL and GH by degrading PGE_2, and PGE_2 may be involved in endocrine disorders caused by prolactinomas or GHomas.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that mediate the degradation of the extracellular matrix. Vascularization and apoptosis was found to be linked to MMP-9 (24,25). GH3 cell proliferation and hormone secretion were inhibited in the presence of the broad range metalloproteinase inhibitor, batimastat (26). Taken together, these reports and our results support the notion that 15-PGDH inhibited MMP-9 expression and hormone secretion. It is possible that the inhibition of hormone secretion and the promotion of apoptosis in GH3 cells by 15-PGDH may be related to MMP-9 suppression.

The Bcl-2 family of proteins regulates various steps in apoptosis. Some of the members of this gene family, such as Bcl-2, block cell death. More than 70% of pituitary

adenomas express Bcl-2 (27,28). In this study, overexpression of 15-PGDH induced cell apoptosis and necrosis as confirmed by flow cytometry, and also downregulated Bcl-2 expression. Therefore, the death-promoting effects of 15-PGDH in GH3 cells may be partly exerted via Bcl-2.

In summary, we verified that 15-PGDH expression is lost or greatly reduced in prolactinomas and GHomas. Transfection of a rat 15-PGDH expression vector into GH3 cells resulted in a substantial inhibition of tumor cell growth, promoted apoptosis and inhibited hormone secretion. The data indicate that 15-PGDH may be regarded as a tumor suppressor in pituitary adenomas. The functional mechanism of 15-PGDH is attributed to the degeneration of PGE_2 and the inhibition of COX-2, MMP-9 and Bcl-2.

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