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1α,25-Dihydroxyvitamin D_3 enhances γ-glutamyl transpeptidase activity in LLC-PK1 porcine kidney epithelial cells

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Abstract. Mammalian γ-glutamyl transpeptidase (GGT) is expressed most highly in the kidney and serves to recover the constituent amino acids of glutathione in the proximal tubules. Serum GGT is used as a marker for obstructive jaundice and alcoholic liver disease and it has been reported that urinary GGT is a potential marker for bone resorption. The present study investigated the effect of derivatives of vitamin D3 on GGT activity in LLC-PK1 porcine renal tubular epithelial cells in order to analyze the biochemical basis of bone metabolism-associated alterations in GGT activity in the kidney. In the presence of 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2VD_3$], GGT activity was observed to be significantly increased in LLC-PK1 cells, with an increase in GGT activity also found in the cell medium. While the stimulatory effect of 1-OH-VD_3 was similar to that of $1,25(OH)_2VD_3$, vitamin D3 and 25-OH-VD_3 had no effect on GGT activity. The increased GGT activity caused by $1,25(OH)_2VD_3$ in LLC-PK1 cells was the result of long-term stimulation of the cells, in contrast to the GGT-induced increase in alkaline phosphatase, which is more transient. Polymerase chain reaction analysis revealed that the $1,25(OH)_2VD_3$-induced increase in GGT activity was due to prolonged GGT turnover, rather than increased GGT expression, as no increase in GGT mRNA expression was observed. Thus, it is likely that the expression of GGT is not entirely constitutive in the kidney, but is altered under certain conditions, including under hormonal regulation.

Introduction

Mammalian γ-glutamyl transpeptidase (GGT) has a role in glutathione metabolism by catalyzing the hydrolysis of a γ-glutamyl moiety of glutathione and associated compounds (1-3). GGT also catalyzes the transfer of γ-glutamyl groups from γ-glutamylated compounds, including that of glutathione to amino acids or dipeptides. The mammalian form of GGT is a membrane-bound glycoprotein with a typical type II membrane protein topology and is anchored to the extracellular surface of cell membranes through a non-cleavable N-terminal signal-anchor domain.

GGT activity is higher in the kidney, intestine and epididymis compared with other tissues and GGT is constitutively expressed in these tissues (4). A previous study using GGT-deficient mice revealed that GGT has an essential role in the kidney in the recovery of cysteine and cystine from glutathione that is excreted into the urine (5). However, while the activity of GGT is undetectable in the adult rat liver, it is relatively high in the fetal rat liver (6). GGT activity can be induced in the adult liver by various chemical compounds, including alcohol, xenobiotics and associated drugs. Furthermore, GGT expression is associated with hepatocarcinogenesis in rats (7-10). Thus, it is well established that GGT is of potential value in clinical chemistry for the diagnosis of certain types of hepatic disease.

Niida *et al* (11) reported that GGT is a bone-resorbing factor. This novel biological function of GGT does not depend on its enzymatic activity. GGT is capable of inducing osteoclasts by stimulating the expression of the receptor activator of nuclear factor-κB, which has further been confirmed using specific antibodies *in vivo* (12). Furthermore, an investigation using transgenic mice indicated that GGT overexpression accelerates bone resorption, leading to the development of osteoporosis (13). It has also been reported that significant increases in urinary GGT activity are associated with elevated bone resorption (14), suggesting that urinary GGT activity has potential to be a marker for bone resorption.

It is well established that among the various animal tissues, GGT is most abundant in the kidney, and that this expression is observed exclusively in the brush border membrane of the proximal tubular epithelial cells (15,16). Thus, increased urinary GGT activity, which may be associated with bone resorption, is likely to be due to enhanced GGT release from epithelial cells into the luminal space of the proximal tubules. However, whether kidney GGT is affected in conjunction with an alteration in extracellular signal molecules and the systemic metabolic status or others, has yet to be elucidated. The present study aimed to investigate the effect of vitamin D_3 and its

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metabolites, which are factors involved in bone metabolism, on GGT activity in LLC-PK1 pig kidney cells, which are cells derived from the proximal tubular epithelium.

Materials and methods

Cell line and cell culture. LLC-PK1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan) and were maintained at 37˚C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin under 5% CO_2 in humidified air.

GGT activity assay. GGT activity was assessed according to the method described by Tate and Meister (17). Transpeptidation reactions were performed at 37˚C using 1 mM γ-glutamyl-*p*-nitroanilide (donor substrate; Wako Pure Chemical Industries Ltd., Osaka, Japan) and 20 mM glycylglycine (acceptor substrate; Wako Pure Chemical Industries Ltd.), in 0.1 M Tris-HCl (pH 8.0). The release of *p*-nitroaniline was monitored spectrophotometrically at 410 nm and the activity was calculated using a molar extinction coefficient of 8.8.

Effect of vitamin D_3 and its derivatives on the enzyme activity of GGT in LLC-PK1 cells. In nearly confluent cultures of LLC-PK1 cells, the medium was changed immediately prior to the addition of the agent to be analyzed. Cells were cultured for the indicated durations in the presence of the agent. Vitamin D3 and its derivatives (Sigma-Aldrich, St. Louis, MO, USA) were added to the cells subsequent to being dissolved in ethanol, which was used as the vehicle. Parathyroid hormone (PTH; American Research Products, Waltham, MA, USA) was dissolved in phosphate buffered saline (PBS). Following culture for the specified durations, the medium was collected and the cells were harvested and washed two or three times using PBS. Cells were centrifuged at 150 x g for 5 min, then resuspended in a small volume of PBS. The cells were lyzed using sonication and were subjected to an enzyme activity assay.

Reverse transcription polymerase chain reaction (RT-PCR) analysis for GGT expression. Total RNA was extracted from the LLC-PK1 cells using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 0.5 μg total RNA using oligo dT primers and a ReverTra Plus RT-PCR kit (Toyobo, Osaka, Japan). PCR amplification of the 495 bp cDNA fragments using specific primers for pig GGT was performed using a Program Temp Control System PC-708 (Astec, Fukuoka, Japan) in a 50 μl reaction volume containing 1 μl cDNA, 1.0 U KOD-Plus (Toyobo), 1 mM $MgSO_4$, 0.2 mM deoxynucleotide triphosphates and 2% dimethyl sulfoxide in the buffer supplied along with the enzyme according to the manufacturer's instructions. The RT-PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide.

Protein concentration determination. Protein concentration was determined using a Bradford protein assay kit (Pierce Chemical Company, Rockford, IL, USA) with bovine serum albumin as a standard.

Statistical analysis. Data were analyzed by a t-test using Prism statistical software (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± standard deviation. $P<0.01$ was considered to indicate a statistically significant difference.

Results

Effect of vitamin D_3 and its derivatives on GGT activity in LLC-PK1 renal tubular cells. LLC-PK1 cells are a well defined renal tubule-derived cell line which exhibit high GGT activity (18,19), similar to the level observed in the proximal tubules in the kidney. As shown in Fig. 1A, the biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2VD_3$], also known as calcitriol, was found to significantly enhance GGT activity in the LLC-PK1 cells, while the non-hydroxylated vitamin D_3 had no effect on GGT activity. The 1α-monohydroxylated form of vitamin D_3 1-OH-VD_3 also appeared to increase GTT activity to an extent similar to that induced by $1,25(OH)_2VD_3$; however, this increase was not statistically significant due to experimental variations. After three days of culture, GGT activity was observed to be significantly higher in the medium of the cells treated with 1-OH-VD_3 and $1,25(OH)_2VD_3$, compared with those treated with the vehicle or the control cells (Fig. 1B).

A precursor of the active form of vitamin D_3, $25(OH)VD_3$, was found to have no effect on GGT activity in LLC-PK1 cells, although it is known that LLC-PK1 cells exhibit some $25(OH)VD_3$ 1α-hydroxylase activity (20). Thus, the levels of 1α-hydroxylase may have been insufficient to cause an increase in the levels of $1,25(OH)_2VD_3$ and stimulate GGT activity. Hydroxylase activity in the renal tubular epithelia is induced by PTH (23); however, the PTH receptor is not expressed in LLC-PK1 cells (21,22). In the present study, PTH was unable to stimulate GGT activity in the LLC-PK1 cells, even in the presence of $25(OH)VD_3$ (data not shown).

Dose-dependent effect of $1,25(OH)_2VD_3$ on GGT activity in LLC-PK1 renal tubular cells. LLC-PK1 cells were cultured for three days in the presence of various concentrations of $1,25(OH)_2VD_3$. The GGT activity in the cells and medium was then assessed. As shown in Fig. 2, GGT activity was found to be significantly increased by the active form of vitamin D_3 $1,25(OH)_2VD_3$ at all concentrations examined, compared with the cells treated with the vehicle. This increase in cellular GGT activity was associated with a significant increase in GGT secretion into the medium. Concentrations of $1,25(OH)_2VD_3$ as low as 10 nM, were sufficient to stimulate GGT activity in the LLC-PK1 cells and to facilitate GGT secretion into the medium.

Time-dependent effect of $1,25(OH)_2VD_3$ on GGT activity and mRNA expression in LLC-PK1 renal tubular cells. To further analyze the stimulation of GGT activity by $1,25(OH)_2VD_3$, a time course for $1,25(OH)_2VD_3$-induced GGT activity was performed. As shown in Fig. 3, $1,25(OH)_2VD_3$ was observed to stimulate an increase in GGT activity in LLC-PK1 cells over a relatively long duration of time. Thus, the stimulatory effect of $1,25(OH)_2VD_3$ was not transient, but appeared to be continuous, in contrast to the $1,25(OH)_2VD_3$-induced alkaline phosphatase (ALP) activity reported in a previous study (24).

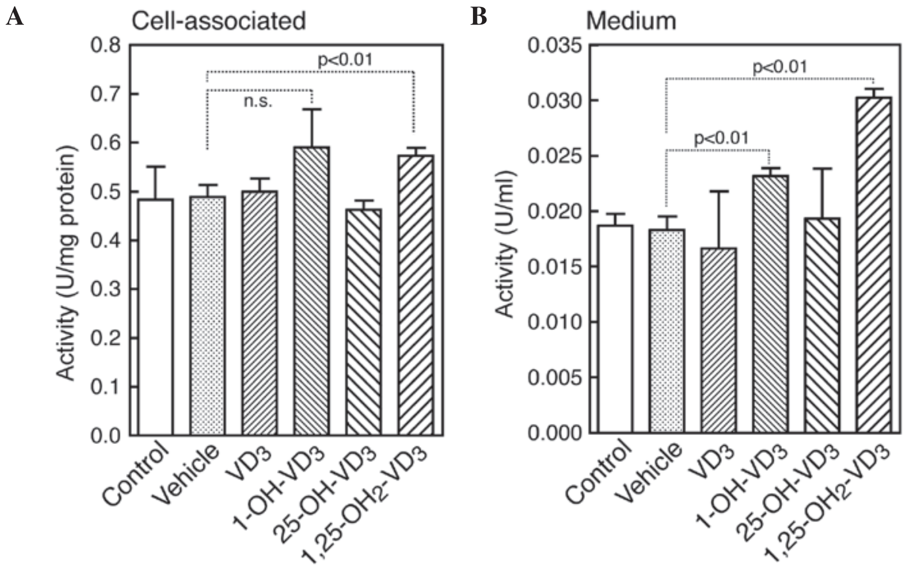

Figure 1. Stimulatory effect of $1,25(OH)_2VD_3$ on GGT activity in LLC-PK1 cells. LLC-PK1 cells were cultured for three days in the presence of various derivatives of vitamin D_3. The vitamin D_3 derivatives were dissolved in ethanol as a vehicle and added to the cells at a final concentration of 100 μM. Ethanol concentrations were 1% (v/v) in all cultures except the control. GGT activities in (A) cell homogenates and (B) cell media were analyzed. Data are presented as the mean ± standard deviation (n=3/group). GGT, γ-glutamyl transpeptidase; $1,25(OH)_2VD_3$, 1α,25-dihydroxyvitamin D3.

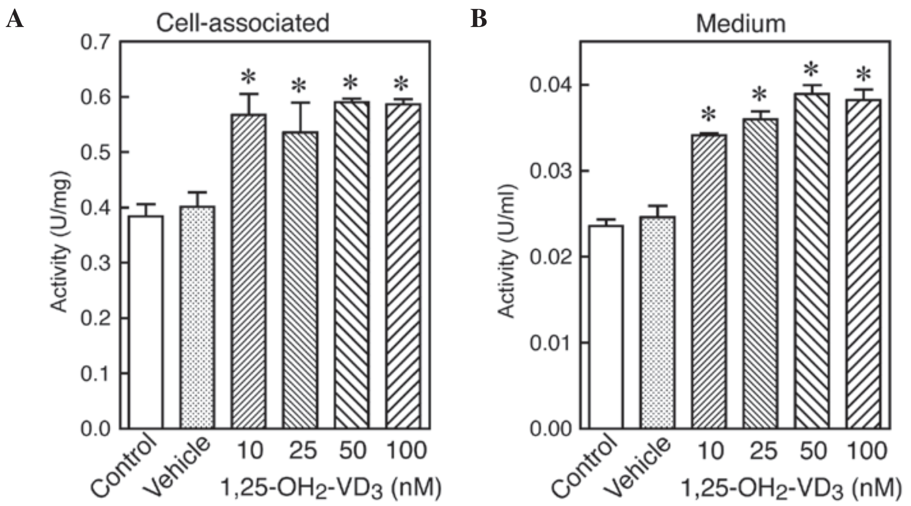

Figure 2. Dose-dependent effect of $1,25(OH)_2VD_3$ on GGT activity in LLC-PK1 cells. LLC-PK1 cells were cultured in the presence of various concentrations of $1,25(OH)_2VD_3$ and GGT activity was assessed in the (A) cell homogenates and (B) cell media. *P<0.01 vs. vehicle. GGT, γ-glutamyl transpeptidase; $1,25(OH)_2VD_3$, 1α,25-dihydroxyvitamin D3.

Furthermore, PCR was used to analyze GGT mRNA expression in the LLC-PK1 cells, 24, 48 and 72 h after the addition of $1,25(OH)_2VD_3$. Fig. 4 shows that after 72 h of culture with $1,25(OH)_2VD_3$, no significant increase in GGT mRNA expression was observed compared with the control and vehicle cells. The same findings were observed for the cells that had been treated with $1,25(OH)_2VD_3$ for 24 and 48 h (data not shown). Thus, it is likely that the increase in GGT activity caused by $1,25(OH)_2VD_3$ is due to a prolonged GGT protein turnover rather than enhanced GGT biosynthesis.

Discussion

GGT expression, which is associated with carcinogenesis in the liver and other tissues, has been extensively investigated (1). By contrast, alterations in GGT activity in the kidney have not been investigated, which is likely to be due to the fact that GGT expression is relatively high and stable in the kidney. It has been reported that urinary GGT may be a potential marker for enhanced bone resorption (14), but little is known about the biochemical basis of the mechanism involved in the stimulation of renal GGT activity and how GGT is secreted into the urine. Therefore, the present study aimed to investigate the effect of factors involved in bone metabolism on GGT activity in renal epithelial cells.

ALP is also expressed in the proximal tubules in the kidney (25,26). Do Thanh *et al* (24) reported that $1,25(OH)_2VD_3$ causes a rapid and transient increase in ALP activity in LLC-PK1 cells, with an almost two-fold increase observed 6 h after the addition of $1,25(OH)_2VD_3$. However,

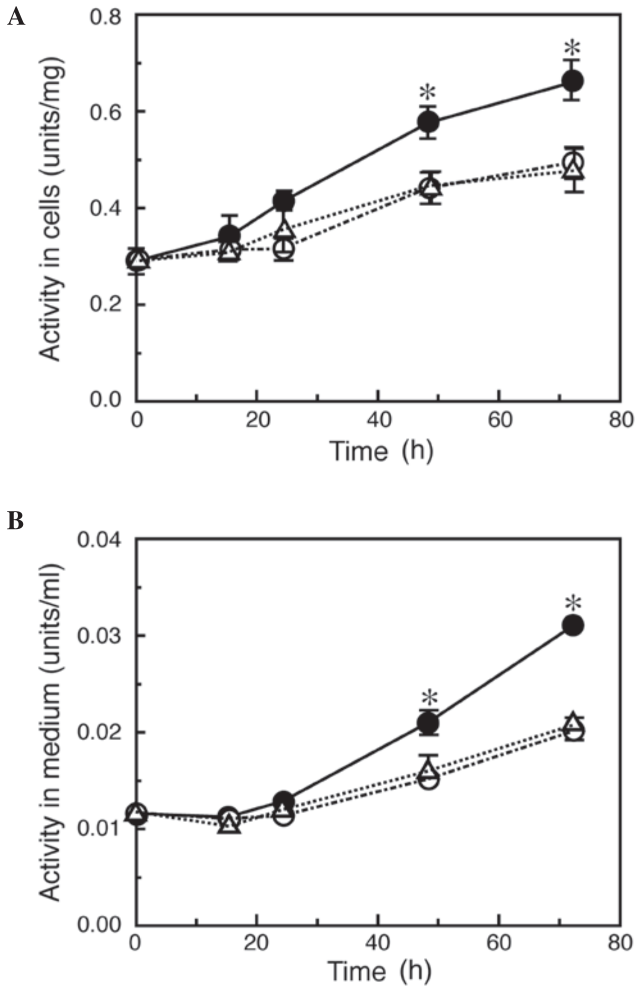

Figure 3. Time course of the effect of 1,25(OH)$_2$VD$_3$ on GGT activity in LLC-PK1 cells. LLC-PK1 cells were cultured in the presence of 100 μM 1,25(OH)$_2$VD$_3$ for the indicated durations and GGT activity was assessed in the (A) cell homogenates and (B) cell media. Open circles, control; closed circles, 1,25(OH)$_2$VD$_3$; and open triangles, vehicle. Data are presented as the mean ± standard deviation (n=3/group). *P<0.01 vs. vehicle. GGT, γ-glutamyl transpeptidase; 1,25(OH)$_2$VD$_3$, 1α,25-dihydroxyvitamin D3.

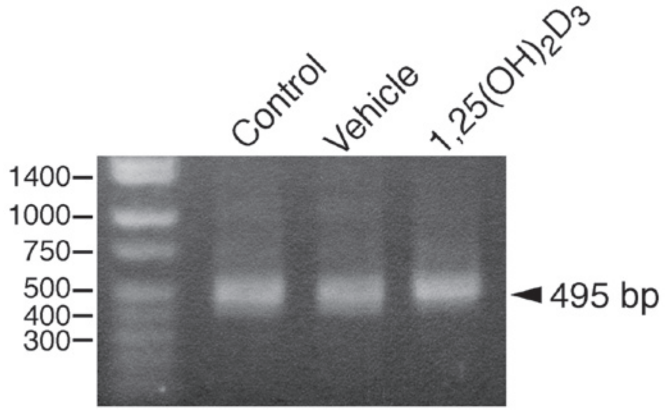

Figure 4. PCR analysis of GGT expression in LLC-PK1 cells. The 495 bp DNA fragments for the coding region of porcine GGT were amplified using PCR with specific primers. The left lane is a DNA size marker. PCR, polymerase chain reaction; GGT, γ-glutamyl transpeptidase; 1,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D3.

no significant increase was observed for GGT activity in the same time course (24). The findings of the present study contradict the lack of GGT activity induced by 1,25(OH)$_2$VD$_3$ reported by Do Thanh *et al* (24). In the present study, GGT activity was found to be stimulated by long-term exposure to 1,25(OH)$_2$VD$_3$. However, the time courses for the enhanced activities induced by 1,25(OH)$_2$VD$_3$ vary between ALP and GGT, suggesting that GGT activity is stimulated by a different mechanism to ALP activity. Although both GGT and ALP are expressed in the proximal tubules of the kidney, their distributions differ (27). Thus, the tubules may be partitioned into four segments according to the distribution of ALP and GGT.

A similar effect of 1,25(OH)$_2$VD$_3$ on GGT activity has been reported in the rat brain (28). In rat astrocytes, 1,25(OH)$_2$VD$_3$ was found to increase GGT activity alone or through potentiating the stimulatory effect of lipopolysaccharides (29). The increased activity of GGT observed in rat astrocytes facilitated glutathione synthesis by supplying constituent amino acids. Thus, increases in GGT activity may have a role in astrocyte detoxification pathways against oxidative stress. Extracellular glutathione is degraded by a series of reactions that are initiated by the hydrolysis of the γ-glutamyl group of glutathione by GGT. The resultant free amino acids are recovered by cells and utilized for the resynthesis of glutathione (1-3). In rat astrocytes, it is likely that GGT activity was stimulated by 1,25(OH)$_2$VD$_3$ either in a short- or long-term manner (28-29).

A previous study analyzed the biomarker potential of urinary GGT using a bone resorption mouse model and revealed that intravenous administration of PTH significantly increased urinary GGT activity (14). This *in vivo* effect of PTH may result from the direct induction of GGT in renal tubules; however, such an effect was not observed in the present study due to the lack of PTH receptor in LLC-PK1 cells (21,22). At present, whether the effect of PTH on GGT activity depends on a mechanism that involves tissues or organs other than the proximal tubular cells has yet to be elucidated. In addition, the specific origin of urinary GGT is unknown, but it is possible that some urinary GGT is derived from circulating GGT in the blood.

The findings of the present study suggested that 1,25(OH)$_2$VD$_3$ stimulates GGT activity in renal proximal tubular cells. This increased activity may, at least in part, modulate glutathione metabolism in the kidney. Furthermore, in the present study, the 1,25(OH)$_2$VD$_3$-induced increases in cellular GGT were associated with increases in GGT secretion into the cell medium. The findings of the present study and those of previous reports suggest that urinary or renal GGT activity may be responsive to the status of bone metabolism. Further investigations are required to provide further evidence for the use of GGT as a biomarker, which may contribute to the diagnosis and monitoring of certain bone diseases, including osteoporosis and abnormal bone resorption.

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