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Increased Activity and Intranuclear Expression of Phospholipase D2 in Human Renal Cancer

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We examined the PLD activities of human renal cancers and found that the PLD2 activity was greatly elevated in almost all cases examined as compared with the adjacent normal region. Western blot analysis showed the increased levels of PLD2 protein, but the PLD1 was not discernible. The oleate-dependent PLD activity was very low but appeared to increase in most cases. Interestingly, the immunohistochemical observations indicated the high expression of PLD2 in the nuclei of clear carcinoma cells. This is the first demonstration which suggests the possible involvement of PLD2 in tumorigenesis of renal cancer. © 2000 Academic Press

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Lipid signaling via various phospholipases plays important roles in the regulation of cell responses to external stimuli, including cell proliferation, transformation and apoptosis. Phospholipase D (PLD, EC 3.1.4.4) has been implicated as a novel player in the transmembrane signaling in a variety of cell types (1, 2). This enzyme hydrolyses phosphatidylcholine (PC) to generate phosphatidic acid (PA) which is subsequently converted to lyso PA (LPA) by phospholipase A_2 (PLA_2) or to diacylglycerol by phosphatidate phosphohydrolase (PAP). Both PA and LPA are mitogenic in several cell lines and are thought to be involved in cell proliferation (3, 4). There are three types of PLD: PLD1, PLD2, and oleate-dependent PLD (putative PLD_{OA}). PLD1 and PLD2 have been characterized at the molecular level (5–7), whereas PLD_{OA} (8) has not been established yet. Both PLD1 and PLD2 activities require phosphatidylinositol 4,5-bisphosphate (PIP_2) as a cofactor. The former is activated by small GTP-binding proteins such as Arf and Rho family proteins and also protein kinase C_α (PKC_α), whereas the latter is not activated by these factors and its activation mechanism remains to be defined. Unsaturated fatty acids, especially oleate, is a stimulator for the PLD_{OA} activity (8–10), but PLD_{OA} has not yet been characterized at the molecular level. Recently, it was reported that the PLD_{OA} activity was elevated in human breast cancer (11), human gastric carcinoma (12) and experimental colon cancer (13). The ratio of its activity in cancer and normal tissue was relevant to the nuclear grade in human breast cancer (11) and to tumor size in human gastric carcinoma (12).

The present study was designed to gain insight into the possible involvement of PLDs in tumorigenesis and promotion, and we demonstrate here for the first time that the PLD2 activity is increased in human renal cancer, and furthermore that PLD2 is highly expressed in the nuclei as judged by immunohistochemical study.

MATERIALS AND METHODS

Samples and reagents. Surgical specimens of human renal cancers and adjacent normal tissues were stored immediately at −80°C and used for PLD assay and Western blot analysis. [2-Palmitoyl-9,10-^{3}H]dipalmitoyl phosphatidylcholine [^{3}H-DPPC] (37.5 Ci/mmol) was obtained from New England Nuclear. PIP_2 and phorbol myristate acetate (PMA) were purchased from Sigma. PC, phosphatidylethanolamine (PE) and sodium oleate were from Funakoshi. GTPγS was from Boehringer Mannheim.

Measurement of PLD activity. For preparation of the membrane fraction, the specimens were washed twice with lysis buffer [5 mM $MgCl_2$, 5 mM Tris HCl, pH 7.4, 5 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 30 μg/ml (L-3-trans-carboxyoxirane-2-carbonyl)-L-leucylagmatine (E64), and 0.3 mM phenylmethylsulfonyl fluoride (PMSF)], and were then homogenized in lysis buffer. Homogenates were subject to centrifugation at 900*g* for 5 min, and the resulting supernatant was further centrifuged at 100,000*g* for 30 min to obtain the membrane fraction (14). The PLD activities *in vitro* (GTPγS-dependent, GTPγS-independent, and oleate-dependent) were measured as previously described (8, 15, 16). Briefly, for the assay of

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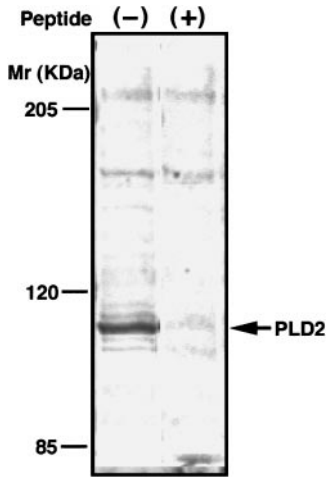

FIG. 1. Western blot analysis of mPLD2-overexpressed COS-7 cells. Cell lysates were subject to Western blotting with anti-PLD2 antibody (Ab-58) preincubated with or without the peptide of PLD2 residues 523–534.

GTPγS-dependent and -independent PLD activities, substrates of mixed lipid vesicles (PE/PIP_2/eggPC, 160/14/10, μM) containing [^{3}H] DPPC were added to homogenates and [^{3}H] phosphatidylbutanol ([^{3}H]PBut) formation was measured in the presence or absence of GTPγS (30 μM) and PMA (100 nM). For the assay at oleate-dependent PLD (PLD_{OA}) activity, substrates of egg PC vesicles containing [^{3}H] DPPC were added to membranes, and [^{3}H]PBut formation was measured in the presence of sodium oleate.

Western blot analysis. Membrane protein samples were separated by SDS–PAGE on 6% gels, and transferred onto nitrocellulose membranes. After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the nitrocellulose membranes were reacted overnight at 4°C with the rabbit polyclonal antibodies against the carboxyl-terminal 15 residues of human PLD1a (Ab-224) and the peptide corresponding to human PLD2 residues 523–534 (Ab-58) (6, 17). The membrane was then washed three times with TPBS and further incubated with alkaline phosphatase-conjugated anti-rabbit antibody (New England Biolabs) at room temperature. The immunoblot was visualized by the enhanced chemiluminescence detection kit (New England Biolabs). To assess the specificity of the anti-PLD2 antibody (Ab-58), PLD2-overexpressed COS-7 cell lysates were subject to Western blotting with anti-PLD2 antibody (Ab-58) preincubated with the peptide which was used to raise the antibody. After this treatment, the dense band corresponding to PLD2 was no longer discernible (Fig. 1).

Immunohistochemistry. The 4-μm sections from paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. After pretreatment of the sections, they were incubated with rabbit polyclonal anti-PLD2 antibody diluted at 1:200 in a moist chamber overnight at 4°C. After washing in 0.01 M PBS, the sections were incubated for 30 min at room temperature with biotinylated goat anti-rabbit immunoglobulin antibody. Then, peroxidase-conjugated streptavidin (Vector Laboratories) was applied. After washing out the excess complex, the localization of immunoreactive complexes was visualized after incubation of the sections for 5–10 min in 50 mM Tris–HCl (pH 7.6) containing 0.02% (w/v) 3,3′-diaminobenzidine tetrahydrochloride and 0.03% (v/v) hydrogen peroxide. To confirm the signal, anti-PLD2 antibody (Ab-58) was preincubated with the peptide corresponding to human PLD2 residues 523–534. Normal rabbit preimmune serum was applied onto slides in each staining as a negative control. Counter staining was performed with hematoxylin.

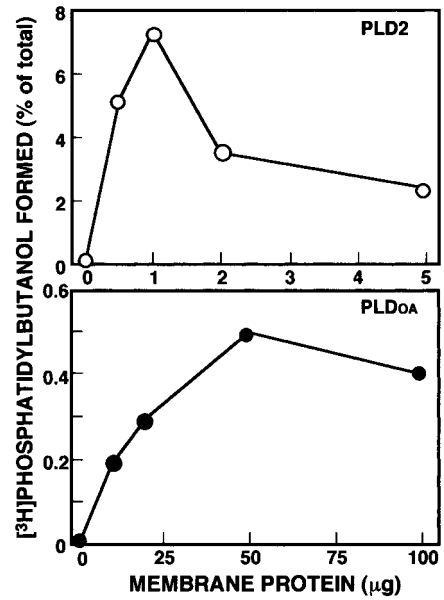

FIG. 2. Activities of membrane-associated PLD2 and PLD_{OA} in normal renal tissues. The activities were measured at indicated amounts of membrane protein as described under Materials and Methods.

RESULTS AND DISCUSSION

We examined which type of PLD activity was present in normal renal tissue. There was no significant GTPγS-dependent/PIP_2-dependent activity (PLD1) (data not shown). Moreover, expression of PLD1 was not detectable by Western blot analysis using anti-PLD1 antibody. In contrast, GTPγS-independent/PIP_2-dependent activity (referred to as PLD2 thereafter) was exceedingly high. Western blotting with anti-PLD2 antibody (Ab-58) exhibited the major dense band of PLD2. The PLD2 activity was increased with a maximum at 1 μg protein (Fig. 2). The oleate-dependent (PLD) (PLD_{OA}) activity was very low but showed a dose-dependent increase up to 50 μg. Thus, we used 1 μg and 50 μg membrane protein to measure the PLD2 and PLD_{OA} activities, respectively. The activities of PLD2 and PLD_{OA} and the clinicopathologic features are

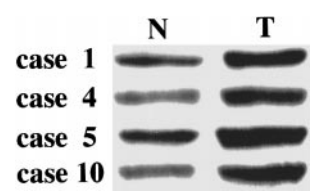

FIG. 3. Western blot analysis of the expression of PLD2 in human renal normal and tumor tissues. The results of the representative cases 1, 4, 5, and 10 are shown. Fifty micrograms of membrane protein was electrophoresed on a 6% SDS–polyacrylamide gel. T, tumor tissues from the patients. N, adjacent normal renal tissues from the same patient.

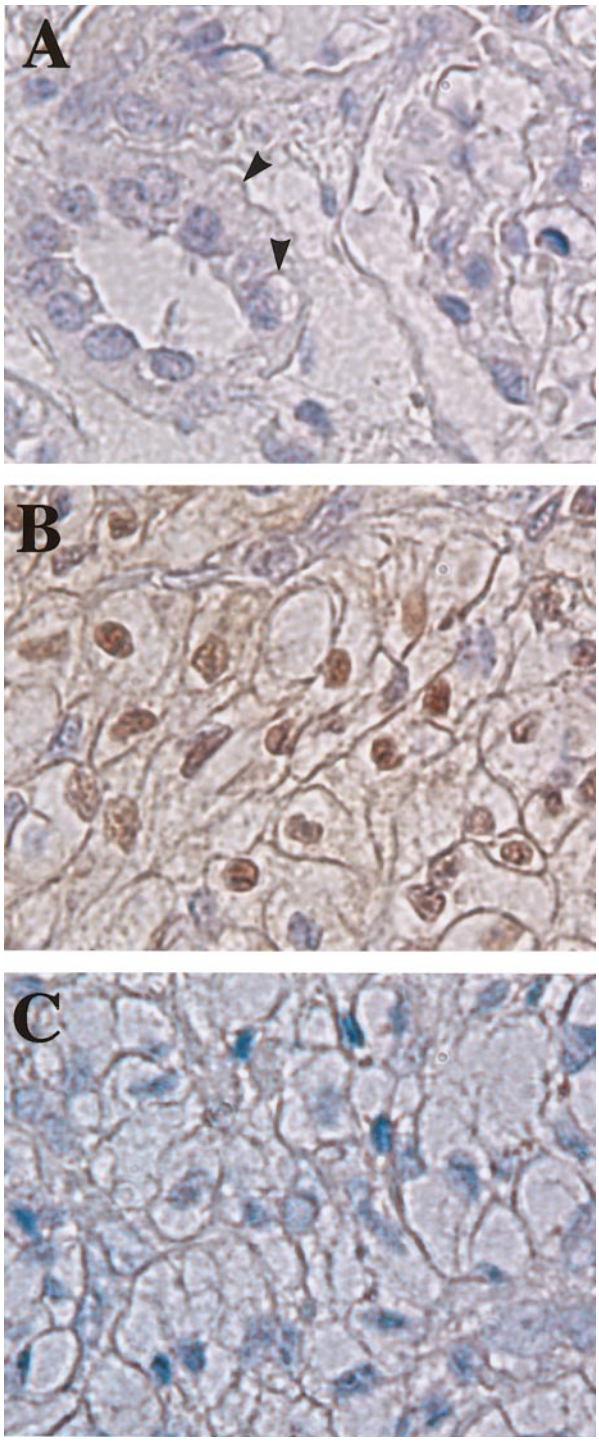

FIG. 4. Immunohistochemical staining of a human renal cancer specimen (case 1) with anti-PLD2 antibody. (A) Noncancerous renal tissue reacted with anti-PLD2 antibody. Note that faint PLD2 specific staining is detectable at the plasma membranes of epithelial cells of a tubulus. Arrowheads indicate the PLD2-staining region in the tubular cells. ×1000. (B) Clear cell carcinoma specimen stained with anti-PLD2 antibody. It is evident that the clear cells are more intensely stained not only at the plasma membrane and in cytoplasm but also in nuclei compared with normal epithelial cells. ×1000. The negative control that was reacted with a rabbit preimmune serum did not show significant staining. (C) The immunoreactive staining of PLD2 completely disappears by the preincubation of the antibody with PLD2 peptide. ×1000.

summarized in Table 1. Histologically, all renal tumors were clear cell carcinomas. The range of nuclear grade was from 1 to 2. Cases 2 and 7 showed metastasis in bone, brain, and/or lung. None had received radiation or chemotherapy prior to resection. The mean PLD2 and PLD_{OA} activities in renal cancer were 2395.9 ± 525.7 (SD) and 17.36 ± 6.2 (SD) pmol/min/mg protein, whereas those in adjacent normal parts were 1159.0 ± 235.5 (SD) and 9.5 ± 3.6 (SD) pmol/min/mg protein, respectively. It was also observed that in all cases examined the PLD2 activities were much higher than those from the normal parts. Western blot analysis showed that the levels of PLD2 expression in the representative cases of 1, 5, 6, and 10 were distinctly increased in tumors compared with their neighbouring normal tissues (Fig. 3). The expression levels well correlated with PLD2 activities. These findings lead us to suggest that PLD2 would be involved in tumorigenesis, but its underlying mechanism remains to be disclosed. PA, the immediate product by PLD activation, has been shown to enhance DNA synthesis in A432 carcinoma cells (18). Also LPA enhances proliferation of PC3 human prostate cancer cells (19).

The immunohistochemical examination of case 1 showed that marginal amounts of the immunoreactive complex with anti-PLD2 antibody were detectable in the borders between cells and cytoplasm in some normal epithelial cells (Fig. 4A). In sharp contrast, large amounts of the immunoreactive complex were found in the nuclei of clear cells, and some were discernible at the plasma membrane and in cytoplasm (Fig. 4B). Similar observations were also obtained in the case 10 (data not shown). When the anti-PLD2 antibody (Ab-58) was preincubated with the peptide corresponding to human PLD2 residues 523–534 which was used to raise the antibody, the immunoreactive complexes were no longer discernible (Fig. 4C). There has been substantial evidence which suggests that lipid signaling in the nucleus may play an important role in nuclear responses such as gene transcription and DNA replication. Almost all of phospholipases are present in the nucleus, i.e., phospholipase C, A_2, D, and sphingomyelinase (20). However, their precise functions have been poorly understood. One of the focal points for the intranuclear lipid signaling is the production of diacylgycerol (DG) which is implicated in the activation of protein kinase C. Besides the phophoinositide hydrolysis by PLC, nuclear DG can also be generated from PC via PLD. Several studies reported the presence of the PLD activity in the nuclei (21, 22) and showed that the DG levels in the nuclei were increased during cell proliferation (22). We have previously shown that the nuclear PLD activity increased in the S-phase of the regenerating rat liver (23).

In summary, the increased activity and expression of PLD2 in human renal cancer cells and also its intranu-

TABLE 1

Activity of Oleate-Dependent PLD and PLD2 and Clinicopathologic Features in the Patients of Renal Cancer

						PLD2 activity (pmol/mg/min)			Oleate-dependent PLD activity (pmol/mg/min)		
Case	Age	Sex[a]	Grade[b]	Stage	DNA ploidy	Normal	Tumor	T/N	Normal	Tumor	T/N
1	50	M	I	I	diploid	665.2	2955.5	4.4	3.9	17.2	4.4
2	73	F	I	IV	diploid	1368.1	N.E.	—	9.9	N.E.	—
3	68	M	I	I	N.E.[c]	822.3	1595.8	1.9	9.4	15.8	1.7
4	76	M	I	I	aneuploid	1533.7	2393.3	1.6	11.8	16.8	1.4
5	62	M	II	I	diploid	1472.4	2944.6	2.0	15.3	29.9	2.0
6	48	M	I	IV	aneuploid	1083.8	2592.6	2.4	10.5	9.5	0.9
7	59	F	II	IV	aneuploid	1193.2	2062.1	1.7	5.8	21.1	3.6
8	47	F	I	I	aneuploid	1117.8	2252.5	2.0	3.6	9.8	2.7
9	74	M	I	I	diploid	1477.0	3190.9	2.2	12.4	20.9	1.7
10	73	F	II	I	N.E.	1065.0	1575.6	1.5	12.9	15.4	1.2

[a] M, male; F, female.
[b] Tumor grade was determined by light microscopy and pathological tumor staging was performed according to the 1997 TNM criteria proposed by the American Joint Committee on Cancer.
[c] N.E., not examined.

clear localization suggest the possible implication of PLD2 in tumorigenesis and promotion.

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