Jpn. J. Cancer Res. 80, 975–980, October 1989

Elevated Concentrations of Brain-type Glycogen Phosphorylase in Renal Cell Carcinoma

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We determined tissue concentrations of brain-type glycogen phosphorylase in normal kidney and renal cell carcinoma by enzyme immunoassay; we also localized it immunohistochemically. Tissue concentration of brain-type glycogen phosphorylase in the renal cortex (n=13) was 1430±709 ng/mg protein (mean±standard deviation) and that in the medulla (n=13) was 1270±635 ng/mg protein. On the other hand, the concentration in renal cell carcinoma (n=26) was 2530±1540 ng/mg protein, ranging from 520 to 6860 ng/mg, significantly higher than those in renal cortex and medulla. Clear cell type tumors contained slightly higher levels of the phosphorylase (2600±1430 ng/mg protein) than granular cell type tumors (2100±1520 ng/mg protein). In renal tissues, brain-type glycogen phosphorylase was immunohistochemically localized in epithelial cells of proximal and distal tubules, collecting tubules, thick and thin limbs of loops of Henle, and Bowman's capsules. In renal cell carcinoma, the phosphorylase was immunohistochemically demonstrated in 97% (34/35) of cases, including one sarcomatoid variant. These findings indicate that renal cell carcinoma cells contain enhanced tissue levels of brain-type glycogen phosphorylase.

Key words: Glycogen phosphorylase — Kidney — Renal cell carcinoma — Immunoassay — Immunohistochemistry

Glycogen phosphorylase (α-1,4-D-glucan: orthophosphate glucosyltransferase EC 2.4.1.1) in animals has three major isozymes: muscle, liver, and brain types.[1-4] They differ from each other functionally, structurally and immunochemically. Recently, several investigators cloned and sequenced the cDNAs encoding human muscle, liver and brain glycogen phosphorylase isozymes, and reported on the homology of the amino acid sequences among the three types of glycogen phosphorylase.[4-6] We recently prepared antibodies specific to human brain glycogen phosphorylase, established a sensitive immunoassay method for measuring immunoreactive brain-type glycogen phosphorylase, and estimated its distribution in various human tissues.[7] We confirmed that the phosphorylase is also distributed in various peripheral tissues, such as in heart, digestive tract, urinary bladder, aorta and testis.[7]

Fetal forms of some isozymes occur during carcinogenesis, and so such isozymes are characterized as oncofetal enzymes.[8] With regard to glycogen phosphorylase, Sato *et al.* found that the major isozyme in rat fetal tissues is the brain-type glycogen phosphorylase, and that hepatoma tissues have significant amounts of brain-type isozyme as well.[9-11] Thus, brain-type glycogen phosphorylase was characterized as a fetal-type isozyme.

Because renal cell carcinoma (RCC) is known to possess a significant amount of glycogen particles,[12] it is of interest to estimate the content of glycogen phosphorylase molecules, a key enzyme participating in the glycogenolysis *in vivo*. This is the first study describing the immunochemical and immunohistochemical localization of the brain-type glycogen phosphorylase in the kidney and RCC tissues.

MATERIALS AND METHODS

Antibodies Antibodies to brain glycogen phosphorylase were raised in New Zealand white rabbits by injecting the purified human brain glycogen phosphorylase with Freund's complete adjuvant as described elsewhere.[7] Antibodies monospecific to brain glycogen phosphorylase were further purified by immunoaffinity column chromatography using brain glycogen phosphorylase-coupled Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), followed by adsorption with muscle glycogen phosphorylase as described previously.[7] The specificity of the purified antibody IgG thus obtained was also reported.[7] For secondary antibodies in immunohistochemistry and immunoblotting, horseradish peroxidase

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[5] Abbreviations used in this paper: RCC, renal cell carcinoma; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

(HRP)-labeled goat IgG Fab′ fragments against rabbit IgG were prepared as described.[13]

Tissue samples Tumor tissues were obtained at surgical operation from 35 patients with RCC, including one with a sarcomatoid variant. Histologically normal renal tissues (n=13) adjacent to the tumor tissues were also prepared. For immunohistochemistry the tissues were fixed in periodate-lysine-4% paraformaldehyde (PLP)[14] for 6 h, washed in phosphate-buffered saline (PBS, pH 7.2) containing increasing concentrations of sucrose, embedded in OCT compound (Tissue-Tek, Naperville, IL), and frozen quickly in dry ice and ethanol.[15] For enzyme immunoassay, the fresh tumor tissues (n=26) and tissues of normal cortex (n=13) and medulla (n=13) were promptly frozen and kept at −80°C until analysis. Frozen tissues (about 0.5 g) were homogenized at 0°C with 10 volumes (v/w) of 50 m*M* Tris-HCl buffer, pH 7.4, using a glass homogenizer. The homogenate was centrifuged at 4°C at 15,000*g* for 20 min, and the supernatant fraction was used for the assays of brain-type glycogen phosphorylase and protein concentrations. Protein concentrations of the extracts were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA), which utilizes the principle of protein-dye binding.[16]

Immunoassay system and assay procedure Concentrations of brain-type glycogen phosphorylase in the extracts were determined by the sandwich-type enzyme immunoassay system specific to brain-type glycogen phosphorylase.[7] In brief, the extract was incubated with a polystyrene ball bearing immobilized anti-brain glycogen phosphorylase antibodies, and then the ball was incubated with the same antibodies labeled with β-D-galactosidase from *Escherichia coli*.[7, 17, 18] The galactosidase activity bound to the ball was assayed with 4-methylumbelliferyl-β-D-galactoside as a substrate. Purified human brain glycogen phosphorylase was used as a standard; the results were expressed as nanograms per milligram of soluble protein, and were compared by means of the Wilcoxon rank-sum test.

Electrophoresis and immunoblotting Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli's method[19] in a 7% polyacrylamide slab gel, and proteins were visualized with Coomassie blue. Immunoblotting was performed by a method similar to that of Towbin *et al.*[20] In brief, proteins were electrophoretically transferred from polyacrylamide gel to a nitrocellulose sheet. The nitrocellulose sheet was incubated in buffers as described previously[7] for 60 min at room temperature, and the purified anti-brain glycogen phosphorylase antibodies (0.1 μg/ml) were added for a further 60 min incubation. After washing, the sheet was incubated with HRP-labeled goat anti-rabbit IgG for 30 min, and then the peroxidase activity on the sheet was visualized with 3,3′-diaminobenzidine and hydrogen peroxide as described previously.[7]

Immunohistochemistry The indirect peroxidase-labeled antibody method was employed for the immunostaining as described previously.[13, 21, 22] In brief, 6 μm-thick cryostat sections were placed on albumin-coated slides and dried at room temperature. The sections were treated with 5 m*M* periodic acid solution for 15 min to inactivate endogenous peroxidase. They were washed in PBS, and then reacted with monospecific anti-human brain glycogen phosphorylase IgG (10 μg/ml) for 120 min. For control sections, antibodies absorbed with purified human brain glycogen phosphorylase antigen were substituted for the primary antibodies. After being washed in PBS, the sections were incubated with the HRP-labeled secondary antibodies for 60 min. After another wash in PBS, they were reacted with 0.025% 3,3′-diaminobenzidine solution containing 10 m*M* hydrogen peroxide and 10 m*M* sodium azide, and then counterstained with methyl green.

RESULTS

Tissue concentrations of brain-type glycogen phosphorylase in normal kidneys and RCCs Table I shows

Table I. Concentrations of Brain-type Glycogen Phosphorylase in Tissues of Normal Kidney and Renal Cell Carcinoma

Tissue	No. of samples	Mean±SD (ng/mg protein)	Range (ng/mg protein)
Normal kidney			
Cortex	13	1430±709	475–2140
Medulla	13	1270±635	484–3030
Renal cell carcinoma	26	2530±1540[a]	520–6860

SD: standard deviation.
a) Significantly higher than renal cortex and medulla ($P<0.01$).

Table II. Concentrations of Brain-type Glycogen Phosphorylase in Tissues of Four Histological Types of Renal Cell Carcinoma

Histological type	No. of samples	Mean±SD (ng/mg protein)	Range (ng/mg protein)
Clear cell type	15	2600±1430	851–6860
Granular cell type	8	2100±1520	520–3690
Mixed type	2	1850, 6010[a]	
Sarcomatoid type	1	2140[a]	

SD: standard deviation.
a) Single value.

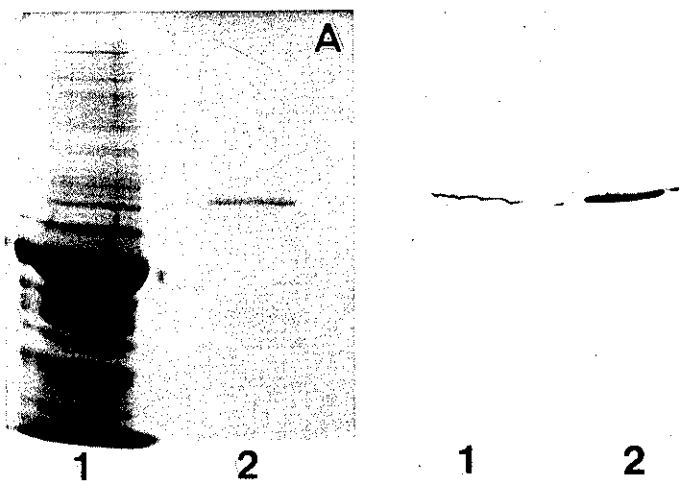

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified brain-type glycogen phosphorylase and a crude extract of renal cell carcinoma (A), and immunoblots with the purified antibodies specific to brain-type glycogen phosphorylase (B). The crude extract of renal cell carcinoma tissue (lane 1) and the purified brain-type glycogen phosphorylase (530 ng, lane 2) were subjected to electrophoresis, followed by immunoblotting. The crude extract contained 360 ng of immunoreactive brain-type glycogen phosphorylase with 100 μg protein.

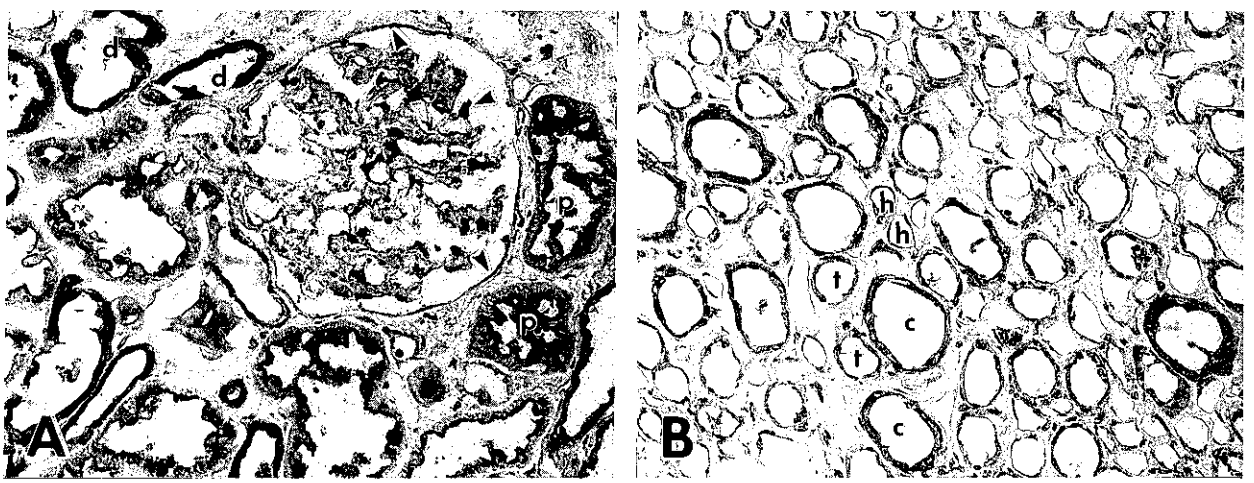

Fig. 2 Localization of brain-type glycogen phosphorylase in normal renal tissues ($\times$165). (A) In the cortex, brain-type glycogen phosphorylase is localized in the cytoplasm of epithelial cells of proximal (p) and distal (d) tubules, and in epithelial cells of Bowman's capsules (arrowheads). The phosphorylase was immunostained intensely in the apical side of the cytoplasm of proximal renal tubules. (B) In the medulla, brain-type glycogen phosphorylase is localized in epithelial cells of collecting tubules (c) and thick limbs of loops of Henle (t). A few thin limbs of loops of Henle were also stained, but faintly (h).

the concentrations of brain-type glycogen phosphorylase in the soluble fractions of the normal cortex and medulla, and in those of RCCs. Significant amounts of brain-type glycogen phosphorylase were found in both cortex and medulla. In RCC, brain-type glycogen phosphorylase amounted to 2530$\pm$1540 ng/mg protein (mean$\pm$standard deviation (SD)), ranging from 520 to 6860 ng/mg. The average value was 1.8 and 2.0 times higher than those in normal cortex and medulla, respectively (both, $P<0.01$). Clear cell type tumors had a slightly higher mean concentration of brain-type glycogen phosphorylase than granular cell type tumors, although there was no statistically significant difference (Table II). No apparent correlation was found between the concentration of brain-type glycogen phosphorylase and histological grade or clinical stage of RCC.

Immunoblotting test To confirm that the immunoreactive substance reactive to the immunoassay is brain-type glycogen phosphorylase, a crude extract of RCC tissue was subjected to electrophoresis, followed by immuno-

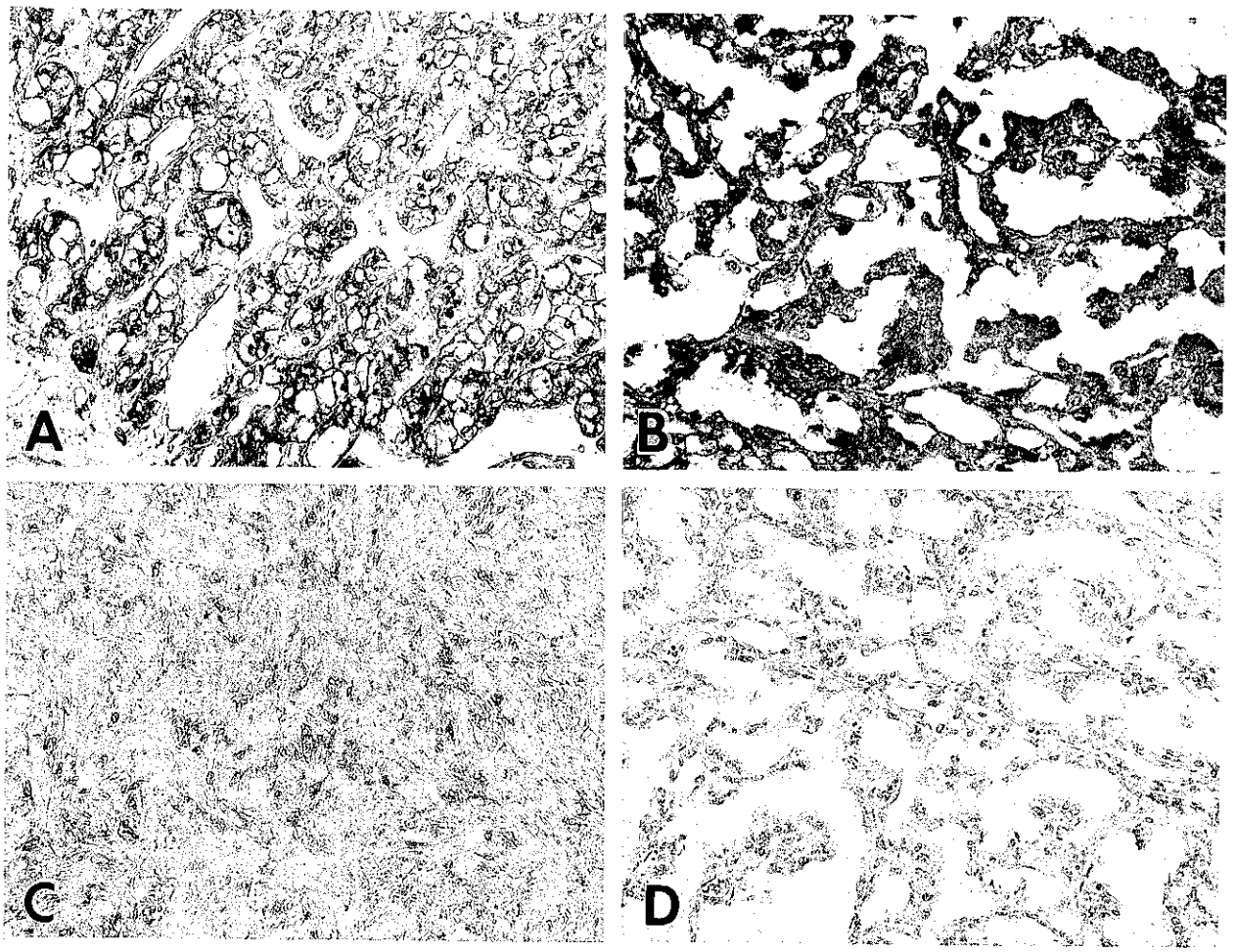

Fig. 3. Localization of brain-type glycogen phosphorylase in three types of renal cell carcinoma (×175). Brain-type glycogen phosphorylase is found in the cytoplasm and nucleus of tumor cells in clear cell (A) and granular cell (B) types. In one case of sarcomatoid variant (C), a few fusiform tumor cells show positive staining for brain-type glycogen phosphorylase. The control section of the granular cell type tumor is uniformly negative (D).

blotting with the antibodies specific to brain-type glycogen phosphorylase. The extract of RCC tissue revealed a single band at a position corresponding to that of brain-type glycogen phosphorylase (Fig. 1). These results indicate that the immunoreactive antigen in the extract of the tumor tissue was the brain-type glycogen phosphorylase.

Immunohistochemical localization of brain-type glycogen phosphorylase in normal renal tubules and RCCs In the cortex, brain-type glycogen phosphorylase was localized in the cytoplasm of epithelial cells of proximal and distal tubules and Bowman's capsules (Fig. 2A). The phosphorylase was immunostained intensely in the apical side of the cytoplasm of proximal renal tubules. In the medulla, the phosphorylase was demonstrated in the cytoplasm of epithelial cells of collecting tubules and thick limbs of loops of Henle. A few thin limbs of loops of Henle were also immunostained, but faintly (Fig. 2B).

Of 35 RCCs studied, 34 (97%) were stained positively for the phosphorylase (Fig. 3), which was localized in the cytoplasm and occasionally in the nucleus of tumor cells. In one case of a sarcomatoid variant, a few fusiform tumor cells showed positive staining for brain-type glycogen phosphorylase (Fig. 3C). Control sections of the tumor tissues treated with the antibody absorbed with the purified brain-type glycogen phosphorylase were uniformly negative (Fig. 3D).

Table III shows the relationship between the histological types and the positivity of immunohistochemical

Table III. Frequency of Positive Tumor Cells for Brain-type Glycogen Phosphorylase in Renal Cell Carcinoma

Histological type	(−)	(+)	(++)	(+++)	Positivity
Clear cell type		1	5	16	22/22 (100%)
Granular cell type	1	2	3	4	9/10 (90%)
Mixed type			1	1	2/2 (100%)
Sarcomatoid type		1			1/1 (100%)
Total	1	4	9	21	34/35 (97%)

(−): no positive cells; (+): fewer than 10% positive cells; (++): 10% to 50% positive cells; (+++): more than 50% positive cells.

staining for brain-type glycogen phosphorylase. According to the frequency of immunohistochemically positive cells, the 35 cases of RCC were divided into four groups: (−), no positive staining; (+), positive in less than 10% of cells; (++), positive in 10 to 50% of cells; and (+++), positive in more than 50% of cells. Clear cell type tumors tended to have more positive cells than granular cell type tumors.

DISCUSSION

We first determined the tissue concentrations of brain-type glycogen phosphorylase in the normal kidney and RCC. Our study showed that tissue levels of brain-type or fetal-type glycogen phosphorylase in RCC were about twice those in normal kidney. Histochemical and electron-microscopic studies revealed that clear cell types of RCC contain larger amounts of particulate glycogen in the cytoplasm than other tumors at other sites and granular cell types of RCC.[12] There seems to be no apparent relationship between the glycogen content and the fetal-type glycogen phosphorylase in RCC, because no significant difference was found between the levels of glycogen phosphorylase in the clear cell type tumors and those of granular cell type tumors. In addition, because the immunoassay method which we employed for determination of brain-type glycogen phosphorylase should be reactive with both the active (or phosphorylated) and inactive (dephosphorylated) forms of the phosphorylase, the values obtained might not directly reflect the glycogenolytic activity in the tissues. Glycogen contents in the tissues are also regulated by glycogen synthetase activity together with various hormonal factors. Therefore, the present results do not offer much information on the mechanism of glycogen accumulation in the tumor cells. Sato *et al.* also found no significant association between the phosphorylase activity of rat hepatoma and the storage of glycogen.[23]

Sato *et al.* showed that in rat fetal liver and hepatoma tissues, brain-type glycogen phosphorylase is the predominant isozyme, in contrast to the liver-type one in normal liver.[9–11] Thus, brain-type or fetal-type glycogen phosphorylase appeared during carcinogenesis.

Shimada *et al.* immunohistochemically localized brain-type glycogen phosphorylase in human gastric cancer, and suggested that the appearance of the brain-type or fetal-type isozyme in gastric cancer is an instance of antigen reversion in cancer.[24] Previous histochemical, immunohistochemical, and ultrastructural studies suggested that RCC is derived from epithelial cells of proximal tubules.[12, 22] Because proximal tubules contain brain-type glycogen phosphorylase, the isozyme in RCC did not newly appear, but just increased.

Sato *et al.* revealed that liver-type glycogen phosphorylase was largely or entirely replaced by fetal-type glycogen phosphorylase with decreased differentiation and increased growth rate of rat hepatomas.[11] The present study found no significant correlation between the concentration of brain-type glycogen phosphorylase and histological grade of differentiation or clinical stage of RCC.

Immunohistochemically, brain-type glycogen phosphorylase was detected in almost all cases of renal cell carcinoma. The frequency of immunohistochemically positive tumor cells for brain-type glycogen phosphorylase varied from case to case, and from site to site in the tumor. Such immunohistochemical heterogeneity among cases, and within each tumor, might explain the wide variation of tissue concentration of brain-type glycogen phosphorylase as determined by immunoassay.

Further studies are necessary to clarify the biological significance of elevated concentrations of brain-type glycogen phosphorylase in RCC tissues and the pathological background of the glycogen deposit in clear cell type of RCC.

(Received June 9, 1989/Accepted August 10, 1989)

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