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Elevated concentrations of γ-enolase in renal cell tumors in rats: similarity to renal cell carcinoma in man

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Abstract Concentrations of enolase isozymes in normal kidney and renal cell tumors in rats were determined using a highly sensitive enzyme immunoassay, and the isozymes were immunohistochemically localized in tissue sections. Levels of α-enolase in renal cell tumors were significantly lower than in normal kidney, whereas those of γ-enolase were significantly elevated (mean ± SD: 211 ± 129 ng/mg protein, $n = 15$, as compared to 27.1 ± 2.9 ng/mg protein, $n = 7$). The proportion of γ-enolase in the total enolases in the tumor tissues (1.6 ± 0.5%) was significantly higher than in normal kidney (0.15 ± 0.05%). Immunohistochemistry revealed epithelial cells of all nephron segments to be positive for the α-isozyme, whereas γ-enolase staining was strongly positive only in the loops of Henle, being faint in the distal tubules and absent in the proximal tubules. Both α- and γ-enolases demonstrated positive immunostaining in all of the seven renal cell tumors studied. These findings indicate that an isozyme switch from α- to γ-enolase occurs during rat kidney carcinogenesis, taking into account the derivation from proximal tubules, consistent with the findings for renal cell carcinomas in man.

Key words Enolase · Isozymes · Rat · Renal neoplasms

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Introduction

Enolase (EC 4.2.1.11), a glycolytic enzyme, is a dimer composed of three immunologically distinct subunits, α, β, and γ, and five forms (αα, ββ, γγ, αβ, and αγ) which are present in mammalian tissues [5, 15]. The α-subunit of enolase (α-enolase) is distributed widely in various tissues while the β-subunit (β-enolase) is localized mainly in the heart and striated muscle. The γ-subunit (γ-enolase), which has been designated neuron-specific enolase, is localized at high levels in neuronal and neuroendocrine cells, as well as tumors derived from these cells. In the last decade, however, immunochemical and immunohistochemical studies have demonstrated that γ-enolase is also localized in other normal tissues and cells [7, 11] and in tumors, including human lung and renal cell carcinomas [6, 8, 18]. We first demonstrated that concentrations of γ-enolase in renal cell carcinoma tissues are 34–55 times higher than those in the normal renal cortex and that serum γ-enolase is a useful biomarker for patients with such neoplasms [8, 18].

Increase or decrease of the activity of several enzymes, including γ-glutamyl transpeptidase and glucose-6-phosphate dehydrogenase, has been reported in renal epithelial tumors induced by chemical agents in rats [14, 22]. Tsuda et al. [21, 23] immunohistochemically demonstrated that the glutathione *S*-transferase and cytochrome P-450 phenotype changes during renal carcinogenesis in rats treated with *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN). To date, however, no quantitative studies have been performed to evaluate changes of any isozyme during rat renal carcinogenesis.

To clarify whether an isozyme switch from α- to γ-enolase occurs during renal carcinogenesis in rats, equivalent to that known in man, we quantitatively determined concentrations of enolase isozymes in normal kidney tissues and renal cell tumors in rats, and localized them immunohistochemically.

Materials and methods

Animals and treatment

EHEN (Sakai Laboratories, Fukui, Japan) was used for induction of renal cell tumors as previously described [20]. In brief, a total of 63 female Sprague-Dawley rats (Charles River Japan, Atsugi, Japan) aged 6 weeks at the beginning of the experiment were kept in a room at 25° ± 2 °C, at a relative humidity of 55 ± 5% and in a 12-h light-dark cycle. The animals were maintained on a basal diet (Oriental M, Oriental Yeast, Tokyo) and tap water *ad libitum*. They were initially given 0.05% EHEN dissolved in their drinking water for 3 weeks, returned to normal tap water for the subsequent 49 weeks, and then killed at the end of week 52.

The kidneys were immediately excised for quantitative assays and immunohistochemical examination. A group of control rats ($n = 7$) not receiving any chemical treatment was also included.

Tissue samples

Tumor and control tissues were obtained from the above rats. Histological examination revealed that the 15 tumors from the 63 rats treated with EHEN were all renal cell tumors, the incidence being 24% [20]. Normal kidney tissues from seven untreated rats were also prepared. For the immunoassays, tissues were promptly frozen and kept at −80 °C until the analysis, when they were homogenized at 0 °C with 10 volumes (V/W) of 50 mM TRIS-HCl (pH 7.4) containing 5 mM $MgSO_4$. Homogenates were centrifuged at 4 °C at 20 000*g* for 20 min, and the soluble fractions were again frozen. They were analyzed after storage at −80 °C for 4 weeks. For histological examination and immunohistochemistry, tissues from five cases of normal kidney and seven cases of renal cell tumors were fixed in periodate-lysine-4% paraformaldehyde for 6 h, washed in phosphate-buffered saline (PBS, pH 7.2) containing increasing concentrations of sucrose, and embedded in OCT compound (Tissue-Tek, Naperville, Illinois, USA).

Antibodies

Antibodies to α- and γ-enolases were raised in New Zealand rabbits by injecting the respective antigens, purified from rat brain, with Freund's complete adjuvant, as described elsewhere [10, 16]. Antibodies to β-enolase were also raised in New Zealand rabbits by injecting the purified antigen from rat skeletal muscles with Freund's complete adjuvant. Antibodies monospecific to the three enolase subunits were purified by immunoaffinity column chromatography using antigen-coupled Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The specificities of the purified antibodies to α- and γ-enolases thus obtained were reported previously [10].

As secondary antibodies for immunohistochemistry, horseradish peroxidase (HRP)-labeled rabbit IgG Fab' fragments against rabbit IgG were prepared [17].

Immunoassay methods

Concentrations of α-, β-, and γ-subunits of enolase isozymes in the soluble fractions of tissues were determined by the sandwich-type enzyme immunoassay system developed by Kato et al. [10]. In brief, extracts were incubated with polystyrene balls bearing immobilized monospecific rabbit antibodies to the respective enolase isozymes, and then the balls were incubated with the same antibodies labeled with β-D-galactosidase from *Escherichia coli*. The bound galactosidase activity was assayed with 4-methylumbelliferyl-β-D-galactoside as a substrate. Purified rat α-, β-, and γ-subunits were, respectively, used as standards, and the results were expressed as homometric enolase antigens equivalent to nanograms per milligram of soluble protein. The assay systems were all highly sensitive, the limit of detection for the three subunits being 3 pg/test tube.

Immunohistochemistry

The indirect HRP-labeled antibody method was employed for the immunostaining as described previously [17, 19]. In brief, 5-μm-thick cryostat sections were placed on albumin-coated slides and dried at room temperature. They were treated with 100% methanol and 0.3% hydrogen peroxide solution for 30 min to inactivate endogeneous peroxidase, washed in PBS, and then incubated with purified anti-α-enolase IgG, anti-β-enolase IgG, or anti-γ-enolase IgG (4 μg/ml) for 12 h at 4 °C. For control sections, antibodies absorbed with the purified respective antigen were substituted for the primary antibodies. After being washed in PBS, all sections were incubated with HRP-labeled secondary antibodies for 60 min at room temperature. After further washing in PBS, they were reacted with 0.025% 3,3'-diaminobenzidine solution containing 10 mM hydrogen peroxide and 10 mM sodium azide, and then counterstained with methyl green.

Other methods

Protein concentrations of the tissue extracts were determined with the aid of a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA, USA), utilizing the principle of protein-dye binding [4]. Quantitative data were expressed as mean ± standard deviation (SD) values and the results compared using the Wilcoxon's rank-sum test.

Table 1 Concentrations of three enolase isozymes in normal kidney tissues and renal cell tumors in rats. Values are means ± SD with range

Tissues	No. of samples	Tissue concentration (ng/mg protein)			$\gamma/(\alpha+\beta+\gamma)$ (%)
		α-Enolase	β-Enolase	γ-Enolase	
Normal kidney	7	19 200 ± 4 400 (13 400–24 800)	0.73 ± 0.16 (0.54–1.03)	27.1 ± 2.9 (22.0–30.4)	0.15 ± 0.05 (0.093–0.22)
Renal cell tumor	15	13 400 ± 4 350[a] (8 100–45 800)	0.40 ± 0.47 (0.16–1.20)	211 ± 129[c] (63.9–522)	1.6 ± 0.5[b] (0.49–2.4)

Significantly different from the normal kidney value ($P < 0.05$[a], 0.01[b], or 0.001[c])

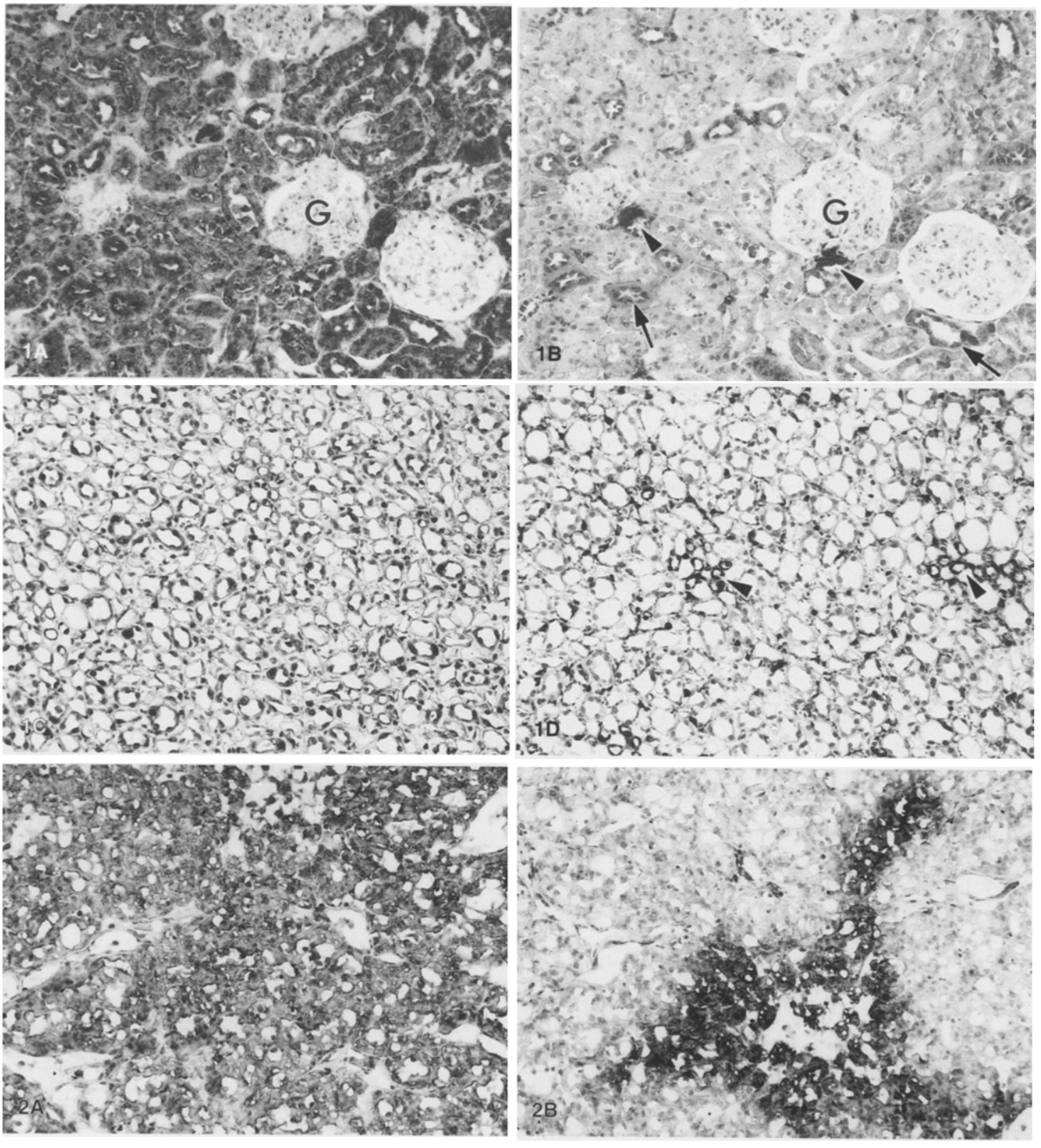

Fig. 1A–D Immunohistochemical localization of α- and γ-enolases in the renal cortex (**A**, **B**) and medulla (**C**, **D**). All of the nephron segments in the cortex (**A**) and medulla (**C**) are positively stained for α-enolase, with varying staining intensities. In contrast, γ-enolase is positive in the macura densa cells (*arrowheads*), only faintly positive for the distal tubules (*arrows*) in the cortex (**B**), and positive for the thin limbs of loops of Henle (*arrowheads*) in the medulla (**D**). *G* glomerulus. Indirect immunoperoxidase method, ×138

Fig. 2A, B Immunohistochemical localization of α- (**A**) and γ- (**B**) enolases in a renal cell tumor in a rat treated with *N*-ethyl-*N*-hydroxyethylnitrosamine. Indirect immunoperoxidase method, ×138

Results

Concentrations of enolase isozymes in rat kidney tissues and renal cell tumors

Table 1 summarizes data for concentrations of the three enolase isozymes in normal rat kidney and renal cell tumors. Values for α-enolase in renal cell tumors were significantly lower than those in normal kidney ($P < 0.05$), while the concentrations of γ-enolase were elevated an average of 7.8-fold ($P < 0.001$). No appreciable β-enolase was found in normal kidneys or renal cell tumors. The proportion of γ-enolase in the total enolase, calculated as $\gamma/(\alpha + \beta + \gamma)$, was significantly higher in renal cell tumors (mean value 1.6%) than in normal kidney (0.15%, $P < 0.01$).

Immunohistochemical localization of enolase isozymes in normal rat kidney and renal cell tumors

Figure 1 illustrates immunohistochemical localization of α- and γ-enolases in normal rat kidney tissue. Immunostaining for α-enolase was found in epithelial cells of all nephron segments, although the staining intensities varied from segment to segment (Fig. 1A, C). Of the nephron segments, only the thin limbs of loops of Henle and the distal tubules were immunohistochemically positive for γ-enolase (Fig. 1B, D), staining being faint in the latter case. In addition, γ-enolase was immunostained in macula densa cells. No positive staining for β-enolase was observed in any nephron segments.

Of the seven cases of renal cell tumor tissues studied, all stained positively for both α- and γ-enolases. Figure 2 illustrates the immunohistochemical localization of α- and γ-enolases in a typical renal cell tumor. In both cases localization was in the cytoplasm and occasionally in the nuclei of the tumor cells. The intensity of staining varied from case to case, and from site to site within individual tumors. Control sections of tissues treated with antibodies pre-absorbed with the respective antigens were uniformly negative.

Discussion

The present study demonstrated that concentrations of γ-enolase are markedly elevated in renal cell tumors in rats, consistent with our previously reported findings for equivalent kidney carcinomas in man [8, 18]. Increase of both absolute concentrations of γ-enolase and its proportion of the total enolase population in rat renal tumors would indicate preferential expression of this isozyme.

Previous histochemical and electron microscopic studies have revealed that human renal cell carcinomas are generally derived from proximal renal tubules [3]. Several investigators have disclosed that the majority of rat renal cell tumors induced by chemical agents also arise from the proximal tubules and only a minority from the collecting ducts [2, 9]. The present study revealed γ-enolase to be immunohistochemically localized only in the loops of Henle and distal tubules, but not in the proximal tubules of the normal rat kidney, suggesting that γ-enolase newly appears during renal carcinogenesis. These immunohistochemical findings are also consistent with those with human renal cell carcinoma [8].

Generally, anaerobic glycolysis is enhanced in neoplastic tissues [26]. However, the biological significance of elevated concentrations of the γ-subunit of enolase, a member of the glycolytic enzyme group, remains unclear, although such an alteration of the isozyme pattern may be a physiological adaptive response conferring a metabolic advantage on the neoplastic cells [23]. Further studies at the gene level are needed to clarify the mechanism of regulation of expression and suppression of genes encoding enolase isozymes.

Oncofetal antigens are defined as antigens found in neoplastic and fetal tissues, but which are generally absent from normal postnatal tissues [1]. In the kidney several studies have revealed that the A-subunit of aldolase, another glycolytic enzyme, is an isozyme having oncofetal characteristics [12, 13, 24, 25, 27]. Thus aldolase A is found in renal cell carcinomas and fetal kidney while aldolase B is expressed in proximal tubules in the normal adult kidney. Because renal cell carcinomas are generally believed to be derived from cells of the proximal renal tubules [3], an isozyme conversion of aldolase B to A occurs during renal carcinogenesis. Regarding enolase isozymes, our preliminary studies indicate that the concentration of γ-enolase in the fetal kidney (gestational days 16 and 19) is about twofold higher than that in the normal adult kidney in rats (data not shown). Further studies are needed to clarify whether enolase is an isozyme having oncofetal characteristics.

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