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Over-expression of glucose transporter isoform GLUT1 and hexokinase I in rat renal oncocytic tubules and oncocytomas

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Abstract Renal oncocytomas, which have previously been shown to originate from the collecting duct system, were induced in male Sprague-Dawley rats by oral administration of N-nitrosomorpholine (NNM) for 7 weeks. The expression of glucose transporter isoforms GLUT1 and GLUT2, and of several enzymes involved in glucose metabolism [hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH)] were studied by cytochemical approaches in serial cryostat sections of the kidney 12, 23 and 34 weeks after withdrawal of NNM. Oncocytic tubules connected with collecting ducts were first observed 23 weeks, and oncocytomas 34 weeks after withdrawal. The cytochemical pattern of oncocytic tubules and oncocytomas was similar, but differed markedly from that of normal collecting ducts in nearly all variables studied; expression of GLUT1 and hexokinase I proteins were strongly increased; activities of HK, PK and MDH were elevated, while LDH activity was reduced. These results suggest that oncocytic transformation is associated with fundamental changes in energy metabolism which differ from those in cell lineages leading to other types of renal cell tumours, such as clear/acidophilic and basophilic cell tumours. The characteristic over-expression of GLUT1 may be used as a diagnostic criterion for the discrimination between oncocytes and acidophilic (granular) cells in clear/acidophilic renal cell tumours which show a reduced expression of this glucose transporter protein.

Key words Renal carcinogenesis
Epithelial kidney tumours · Energy metabolism

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Introduction

The oncocytoma is a characteristic type of tumour appearing in various human tissues including the kidney [19, 24] Irrespective of their site, oncocytomas are composed of large epithelial cells (oncocytes) which show a granular-acidophilic cytoplasm due to abundant atypical mitochondria [31, 47]. Renal oncocytomas induced in rats by chemicals such as N-nitrosomorpholine (NNM) [9] or N-ethyl-N-hydroxyethylnitrosamine [49] represent excellent experimental models for studying this unique tumour type [7, 42]. In both rat and man renal oncocytomas originate from the collecting duct system and have a benign biological behaviour [41, 43, 46, 55]. The reason for this benign behaviour is poorly understood, especially since the development of renal oncocytomas induced in rats by chemicals is frequently accompanied by the appearance of other types of renal cell tumour such as clear/acidophilic and basophilic cell tumours which are potentially malignant [7].

Previous studies suggested that variations in metabolic aberrations emerging during carcinogenesis may play in important role in the development of the different types of renal cell tumour [1, 2, 10, 45, 49]. Thus, in contrast to the rapidly growing basophilic, clear/acidophilic or acidophilic (granular) rat renal cell tumours, the slowly growing lesions consisting predominantly of clear cells or exclusively of oncocytes, do not usually show an increased butanormal or decreased activity of the glucose-6-phosphate dehydrogenase. Interesting variations between different types of renal cell tumour have also been observed in the expression of glucose transporter proteins [1, 2] and in the activities of key glycolytic enzymes [1, 2, 49].

The cortical collecting duct system, from which the renal oncocytoma derives, utilizes glucose as a preferred respiratory substrate [51]. The transport of glucose across the plasma membrane is mediated by a family of structurally related transporter proteins [12, 22]. Among five well-characterized glucose transporter isoforms, the erythrocyte-type (GLUT1) and the liver-type (GLUT2)

are mainly responsible for the transport of glucose in the kidney. The level of the high affinity GLUT1 correlates well with the glycolytic activity of different segments of the tubular system, whereas the expression of low affinity GLUT2 is confined to the proximal convoluted tubules characterized by high gluconeogenic activity. Here we report a striking over-expression of GLUT1 and hexokinase I in rat renal oncocytic tubules and oncocytomas. The over-expression of GLUT1 is of particular interest since this glucose transporter isoform is strongly reduced in other types of rat renal cell tumours [1, 2].

Materials and methods

Male Sprague-Dawley rats weighing about 180 g were purchased from Zentralinstitut für Versuchstierzucht (Hannover, Germany). The animals were housed in sanitary, ventilated animal rooms and maintained under constant conditions (22° C, 12 h light/dark cycle) with free access to food. Fourty-seven rats were administered NNM dissolved in the drinking water at a concentration of 120 mg/l for 7 weeks as previously described [8]. Twenty-eight animals having received tap water were used as controls. Groups of 15 to 17 treated rats and 7 to 14 control rats were sacrificed 12, 23 and 34 weeks after withdrawal of the carcinogen. A median longitudinal slice of each kidney was snap-frozen in isopentane at −150° C and stored at −80° C until used. Frozen sections (6 µm thick) were cut on a cryostat and stained with PAS-toluidine blue to identify oncocytic lesions. Serial cryostat sections adjacent to those selected by PAS-toluidine blue staining were used for the subsequent histochemical study. Intensities of histochemical staining reactions in the cortical collecting duct and oncocytic lesions were estimated semi-quantitatively using three grades as indicated in Table 1. With the exception of the immunohistochemical reaction for GLUT1 all histochemical reactions were largely homogeneous throughout the oncocytic lesions.

Antibodies against hexokinase I were prepared by immunizing Chincilla rabbits with the purified rat brain hexokinase I emulsified in complete Freund's adjuvant. About 150 µg protein was injected subcutaneously, followed by three further injections each of 100 µg protein in incomplete Freund's adjuvant at approximately 3 weeks intervals. The presence of antibodies was demonstrated by the Ouchterlony double-immunodiffusion assay [21]. Isolation of immunoglobulins from the antiserum as well as from the serum of the non-immunized rabbit was carried out by chromatography on protein A-sepharose [25]. Anti-rat GLUT1 and GLUT2 antibodies [48] were a kind gift of Dr. Thorens (University of Lausanne, Switzerland).

Expression of GLUT1, GLUT2 and hexokinase I were examined by light microscopy using the immunogold-silver staining procedures as described previously [1]. Briefly, cryostat sections (6 µm thick) were fixed with 0.1% alcoholic periodic acid for 5 min at 4° C, treated for 20 min with 50 mM L-glycine to inactivate free aldehyde groups. After incubation for 15 min with 1% (v/v) bovine serum albumin (BSA)/phosphate buffer saline (PBS), the sections were incubated first with the primary antibodies (diluted 1:100 for GLUT1 and GLUT2, 1:500 for hexokinase I in 1% BSA/PBS) for 2 h at room temperature, then with 0.8 nm colloidal gold conjugated anti-rabbit antibodies (Aurion, The Netherlands). The sections were subjected to a silver enhancement procedure by incubation of the samples with the R-GENT kit (Aurion, The Netherlands) for 30 min at room temperature. Control sections were incubated in the absence of primary antibodies.

Activities of hexokinase, pyruvate kinase, lactate dehydrogenase and malate dehydrogenase were studied on serial cryostat sections (6 µm thick) using histochemical techniques. Semi-permeable membranes and membrane vessels were used to improve the localization of the reaction product. Hexokinase activity staining was performed using a modified version of the method described by [33]. Basal medium for demonstration of hexokinase activity contained 100 mM morpholinopropane sulphonic acid (MOPS), pH 7.3, 3 mM glucose, 13 mM adenosine 5′-triphosphate, 2 mM nicotinamide adenine dinucleotide phosphate, 20 mM magnesium chloride, 25 mM sodium arsenate and 25 mM sodium azide. To 4 ml basal medium 1 ml 7.7 mM methylphenazine methosulphate and 1 ml 120 mM nitrobluetetrazolium were added. After mixing with 4 ml of warm 30% gelatine, the medium was poured into membrane vessels and allowed to harden. As auxiliary enzyme 50 µl of glucose-6-phosphate dehydrogenase (G6PDH) (10 µl of 350 U G6PDH diluted with 40 µl 0.1% BSA/100 mM MOPS; G6PDH from yeast, Boehringer, Germany) were dropped on the membrane of each incubation vessel and spread as evenly as possible. The enzyme solution was allowed to dry completely in air before sections were mounted on the membrane. After incubation for 2 h at 20° C the incubation medium was removed from the vessels and the sections attached to membranes were fixed in 4% buffered formalin for 30 min and mounted in Kaisers glycerin. For the histochemical demonstration of pyruvate kinase activity, the same procedures as described by [29] were employed. Lactate dehydrogenase activity was determined using a modification of the method described by [36]. Basal medium for lactate dehydrogenase staining contained 100 mM HEPES (pH 7.6), 100 mM DL-lactic acid (sodium salt), 4 mM nicotinamide adenine dinucleotide, 12 mM magnesium chloride, 25 mM sodium arsenate and 25 mM sodium azide. Incubation medium was prepared as described for hexokinase, and sections were incubated for 1 h at 20° C. Malate dehydrogenase activity was demonstrated by the same method applied for lactate dehydrogenase activity except that 50 mM L-malic acid (sodium salt) was used as a substrate.

Table 1 Histochemical pattern of renal oncocytic lesions when compared with that of the normal collecting duct epithelium (−, negative; +, positive; ++, strongly positive; ↓, decrease; *NC*, no change; ↑, increase; *PAS*, periodic acid-Schiff; *HK*, hexokinase; *PK*, pyruvate kinase; *LDH*, lactate dehydrogenase; *MDH*, malate dehydrogenase; *NCD*, normal collecting duct; *n*, number of lesions examined)

Histochemical variables	NCD	Oncocytic lesions (n=42)
Glycogen	−	NC
GLUT1	++	↑
GLUT2	−	NC
HK[a]	++	↑
HK[b]	++	↑
PK	++	↑
LDH	++	↓
MDH	+	↑

[a] Content of hexokinase I
[b] Activity of hexokinase

Results

Normal collecting duct epithelium

The cortical collecting duct (CCD) is made up of two predominant cell types, principal and intercalated cells. These cell types displayed distinct histochemical patterns. CCD was heterogeneously labelled with antibodies against GLUT1. According to [48] the cells with a narrow band of basal staining are principal cells, while intercalated cells express GLUT1 at a high level in the basolateral membranes. GLUT2 was not found in both CCD and oncocytes but was present in the proximal convoluted tubules. Hexokinase activity in different seg-

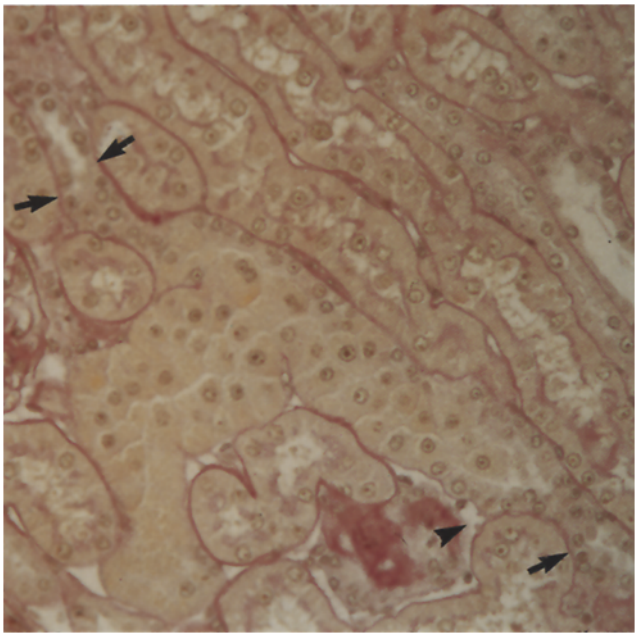

Fig. 1 Oncocytic tubule composed of oncocytes with finely granular acidophilic cytoplasm. Note the connection (*arrows*) of oncocytic tubule to collecting duct at the upper and lower ends in the section. PAS ×290

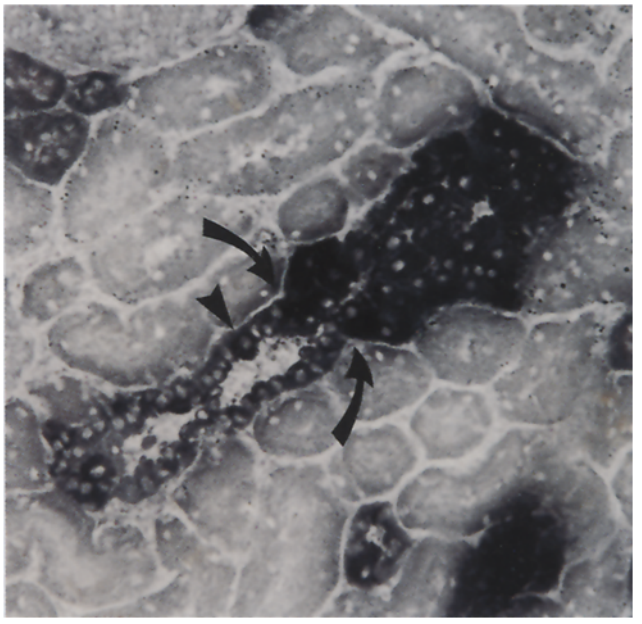

Fig. 2 Demonstration of increased hexokinase activity in oncocytic tubule directly connected to collecting duct segment of normal appearance (*arrows*). Higher hexokinase activity in intercalated cell (*arrowhead*) compared with that in principal cells. ×290

ments of the renal tubular system correlated well with the level of this enzyme as demonstrated by immunohistochemistry. Hexokinase was mainly expressed in the thick ascending limb of Henle, distal tubules and collecting duct cells as described by others [35]. The most intense staining for hexokinase was found in the intercalated cells of connecting tubules and CCD. Distribution of pyruvate kinase along the renal tubular system was very similar to that of hexokinase. There was a high activity of lactate dehydrogenase in CCD, whereas malate dehydrogenase activity was relatively weak in this tubular segment.

Oncocytic lesions

A small number of oncocytic tubules (Fig. 1) was found at 23 weeks after withdrawal of NNM, but the majority of oncocytic tubules and all oncocytomas were observed at 34 weeks after cessation of treatment with the carcinogen. There was no significant difference in the histochemical profile between oncocytic tubules and oncocytomas (Figs. 2 and 3c) indicating that no further fundamental changes in the metabolic pathways investigated occur during the development of oncocytomas from oncocytic tubular lesions. In comparison with CCD, oncocytes exhibited a clear elevation in the expression of GLUT1 (Table 1). The majority of oncocytes in tubular lesions expressed GLUT1 in the basolateral membranes. However, some oncocytes showed positive immunoreactivity for GLUT1 only in the basal membranes like principal cells of normal CCD. Cells forming the periphery of oncocytomas showed an expression of GLUT1 in a pattern identical to that of oncocytes in tubular lesions (Fig. 3a). The other cells of oncocytomas expressed variable amounts of GLUT1 ranging from low to intermediate levels. Both contents (Fig. 3b) and activity (Fig. 3c) of hexokinase in oncocytes were markedly increased compared with those in CCD. A slightly elevated activity of pyruvate kinase (Table 1) was observed in oncocytes. Oncocytes exhibited a marked elevation in malate dehydrogenase activity, while lactate dehydrogenase activity was reduced in these cells (Table 1).

Discussion

In human beings, oncocytes may arise from several epithelial cell types, but share characteristic features such as cytomegaly and acidophilic-granular cytoplasm containing large numbers of atypical mitochondria, independent of their cellular origin [19, 24]. Corresponding to the abundance of mitochondria in oncocytes, high levels of several mitochondrial enzymes were observed in these cells [15, 20, 43, 55]. Oncocytes induced in rat kidney with various carcinogens show many morphological and histochemical similarities to human oncocytes [11, 31, 38]. The present study revealed that rat renal oncocytes exhibit an elevation in the expression of GLUT1 and hexokinase I proteins, and in the activities of glycolytic and mitochondrial enzymes, while lactate dehydrogenase activity of oncocytes is decreased. In preneoplastic and neoplastic lesions of the rat liver, we have previously shown that the histochemical evaluation of the activities

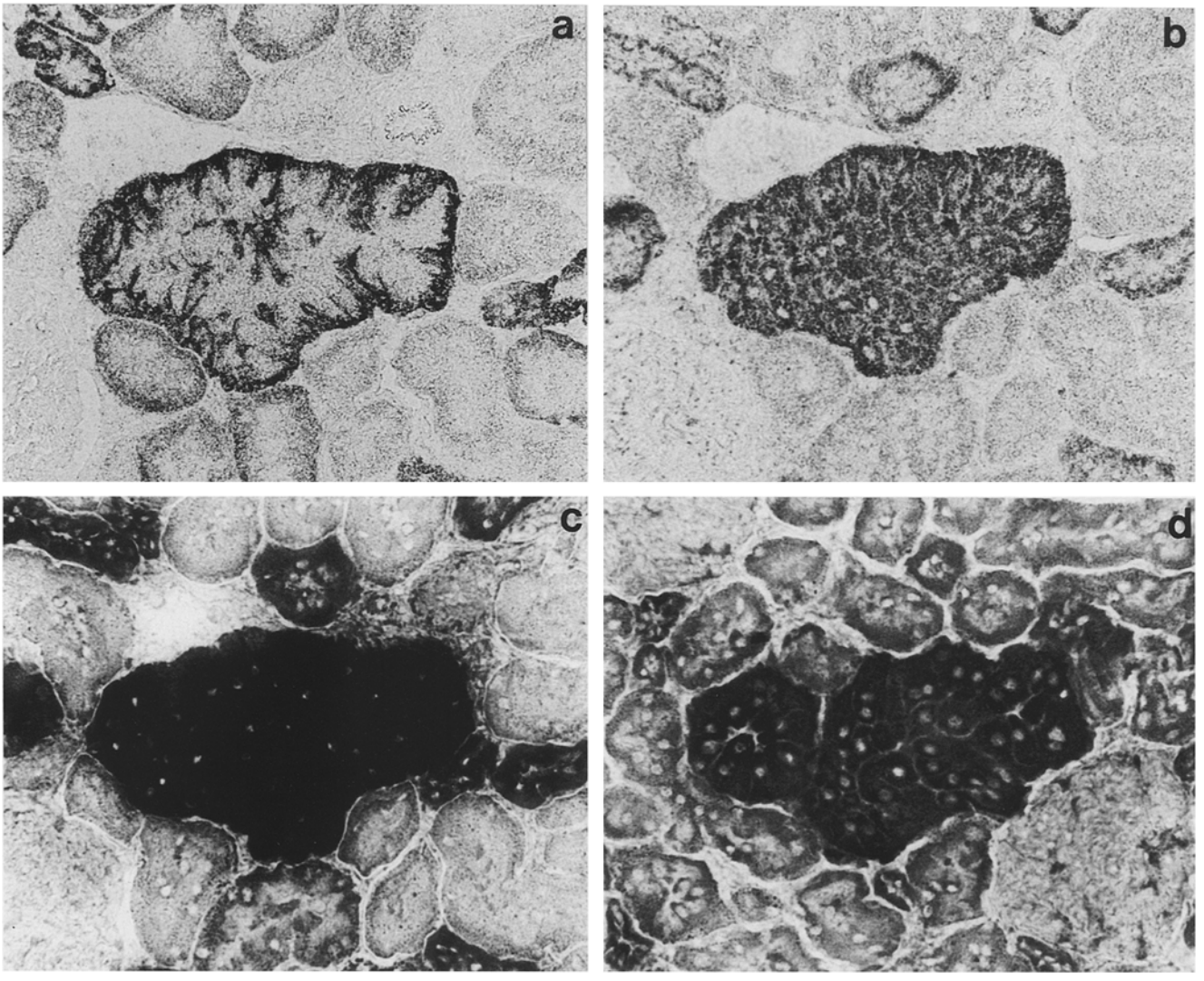

Fig. 3 Histochemical pattern of small renal oncocytoma. Serial sections through oncocytoma exhibiting increased content of GLUT1 (**a**), hexokinase I (**b**), and elevated activities of hexokinase (**c**), and pyruvate kinase (**d**). a–d ×290

of a number of enzymes, particularly hexokinase [27], pyruvate kinase [26], and glucose-6-phosphate dehydrogenase [28] correlate well with biochemical data determined in microdissected tissue samples. In addition, enzyme histochemical findings in specimens of human renal clear cell carcinomas [10] were also largely confirmed by subsequent biochemical investigations [37, 45]. Hence, the biochemical changes demonstrated in situ by histochemical approaches in rat renal oncocytic tubules and tumours appear to provide a reliable basis for considerations on the metabolism and growth behaviour of these lesions.

The level of GLUT1 may reflect the rate of glucose transport into cells, since this transporter exhibits kinetic asymmetry, with a lower K_m value for net influx than for either equilibrium exchange or net efflux [34, 53]. High levels of GLUT1 and hexokinase I allow oncocytes to produce large amounts of glucose-6-phosphate, the substrate for various metabolic pathways. This intermediate metabolite appears to be mainly channelled into the formation of pyruvate, because pyruvate kinase activity is elevated in oncocytes, whereas activities of glucose-6-phosphate dehydrogenase and glycogen synthase were found to be unchanged or, in the case of glucose-6-phosphate dehydrogenase, often even decreased [49]. In contrast to fast growing tumour cells characterized by high aerobic glycolysis [6, 14, 16, 44, 52], the reduced lactate dehydrogenase activity in oncocytes suggests that the glycolytic pyruvate is not preferentially converted to lactate, but is mainly introduced into the Krebs cycle. An elevation in channelling the glycolytic pyruvate to the oxidative phosphorylation may be required to meet the enhanced demand for oxidative substrate indicated by abundant mitochondria and hyperactivities of several mitochondrial enzymes in oncocytes.

Renal oncocytoma was distinct from rat renal clear/acidophilic, and basophilic cell tumours by virture of metabolic changes during its development. A marked reduction in the activities of both glycolytic and mitochondrial enzymes occurs during the development of

preneoplastic clear cell (glycogenotic) tubules. However, activities of these enzymes are elevated in the progression from glycogenotic tubules to clear/acidophilic cell tumours [1]. During the development of renal basophilic cell tumours, activities of glycolytic enzymes gradually increase from preneoplastic tubules to tumours, whereas activities of glucose-6-phosphatase, succinate dehydrogenase and marker enzymes for the proximal tubules such as alkaline phosphatase decrease [2, 49]. In contrast, we did not find any further significant metabolic alterations during the development of oncocytomas from oncocytic tubules. GLUT1 expression was concurrently increased with the glycolytic enzyme activities in oncocytes, whereas clear/acidophilic, and basophilic cell tumours express GLUT1 and GLUT2 at barely detectable levels in spite of their high glycolytic enzyme activities [1, 2]. It is difficult to understand the obvious differences in the behaviour of GLUT1 expression in oncocytoma and other types of renal cell tumour, but the overexpression of GLUT1 in renal oncocytes correlates well with the increased content and activity of hexokinase in these cells. The characteristic over-expression of GLUT1 in renal oncocytomas may be used as a diagnostic criterion for separating oncocytes from other types of acidophilic (granular) cells in renal cell tumours, which show a reduced expression of this glucose transporter protein [1].

It is well-known that a fraction of isozyme types I and II of hexokinase is bound to the outer mitochondrial membrane [3]. Preliminary immunoelectronmicroscopical studies in our laboratory suggest that this may also be true for hexokinase I in renal oncocytes. In comparison with the soluble form of hexokinase, the bound form exhibits advantages in the kinetic properties such as higher affinity for ATP [54] and less sensitivity to the feedback inhibition by glucose-6-phosphate [50]. There is also increasing evidence that mitochondrially bound hexokinase preferentially utilizes intramitochondrially generated ATP, even in the presence of high extramitochondrial ATP concentration [3, 13]. In accordance with these advantageous catabolic features of mitochondria-bound hexokinase, high activity of bound hexokinase is mainly detected in high glucose-utilizing cells such as normal cerebral cells and neoplastic cells [4]. Furthermore, a good correlation between mitochondrial bound hexokinase activity, aerobic glycolysis and growth rate has been reported for various tumour cell lines [17, 30, 40].

In light of these findings, the high activities of glycolytic enzymes in oncocytes are striking, since renal oncocytoma is a slowly growing, benign neoplasm in both man and laboratory animals. This discrepancy may be related to an unusual behaviour of glucose-6-phosphate dehydrogenase in oncocytes. Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway which supplies precursors for the biosynthesis of nucleic acids and phospholipids. An increased synthesis of these molecules appears to be required for cell division and membrane biosynthesis during tumour growth. Thus, enhanced activity or protein content of glucose-6-phosphate dehydrogenase has been observed in preneoplastic and neoplastic lesions induced in different organs [5, 18, 28, 39] and activity of this enzyme is closely correlated with the proliferative activity in certain tumours [5]. It has been shown that, in contrast to other types of renal cell tumour [1, 49], oncocytomas display a normal or even clearly decreased activity of glucose-6-phosphate dehydrogenase [49]. This metabolic feature may be responsible for the slow growth of renal oncocytoma, despite the high glycolytic enzyme activities.

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