INCREASED GLUCOSE 6-PHOSPHATE DEHYDROGENASE CONCENTRATION IN HEPATOMA 3924A: ENZYMIC AND IMMUNOLOGICAL EVIDENCE

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1. Introduction

Previous work from this laboratory demonstrated that there is an ordered pattern of metabolic imbalance in the spectrum of hepatomas of different growth rates that is expressed in enzymatic alterations linked with malignant transformation and progression [1,2]. Recent investigations showed that phosphofructokinase, a proliferation-linked enzyme (in this class are enzymes the activity of which is elevated or decreased in parallel with tumor growth rate and malignancy), was increased in both activity and concentration in hepatomas [3,4]. It appears that no evidence has been published that malignant-transformation-linked enzymes (in the class are grouped enzymes that are altered in the same direction in all the hepatomas) also exhibited an increase in enzyme concentration. This work brings such evidence reporting that glucose 6-phosphate dehydrogenase (G6P DH) (EC 1.1.1.49), the first committed enzyme of pentose phosphate biosynthesis, was present in increased concentration in the hepatomas. This evidence is provided by separate techniques independently assaying the total enzyme activity and titrating with anti-enzyme serum the immunoprecipitable enzyme protein concentration. Thus, the enzymatic alterations linked with tumor progression (phosphofructokinase) and linked with transformation (G6P DH) represent the reprogramming of gene expression as manifested in the hepatomas.

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2. Materials and methods

2.1. *Animals and preparation of tissue extracts*

Normal male Wistar rats weighing 200 to 240 g and normal male ACI/N and tumor-bearing ACI/N rats (bilateral subcutaneous transplants) weighing 160–180 g were used. The animals were kept in individual cages. Purina laboratory chow and tap water were available ad libitum, except when the animals were put on starvation or on a high carbohydrate diet to induce G6P DH [5]. New Zealand adult male albino rabbits were used for production of the antiserum. Rats were stunned, decapitated and exsanguinated. Liver or tumor was immediately removed and minced. A homogenate was made in 3 vol of a medium containing KCl (154 mM), Tris–HCl (50 mM, pH 8.0) and EDTA (1 mM) [6]. The homogenate was centrifuged at 25 000 *g* for 1 h and the resulting supernate was used for enzyme purification. For immunochemical titration the 100 000 *g* (30 min) supernate was used.

2.2. *Purification of liver and hepatoma G6P DH*

Previously published methods [7] were adapted for the extraction of the enzyme in our liver and hepatoma systems. The modifications we

introduced are given below. In the present extraction procedure instead of DEAE-cellulose and CM-cellulose, DEAE-Sephadex and CM-Sephadex columns were used. The DEAE Sephadex column was equilibrated with Tris–HCl buffer (50 mM, pH 8.0). After the sample application the column was washed with 100 mM of NaCl gradient in the equilibrating buffer and the enzyme was eluted with 350 mM NaCl in the same buffer. The ammonium sulfate-treated redissolved precipitate was dialyzed against the Tris buffer containing $NADP^+$ (0.12 mM) instead of distilled water. Na-acetate buffer (50 mM, pH 5.5) containing $NADP^+$ (0.12 mM) was used for the equilibration of the CM-Sephadex column. The enzyme was eluted with 100 mM NaCl containing G6P (0.15 mM) in Na-acetate buffer (50 mM, pH 6.5). Before the enzyme elution, the column was washed with 50 mM NaCl in the equilibrating buffer. The conditions for the second DEAE-Sephadex chromatography were the same as for the first one except that both the equilibrating buffer and the gradients contained $NADP^+$ (0.12 mM) and before the final elution (350 mM NaCl) the column was washed with 100 and 200 mM of NaCl gradient, respectively. The procedure for the hepatoma enzyme extraction and purification was identical.

2.3. *Enzyme assays and protein*

G6P DH activity was determined spectrophotometrically at 37°C as cited elsewhere [8]. The enzyme unit is the activity that metabolizes 1 μmol of substrate per min at 37°C. Protein content was measured by the method of Lowry et al. [9].

2.4. *Production of antiserum*

Fifty units of the purified enzyme were emulsified with an equal vol of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and injected subcutaneously into the scapular region of rabbits on the 1st day and into the thigh on the 7th day. Three weeks after the first injection a booster injection of the same amount of enzyme was given intravenously without the adjuvant. One week after the last injection blood samples were taken from the ear and when antibody titer was sufficiently high, the antiserum was collected and stored at −20°C.

2.5. *Double-diffusion method*

This procedure was performed as described by Ouchterlony [10] using 0.75% agarose (Bio-Rad Laboratories, Richmond, Calif.) suspended in Tris buffer (50 mM, pH 8.0). The plates were incubated for 24 h at room temperature and the precipitine lines were stained either for proteins (Buffalo black) or enzyme activity.

2.6. *Immunochemical titration*

Enzyme samples were incubated in duplicates for 45 min at 37°C in a final vol of 0.5 ml in presence of Tris–HCl buffer (5 mM, pH 8.0), $NADP^+$ (0.24 mM) and different amounts of antiserum or control serum. Hepatoma extracts were brought to about the same activity as that of the liver to achieve complete neutralization and values were corrected for this dilution. The samples were kept at 5°C overnight and centrifuged the next morning (40 000 *g* for 20 min); activities were assayed in the supernates.

3. Results and discussion

3.1. *Purification of G6P DH from normal and neoplastic liver*

Purification methods were adapted to the liver and hepatoma systems in this Laboratory. The enzyme was purified from the liver of refed rats 176-fold and from hepatoma 3924A, 74-fold. The final specific activities for the liver and the hepatoma enzyme were 55.3 and 11,9 μmol/mg protein/min, respectively.

3.2. *Comparison of kinetic properties of purified G6P DH from liver and hepatoma 3924A (table 1)*

According to studies carried out on purified G6P DH from liver and hepatoma the enzyme preparations were indistinguishable on the basis of kinetic properties.

3.3. *Inhibition by hemoglobin, methemoglobin and myoglobin*

Earlier reports indicated that hemoglobin may affect the structure [11] or inhibit the activity [12] of G6P DH. The observations in table 1 indicate that hemoglobin (from beef blood, Type 1,

Table 1
Comparison of the kinetic properties of purified rat liver and hepatoma glucose 6-phosphate dehydrogenase

Modulators	Liver	Hepatoma 3924A
	μM	μM
Glucose 6-phosphate, K_M	54	47
$NADP^+$, K_M	13	10
Hemoglobin, I_{50}	48	36
Inhibition by NADPH	52.5%	43.9%

Sigma) markedly inhibited this enzyme from both tissues. At hemoglobin concentrations of 80 μM and higher a complete inhibition of the activity of both purified enzymes was observed. Methemoglobin was equally effective and myoglobin was also inhibitory; however, albumin, ferrous sulfate or ferric chloride in the same concentrations did not affect either enzyme activity.

3.4. *Inhibition by NADPH*

NADPH is a powerful competitive inhibitor of normal liver G6P DH and the inhibition depends on the ratio of $NADP^+$/NADPH [13]. Table 1 shows that both liver and hepatoma purified G6P DH was inhibited to a similar extent by NADPH in a reaction mixture where the concentration of NADP was constant (21.6 μM). At a NADPH/$NADP^+$ ratio of 8.6 the inhibition for liver and hepatoma enzymes was 52.5 and 43.9%, respectively.

3.5. *Immunochemical studies*

The antiserum produced against the purified liver enzyme reacted with both the crude and purified G6P DH preparations from normal liver and hepatoma 3924A, resulting in a single precipitine line that fused and was stained for G6P DH activity. Similar reactions were observed with antiserum against purified enzyme from hepatomas 3924A or 7777.

3.6. *Titration of liver and hepatoma supernate G6P DH activity by liver G6P DH antiserum*

The immunodiffusion studies showed that the purified G6P DH from liver and hepatoma were immunologically identical (fig.1). Neutralization studies demonstrated that with suitable concentration of the antiserum both liver and tumor G6P DH activities were completely neutralized. The fact that the slopes of the neutralization lines were similar supports the evidence of the immunodiffusion studies indicating that the enzymes are identical. Although valuable studies have been carried out with immunochemical techniques on the behavior of G6P DH in rat liver [6,14,15], the present report appears to be the first on the application of this method to the examination of the concentration of this enzyme in neoplastic liver. The representative experiment reported in fig.1 also indicated that the G6P DH specific

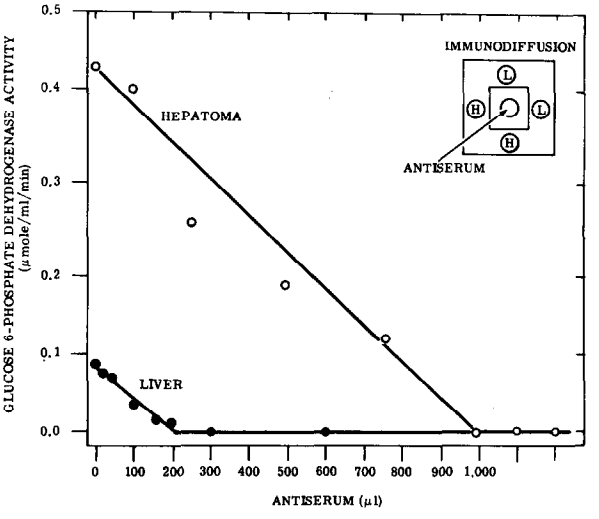

Fig.1. Immunochemical titration of G6P DH from rat liver and hepatoma 3924A supernates. Duplicate samples were incubated in the presence of different amounts of antiserum in Tris buffer (5 mM, pH 8.0) for 45 min at 37°C and stored overnight at 5°C. The supernates were centrifuged at 40 000 *g* for 20 min and assayed for the enzyme activity. Each point represents the average of two duplicate determinations.

activity purified from hepatoma 3924A was approx. 5-fold of that isolated from the normal liver here by the same procedure. In turn, the hepatoma extract required about 5 times more antiserum for complete neutralization of the G6P DH activity than was necessary for the normal liver. Thus, in the hepatoma 3924A there was a 5-fold increase in G6P DH concentration.

3.7. *Increased G6P DH amount in hepatoma: evidence for reprogramming of gene expression in cancer cells*

The objective of these investigations was to analyze the evidence for an increase in the activity of gene expression in hepatomas as manifested in production of an increased amount of the specific catalytic protein, G6P DH. Earlier studies from this Laboratory indicated that the G6P DH activity, as determined in crude extracts in a spectrum of hepatomas of vastly different growth rates [8,16,17], was increased in every neoplasm. The observations in talbe 1 and fig.1 provided evidence that the purified G6P DH from rat liver and rapidly growing hepatoma 3924A was immunologically identical and the enzyme concentration was 5-fold elevated in this tumor. Kinetic studies of the purified enzyme from liver and hepatoma 3924A further confirm that the enzyme preparations were indistinguishable on the basis of kinetic properties for substrates and inhibitors.

An isotope estimation of the overall behavior of the oxidative pathway of pentose phosphate biosynthesis indicated that there was an increased potential in the operation of this pathway in the hepatomas, especially in the rapidly growing tumors such as hepatoma 3924A (18). These isotope data are in line with the increased potential provided by increased concentration of G6P DH which is the rate-limiting enzyme of the direct oxidative pathway [8,16,17].

4. Conclusions

The results obtained in these studies by employment of independent techniques for measuring enzyme concentrations support the interpretation that the alteration in the biochemical pattern of tumor cells entails, in part, a reprogramming of gene expression. Earlier work demonstrated that the proliferation-linked enzyme phosphofructokinase was increased in concentration in hepatomas [3,4]. The present study provides evidence that the transformation-linked enzyme, G6P DH, was also increased in concentration in the hepatoma. This imbalance in gene expression coupled with an increased activity of transaldolase (EC 2.2.1.2) [17], phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) [19], and glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14) [20,21] increase the potential of the tumors to channel metabolites into purine and pyrimidine biosynthesis. This pattern of reprogramming gene expression should provide selective advantages for the cancer cells.

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