Activities and Isoenzyme Patterns of Some Enzymes of Glucose Metabolism in Human Primary Malignant Hepatoma

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SUMMARY

The levels of a number of enzymes of carbohydrate metabolism were assayed in the cancerous and morphologically normal (host) portions of livers from nine primary malignant hepatoma cases obtained at autopsy. The levels of these enzymes in two normal human adult and two fetal livers were also determined. The activities of phosphoglucomutase, fructose 1,6-diphosphatase and α-glycerophosphate dehydrogenase were considerably lower in the tumor tissue than in host tissue or tissue from normal adult livers. Other enzymes that showed decreased activity in tumor relative to host tissue included triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and lactate and malate dehydrogenases.

Pyruvate kinase activity was somewhat elevated in the tumor. More striking changes were seen on electrophoresis, which showed that the major L isoenzyme of liver was replaced by the more positively charged M band. Hexokinase activity showed no consistent changes in level in hepatoma, but starch gel electrophoresis showed the appearance of hexokinase II and disappearance of hexokinase III in tumor tissue. Glucokinase was not detected in any preparations.

An increase in the proportion of lactate dehydrogenase Isoenzyme 3 was found for one cancer patient. No differences in the electrophoretic patterns of α-glycerophosphate dehydrogenase or triosephosphate isomerase were seen between cancer, host, or normal liver tissue. The ratio of activity of tumor aldolase with fructose 1,6-diphosphate to that with fructose 1-phosphate was considerably increased, showing a change from the liver type B to admixture with muscle type A.

INTRODUCTION

Warburg et al. (21) first made the generalization that tumorous tissues show increased glycolysis. Subsequent work with “minimum-deviation hepatoma” in rats has shown that these slow-growing tumors may in fact have unaltered or decreased glycolysis. A correlation has been shown, however, between increasing growth rate of rat tumors and increase of glycolysis or decrease in gluconeogenesis (22). This has been attributed to altered activity of key gluconeogenic and glycolytic enzymes (22). The activities of the key glycolytic enzymes, hexokinase, P-fructokinase, and pyruvate kinase, increase with increasing growth rate and dedifferentiation, whereas key gluconeogenic enzymes, namely, glucose 6-phosphatase and fructose 1,6-diphosphatase, decrease with increased growth rate (22). The levels of the bifunctional enzymes were unrelated to growth rate in these hepatomas (22).

In addition, the replacement of regulatory liver isoenzymes for those that predominate in muscle and fetal tissue has been shown to occur in rat hepatoma. For example, glucokinase is replaced by hexokinase II (15), type L pyruvate kinase by type M pyruvate kinase (20), and aldolase type B by aldolase type A (16).

Studies on 10 primary human hepatomas obtained from diverse geographical areas revealed that the direction of change of some of these key regulatory enzymes resembled those occurring in rat hepatoma (2).

This paper presents a comparative study of the levels and electrophoretic patterns of various glucose-metabolizing enzymes in cancerous and host tissue of primary malignant human hepatomas as well as in 2 normal human adult and fetal livers. The subjects were Bantu-speaking Negro people from Mozambique, where there is a high incidence of hepatoma (1:1100) (12) of an etiology presumably distinct from that for the subjects of Boxer and Shonk (2). The data on the enzyme levels reflect a pattern similar to that reported by Boxer and Shonk (2) for primary human hepatomas, although with less variability. The hexokinase and pyruvate kinase isoenzyme patterns resembled those of fast-growing rat hepatomas.

MATERIALS AND METHODS

Substrates. Trisodium 2-P-glycerate, trisodium 3-P-glycerate, monosodium P-enolpyruvate, trisodium ADP, disodium ATP, oxaloacetic acid, and dihydroxyacetone-P (dimethylketal dicyclohexylammonium salt) were obtained from Biochemica Boehringer, Mannheim, Germany. Disodium fructose-1,6-di-P, disodium fructose-1-P, barium fructose-6-P, disodium glucose-6-P, glyceraldehyde-3-phosphoric acid, NADH, and NADP...
were obtained from Sigma Chemical Co., St. Louis, Mo. Potassium pyruvate and trisodium glycerol-1-P were obtained from Fluka AG Chemische Fabrik, Buchs, Switzerland.

**Auxiliary Enzymes.** All auxiliary enzymes were obtained from Biochemica Boehringer.

**Other Chemicals.** Dithiothreitol was obtained from Calbiochem, Los Angeles, Calif. Monosodium AMP and phenazine methosulfate were obtained from Sigma; 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-nitrophenyl tetrazolium salt was from the National Biochemical Corp., Cleveland, Ohio; and agarose was from Miles-Seravac Laboratories, Cape Town, South Africa. Connaught hydrolyzed starch was purchased from the Connaught Medical Research Laboratory, Toronto, Canada. Other standard reagents were obtained from British Drug Houses, Poole, England, and E. Merck, Darmstadt, Germany, and were of analytical grade.

**Source of Material.** The Bantu-speaking Negro men, from whom the liver material used in this study was obtained postmortem, were Mozambique citizens working as mine laborers in South Africa. In all cases the diagnosis of primary malignant hepatocellular hepatoma was histologically confirmed. The disease was rapidly progressive with an average survival time of 60 days following initial diagnosis. The drugs used in the treatment of these patients included 5-fluorouracil; C.63 (a-W-butyryloxyethylglyoxal dithiosemicarbazone); 5-fluorouracil + imidazole-4-carboxamide 5-{3,3-dimethyltriazeno} + vincristine + 1,3-bis(2-chloroethyl)-1-nitrosourea. The only side effect noted was alopecia in 1 case. Due to epigastric discomfort with meals, the patients could eventually eat very little food. Some were only moderately emaciated but in others emaciation was extreme.

Death was due to i.p. and/or gastrointestinal hemorrhage in 7 cases; to massive pulmonary metastases in 1 case; and probably to cachexia, inanition, and liver failure in 2 others. Four of the autopsies gave evidence of liver cirrhosis, and 4 showed distant metastases in lungs and/or portal glands. Material was obtained within 6 hours of death. Gross liver weight varied from normal size in 1 case to approximately 5 kg in several others. Histopathological examination confirmed that the liver tumor was of the hepatocellular type in all cases. Microscopic examination showed the presence of less than 10% necrotic tissue in areas adjacent to those used for the assays. Assessment of the degree of differentiation of tumor cells is highly subjective and was therefore not recorded.

The cancerous sections of the tumorous livers were dissected and separated from the sections that appeared morphologically normal; the latter were labeled "host tissue." The normal livers were obtained from patients who died of pneumonia. Two fetal livers were obtained from aborted fetuses at approximately 20 weeks of gestation. All tissues were used immediately or stored at —20°C.

**Preparation of Homogenates.** Small pieces of tissue (0.5 to 1 g) were removed from the frozen material, weighed, and homogenized in an Ultra-Turrax homogenizer in a solution containing 0.15 M KCl, 0.05 M KHCO₃, 0.006 M disodium EDTA, 0.001 M dithiothreitol [cf. Shonk and Boxer (18)]. The homogenates were centrifuged at 36,000 X g for 30 min at 4°C, and the clear supernatant was used in the assays.

**Assay Procedures.** The supernatants were diluted with homogenizing medium. Most of the enzymes were assayed by coupling to systems that involved the oxidation or reduction of pyridine nucleotides and following the change of absorbance at 340 nm. Exceptions were enolase and P-glycerate mutase, which were assayed by following P-enolpyruvate formation at 230 nm. A Unicam SP 800 or Beckman DB recording spectrophotometer was used, with the cuvet holder thermostatted at 30°C. The assay procedures used were those of Shonk and Boxer (18) with slight modifications as indicated below.

**Glucose-6-P dehydrogenase.** P-glucocutamatase, fructose-1,6-diphosphatase, aldolase, triose-P isomerase, α-glycerophosphate dehydrogenase, glyceraldehyde-P dehydrogenase, P-glycerate mutase, pyruvate kinase, and malate dehydrogenase were assayed as described by Shonk and Boxer (18), except that 0.15 mM NADH was used. Concentrations of 300 mM and 1.5 mM glucose were used in the assay procedures for glucokinase and hexokinase, respectively. The assay procedure for P-glucose isomerase was modified in that a lower concentration of fructose-6-P (0.75 mM) was used because of a glucose-6-P impurity in the fructose-6-P preparation, leading to a considerable production of NADPH. The glucose-6-P oxidation was allowed to reach completion before addition of homogenate. The lactate dehydrogenase assay procedure was modified by the use of 2.9 mM pyruvate.

Fructose-1-P aldolase was assayed similarly to fructose-1,6-di-P aldolase except that 43.5 mM fructose-1-P replaced the fructose-1,6-di-P and triose-P isomerase was omitted from the assay mixture. Reaction mixtures for the assay of enolase activity contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, 2.8 mM 2-P-glycerate, and homogenate. For the assay of P-glyceraldehyde mutase, reaction mixtures contained 50 mM Tris-HCl buffer (pH 7.4), 2.7 mM 3-P-glycerate, 1 unit enolase, and the homogenate. A unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 μmole of substrate per min.

**Electrophoretic Procedures.** Starch gel electrophoresis was used for separating the various hexokinase isoenzymes, and the procedure used to stain them was essentially that of Katzen and Schimke (8). The pyruvate kinase isoenzymes were separated by the method of Filides and Harris (6) for adenylate kinase and the staining procedure was modified to demonstrate pyruvate kinase activity. The staining mixture consisted of 0.2 M Tris-HCl buffer (pH 8.0), 20 mM MgCl₂, 1.5 mM ADP, 3 mM P-enolpyruvate, 12 mM AMP (to inhibit the adenylate kinase reaction), 0.4 mM NADP, 10 mM glucose, 7 units hexokinase, 7 units glucose-6-P dehydrogenase, 2 mg 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-nitrophenyl tetrazolium salt per ml, and 0.04 mg phenazine methosulfate per ml. A blank consisting of all reagents except P-enolpyruvate was set up. Another layer of the same starch gel was also stained with all the above reagents except that both P-enolpyruvate and AMP were omitted so that any adenylate kinase activity could be detected. By comparison of the isoenzyme patterns obtained with these 3 stains, the position of the pyruvate kinase isoenzymes could be determined.

Lactate dehydrogenase, α-glycerophosphate dehydrogenase, and triose-P isomerase were separated by horizontal electrophoresis on starch gels. The gel buffer was 0.076 M Tris-0.005 M citrate...
buffer, (pH 8.6), and the bridge buffer was 0.3 M borate buffer, pH 8.0. Electrophoresis was carried out for 3 hr at 4° and 20 ma. The lactate dehydrogenase isoenzymes were then detected with the stain described by Raabo (13). The stains for α-glycerophosphate dehydrogenase and triose-P isomerase were similar to those described by Shaw and Koen (17).

RESULTS AND DISCUSSION

Enzyme Levels. Chart 1 shows the levels of glucose-metabolizing enzymes measured in 2 normal and 2 fetal livers, as well as in host and cancerous tissue obtained from several hepatoma patients. A solid line is used to join the activity of the enzyme in the cancerous tissue (C) with that of its corresponding host tissue (H). The levels of the enzymes in the cancerous tissues were expressed as a percentage of those in the corresponding host and subjected to statistical analysis with Student’s t test (Table 1). Some enzymes changed greatly in level in hepatoma, while others changed slightly or not at all. Table 1 also shows the mean enzyme activities for host and cancerous tissues.

In this Discussion we compare the results of the present study to those for humans and rats obtained by Boxer and Shonk (2). They used gliceraldehyde-P dehydrogenase as a base line (= 100%) and formulated their results relative to that. It can be seen by studying both their results and Chart 1 that there is a great variation in the level of this enzyme; thus their results were recalculated for units/g wet weight, in order to compare them to the present results and to determine the statistical significance of differences. They compared cancer enzyme levels to those of normal liver, whereas we considered the host to be more reliable for comparative purposes, since factors such as effects of drugs and diet are eliminated.

It can be seen from Chart 1 and Table 1 that hexokinase and glucose-6-P dehydrogenase showed no significant increases in tumors, as was also shown by Boxer and Shonk (2). This should be compared with the low levels of hexokinase found only in slow-growing rat hepatomas, while considerably elevated levels occur in fast-growing hepatomas (15, 23), and with the elevation of glucose-6-P dehydrogenase that was found in all but 1 rat hepatoma tested (22). Glucokinase, the hexose:ATP phosphotransferase with a high Michaelis constant for glucose (11), which disappears in rat hepatomas (15, 23), was absent from host and cancer tissues and from normal liver in the present study. Similar results were found by others for human autopsy material (2, 9), but not in well-nourished humans (3); the activity has been found in biopsy specimens of normal human livers. Since this enzyme is very unstable at 37° and while freezing and thawing (9), its absence in the present study can be understood.

P-glucomutase, fructose 1,6-diphosphatase, and α-glycerophosphate dehydrogenase were considerably reduced in all tumors in this study. The differences between host and cancer were highly significant on statistical analysis (p < 0.005). Boxer and Shonk (2) also showed consistent reduction in tumor levels of P-glucomutase and α-glycerophosphate dehydrogenase, while fructose 1,6-diphosphatase was reduced in only 6 of 10 cases. All rat hepatomas studied have shown considerably lower activities of these 3 enzymes compared to levels in the host (2, 19, 22).

Pyruvate kinase was the only enzyme that was statistically significantly raised in hepatoma relative to the corresponding host tissue (p = 0.005). Previous studies on human hepatomas showed no statistically significant increase in this enzyme (2), but rat hepatomas showed a correlation between increasing growth rate and raised pyruvate kinase activity (5).

Isoenzymes. The level of aldolase is not significantly different in tumor tissue as compared to host, although the relative amounts of aldolase A and B appear to alter, as evidenced by a raised fructose-1,6-di-P:fructose-1-P activity ratio. Muscle aldolase A has a low activity with fructose-1-P, giving fructose-1,6-di-P:fructose-1-P activity ratios of 50, whereas liver aldolase B is less specific and gives a ratio of 1 (14). The elevated ratio in hepatoma points to the presence of a mixture of aldolases A and B. This confirms our earlier results (1) and those of Schapira et al. (16). Rat hepatomas, too, showed elevated fructose-1,6-di-P:fructose-1-P activity ratios (10). Fetal liver showed only slightly higher ratios than did adult liver; this differs from the results of Schapira et al. (16).

Fig. 1A shows the hexokinase isoenzyme patterns for normal, host, and cancerous human liver. All the isoenzymes migrated toward the anode and are numbered I, II, and III in order of increasing mobility (8). Type I hexokinase was the predominant isoenzyme in all the tissue types tested. Type II hexokinase was absent from normal adult liver, which has also been found by others (3) but was present in the hepatomas and in some of the host livers. Type III hexokinase occurred in normal host and cancerous liver, but was more faintly stained than Band II in the cancers. It appears that hexokinase II occurs in precancerous liver and gradually replaces Type III in cancerous liver. Fast-growing rat hepatomas also show disappearance of Band III and increase of Band II hexokinase (15). The absence of glucokinase was confirmed by staining the gels in 0.1 M glucose, when no additional bands appeared. No bands appeared in the absence of glucose.

Fig. 1B shows the electrophoretic patterns obtained for pyruvate kinase from normal human adult, host, and cancerous tissue. The farther-moving anodal band represents adenylyl kinase, which also shows up with this stain, although it is partially inhibited by AMP in the staining mixture. The slower-moving anodal band (type L) (20) predominates in normal liver, which shows only traces of the cathodal band (type M) (20). In hepatoma tissue, type M predominates, with type L faint or absent. The host tissue showed rather faint bands, but both types L and M appeared to be present in approximately equal amounts. Fast-growing rat hepatomas also showed the predominance of Isoenzyme M (5). A new isoenzyme occurring only in fast-growing rat hepatoma has been shown by isoelectric focusing (4). No bands appeared in
Chart 1. Levels of enzymes of glucose metabolism in cancerous (C) and corresponding host (H) portions of livers from patients with primary hepatoma. In addition, activities in normal adult (a) and fetal liver (f) are shown. F1P, fructose-1-P; FDP, fructose 1,6-diphosphate.

The enzymes assayed are: hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) (HK); glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2); glucose-6-P dehydrogenase (D-glucose-6-P:NADP oxidoreductase, EC 1.1.1.49) (G6PDH); P-glucomutase (α-D-glucose-1,6-di-P:α-D-glucose-1-P phosphotransferase, EC 2.7.5.1) (PGM); P-glucose isomerase (D-glucose-6-P ketol isomerase, EC 5.3.1.9) (PGI); fructose diphosphatase (D-fructose-1,6-di-P-1-phosphohydrolase, EC 3.1.3.11) (FDPase); aldolase (fructose-1,6-di-P-D-glyceraldehyde 3-P lyase, EC 4.1.2.13) (ALD); triose-P isomerase (D-glyceraldehyde-3-P ketol isomerase, EC 5.3.1.1) (TP); α-glycerophosphatase (L-glycerol-3-P:NAD oxidoreductase, EC 1.1.1.8) (α-GDH); glyceraldehyde-P dehydrogenase (D-glyceraldehyde-3-P:NAD oxidoreductase phosphorylating), EC 1.2.1.12); P-glycerate kinase (ATP:3-P-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (PGK); P-glycerate mutase (2,3-di-P-D-glycerate:2-P-D-glycerate phosphotransferase, EC 2.7.5.3) (PGM); enolase (2-P-D-glycerate hydroxylase, EC 4.2.1.11) (ENO); pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (PK); lactate dehydrogenase (L-lactate: NAD oxidoreductase EC 1.1.1.27) (LDH); malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) (MDH).
the absence of P-enolpyruvate.

Lactate dehydrogenase showed the same isoenzyme pattern for cancer tissue as for normal liver, although in some cases an increase in Isoenzyme 3 was seen (Fig. IC). Tumors of other tissues, e.g., colon, show an increase in Isoenzyme 5 (7), but since this is the predominant liver isoenzyme, it would not be expected to increase. No differences in isoenzyme patterns were found for triose-P isomerase or α-glycero-P dehydrogenase between normal and hepatoma tissue.

Conclusion. Of the enzymes of carbohydrate metabolism investigated, the activities of P-glucomutase, fructose 1,6-diphosphatase, and α-glycero-P dehydrogenase were considerably reduced in all hepatomas as compared to the corresponding host tissue. These differences could indicate reduced glycolysis, gluconeogenesis, and glycogen synthesis, respectively. Similarly, the higher levels of pyruvate kinase could indicate increased glycolytic activity in cancers. Moreover, the change to Isoenzyme M of pyruvate kinase in cancer is significant, since this isoenzyme has a lower Michaelis constant for the substrate P-enolpyruvate than Isoenzyme L (D. Balinsky, E. Cayanis, and I. Bersohn, submitted for publication), and can therefore utilize P-enolpyruvate at lower concentrations. The lower levels in cancer of lactate, malate, and glyceraldehyde-P dehydrogenases, P-glyceraldehyde kinase and mutase, and triose-P isomerase cannot be explained metabolically as clearly but emphasize that multiple enzyme changes occur in hepatoma. The appearance of hexokinase II in cancer and the altered specificity of hepatoma aldolase also stress this point.

REFERENCES


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Fig. 1. Isoenzymes separated by starch gel electrophoresis as described in the text. A, hexokinase. Left to right, cancer 1, host 1, cancer 2, host 2, cancer 3, host 3, normal liver. B, pyruvate kinase. Left to right, normal liver, host 1, cancer 1, host 2, cancer 2, host 3, cancer 3. C, lactate dehydrogenase. Left to right, normal liver, host 3, cancer 3, host 1, cancer 1, host 2, cancer 2.
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