GANN Monograph on Cancer Research 13

ISOZYMES AND ENZYME REGULATION IN CANCER

JAPANESE CANCER ASSOCIATION

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Tomizo Yoshida, M.D.

species in slow-growing, well-differentiated hepatomas, but near or complete loss of those enzymes and their replacement in some instances by nonhepatic enzyme species in fast-growing, poorly differentiated hepatomas. A striking feature of the poorly differentiated tumors was the occurrence of certain i.ozymes found in fetal liver, but low or absent in adult liver. Thus, neoplasia appears to involve activation of genes normally repressed during embryonic development and the repression of genes normally expressed in the functional organ. Further understanding of these phenomena must await a deeper exploration of those factors which regulate gene expression in eukaryotic cells.

Other noteworthy observations reported at this conference were a striking correlation between growth rates of hepatomas and their ability to incorporate thymidine into DNA, and some significant effects of an implanted tumor on the metabolism and properties of host tissues.

The participants unanimously agreed that this conference was a uniquely beneficial experience in international cooperation toward common objectives. The conferences expressed their special thanks to Drs. Morris and Yoshida for the hepatomas which their researches have made available to the scientific community.

Thanks are also expressed to the U. S. and Japan sponsoring agencies for their generous support, to *GANN* and its Board for publishing the proceedings, and to Margaret Foti, Managing Editor of *Cancer Research*, and her staff for valuable editorial assistance with the manuscripts.

January, 1972

Sidney Weinhouse
Tetsuo Ono

CONTENTS

ISOZYMES IN RELATION TO DIFFERENTIATION IN TRANSPLANTABLE RAT HEPATOMAS*[1]

Sidney WEINHOUSE, Jennie B. SHATTON, Wayne E. CRISS,*[2]
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To determine the degree of retention and deletion of isozymes involved in normal liver function in liver neoplasms, a series of Morris hepatomas has been studied. Illustrative data on four isozyme systems reveal the following: With glucose-ATP phosphotransferases, aldolases, pyruvate kinases, and adenylate kinases, the highly differentiated, slow-growing hepatomas display the same isozyme pattern as normal adult liver; with decreased differentiation and increased growth rate, there is a variable loss of the "liver-specific" isozymes, gluokinase, aldolase B, pyruvate kinase II, and adenylate kinase III. With a series of poorly differentiated, fast-growing tumors, these liver-specific isozymes are virtually completely lost. In poorly differentiated hepatomas the liver-specific phosphotransferase, aldolase, and pyruvate kinase are each replaced by an isozyme which is very low in the adult liver. These findings reveal that retention of differentiated tissue function is not incompatible with the neoplastic transformation, but suggest that replacement of highly regulated isozymes by others not subject to host regulation may account for the lack of growth control of poorly differentiated tumors. These as well as other isozyme studies point to an instability of gene expression as a characteristic feature of the neoplastic cell.

There is an astonishing resemblance between poorly differentiated hepatomas and fetal liver in the isozyme patterns thus far studied. This observation may be added to a growing body of evidence for a "switching on" of fetal protein synthesis following or accompanying the "switching off" of gene products of the differentiated cell. These findings further suggest that impairment of

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gene control, rather than alteration of gene structure, may be a crucial factor in the neoplastic transformation.

At the present time, exciting new findings in virology and immunology overshadow many of the more traditional disciplines which share in the battle against cancer. While these now occupy the center of the stage, rightly so because of the possible immediacy of their application to the prevention or cure of human cancer, nevertheless there are certain other characters in this drama whose role may assume great importance in the future. One of these is the subject we have chosen to discuss at this meeting, that of isoenzymes and their regulation. This relatively new area of biochemistry holds great promise in furthering our understanding of such basic biological processes as gene activation and expression, disorders of which loom as possible etiological vectors in the cancer problem.

In this introductory presentation, it is appropriate to discuss briefly the theoretical background of our common field of interest. Although heterogeneity of enzyme structure had been occasionally suggested in the early years of biochemistry, it was not until simple and powerful methods of protein separation and identification were developed that the science of what we might call isoenzymology was created. The molecular heterogeneity of certain esterases and lactate dehydrogenase was demonstrated by Markert and Moller (*31*) hardly more than a decade ago. In the intervening years, the field has blossomed and borne much good fruit. An examination of the literature, which is now of overwhelming magnitude, reveals that the study of isozymes has become an unparalleled boon to the geneticist by furthering our understanding of genetic diseases and is assuming increasing practical utility in general medical diagnosis (*28*, *46*, *52*, *55*).

TABLE I. Experimental Procedures for Identification of Isozymes

1. Kinetic	4. Isoelectric focusing
2. Electrophoresis	5. Immunological
3. Chromatographic	

Table I lists the experimental techniques that have been most often employed for the identification of multiple forms of enzymes. Perhaps no method is more generally useful than that of zone electrophoresis on a solid matrix such as starch or polyacrylamide gel, combined with specific enzymatic staining methods. Kinetic differences can be employed to good advantage when circumstances permit. We, as well as others, have taken advantage of this method to determine isozymes of glucose-ATP phosphotransferases (*44*, *45*), aldolases (*2*), and lactate dehydrogenases (*38*) and are now applying it to pyruvate kinase isozymes.*

Methods of column chromatography, particularly the newly developed procedure of isoelectric focusing (*22*), are not easily adaptable to routine estimations but are extremely useful in specific applications. The same is true of immunological techniques, whose sensitivity and specificity have been thoroughly documented. Needless to say, combinations of these procedures complement and reinforce the individual methods.

* Unpublished work of F. Farina, S. Weinhouse, and H. P. Morris.

TABLE II. Molecular Basis for Multiple Forms of Enzymes

1. Multiple genes	4. Protein modification
2. Aggregation	5. Conformational isomerism
3. Partial proteolysis	

The voluminous isozyme literature has been adequately reviewed in recent books and monographs (*28*, *46*, *52*, *55*), but we might consider briefly the nature and origins of isozymes. Basically, multimolecular forms may arise in two ways; either by synthesis at different gene loci, giving rise to structurally different polypeptides, or by modification of preexisting proteins (Table II). In the former instance, individual polypeptides may be active, as with the glucose-ATP phosphotransferases (*21*), or inactive subunits may combine in different proportions to form a series of isozymes. For example, the two subunits of lactate dehydrogenase combine to form 5 active tetramers (*21*) and the three subunits of aldolase A, B, and C likewise combine to form active tetramers consisting of combinations of A with B and C (*39*).

There are so many isozymic forms arising by modification of proteins that we can barely scratch the surface of this subject. If the isozyme concept can validly be extended to all of the allosteric enzymes whose kinetic properties are profoundly affected by combination with various substances, there would hardly be an enzyme that had no isozymic forms. Of the many well-recognized examples of this type, there are the glutamine synthetases of *Escherichia coli* studied by Stadtman (*47*), which differ in their attachment to AMP; the glycogen synthetases and phosphorylases which exist in phosphorylated and dephosphorylated forms; the proteases, which are activated by partial proteolysis; and the glutamate dehydrogenases, which have different substrate specificities depending on the state of aggregation.

With this background, we should like to explore with you some issues which arise from isozyme studies carried out in our laboratory during the past eight or nine years. These studies were all carried out with the pleasant and profitable collaboration of Dr. Harold P. Morris, who will provide a more detailed description of these tumors. The work we have chosen to discuss covers the study of isozyme patterns of four enzymes in liver under a variety of dietary and hormonal influences and in a " spectrum " of Morris tumors varying in growth rate and in degree of differentiation. The degree of differentiation is based on cellular morphology and tissue architecture from histological examinations carried out in our laboratory by Dr. David Meranze. For convenience, these have been divided into three groups, characterized as highly, well-, and poorly differentiated. In gross appearance, the highly and well-differentiated hepatomas resemble liver in color and consistency, the cells are large and contain abundant pale-staining eosinophilic cytoplasm, and they contain variable quantities of glycogen. The nuclei are round, with large nucleoli, and there are occasional double-nucleated cells. The cells are arranged in sheets, often in a lobular pattern. There are canaliculi, sometimes with bile pigment and sinusoids are conspicuous, with lining cells resembling Kuppfer cells. There may be bands of connective tissue, with macrophages containing hemosiderin, ceroid, lipid, and bile pigment. There are

TABLE III. General Properties of Morris Hepatomas (*28*, *44*, *57*)

Property	Degree of differentiation		
	High	Well	Poor
Growth rate	Very low	Low	Rapid
Chromosome number	Normal	Nearly normal	Abnormal
Chromosome karyotype	Normal	Nearly normal	Abnormal
Respiration	High	Moderate	Moderately low
Glycolysis	Low	Low	High
Enzyme pattern	Liver-like	Some deletions	Many deletions

many transitional stages between different hepatocellular carcinomas, based on architecture, cytology, and staining characteristics, often within a particular hepatoma. The choice between well- and highly differentiated is a matter of judgment based on the above-mentioned criteria.

The poorly differentiated hepatomas are grossly firm and gray or white in color. The cells are smaller, with great variations in size and pattern, and are laid down in crowded sheets. There is a complete loss of hepatocellular and lining cell pattern. Mitoses are frequent, with only rare canalicular formation and pigment deposition. A more detailed description of the gross and microscopic characteristics may be found in earlier reviews (*33–35*).

A summary of the properties of these hepatic tumors is shown in Table III. Closely correlated with the degree of differentiation is the growth rate. It is very low in the few highly differentiated tumors, extending from three or four months to a year for a transplant generation (*33*, *34*). Growth rates are considerably faster, at two to six months, for well-differentiated tumors, and are extremely rapid, at one month or less, for the poorly differentiated hepatomas. Some of the highly differentiated tumors have the normal liver chromosome number and karyotype. Some of the well-differentiated tumors have the diploid number of chromosomes but differ slightly in karyotype. The poorly differentiated tumors deviate markedly in chromosome karyotype and number from those of rat liver. Respiration decreases moderately with loss of differentiation, but the striking feature of these tumors is the low or negligible glycolysis in the well-differentiated, in contrast with the usual high level of glycolysis in the poorly differentiated tumors (*4*, *54*). These well-differentiated tumors, with their low glycolytic activity, make it clear that high lactic acid production is not an absolute requirement for tumor survival. These well-differentiated low-glycolyzing tumors can grow, albeit slowly, and they can also metastasize and ultimately kill their hosts. With decreased differentiation there is also a decrease or loss of certain enzymes which play a unique role in liver function, the so-called liver marker enzymes. We will elaborate on this aspect shortly.

Of the four enzymes whose isozyme patterns we plan to discuss, three are key enzymes of carbohydrate metabolism, the glucose-ATP phosphotransferases, the aldolases, and the pyruvate kinases. The fourth enzyme is adenylase kinase. Though not specifically involved in a metabolic pathway, it has the important function of maintaining the equilibrium among the three adenine nucleotides,

AMP, ADP, and ATP, substances which are either substrates, products, or effectors of a host of metabolic processes.

Glucose-ATP Phosphotransferases

This enzyme, as seen in Fig. 1, exists in multiple forms, with each normal tissue having its individual pattern. Liver has four forms, three of which, marked I, II, and III, are collectively called hexokinase. The fourth form is called glucokinase. In this figure is shown the relative intensities of the bands on starch gel, though it is important to point out that the intensity is not a reliable index of the activity. In fetal and neonatal liver, the preponderant forms are the three hexokinases, and the presence of glucokinase is undetectable by spectrophotometric assay, although a very faint band denotes its presence in extremely low activity.

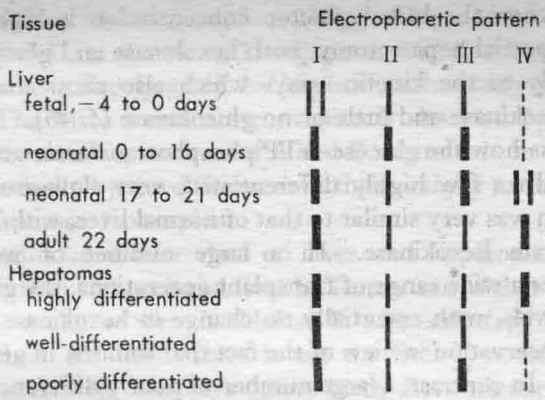

FIG. 1. Starch gel electrophoretic patterns of fetal and neonatal liver, and hepatoma glucose-ATP phosphotransferases

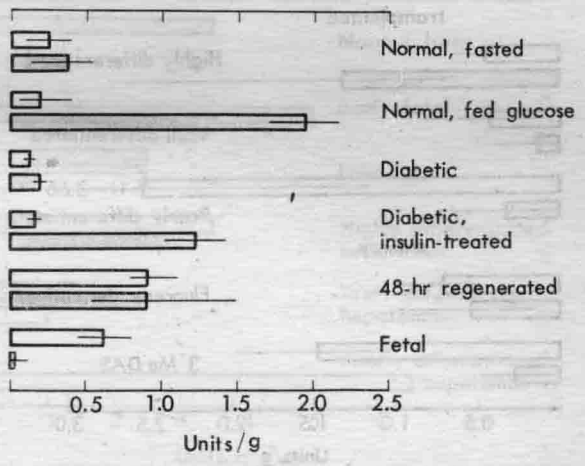

FIG. 2. Values for hexokinase (□) and glucokinase (■) determined by kinetic assay

Hexokinase includes all three low K_m isozymes I, II, and III.

Not until 17 days after birth does the glucokinase make itself evident, and by 20 or 21 days after birth it is at the normal adult level. In the lower portion of this figure are patterns for the various classes of hepatomas. All three hepatoma types have the same three hexokinases. However, the glucokinase varies markedly with the state of differentiation.

Kinetic assays supplement the electrophoretic results in providing quantitative data, as shown in Fig. 2. The hexokinase isozymes, I, II, and III, collectively shown in the clear bars, are present at a low total activity which does not change with diet or hormonal conditions. On the other hand, the fourth isozyme, called glucokinase, is highly responsive to dietary and hormonal conditions, being low in fasted normal animals and high in carbohydrate-fed animals (*5*, *10*, *40*, *44*, *45*, *53*). It is also insulin-dependent, being extremely low in diabetes, and is restored by insulin injection (*10*, *40*). This isozyme has an important physiological function in hepatic glucose utilization. It has a very high K_m for glucose, and it is this property that is responsible for the fact that the liver takes up glucose only when the blood glucose concentration is high. During liver regeneration after partial hepatectomy, both hexokinase and glucokinase are high. We point especially to the kinetic assays which also show that fetal liver has essentially only hexokinase and little or no glucokinase (*5*, *45*).

Figure 3 shows how the glucose-ATP phosphotransferase activity changes in liver neoplasms. In a few highly differentiated, very slow-growing hepatomas the isozyme pattern was very similar to that of normal liver, with high glucokinase and low-to-moderate hexokinase. In a large number of well-differentiated tumors studied over a wide range of transplant generations, the glucokinase dropped to very low levels, with essentially no change in hexokinase (*41*, *45*). This is a very striking observation in view of the fact that tumors, in general, have high hexokinase levels. In contrast, a large number of poorly differentiated hepatocarcinomas do exhibit high hexokinase activity, but with little or no glucokinase.

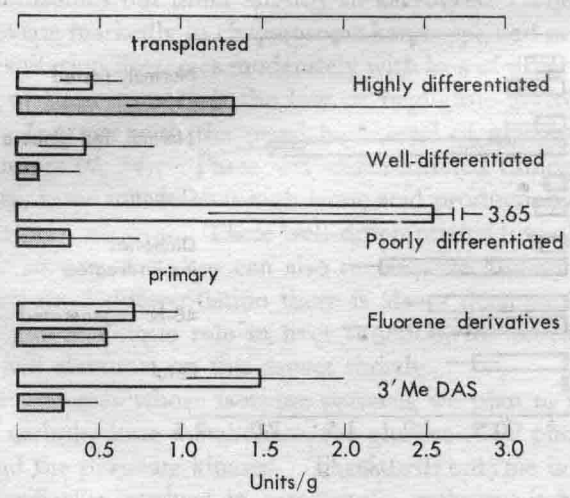

FIG. 3. Values in primary and transplanted hepatomas for hexokinase (□) and glucokinase (▩), determined by kinetic assay

Thus, loss of differentiation in hepatic tumors, which is accompanied by greatly increased growth rate, results in virtual loss of a glucose-ATP phosphotransferase which is physiologically functional and under regulation by diet and hormones, being replaced by high activities of three isozymes which are ordinarily low in normal liver. In the poorly differentiated Novikoff and 3924A hepatomas, all three hexokinase isozymes share in the marked rise, but predominant activity is present in Isozyme III. We call attention particularly to the striking resemblance of the isozyme pattern of the poorly differentiated tumors to that of the fetal liver.

Aldolase

Aldolase also exists in multiple forms which, like lactate dehydrogenase are tetramers of subunits with different primary structures distinguishable by immunological or kinetic criteria (*39*). In addition to aldolase A, which is the sole form of muscle aldolase, and aldolase B, which is the major form in liver, a new form termed aldolase C, as shown by Dr. Sugimura and others, has been found in brain, where it exists largely as an A-C hybrid with a large preponderance of A (*36*, *39*, *43*). The three forms are conveniently detected by starch gel electrophoresis, but the relative quantities of A and B subunits in liver and hepatomas can be assayed kinetically by the differences in their activity towards fructose 1, 6-diphosphate and fructose 1-phosphate.

Figure 4 contains composite data on liver and hepatomas from our own studies and data on fetal and perinatal liver by Rutter and Weber (*39*). In normal rat liver the almost exclusive form is aldolase B. In early fetal liver, up to about ten days before birth, the major form is not the liver-type aldolase B, but the nonhepatic aldolase A. At four days before birth there are approximately equal quantities of A and B, and the adult pattern is reached by one or two days before

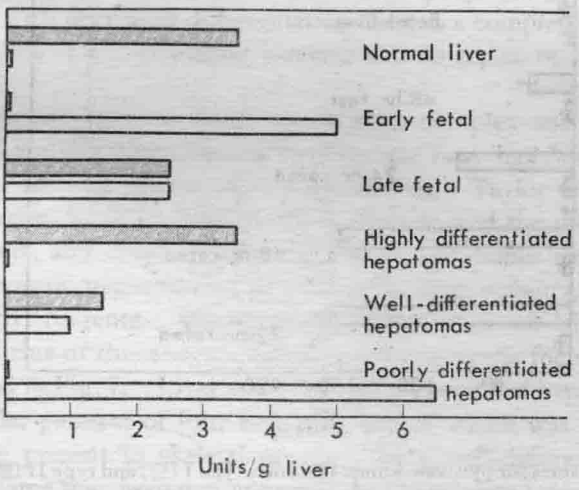

FIG. 4. Values for aldolase A (□) and aldolase B (▩) in liver and hepatomas, as determined by ratio of activities toward FDP and F1P

birth. Again, the highly differentiated hepatomas exhibit essentially only aldolase B activity.

In the well-differentiated, slow-growing tumors, the A form becomes evident, but the B form is still preponderant. However, in the poorly differentiated tumors, the B form has been essentially completely replaced by the A form. Similar observations have been made by George and Fanny Schapira in their extensive studies of aldolase isozymes in neoplasia (*42*, *43*, *48*). An interesting example of the persistence of the C form in brain tumors has been provided recently by Sugimura *et al.* (*48*), and these authors as well as Schapira *et al.* (*43*) found aldolase C to be present also in certain poorlydifferentiated hepatomas. Again we observe a striking similarity between the poorly differentiated tumors and the fetal liver.

Pyruvate Kinase

A crucial enzyme in the glycolytic pathway is the transphosphorylase which catalyzes the transfer of phosphate from phosphoenolpyruvate to ADP. This enzyme, like the glucose-ATP phosphotransferases, also exists in multiple forms. Two major forms are found in liver; type I, similar in kinetic properties to the muscle enzyme, and type II, which is the predominant form in normal liver (*9*, *30*, *49*). The latter is highly responsive to carbohydrates in the diet and has a number of distinctive kinetic properties, such as inhibition by ATP and activation by fructose diphosphate, which differentiate it sharply from the former. It is also reported by Weber *et al.* (*56*) to be low in diabetic rats, and its activity is restored by insulin treatment. Thus, it shares, in common with certain other liver

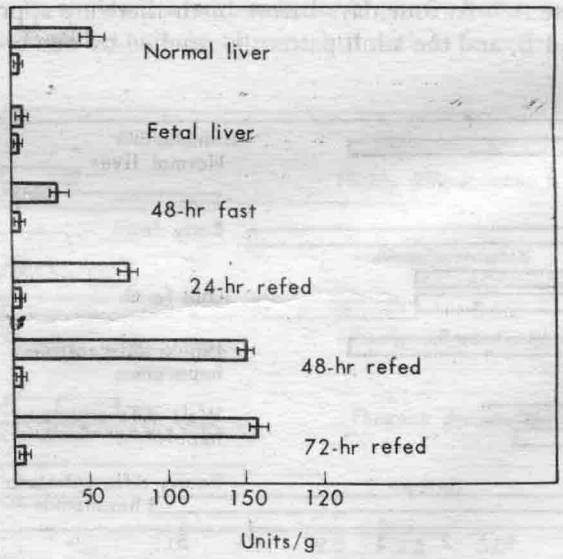

FIG. 5. Values for pyruvate kinase isozymes type I (□) and type II (■) in rat liver, as determined by differential absorption on DEAE-cellulose

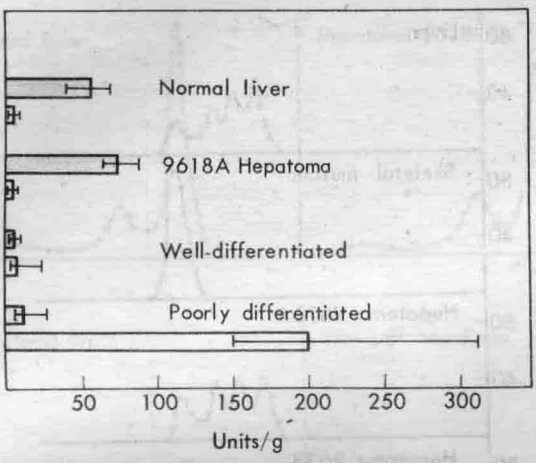

FIG. 6. Values for pyruvate kinase isozymes type I (□) and type II (■) in normal liver and in rat hepatoma, as determined by differential absorption on DEAE-cellulose

" marker " enzymes, specific functions that give the liver cells their unique metabolic capabilities. Figure 5 shows how the activities of forms I and II vary in liver. In normal liver form II is predominant, but as with other liver marker enzymes, is very low in fetal liver. The level of Isozyme II is lowered by about two-thirds by fasting and is restored readily by feeding carbohydrates. Throughout the dietary and hormonal manipulations, Isozyme I remains low and unchanged (*14*).

As shown in Fig. 6, a single, highly differentiated tumor, the 9618A, has the same isozyme pattern as liver, with a predominance of type II. However, a sharp distinction was observed between the well-differentiated and the poorly differentiated tumors. The former had very low levels of both isozymes, whereas the rapidly growing, poorly differentiated Novikoff and 3924A tumors had extremely high levels of an isozyme which has the properties of type I. Here we see again that with decreased differentiation there is a complete switch in isozyme pattern, with loss of a liver marker isozyme and its replacement by a nonhepatic type.

It now appears that the liver type is more complex and exists in multiple forms. Tanaka *et al.* (*49*) reported that normal liver has four pyruvate kinase isozymes detectable by starch gel electrophoresis. Taylor *et al.* (*50*) partially purified the major isozymes from rat liver, muscle, and the poorly differentiated 3924A hepatoma, and observed that the muscle and tumor isozymes differed in their electrophoretic migration on starch gel, in their stability, and in their susceptibility to SH reagents. More recently, Criss (*7*) in our laboratory separated the multiple forms of this enzyme by means of isoelectric focusing and obtained results depicted in Fig. 7. Liver and a highly differentiated hepatoma, the 9618A, exhibited similar patterns of four isozymes, one of which was identical with the single isozyme present in skeletal muscle. In a well-differentiated hepatoma, the 9633, the same four isozymes appeared, but they were accompanied by a fifth