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Prolyl Hydroxylase Activity in Normal and Diseased Human Liver (39143)[1]

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Several authors have documented the increased rate of collagen synthesis that leads to cirrhosis and eventual loss of function in liver disease (1, 2). The enzyme prolyl hydroxylase (EC 1.14.11.2; proline, 2-oxoglutarate dioxygenase) has been used frequently as a marker for collagen synthesis (3) and large increases in enzyme activity have been reported in association with experimental injury-induced liver fibrosis (4). Since its identification, this enzyme has been measured under numerous conditions of accelerated collagen biosynthesis, and large increases in enzyme activity have been reported in association with increased collagen biosynthesis in a variety of tissues responding to injury-induced damage (3, 6). Limited information with human liver disease is also available which suggests that increases in prolyl hydroxylase activity occur which are proportional to injury-induced collagen formation (5).

Prolyl hydroxylase synthesizes the hydroxyproline in collagen in a unique pathway in which proline is hydroxylated after incorporation into a large polypeptide precursor of collagen (3, 6). In this investigation we demonstrated that prolyl hydroxylase can be measured in the minute quantities of tissue available through needle biopsy while allowing for simultaneous histopathological examination of the specimen. Further, the rapidity of the procedure is compatible with clinical laboratory requirements for routine screening, and on the basis of the patients used in this study, the level of liver prolyl hydroxylase appears to be a sensitive indicator of active fibrogenesis.

Methods. Human liver samples were obtained from 10 adult males by use of the Menghini needle technique (7). In every instance the biopsy was performed for medical indications, and enzyme assays were carried out on excess tissue obtained incidental to the tissue used for histopathology required for patient care. The specimens were quick frozen within 90 sec in a cryostat and then stored at −40° for a maximum period of 5 days before enzyme assays were performed. Portions of each sample were prepared and stained for standard histopathological examination. The liver biopsy specimens were homogenized and assayed for prolyl hydroxylase in a single blind study where the laboratory identified the samples only by an assigned number until the completion of the experiment.

The samples of available liver tissue (2 to 10 mg) were homogenized in a glass–glass motor driven coaxial homogenizer (Duall size AA, Kontes Glass Co.) at 4° in 0.2 to 0.4 ml of buffer composed of 0.05 *M* Tris–HCl (pH 7.4), 0.25 *M* sucrose, 10^{-5} *M* ethylenediaminetetraacetic acid, and 10^{-3} *M* dithiothreitol. Enzyme assays were conducted immediately on whole homogenates. Prolyl hydroxylase activity was measured by a method based on the stoichiometric formation of [^{3}H]water and [^{3}H]hydroxyproline when a substrate consisting of a polypeptide rich in [4-^{3}H]proline was incubated with enzyme and cofactors as described by Hutton *et al.* (8).

Incubations were carried out at 30° under

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air in a 1 ml mixture containing substrate (350,000 dpm), 0.5 m*M* ascorbic acid, 0.1 m*M* α-ketoglutarate, 0.1 m*M* Fe as ferrous ammonium sulfate, bovine serum albumin 2%, 0.01 ml Catalase (15,000 units), 0.05 *M* tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4), and 0.1 or 0.2 ml of whole homogenate as the enzyme source. Incubations were ended after 30 min by the addition of 0.1 ml of trichloroacetic acid, tritiated water was collected by vacuum distillation, and the radioactivity was counted in a liquid scintillation spectrometer. Because of variation in the specific activity of the peptide substrate from batch to batch (due to the *in vitro* chick embryo system), it is not possible to make comparisons of total enzyme activity outside of individual experiments or between laboratories where different preparations of substrate are used.

The same batch of substrate was used throughout this investigation under assay conditions known to be linear over the range used. When possible because of the amount of tissue available, the linearity of the assay was confirmed by assaying aliquots of the same homogenate. The protein content of the whole homogenate was determined by the method of Lowry *et al.* (9). Values are expressed as counts per minute per milligram of protein with the mean ± standard error shown with each group. Student's *t* test was used to compare these means.

Results. After the values for prolyl hydroxylase were obtained it became apparent that they were distributed in three numerical populations representing low, intermediate, and high levels of enzyme activity (Table I). Each group is significantly different from the others, and corresponds to a clinical grouping representing clinically normal liver function, active liver disease, and active aggressive fibrosis.

The three values in the group with lowest enzyme activity shown in Table I (patients 1, 5, and 8) had liver function classified as normal based on clinical and/or histological observations. Patient 1 had a fatty liver but histopathology showed no evidence of inflammation or portal fibrosis. Patient 5 was convalescing from mild alcoholic hepatitis, and had normal liver function. A slight increase in fibrous tissue was observed even though this patient has been recovering for approximately 1 yr. Patient 8 was biopsied for suspected Sarcoidosis, which was not found in the histological specimen.

The liver prolyl hydroxylase values in patients with active hepatitis appear to break into two numerical populations based on the extent of elevation over control. The first of these groups includes those with enzyme levels elevated approximately 2.5-fold over normal. Included in this group are patients with active (but nonaggressive) hepatitis (Patient 2 and 4) and those where advanced portal fibrosis is already established (6 and 9).

The second group, where prolyl hydroxylase was elevated approximately 9-fold, was comprised of two patients with advanced clinical symptoms of active alcoholic hepatitis with evidence of aggressive cirrhosis with only early minimal evidence of existing fibrosis.

Discussion. For some time evidence has been available demonstrating that the excessive synthesis and deposition of collagen and other components of the extracellular matrix in liver disease is a requirement for these conditions to progress to cirrhosis (1, 5). The present procedures used for morphological examination of tissue are sensitive for the detection of hepatic fibrosis after this has occurred, but unfortunately these methods cannot identify the liver which is in a state of active fibrogenesis. The data presented here and those of McGee *et al.* (5) show that the procedure used here provides a rapid and convenient means of estimating the extent of active fibrogenesis by measuring liver prolyl hydroxylase activity, an enzyme in the biosynthetic pathway for collagen.

Collagen synthesis occurs in a series of sequential steps consisting of assembly of a proline-rich and lysine-rich polypeptide precursor of collagen (procollagen α-chains), enzymatic hydroxylation of some of the prolyl and lysyl residues, and glycosylation of some of the hydroxylysyl residues (6). Since proline residues are not hydroxylated before they are in peptide-bound form in protocollagen, the conversion of isotopi-

TABLE I. PROLYL HYDROXYLASE ACTIVITY IN HUMAN LIVER SAMPLES.

Patient	Age	Clinical impression	Histopathological diagnosis	Prolyl hydroxylase (cpm/mg protein)[a]
1	61	Hepatomegaly-normal liver function	Fatty metamorphosis	636
5	60	Convalescent mild alcoholic hepatitis, normal liver function	Lymphocyte infiltration of portal tracts	416
8	42	Sarcoidosis	Normal	614
				$\bar{x}$ 555 ± 70
2	27	Chronic active hepatitis	Chronic active nonaggressive hepatitis	1691
4	46	Mild alcoholic hepatitis	No evidence of cirrhosis	1078
6	52	Advanced portal cirrhosis	Advanced portal cirrhosis	1027
7	60	Chronic passive congestion	Chronic passive congestion	1432
9	56	Advanced portal cirrhosis	Advanced portal cirrhosis	1615
				$\bar{x}$ 1369 ± 136
3	54	Chronic active hepatitis	Lymphocyte infiltration of portal tracts early minimal fibrosis (aggressive)	4109
10	53	Advanced portal cirrhosis	Portal cirrhosis (aggressive)	4595
				$\bar{x}$ 4352 ± 243

[a] Enzyme activity is expressed as the amount (cpm) of [^{3}H] H_2O formed from [4-^{3}H] proline per milligram of protein per 30 min. Student's t test was used to test for differences between activity between each group. Statistical difference between each group = $P < .001$.

cally labeled proline to radioactive hydroxyproline can be taken as one parameter reflecting the rate of collagen formation. This type of parameter has been used in chemical and audioradiological studies which have shown increased collagen synthesis in human cirrhotic liver (2, 10). Although the importance of prolyl hydroxylase as a controlling factor in collagen synthesis is unclear, large increases in enzymatic activity have been reported in a variety of tissues responding to injury-induced damage associated with collagen synthesis (3). It is this parallelism that has generated interest in the use of prolyl hydroxylase as a marker for the rate of collagen synthesis.

The changes in levels of prolyl hydroxylase activity found in the livers of the patients reported here are in agreement with the findings of McGee *et al.* (5). These authors reported three- to eightfold increases in four cirrhotic livers and smaller but consistant increases in five patients with hepatic dysfunction in the absence of observable cirrhosis. Our data are similar in that patients with active mild (nonaggressive) alcoholic hepatitis have levels of prolyl hydroxylase activity which are elevated two- to threefold over normal. A similar modest increase (two- to threefold) was observed in two patients (6 and 9) with extensive portal fibrosis already established with marked replacement of normal tissue with collagen nodules. This observation would suggest that these livers have reached an end-point with regard to fibrogenesis and that the biosynthetic pathway for collagen is returning toward a normal value when compared to the large increase (ninefold) observed in those patients with only early minimal histopathological evidence of existing fibrosis in the presence of aggressive alcoholic hepatitis.

The well-documented parallelism between prolyl hydroxylase and collagen during fibrogenesis, and the convenience with which the enzyme can be measured makes the use of this assay a desirable and feasible marker for clinical fibrogenesis. Since the tissue levels of enzyme activity increase prior to observable accumulation of collagen (4, 11) the use of this procedure on available biopsy material (i.e., that remaining after histopathology requirements)

would be a valuable diagnostic tool since it would predict the progression to cirrhosis in patients with chronic liver disease.

Summary. Prolyl hydroxylase activity was determined in liver biopsy samples obtained from 10 patients. The liver prolyl hydroxylase values in patients with active hepatitis distribute into two numerical populations based on the extent of elevation over control. The first of these groups includes those with enzyme levels elevated approximately 2.5-fold over normal. Included in this group are patients with active (but nonaggressive) hepatitis and patients where advanced portal fibrosis is already established. The second group where prolyl hydroxylase is elevated approximately ninefold is comprised of two patients with advanced clinical symptoms of active alcoholic hepatitis with evidence of aggressive cirrhosis but with only early minimal evidence of existing fibrosis.

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