Evaluation of ^{13}C-Phenylalanine and ^{13}C-Tyrosine Breath Tests for the Measurement of Hepatocyte Functional Capacity in Patients with Liver Cirrhosis

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Liver disease is associated with an abnormal elevation of the plasma concentrations of the aromatic amino acids phenylalanine and tyrosine. The liver is the main site of aromatic amino acid metabolism, particularly the hydroxylation of phenylalanine to tyrosine and further tyrosine degradation. In the present study, we have examined the usefulness of the L-[1-^{13}C]phenylalanine breath test (^{13}C-PheBT) and L-[1-^{13}C]tyrosine breath test (^{13}C-TyrBT) for the detection of hepatic damage in patients with liver cirrhosis. First, the time courses of $^{13}CO_2$ excretion after the administration of L-[1-^{13}C]phenylalanine and L-[1-^{13}C]tyrosine were compared. The peak times (the time expressed in minutes at which $^{13}CO_2$ excretion was maximal) were 20 min in both breath tests, but ^{13}C-TyrBT gave a higher peak than ^{13}C-PheBT. Next, the parameters of ^{13}C-PheBT and ^{13}C-TyrBT were compared with biochemical liver function test values. These parameters were well correlated with several liver blood test values conventionally regarded as measures of hepatocyte functional reserve. Therefore, ^{13}C-PheBT and ^{13}C-TyrBT may be useful to assess the degree and progression of hepatic dysfunction.

Key words ^{13}C-phenylalanine; ^{13}C-tyrosine; liver function; breath test; liver cirrhosis

The liver is the principal site of aromatic amino acid metabolism, and the plasma concentrations of these amino acids are largely dependent upon liver function.[1)] Phenylalanine is mostly converted into tyrosine by phenylalanine hydroxylase in the liver. Subsequently, tyrosine is mostly transaminated in the liver to *p*-hydroxyphenylpyruvic acid, which is further metabolized with loss of CO_2. The synthesis and metabolism of tyrosine are accomplished mostly in the liver.[2)] The activity of phenylalanine hydroxylase in patients with liver cirrhosis was estimated to be 20% of the normal value, suggesting that reduced enzyme levels accounted for the decreased metabolism of phenylalanine in these patients.[3)] Tyrosine clearance has also been found to be decreased in liver cirrhosis.[2,4)]

A breath test, in which a ^{13}C-labeled compound is administered and the metabolized $^{13}CO_2$ is measured in exhaled air, is a valuable clinical test.[5a—c)] In a previous paper,[6)] we reported optimization studies to develop a superior ^{13}C-phenylalanine breath test for the diagnosis of liver disease. First, we examined the optimum ^{13}C-labeling position in phenylalanine for use in a breath test based on infrared (IR) spectroscopic detection of $^{13}CO_2$ in exhaled air. L-[1-^{13}C]Phenylalanine gave the best result. Next, a suitable dosage to give a short peak time (the time expressed in minutes at which $^{13}CO_2$ excretion is maximal) after administration was determined in normal healthy subjects. The $^{13}CO_2/^{12}CO_2$ ratio in exhaled air after administration of 100 mg per subject of L-[1-^{13}C]phenylalanine peaked sharply at 15 min. We also examined the effect of food on the hepatic metabolism of L-[1-^{13}C]phenylalanine. We found that a fasting period of greater than 7 h before the test resulted in a higher $^{13}CO_2$ peak excretion. In the present study, we have examined the usefulness of the L-[1-^{13}C]phenylalanine breath test (^{13}C-PheBT) and the L-[1-^{13}C]tyrosine breath test (^{13}C-TyrBT) for the detection of hepatic damage in patients with cirrhosis and analyzed the correlations between the parameters of these breath tests and standard liver blood tests.

Results and Discussion

L-[1-^{13}C]Phenylalanine and L-[1-^{13}C]tyrosine were administered to healthy volunteers and patients with cirrhosis. The $^{13}CO_2$ exhaled was measured ($^{13}CO_2/^{12}CO_2$) as the Δ^{13}C permil (‰) using IR spectroscopy.[7)] The Δ^{13}C ‰ was calculated from the IR absorption intensities of $^{13}CO_2$ ($2280\pm10\,cm^{-1}$) and $^{12}CO_2$ ($2380\pm10\,cm^{-1}$).[5c)] Results of the $^{13}CO_2$-breath tests were expressed as the Δ^{13}C ‰ and the cumulative percentage of the administered dose of ^{13}C recovered over 1 h (% ^{13}C cumulative dose).

^{13}C-PheBT was performed on 13 patients with cirrhosis and 3 control subjects. The characteristics of the patients and controls are summarized in Table 1. The % ^{13}C cumulative dose were 4.3 ± 0.7 in patients and 8.8 ± 1.0 in control subjects. The peak Δ^{13}C were 8.0 ± 1.4 in patients and 19.4 ± 2.2 in control subjects. ^{13}C-TyrBT was performed on 8 patients with cirrhosis, and 2 control subjects. The characteristics of the patients and controls are summarized in Table 2. The % ^{13}C cumulative dose were 7.0 ± 1.0 in patients and 14.1 in control subjects. The peak Δ^{13}C were 10.6 ± 1.8 in patients and 22.1 in control subjects.

The results for ^{13}C-PheBT and ^{13}C-TyrBT are compared in Fig. 1. To avoid the effects of interindividual differences, all data shown were obtained from common subjects ($n=7$). The time interval between ^{13}C-PheBT and ^{13}C-TyrBT was almost 4 weeks. Most phenylalanine is known to be absorbed at the brush border membrane of the proximal small intestine after oral administration.[8)] During the above time interval, no clinically problematic finding, such as diarrhea, acute abdominal complaint, gastrointestinal bleeding, gastritis, peptic ulcer or deterioration of liver blood test values, was observed in these

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Table 1. Characteristics of Control Subjects (n=3) and Patients (n=13) with Cirrhosis Who Participated in the ^{13}C-PheBT

	Controls (n=3)	Patients (n=13)
Age	41.7±3.8	64.5±1.5
Wt (kg)	69.2±3.6	58.0±3.2
MR ratio	3.7±0.3	1.9±0.1
Tyr (nmol/ml)	65.2±1.3	123.8±13.8
Phe (nmol/ml)	65.2±1.0	96.8±16.7
TP (g/dl)	7.3±0.3	7.4±0.1
ALB (g/dl)	4.6±0.1	3.4±0.1
AMO (μg/dl)	72±35	91.6±13.7
T-B (mg/dl)	0.5±0.2	1.2±0.3
D-B (mg/dl)	0.2±0.1	0.6±0.2
GOT (IU/l)	18.7±2.3	75.4±11.1
GPT (IU/l)	30±9.1	64.1±7.6
T-CHO (mg/dl)	175.7±16.7	156.7±7.4
CHE (IU/l)	4309±506.6	2597±291.4
HPT (%)	116±8.7	73.9±6.2
PLT ($\times 10^4/\mu$l)	22.8±2.1	9.7±1.2
γ-GTP	30.7±10.5	60.0±19.6

Values are expressed as means±S.E.M.

Table 2. Characteristics of Control Subjects (n=2) and Patients (n=8) with Cirrhosis who Participated in the ^{13}C-TyrBT

	Controls (n=2)	Patients (n=8)
Age	42.5±6.5	65.5±1.5
Wt (kg)	72.8±0.7	57.2±4.9
MR ratio	3.9±0.2	1.6±0.2
Tyr (nmol/ml)	64.7±2.1	127.4±14.7
Phe (nmol/ml)	64.2±0.6	83.4±3.8
TP (g/dl)	7.2±0.4	7.4±0.2
ALB (g/dl)	4.5±0.1	3.6±0.2
AMO (μg/dl)	40.1±3.5	97.9±20.2
T-B (mg/dl)	0.3±0.0	1.4±0.4
D-B (mg/dl)	0.1±0.0	0.8±0.2
GOT (IU/l)	20.5±2.5	65.4±10.5
GPT (IU/l)	33.5±14.5	57.5±9.3
T-CHO (mg/dl)	182.5±26.5	159.0±8.0
CHE (IU/l)	4666±623.5	2552±320.2
HPT (%)	113.5±14.5	75.3±8.1
PLT ($\times 10^4/\mu$l)	21.9±3.3	7.3±0.8
γ-GTP	29.0±18.0	28.8±7.2

Values are expressed as means±S.E.M.

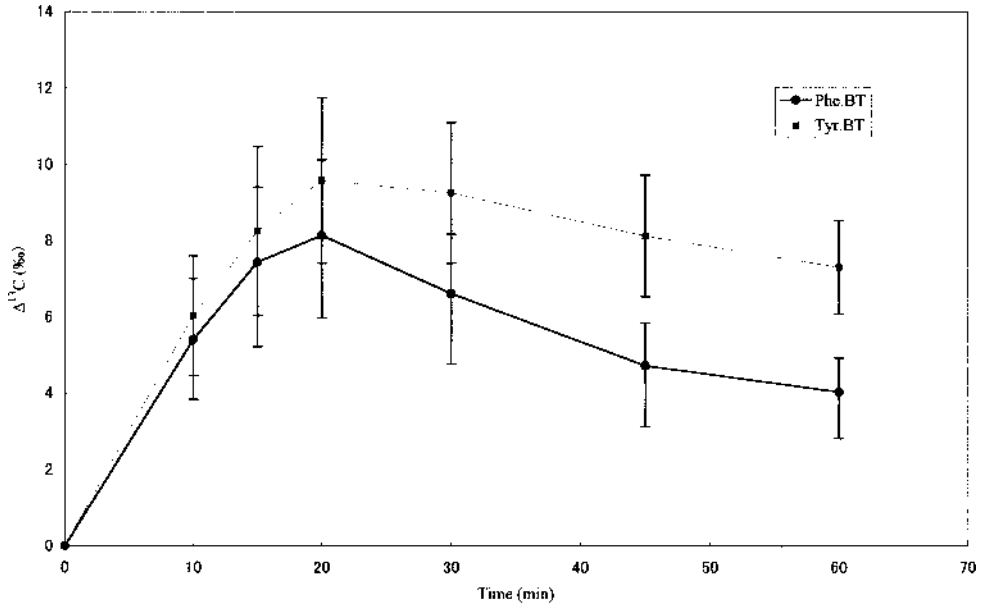

Fig. 1. Time Courses of $^{13}CO_2$ Excretion by Subjects in the ^{13}C-PheBT and ^{13}C-TyrBT (n=7)
Values are expressed as means±S.E.M.

subjects. A significant difference in the % ^{13}C cumulative dose between ^{13}C-PheBT and ^{13}C-TyrBT was found ($p<$ 0.05). The peak times in these breath tests were both 20 min, but ^{13}C-TyrBT produced a higher peak than ^{13}C-PheBT. A significant difference in the peak Δ^{13}C between ^{13}C-PheBT and ^{13}C-TyrBT was found ($p<0.02$). Liver disease is associated with abnormal elevations of the plasma concentrations of phenylalanine and tyrosine. The alterations of metabolism of phenylalanine and tyrosine in cirrhosis involve impairment at several steps in the metabolic pathways of these amino acids. Phenylalanine participates in a number of metabolic pathways, the major one being irreversible hydroxylation to form tyrosine. Three other pathways of phenylalanine metabolism, normally of minor quantitative importance, are transamination to phenylpyruvic acid, decarboxylation to form β-phenylethylamine, and acetylation of the amino group. Tyrosine may be hydroxylated to form dihydroxyphenylalanine (DOPA), iodinated to form triiodothyronine or decarboxylated to form tyramine. The major metabolic pathway for tyrosine, however, is transamination to *p*-hydroxyphenylpyruvic acid. Most *p*-hydroxyphenylpyruvic acid is converted to homogentisic acid and, at this step, the label of L-[1-^{13}C]phenylalanine and L-[1-^{13}C]tyrosine is released as $^{13}CO_2$.[9] This stepwise oxidation of tyrosine through *p*-hydroxyphenylpyruvic acid, homogentisic acid, and fumaroylacetoacetic acid takes place within the liver and would be expected to be suppressed in patients with liver dysfunction.[4] In hepatic encephalopathy, tyrosine levels increase markedly. Most of this increased tyrosine is decarboxylated to tyramine by DOPA decarboxylase.[10] Thus, $^{13}CO_2$ production in ^{13}C-PheBT may be somewhat influenced by minor pathways of phenylalanine metabolism and this may be why

the peak $\Delta^{13}C$ in ^{13}C-TyrBT was higher than that in ^{13}C-PheBT.

Table 3 shows the correlation between the % ^{13}C cumulative dose expired up to 60 min in ^{13}C-PheBT and the corresponding biochemical liver function test values. Serum albumin (ALB), hepaplastine test (HPT) and serum choline esterase activity (CHE) were most highly correlated with the % ^{13}C cumulative dose in ^{13}C-PheBT, followed by Fischer's amino acid molar ratio (MR ratio), serum total cholesterol (T-CHO), platelet count (PLT), serum total bilirubin (T-B), and serum direct bilirubin (D-B). Serum total protein (TP), serum tyrosine (Tyr), serum phenylalanine (Phe), serum ammonia (AMO), serum glutamic oxaloacetic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), and serum γ-glutamyltranspeptidase (γ-GTP) showed no correlation with the % ^{13}C cumulative dose in ^{13}C-PheBT. In summary, the ^{13}C-PheBT parameter is correlated with several liver blood test values considered to be measures of hepatocyte functional reserve (that is, ALB, CHE, HPT, T-CHO, T-B, D-B, and PLT). Table 4 shows the correlation between the % ^{13}C cumulative dose up to 60 min in ^{13}C-TyrBT and biochemical liver function tests. ALB, CHE, T-B, D-B, and Tyr were most highly correlated with the % ^{13}C cumulative dose in ^{13}C-TyrBT, followed by MR ratio and HPT. The PLT, Phe, TP, AMO, GOT, GPT, T-CHO, and γ-GTP showed no correlation. This indicates that the parameter of ^{13}C-TyrBT is correlated with hepatocyte functional reserve (that is, ALB, CHE, HPT, T-B, D-B, and PLT), except for T-CHO. There were a few significant differences between ^{13}C-PheBT and ^{13}C-TyrBT in their correlations with several hepatic synthetic function tests, presumably because the label of L-[1-^{13}C]phenylalanine is mainly released as $^{13}CO_2$ *via* 3 steps (hydroxylation of phenylalanine to tyrosine, transamination of tyrosine to *p*-hydroxyphenylpyruvic acid and dioxygenation of *p*-hydroxyphenylpyruvic acid to homogentisic acid), while the label of L-[1-^{13}C]tyrosine is released as $^{13}CO_2$ *via* only the latter 2 steps. In mammalian phenylalanine metabolism, hydroxylation is the rate-limiting step, and is conducted exclusively in the liver.[11] Therefore, the ^{13}C-PheBT parameter seems more likely to be strongly correlated with liver blood test values, even though ^{13}C-TyrBT produced a higher peak $\Delta^{13}C$ than ^{13}C-PheBT. On the other hand, in the stepwise oxidation of tyrosine through *p*-hydroxyphenylpyruvic acid, homogentisic acid, and fumaroylacetoacetic acid to CO_2 in the liver, the initial transamination reaction of tyrosine is rate-limiting under conditions of liver failure.[12] The alternative synthetic pathways (tyramine and dopamine synthesis), although relatively minor in the normal state, may become more important in liver failure. For example, serum tyramine concentrations and turnover have been shown to increase significantly in patients with cirrhosis in relation to degree of encephalopathy.[13] Tyramine has also been shown to be the direct precursor of the false neurotransmitter, octopamine.[14] The patients subjected to both breath tests showed no change in mental condition, but Tyr levels were more highly correlated with the % ^{13}C cumulative dose in ^{13}C-TyrBT than that in ^{13}C-PheBT. High Tyr levels or abnormal tyrosine tolerance test by oral administration have been detected in several conditions other than liver failure, such as tyrosinosis, thyrotoxicosis, scurvy and renal failure.[2] This suggests that ^{13}C-TyrBT might be of value for follow-up studies in patients with the above diseases, if there is no deterioration in liver function. Our results justify further investigations to assess the clinical usefulness and pitfalls of these breath tests with larger groups of patients.

Table 3. Correlation Coefficients between the % ^{13}C Cumulative Dose in the ^{13}C-PheBT and the Standard Liver Blood Test Values in Subjects ($n=16$)

	Coefficient of correlation ($n=16$)	Significance
MR ratio	0.64	<0.01
Tyr (nmol/ml)	-0.43	<0.1
Phe (nmol/ml)	-0.21	N.S.
TP (g/dl)	0.42	<0.1
ALB (g/dl)	0.85	<0.001
AMO (μg/dl)	-0.31	N.S.
T-B (mg/dl)	-0.54	<0.05
D-B (mg/dl)	-0.52	<0.05
GOT (IU/l)	-0.31	N.S.
GPT (IU/l)	-0.18	N.S.
T-CHO (mg/dl)	0.62	<0.01
CHE (IU/l)	0.77	<0.001
HPT (%)	0.80	<0.001
PLT ($\times10^4/\mu$l)	0.58	<0.02
γ-GTP	0.22	N.S.

N.S., not significant ($p>0.1$).

Table 4. Correlation Coefficients between the % ^{13}C Cumulative Dose in the ^{13}C-Tyr-BT and the Standard Liver Blood Test Values in Subjects ($n=10$)

	Coefficient of correlation ($n=10$)	Significance
MR ratio	0.68	<0.05
Tyr (nmol/ml)	-0.78	<0.01
Phe (nmol/ml)	-0.5	N.S.
TP (g/dl)	0.05	N.S.
ALB (g/dl)	0.81	<0.01
AMO (μg/dl)	-0.34	N.S.
T-B (mg/dl)	-0.82	<0.01
D-B (mg/dl)	-0.86	<0.01
GOT (IU/l)	-0.2	N.S.
GPT (IU/l)	0.08	N.S.
T-CHO (mg/dl)	0.36	N.S.
CHE (IU/l)	0.78	<0.01
HPT (%)	0.74	<0.05
PLT ($\times10^4/\mu$l)	0.59	<0.1
γ-GTP	0.21	N.S.

N.S., not significant ($p>0.1$).

Burke *et al.* have reported that ^{13}C-PheBT is correlated with scores of the Child Pugh classification, which is widely accepted as a predictor of the severity of liver disease.[15] In our study, patients with cirrhosis were classified by their score in ^{13}C-PheBT into either class B ($n=10$) or class C ($n=3$). The % ^{13}C cumulative dose of patients classified into class B was 4.9 ± 0.7, and that of class C was 2.2 ± 1.0. One-way analysis of variance (ANOVA) of the % ^{13}C cumulative dose in ^{13}C-PheBT showed significant ($p<0.05$) differences among the normal, class B and class C groups. The time-courses of $^{13}CO_2$ excretion in ^{13}C-PheBT in normal subjects, and class B and class C patients are compared in Fig. 2. The peak of $^{13}CO_2$ excretion in class C was more poorly defined. We also classified patients with cirrhosis by their score in ^{13}C-TyrBT into either class B ($n=5$) or class C ($n=3$). The %

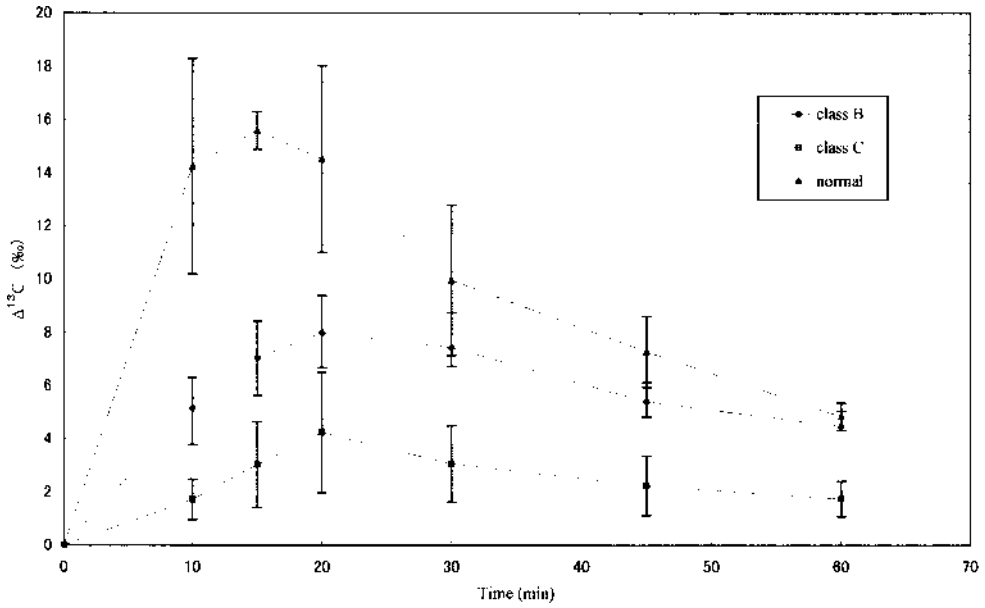

Fig. 2. Time Courses of $^{13}CO_2$ Excretion in ^{13}C-PheBT by Normal Subjects (n=3), and class B (n=10) and class C (n=3) Patients

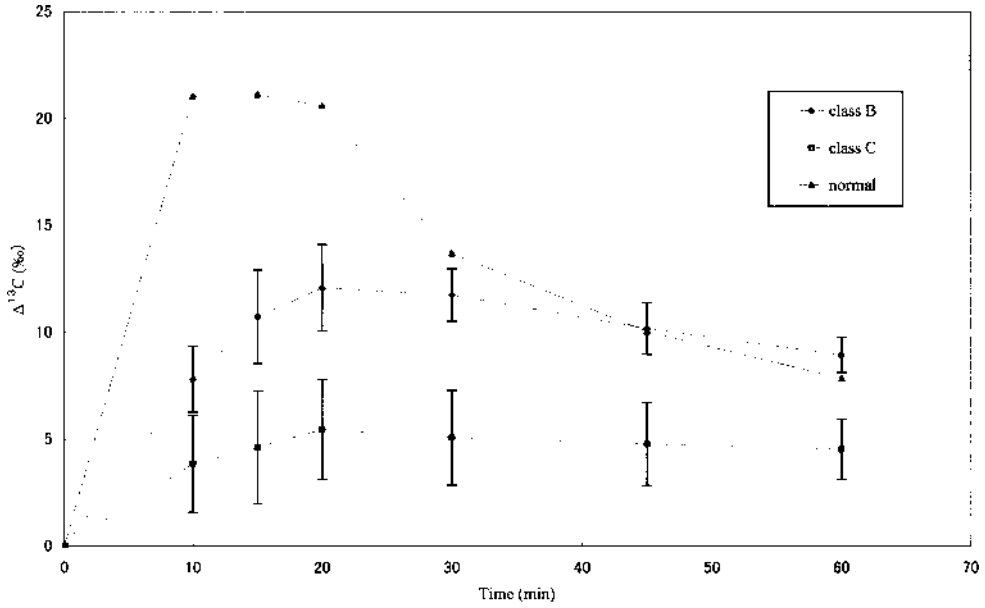

Fig. 3. Time Courses of $^{13}CO_2$ Excretion in ^{13}C-TyrBT by Normal Subjects (n=2), and class B (n=5) and class C (n=3) Patients

^{13}C cumulative dose of patients classified into class B was 8.4±0.7, and that of class C was 4.6±1.7. One-way ANOVA of the % ^{13}C cumulative dose values in ^{13}C-TyrBT also revealed significant ($p<0.001$) differences between class B and class C groups. The time-courses of $^{13}CO_2$ excretion in ^{13}C-TyrBT for normal subjects, and class B and class C patients are compared in Fig. 3. The peak of $^{13}CO_2$ excretion in class C was again more poorly defined. Patients with cirrhosis who maintain very low % ^{13}C cumulative dose values should be observed carefully. The central plasma amino acid clearance rate in liver has been shown to differentiate between survivors and nonsurvivors within a given Child Pugh classification.[16] More importantly, the ability of the liver to clear amino acids has been shown to be impaired in patients with cirrhosis and to be predictive of mortality after surgery.[17] In particular, patients with hepatocellular carcinoma combined with cirrhosis or chronic hepatitis, because of a decline in the hepatocyte functional capacity, may die from severe complications after surgery, or from multiple organ failure caused by hepatic failure. Therefore, it is important to assess accurately the hepatocyte functional capacity in patients. As reported by Burke *et al.*, ^{13}C-PheBT shows a correlation with Child Pugh classification,[15] reflecting its ability to predict hepatocyte functional capacity. ^{13}C-PheBT may be capable of predicting mortality after hepatic resection for hepatocellular carcinoma. In future, it will be necessary to investigate whether there is a correlation between these breath test values, the clinical stage, and the survival rate after hepatic resection in cirrhosis patients, or whether these breath tests are useful for planning hepatic transplantation.

In phenylalanine metabolism, the hydroxylation of phenylalanine to tyrosine is rate-limiting,[11] so ^{13}C-PheBT may allow estimation of the *in vivo* rate of phenylalanine hydroxylation. In tyrosine metabolism, the transamination of tyrosine to *p*-hydroxyphenylpyruvic acid is rate-limiting,[12] and about 99% of the daily degradation of tyrosine normally flows through *p*-hydroxyphenylpyruvic acid to homogentisic acid.[2] Therefore ^{13}C-TyrBT may allow estimation of the *in vivo* rate of tyrosine transamination. The comparative analysis of ^{13}C-PheBT and ^{13}C-TyrBT should provide better kinetic estimates of these components of phenylalanine metabolism. In the present study, we examined the usefulness of ^{13}C-PheBT and ^{13}C-TyrBT for the detection of hepatic damage in patients with cirrhosis. Both ^{13}C-PheBT and ^{13}C-TyrBT could be used to evaluate hepatocyte functional reserve in patients with cirrhosis. Several other specific quantitative tests for measuring liver function have been developed to supplement the blood screening tests. ^{13}C-Phenacetin,[5b,c] ^{13}C-aminopyrine[18] and ^{13}C-methacetin[19] are microsomal substrates, which have been used in breath tests to assess the degree of hepatic dysfunction. However, these tests are measuring drug metabolism instead of degradation of natural metabolites. The ^{13}C-Phe or ^{13}C-Tyr BT offers a safe, sensitive and relatively non-invasive means of measuring liver function in patients with cirrhosis. These tests can provide quantitative information allowing us to evaluate the degree and progression of hepatic dysfunction. We are now examining the usefulness of these tests for the detection of hepatic damage in patients with chronic hepatitis. These tests would be helpful in deciding the prognosis and treatment of patients with liver disease, and provide information on phenylalanine and tyrosine kinetics in liver disease.

Experimental

Materials L-[1-^{13}C]Phenylalanine (99 atom% ^{13}C) and L-[1-^{13}C]tyrosine (99 atom% ^{13}C) were obtained from Cambridge Isotope Laboratories. Shiseido Co., Ltd. supplied aluminized bags.

Instruments IR spectra for the $^{12}CO_2/^{13}CO_2$ breath test were measured with a $^{13}CO_2$ analyzer (HBP-100, JASCO, Tokyo, Japan). The $\Delta^{13}CO_2$ ‰ was calculated from the IR absorption intensities of $^{13}CO_2$ ($2280 \pm 10\,cm^{-1}$) and $^{12}CO_2$ ($2380 \pm 10\,cm^{-1}$). The cumulative percentage of administered dose of ^{13}C recovered over 1 h (% ^{13}C cumulative dose) was also calculated.

Subjects ^{13}C-PheBT was performed on 13 hospital outpatients (3 males and 10 females) with cirrhosis, and 3 control subjects (3 males) who were in good health and had no history of liver disease. The ages of the cirrhosis patients ranged between 57 and 74. The types of cirrhosis were hepatitis C virus (HCV) in 10 patients and non-B, non-C in 3 patients, and the diagnoses were confirmed by liver biopsy.

^{13}C-TyrBT was also performed on 1 hospital outpatient (female), and 9 of the subjects for ^{13}C-PheBT, *i.e.*, 7 (2 males and 5 females) of the 13 patients and 2 (2 males) of the 3 control subjects. The ages of the cirrhosis patients ranged between 60 and 73. The types of cirrhosis were HCV in 7 patients and non-B, non-C in one patient (again diagnoses were confirmed by liver biopsy). These patients were classified into class B or class C based on the Child Pugh classification scores. Patients were clinically stable, and none had tyrosinosis, thyrotoxicosis, scurvy or renal disease. The absence of peptic ulcer, gastroesophageal reflux disease, and esophageal varices was confirmed by an experienced observer. Informed consent was obtained from all subjects.

^{13}C-PheBT and ^{13}C-TyrBT by IR Spectroscopy The subjects had fasted overnight and then drank a suspension of L-[1-^{13}C]phenylalanine or L-[1-^{13}C]tyrosine, 100 mg per subject in 100 ml of water. The container was then rinsed with 100 ml of water, which the subjects also drank. The breath samples for baseline were collected in duplicate before each breath test administration. Alveolar breath samples were collected in a resting position by normal exhalation into 250 ml aluminized bags at 10, 15, 20, 30, 45, and 60 min after ingestion of L-[1-^{13}C]phenylalanine or L-[1-^{13}C]tyrosine. The content of $^{13}CO_2$ in the exhaled air was determined by IR spectroscopy.

Statistical Methods Results were expressed as the mean±standard error of the mean (S.E.M.). Differences in the % ^{13}C cumulative dose between patients with cirrhosis and control subjects were examined by using the two-tailed two-sample *t*-test. Equality of variance was tested by an *F* test for two-group comparisons. Differences in the % ^{13}C cumulative dose between ^{13}C-PheBT and ^{13}C-TyrBT were examined by using the two-tailed one-sample *t*-test. Comparisons among control subjects, class B patients and class C patients were made using one-way ANOVA. A p value of 0.05 or less was considered to be significant.

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