Table 1
Frequency of the *GAA* haplotype in the controls and the patients.

Haplotype[a]	Controls		Patients	
	Number	Frequency	Number	Frequency
GG	1017	0.71	21	0.58
AA	274	0.19	15	0.42
GA	139	0.10	0	0.00
AG	0	0.00	0	0.00
Total	1430	1.00	36	1.00

Statistically significant differences between controls and patients in each haplotype; GG, $p = 0.145$; AA, $p = 0.004$; GA, $p = 0.053$.

[a] Abbreviations for haplotype are depicted as follows; GG, c.[1726G; 2065G]; AA, c.[1726A; 2065A]; GA, c.[1726G; 2065A]; AG, c.[1726A; 2065G].

Statistical analysis

The measured values are expressed as means ± SD unless otherwise indicated. Two-sample independent-groups *t*-test was used for data comparison between the two groups. Data comparison among three or more groups was based on analysis of variance (ANOVA) with respective all pair-wise multiple comparison post-hoc analysis utilizing the Bonferroni's method. Categorical variables were compared with chi-square analysis. Results were considered to be significant at $p < 0.05$.

Results

Validation of the measurement of AαGlu activity in DBSs

The within-imprecision of the present method was estimated by repeated analysis of some DBSs with different activities. The within-run CVs ($n = 12$) were 1.9–5.6%. To estimate between-day imprecision, the DBSs in sealed plastic bags were stored at −20 °C and then assayed with the present method over 12 days. The between-day CVs ($n = 12$) were 3.4–6.8%. The stability of the enzyme activity in DBSs was assessed by determining the average activity of 5 different DBSs stored at room temperature, at 4 °C and at −20 °C for 2, 4, 8 and 16 weeks. The activity change was less than 10% for 16 weeks at either 4 or −20 °C.

AαGlu activity in DBSs from healthy newborns and patients with GSDII

The AαGlu activity in DBSs from 715 healthy Japanese newborns (controls) and 18 patients with GSDII was measured with 4MU-αGlc in the presence of acarbose. As shown in Fig. 1 there was no normal Gaussian distribution of activities in the control group, instead there appeared to be a bimodal distribution: a minor group with less than 9.0 pmol/h/disk of enzyme activity and a major group with activities of more than 9.0 pmol/h/disk. Four individuals in the minor control group fell into the range of activities measured in the patient group (0–2.8 pmol/h/disk). The poor separation between the minor control group and the patient group was not improved by taking the ratio of acarbose-inhibited over uninhibited activity into account (data not shown).

Genotyping with DBSs from healthy newborns and patients with GSDII

We then examined the allele frequencies of 4 *GAA* haplotypes and 10 diplotypes as determined by two SNPs (c.1726G>A and c.2065G>A) using DNA extracted from the same blood spots that were used to measure the AαGlu activity (Tables 1 and 2). Significant correlation was observed between the enzyme activity in the DBS and the diplotype of the DNA from the same spot. Twenty-seven of the 28 individuals homozygous for c.[1726A; 2065A] (3.9% of the total number of controls) belonged to the minor group with low enzyme activity, whereas individuals heterozygous for c.[1726G; 2065G] and c.[1726A; 2065A] formed together a broad range of activities overlapping with those from c.[1726A; 2065A] or c.[1726G; 2065G] homozygotes (Table 2). The mean activity of c.[1726G; 2065A] homozygotes was only slightly lower (82%) than that of c.[1726G; 2065G] homozygotes, but c.[1726A; 2065A] homozygotes had markedly lower activity (12%).

The c.[1726A; 2065A] allele had a significantly higher frequency (42%) in the patient group than in the control group (19%). Twelve of the 18 patients had either one (9 cases) or two (3 cases) c.[1726A; 2065A] allele(s) indicating linkage disequilibrium of the two SNPs between controls and patients. None of the controls nor patients had a c.[1726A; 2065G] allele.

Validation of genotyping with DBSs

Six of the diplotypes that we identified with the present method were confirmed by sequence analysis of genomic DNA from 18 DBS samples (six diplotypes, $n = 3$ each). There was no discrepancy between the results obtained by either method (data not shown).

Table 2
Frequency of the *GAA* diplotype and AαGlu activity in the controls and the patients.

Diplotype[a]	Controls				Patients			
	Number	Frequency	Enzyme activity[b]		Number	Frequency	Enzyme activity[b]	
			Mean ± SD	Range			Mean ± SD	Range
GG/GG	360	0.503	36.4 ± 11.2	12.4–102.1	6	0.333	0.5 ± 0.5	0–1.1
GG/AA	194	0.271	19.5 ± 6.9	7.7–47.4	9	0.500	1.0 ± 0.9	0–2.8
AA/AA	28	0.039	4.4 ± 1.7	1.4–10.1	3	0.167	0.5 ± 0.3	0.2–0.8
GG/GA	103	0.144	32.8 ± 9.6	6.7–58.0	0	0	–	–
GA/GA	6	0.008	29.8 ± 9.3	16.0–41.2	0	0	–	–
GA/AA	24	0.034	17.4 ± 4.1	11.3–28.6	0	0	–	–
AG/AG	0	0	–	–	0	0	–	–
GG/AG	0	0	–	–	0	0	–	–
GA/AG	0	0	–	–	0	0	–	–
AG/AA	0	0	–	–	0	0	–	–
Total	715	1.000	29.4 ± 13.2	1.4–102.1	18	1.000	0.8 ± 0.8	0–2.8

[a] Abbreviations for diplotype are depicted as the combination of the haplotypes described in Table 1.

[b] AαGlu activity in a 3.2-mm diameter disk from DBSs of Japanese newborns (controls) and Japanese patients with GSDII was measured in duplicate with 4-methylumbelliferyl α-D-glucopyranoside as substrate in the present of 3 μmol/L acarbose. The activity was expressed as pmol methylumbelliferone/h/disk. Statistically significant differences between GG/GG and other diplotypes; GG/AA, $p < 0.001$; AA/AA, $p < 0.001$; GG/GA, $p = 0.052$; GA/GA, $p = 0.057$; GA/AA, $p < 0.001$.

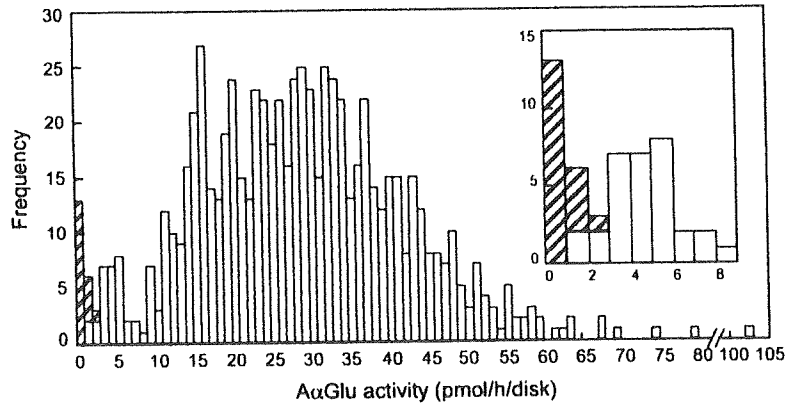

Fig. 1. Distribution of AαGlu activity in DBSs from controls and patients. The AαGlu activity was measured with 4MU-αGlu as substrate in the presence of acarbose using DBSs from 715 Japanese healthy newborns (controls) and 18 Japanese patients with GSDII. Open and hatched bars represent the controls and the patients, respectively. The enzyme activities (mean ± SD, pmol/h/disk) were 29.4 ± 13.2 for the controls and 0.8 ± 0.8 for the patients, and the range of the activities were 1.4–102.1 for the controls and 0–2.8 for the patients. The measurement was performed as described in Subject, materials and methods, and the data were expressed as an average of duplicate determinations. Inset indicates the area of low activities enlarged.

Discussion

In this study, we measured the AαGlu activity in 715 randomly collected DBSs from Japanese newborns, and obtained a bimodal distribution of the activities (a major group with 684 individuals and a minor group with 31 individuals). We could then demonstrate by ARMS that 27 of 31 control individuals (minor group) with an activity of less than 9.0 pmol/h/disk (30.6% of total mean) were c.[1726A; 2065A] homozygotes. Three other individuals with very low activity (7.7, 7.9 and 8.8 pmol/h/disk) were c.[1726G; 2065G]/c.[1726A; 2065A] heterozygotes and one individual (6.7 pmol/h/disk) was c.[1726G;2065G]/c.[1726G;2065A] heterozygote. One c.[1726A; 2065A] homozygote had slightly higher activity (10.1 pmol/h/disk) (Table 2). The 28 homozygotes with c.[1726A; 2065A] representing 3.9% of the study group (Table 2) had on average a markedly lower AαGlu activity (12%) than the homozygotes with c.[1726G;2065G] forming approximately 50% of the study group. Thus, we observed a close correlation between the enzyme activities and the genotypes of the donors. The actual diplotype frequency of c.[1726A; 2065A]/c.[1726A; 2065A] in our sample collection (3.9%) comes close to what we could calculate from the published c.[1726A; 2065A] allele frequency in the Japanese population based on the allele frequencies in the NCBI and in our own sample set [24]. Four of the 28 (14%) homozygotes with c.[1726A;2065A] had an AαGlu activity that fell in the patient range (0–2.8 pmol/h/disk). This poses a serious problem for newborn screening in Japan. If 3.9% of the population is homozygote with c.[1726A; 2065A] and 14% of them has very low activity it means that newborn screening potentially results in 0.56% false positive cases, which is too many to handle. Heterozygotes with genotype c.[1726G; 2065G]/c.[1726A; 2065A] had about half the activity (54%) of c.[1726G; 2065G] homozygotes. None of the DBSs from these heterozygotes or those from individuals with other diplotypes overlapped with the patient range.

The AαGlu activity distribution curves depicted in Fig. 2 are based on the frequencies of the three *GAA* genotypes, c.1726G/G, c.1726G/A and c.1726A/A, in the Japanese population and illustrate the problem encountered in newborn screening. Obviously, the high number of false positives is caused by the high frequency of the c.1726A/A allele in the Japanese population. The same problem will be encountered in other Asian countries [24]. Especially, in Taiwan higher frequency of c.1726A/A (14% of normal individuals) was reported as compared to that in Japan (3.9%) [29]. In practice, the very first large scale newborn screening for GSDII in Taiwan

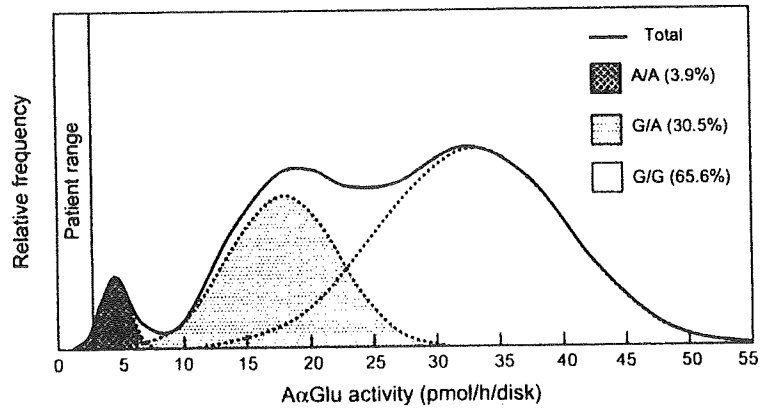

Fig. 2. Estimated distribution of AαGlu activity in DBS in Japan based on the frequency of the three genotypes, c.1726G/G, c.1726G/A and c.1726A/A. Gaussian curves illustrating the distribution of AαGlu activities in DBS were drawn for the Japanese populations based on the frequencies of the three relevant *GAA* genotypes, and their mean and SD of the enzyme activity; c.1726G/G, 33.0 ± 10.0 (n = 469); c.1726G/A, 18.5 ± 5.5 (n = 218); and c.1726A/A, 4.4 ± 1.7 pmol/h/disk (n = 28).

encompassing 132,538 newborns required a second DBS from 1093 newborns (0.82% of total) when the cut-off value was set at 25% of normal mean AαGlu activity [4]. Most of these recalls might have been c.[1726A; 2065A] homozygotes. After publication of the Taiwanese study, we tried to improve the feasibility of the screening procedure by adopting the assay conditions described by Chien et al. [4]. Forty DBSs from 12 patients and 28 c.[1726A; 2065A] homozygotes were subjected to the screening procedure with the following cut-off values; the AαGlu activity, less than 8% of normal mean; the ratio of neutral over acid α-glucosidase activities, more than 60; and the percentage inhibition of total α-glucosidase activity by acarbose, more than 80% [4]. Using the combination of these criteria, none of the c.[1726A; 2065A] homozygotes was identified as false positive, but 2 of the 12 patients were misdiagnosed (unpublished data). The use of a different substrate coupled to a different assay procedure (e.g., the newly developed substrates for tandem mass spectrometry) might solve the problem [10–13]. Tandem mass spectrometry worked remarkably well in a pilot study in 10,279 Austrian newborns. The calculated recall rate would have been only 0.039% [5]. But, it must be emphasized that the great majority of Austrians is Caucasian, and that the frequency of c.[1726A; 2065A] homozygotes in that population is probably very low.

As it stands, our method based on the measurement of AαGlu activity in DBSs using 4MU-αGlc in the presence of 3.0 μmol/L acarbose detects 1.5% of Japanese newborns as potential patients at a cut-off value of 4.0 pmol/h/disk without false negative outcomes. But, at lower cut-off value some patients will be missed (Tables 2 and 3). We have demonstrated the feasibility of obtaining *GAA* genetic information from the DNA that remains on the DBSs, but the application is not sufficient as second tier test. If an activity of less than 3 pmol/h/disk is found in an individual with any diplotype other than c.[1726A; 2065A] homozygote, this individual is very likely to have GSDII. However, a c.[1726A; 2065A] homozygote with low activity remains to be diagnosed.

Notably, there were three c.[1726A; 2065A] homozygotes among the 18 proven patients. Although the sample size is small, it seems that the frequency of the c.[1726A; 2065A] allele is higher in the patient (42%) than in the control population (19%), which is suggestive for a founder effect. This hypothesis can be investigated by *GAA* sequence analysis of the 12 patients that are either homozygote or heterozygote for c.[1726A; 2065A]. They are then expected to share a common pathogenic mutation besides the linked c.1726G>A and c.2065G>A SNPs. Gene sequencing is in progress to clarify this possibility. Interestingly, c.1935C>A leading to amino acid substitution D645E is the most common pathogenic mutation in the Southeastern part of China and in Taiwan. This mutation was first reported by Shieh and Lin [28] to be linked to c.2065G>A, whereas linkage to c.1726G>A was not investigated at that time. Recently, Wan et al. reported that they could not find any specific polymorphism that links to the pathogenic c.1935C>A mutation in the same Chinese population [29]. This would mark position c.1935 as a mutational hotspot, although c.1935C>A is not very common in Japan among patients with GSDII (6.8% of the mutant alleles) [30,31].

In conclusion, our findings illustrate that homozygosity for the c.[1726A; 2065A] allele, resulting in "pseudodeficiency" of AαGlu, complicates newborn screening for GSDII in the Japanese and other Asian populations. Our findings also suggest that one or more pathogenic mutations are associated with this allele. Further investigations are required to optimize the selectivity of the newborn screening procedure and to minimize the number of cases that have to be recalled for second or third tier testing.

Table 3
Diagnostic property of newborn screening with different cut-off values on DBSs from 715 controls and 18 patients in Japanese populations.

Cut-off value (pmol/h/disk)	Normal mean activity (%)	False positive (%)	False negative (%)
10.0	34.0	39/715 (5.5)	0/18 (0)
9.0	30.6	31/715 (4.3)	0/18 (0)
8.0	27.2	30/715 (4.2)	0/18 (0)
7.0	23.8	28/715 (3.9)	0/18 (0)
6.0	20.4	26/715 (3.6)	0/18 (0)
5.0	17.0	18/715 (2.5)	0/18 (0)
4.0	13.6	11/715 (1.5)	0/18 (0)
3.0	10.2	4/715 (0.6)	0/18 (0)
2.0	6.8	2/715 (0.3)	1/18 (5.5)

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Effects of bezafibrate on dyslipidemia with cholestasis in children with familial intrahepatic cholestasis–1 deficiency manifesting progressive familial intrahepatic cholestasis

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Abstract

No appropriate pharmaceutical therapy has been established for dyslipidemia with cholestasis in progressive familial intrahepatic cholestasis (PFIC) 1. We evaluated the efficacy of bezafibrate in PFIC-1. We monitored the clinical presentation and lipoprotein metabolism of 3 patients, aged 3, 4, and 8 years, with FIC1 deficiency, manifesting PFIC-1, over 12 months of bezafibrate therapy. Pruritus was substantially alleviated in the 3 patients after initiation of bezafibrate. Cholestasis was alleviated in 2 of them. Serum high-density lipoprotein cholesterol and low-density lipoprotein cholesterol increased 1.6- to 2.0-fold and 1.1- to 1.2-fold, respectively; but the values remained low and normal, respectively. Serum lipoprotein X, which was at normal levels before treatment, was elevated to levels above the upper limit of the reference range. High serum triglyceride levels decreased by 15% to 30%, to normal levels, after treatment initiation. The activities of lipoprotein lipase and hepatic triglyceride lipase were increased, but those of high-density lipoprotein regulators remained unchanged. Liver expression of multidrug resistance protein 3, which regulates lipoprotein X synthesis, was enhanced by bezafibrate therapy. Bezafibrate treatment favorably affected pruritus, dyslipidemia, and cholestasis in PFIC-1.
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1. Introduction

Progressive familial intrahepatic cholestasis (PFIC), formerly called *Byler syndrome*, is a congenital cholestatic liver disorder with normal γ-glutamyl transpeptidase (GGT) levels [1-3]. This disorder is classified into PFIC-1 and PFIC-2, depending on the mutation. Progressive familial intrahepatic cholestasis 1 is caused by mutations in the *ATP8B1* gene, encoding the FIC1 protein; and PFIC-2 is caused by mutations in the adenosine triphosphate binding cassette B 11 gene (*ABCB11*) [1-3].

Cholestasis in PFIC usually manifests itself in the first few months of life and is unremitting thereafter, with normal GGT levels. The liver disease typically progresses to cirrhosis before the end of the second decade of life. Markedly short stature, osteoporosis, and severe pruritus due to cholestasis have been noted as serious problems over a long-term follow-up period [1-3].

Unlike other cholestatic liver disorders, serum total cholesterol (TC) levels are normal or low in PFIC patients. However, our previous work provided evidence that PFIC patients have dyslipidemia, characterized by the

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accumulation of triglyceride (TG)-rich low-density lipoprotein (LDL) and depleted high-density lipoprotein (HDL), and a predisposition to cardiovascular disorders involving atherosclerosis [4-11].

To date, no appropriate pharmaceutical therapy has been established for dyslipidemia with cholestasis in PFIC. Bezafibrate, a fibrate, is commonly administered in Japan as an antihyperlipidemic agent. Bezafibrate is known as a ligand of the peroxisome proliferator activator receptor (PPAR) γ, a nuclear receptor regulating lipoprotein metabolism. Through this pathway, bezafibrate lowers serum TG levels and raises serum HDL cholesterol (HDL-C) levels [12-15]. PPAR γ also enhances hepatic expression of liver multidrug resistance protein 3 (MDR3), which promotes phospholipid secretion into bile [16,17]. Clinically, bezafibrate has recently been used to treat primary biliary cirrhosis; it improved cholestasis and liver function [18-21]. However, the effects of bezafibrate on congenital cholestatic liver disorders have not been studied sufficiently.

We treated 3 PFIC-1 children with bezafibrate for 12 months and compared their clinical presentation and lipoprotein metabolism with those of 2 PFIC-1 patients not receiving bezafibrate therapy. This report describes the beneficial effects of bezafibrate on dyslipidemia and cholestasis in PFIC-1.

2. Subjects and methods

2.1. Subjects

We enrolled 5 patients with PFIC-1 (aged 3-8 years) in this study. Of the 5, 3 patients (patients [Pts] 1, 2, and 3) received bezafibrate treatment, whereas the others (Pts 4 and 5) did not, during the study period. For all patients, the diagnosis of PFIC-1 was established based on their clinical course, liver histopathology (light and electron microscopy), and genetic analyses [1-3] (Table 1).

To confirm the abnormality in FIC1 at the protein level, we performed Western blot analyses of frozen liver biopsy samples using a rabbit polyclonal antibody (Sawady Technology, Tokyo, Japan) against amino acid residues 828 to 850 of the human *ATP8B1* sequence (GenBank accession no. AF038007). As a control, the same Western blot analysis was conducted using frozen liver biopsy samples obtained from 3 healthy men aged 31, 36, and 37 years. They were donors for living-related liver transplants for their children with extrahepatic biliary atresia and resulting liver failure. We confirmed that the liver expression of FIC1, encoded by *ATP8B1*, was strikingly decreased in all patients, supporting the diagnosis of PFIC-1 (Fig. 1).

Table 1
Backgrounds of patients with PFIC-1

Patient no. (current age)	M/F	FIC1 gene mutations	Recent liver histology		
			Expansion of portal area	Fibrosis	IHBD degeneration
Pt 1 (3 y, 11 mo)	F	I 800 I 842Q	No	Mild	No
Pt 2 (3 y, 10 mo)	M	G1238C I 2021C	No	Mild	No
Pt 3 (8 y, 2 mo)	F	G1208A or 2357insTG	Yes	Moderate	Yes
Pt 4 (7 y, 2 mo)	M	G858A G1361T	Yes	Moderate	Yes
Pt 5 (5 y, 5 mo)	M	3840 A1604T	No	Mild	No

F indicates female; M, male; IHBD, intrahepatic bile duct.
Three patients (Pts 1, 2, and 3) received bezafibrate for 12 months.

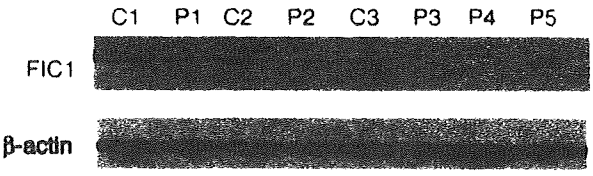

Fig. 1. Hepatic FIC1 protein levels in PFIC-1 children and control subjects. The FIC1 protein levels in the liver were determined by Western blotting, as described in the "Subjects and methods." Liver specimens were obtained from 5 PFIC-1 children (Pts 1-5) and 3 healthy controls. C1, C2, and C3 indicate control 1, control 2, and control 3, respectively.

The PFIC-1 children presented with severe pruritus, short stature, low body weight, and mild jaundice. Liver histologies revealed that 3 of the 5 patients had only mild fibrosis (Table 1). They received adequate lipid-soluble vitamins to maintain blood levels within the reference ranges after diagnosis.

Bezafibrate was administered at 5 mg/kg/d (a typical dose for the treatment of dyslipidemia) to Pts 1, 2, and 3. Their liver function and lipoprotein metabolism, as well as their general clinical presentation, were monitored during 12 months of bezafibrate therapy at this dosage [12-15]. Their clinical courses were compared with those of Pts 4 and 5.

Institutional review boards approved this study. Informed consent for treatment with bezafibrate was obtained from the patients and their parents.

2.2. Liver function tests and pruritus scores

Serum levels of aspartate aminotransferase, alanine aminotransferase, total protein, and albumin were determined. As hallmarks of cholestasis, serum levels of GGT, total bile acids (TBAs), and total bilirubin (T-B) were also determined.

Pruritus severity was scored as described in a previous report [22]: 0, none; 1, mild scratching when undistracted; 2, active scratching without abrasion; 3, abrasions; or 4, cutaneous mutilation, with bleeding and scarring.

2.3. Lipids and apolipoproteins

Serum levels of TC, TG, and phospholipids were measured using enzymatic methods with commercial kits. Serum LDL cholesterol (LDL-C) levels were determined

using a homogeneous method with a commercial kit (Cholestest LDL; Daiichi Pure Chemicals, Tokyo, Japan). Serum levels of apolipoprotein (apo) A-I, apo B, and apo C-II were determined using a turbidimetric immunoassay (Apo A-I, Apo B, and Apo C-II Auto N Daiichi; Daiichi Pure Chemicals). Lipoprotein X (LpX) was determined by selective immunoprecipitation as described previously [23]. All measurements were carried out using autoanalyzers (models 7310 and 7170; Hitachi, Tokyo, Japan).

Serum levels of total HDL-C were measured as described previously [4,11]. Briefly, the serum sample was mixed with an equal volume of aqueous 13% polyethylene glycol (PEG 6000; Wako Pure Chemical Industries, Osaka, Japan); and the mixed sample was centrifuged (2000*g*, 15 minutes, room temperature). The cholesterol level was determined in the supernatant as described above.

The chemical composition of LDL was examined according to a method using ultracentrifugation and gel filtration as described previously [4,11]. The chemical compositions of the other lipoproteins (HDL and very low-density lipoproteins) could not be examined because so little was recovered from blood samples, especially before bezafibrate therapy.

2.4. Enzymes related to lipoprotein metabolism

Lecithin-cholesterol acyltransferase (LCAT) activity was determined using an exogenous substrate method with liposomes composed of cholesterol and lecithin (Anasorb LCAT, Daiichi Pure Chemicals). The cholesteryl ester transfer protein (CETP) level was measured using a sandwich enzyme immunoassay.

Activities and protein levels of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) were determined using postheparin samples. Briefly, a citrated plasma sample was obtained 10 minutes after intravenous injection of heparin (30 IU/kg body weight). The LPL and HTGL activities were measured using nonradioisotopic determination of the amount of released free fatty acid from the substrate triolein, as described previously [24]. The LPL protein level was determined using a sandwich enzyme immunoassay with a commercial kit (LPL-ELISA kit; Dainippon Pharmaceutical, Tokyo, Japan) [25]. The HTGL protein level was determined with a sandwich enzyme immunoassay using monoclonal antibodies [24,25].

2.5. Immunoblot of MDR3 using liver samples

Liver samples were obtained from Pts 1 and 2 by percutaneous liver biopsies before and after 4 months of bezafibrate therapy. Percutaneous liver biopsy was also performed for Pts 4 and 5 before and at 6 to 7 months of this study, and the liver samples were obtained. First, 100 mg of each frozen liver sample were homogenized in 1 mL of lysis buffer containing 10 mmol/L Tris-HCl, 200 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, 5 mmol/L 2-mercaptoethanol, 1 mmol/L MgCl2, and 0.5 mmol/L phenylmethylsulfonyl fluoride, a protease inhibitor. After centrifugation (1500*g*, 1 minute), the protein concentration in the supernatant was determined using the Bradford reagent (Sigma Chemical, St Louis, MO). The supernatant was mixed with an equal volume of loading buffer containing 125 mmol/L Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred by electroblotting to nitrocellulose membranes (Millipore, Tokyo, Japan). The membranes were blocked with 10% nonfat dried milk in Tris phosphate-buffered saline. They were then reacted with a commercial antibody for MDR3 (P3II-26; Alexis, Lausen, Switzerland). After washing the membrane, the secondary immunoglobulin G antibody conjugated with peroxidase (The Binding Site, Birmingham, UK) was added to the membranes for 1 hour. The membranes were washed, incubated in enhanced chemiluminescent reagents (Amersham, Buckinghamshire, United Kingdom), and exposed to chemiluminescent film according to the manufacturer's instructions.

3. Results

3.1. Effects on pruritus, cholestasis, and liver function

In Pts 1, 2 and 3, the pruritus scores were highest at baseline and were reduced by bezafibrate therapy. In Pts 1 and 2, the maximum reduction was observed after 4 and 6 months of bezafibrate treatment, respectively. In Pt 3, the slight reduction of the pruritus score was observed after 4 months of treatment; but further improvement was not observed by 12 months (Fig. 2A).

The baseline levels of T-B and TBAs were elevated in these patients, although their GGT levels were normal or low. In Pts 1 and 2, both the T-B and TBA levels decreased after 2 months of treatment but did not change further by 12 months.

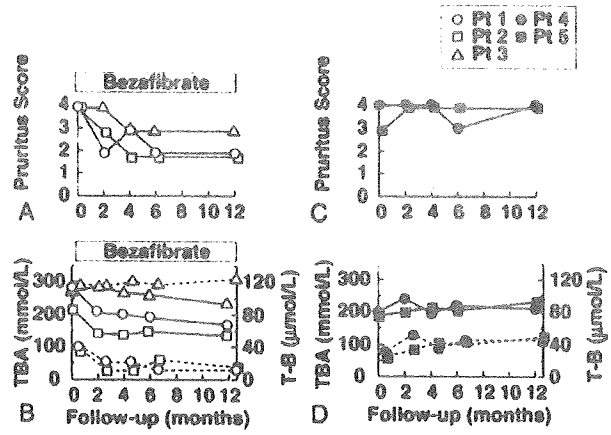

Fig. 2. Effects of bezafibrate on pruritus score, T-B, and TBAs in PFIC-1 children. The PFIC-1 children were followed for 12 months with (A and B) or without (C and D) bezafibrate treatment. TBAs, solid lines; T-B, dotted lines.

In contrast, a slight decrease was observed only in the TBA level in Pt 3 after 12 months of treatment (Fig. 2B).

Increases in the levels of total protein and albumin were also observed in Pts 1 and 2 at 2 months, and the levels increased further over time. To the contrary, no improvement in these liver function tests was seen in Pt 3. Decreases in the aspartate aminotransferase and alanine aminotransferase levels were observed in Pts 1 and 2 after 2 months of therapy; further improvements in liver function were apparent in these patients as therapy continued (data not shown).

None of the possible adverse effects caused by bezafibrate, such as rhabdomyolysis, mucocutaneous syndrome, or any exacerbation of liver dysfunction, was observed in any patient.

Patients 4 and 5, who did not receive bezafibrate therapy, showed no improvement in liver function or pruritus (Fig. 2C-D).

3.2. Effect on lipids, lipoproteins, and apolipoproteins

At baseline, all PFIC-1 children (Pts 1-5) had very low HDL-C levels, slightly high TG levels, and normal LDL-C and LpX levels (Fig. 3A-F). In the patients treated with bezafibrate (Pts 1, 2, and 3), the LDL-C and HDL-C levels increased 1.1- to 1.2-fold and 1.6- to 2.0-fold, respectively, after 2 months of therapy (Fig. 3A). Nevertheless, the posttreatment levels of HDL-C remained below the reference range. In contrast, the TG levels decreased to within the reference range in these patients (Fig. 3B). Although the baseline levels of LpX were normal, the LpX levels increased 2.2- to 4.5-fold, exceeding the upper limit of the reference range, after 12 months of bezafibrate treatment (Fig. 3C).

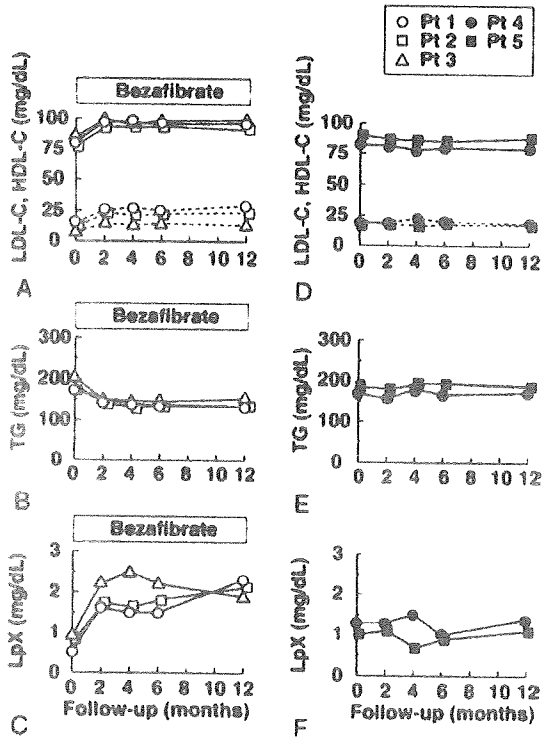

Fig. 3. Effects of bezafibrate on lipids and apolipoproteins in PFIC-1 children. The PFIC-1 children were followed for 12 months with (A, B, and C) or without (D, E, and F) bezafibrate treatment. N indicates reference ranges obtained from 60 age-matched controls consisting of 30 girls and 30 boys with ages ranging from 2 to 8 years. LDL-C, solid lines; HDL-C, dotted lines.

Table 2
Chemical composition of LDL before and after bezafibrate therapy

Patient no.	CE (%)	FC (%)	TG (%)	PL (%)	Protein (%)
Pt 1					
Baseline	22.1	6.7	20.3	26.4	24.5
6 mo	30.2	6.9	15.2	25.2	22.5
Pt 2					
Baseline	19.1	5.9	23.3	25.5	26.2
6 mo	28.9	6.5	16.3	24.4	23.9
Pt 3					
Baseline	12.5	6.2	29.5	26.9	25.9
6 mo	20.3	6.5	23	15.5	24.3
Age-matched control*	39.1 ± 1.8	7.2 ± 0.8	8.4 ± 1.5	22.4 ± 0.5	21.3 ± 1.3

Blood samples were obtained at baseline and 6 months after bezafibrate therapy. Data are presented as percentages of the total weight of LDL. CE indicates cholesteryl ester; FC, free cholesterol; PL, phospholipids.

* Age-matched control values (mean ± SD) were obtained from 20 children with ages ranging from 2 to 6 years [4].

In the patients treated with bezafibrate, the apolipoprotein levels changed similarly to the HDL-C and TG levels. As for HDL-C, the apo A-I and apo C-II levels at baseline were low in Pts 1, 2, and 3. Both levels increased 1.2- to 1.6-fold but remained below the reference ranges after 2 months of therapy. No further changes were observed thereafter. In contrast, the apo B and apo E levels at baseline were high in all patients. Like the TG levels, the levels of both apo B and apo E decreased by 15% to 30% after bezafibrate treatment and normalized in 2 of the 3 patients (data not shown).

In Pts 4 and 5, who did not receive bezafibrate, the lipid levels showed no significant change (Fig. 3D-F).

Before bezafibrate therapy, the LDL particles from Pts 1, 2, and 3 were rich in TG and proteins but poor in cholesteryl esters, consistent with our previous report [5]. Bezafibrate treatment decreased the TG and protein contents but markedly increased the cholesteryl ester content of the LDL particles (Table 2).

3.3. Effects on LCAT, CETP, LPL, and HTGL activity and protein levels

Before bezafibrate therapy, the LPL activity and protein level were low in Pts 1, 2, and 3. The LPL activity in Pt 3 was disproportionately low, relative to the LPL protein level, reflecting that patient's very low levels of apo C-II, a coenzyme of LPL. The HTGL activity was also reduced in all patients, consistent with its protein level. The LCAT activity and CETP protein level were normal in Pts 1 and 2 but were quite low in Pt 3 (Table 3).

Table 3
Lipoprotein regulators before and after bezafibrate therapy

Patient no	LPL activity (mmol/h mL)	LPL protein (ng/mL)	HTGL activity (mmol/h mL)	HTGL protein (ng/mL)	LCAT activity (nmol/h mg)	CETP protein (μg/mL)
Pt 1						
Baseline	3.08	61	1.96	189	69.5	1.2
4 mo	4.03	81	2.77	261	70.5	1.3
Pt 2						
Baseline	3.66	70	2.11	202	70.4	1.6
4 mo	4.52	96	2.87	285	71.3	1.4
Pt 3						
Baseline	2.13	56	0.71	85	50.2	0.8
4 mo	2.55	63	1.11	101	49.1	0.8
Normal	7.34-14.10	130-300	8.55-12.21	720-2015	67.3-108.2	1.1-3.5

Blood samples were obtained at baseline and 4 months after bezafibrate therapy.

Bezafibrate therapy increased both the LPL and HTGL activities and the respective protein levels, especially in Pts 1 and 2. On the other hand, the LCAT activity and CETP protein level were not changed, remaining at normal levels in Pts 1 and 2 and at a low level in Pt 3 (Table 3).

3.4 Immunoblot of MDR3 using liver samples

Immunoblot analysis revealed that bezafibrate apparently increased the liver MDR3 expressions in Pts 1 and 2, compared with the baseline level, after 4 months of treatment (Fig. 4). On the other hand, the liver MDR3 expressions in Pts 4 and 5, who did not receive this treatment, were not changed.

4. Discussion

The lipid and lipoprotein profiles changed considerably in all 3 PFIC patients after the initiation of bezafibrate. In 2 of the 3 patients, bezafibrate improved pruritus, cholestasis, and liver dysfunction. High TG levels and low TC levels, in addition to low LDL-C, returned to normal after bezafibrate treatment. The serum levels of HDL-C also increased but remained low.

The improvement of liver dysfunction, along with cholestasis, in our 2 patients may be attributable to the enhancement of MDR3 expression by bezafibrate. The results of recent studies indicate that bezafibrate enhances the expression of MDR3 (ABCB4), a canalicular phospholipid transporter in hepatocytes, via the nuclear receptor PPARα [16,17,20,21]. Phospholipids attenuate the cytotoxicity of hydrophobic bile acids, thereby engendering the development and progression of hepatocholangiopathy [17-19].

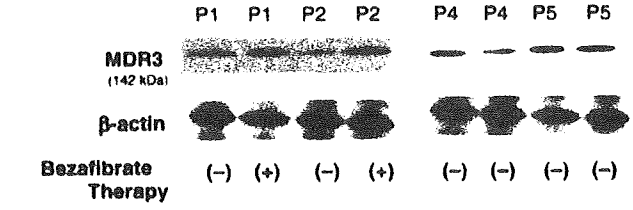

Fig. 4. Hepatic MDR3 levels before and after bezafibrate therapy. In 3 PFIC-1 children (Pts 1, 2, and 3), liver biopsy specimens were obtained immediately before and 2 months after the initiation of bezafibrate therapy. Western blot analysis was carried out as described in "Subjects and methods."

Recently, Shoda et al [17] reported that bezafibrate enhanced the expression of 2 other major canalicular transporters: MRP2 (ABCC2), a transporter of multispecific organic anions involving bilirubin, and BSEP (ABCB11), a transporter of bile acids. Increases in the expression of these transporters may have contributed to the improvement in cholestasis and liver dysfunction in our 2 patients, but we did not directly examine this.

Mutations in the *ATP8B1* gene, encoding FIC1, are responsible for PFIC-1; but the exact role of this protein remains unclear [1,3]. The results of the present study suggest that this protein is associated with many proteins involved in bile acid and bilirubin transport systems, the expressions of which might be stimulated by bezafibrate.

Changes in the lipid and lipoprotein profiles of our patients should be interpreted as a direct effect of bezafibrate on lipoprotein metabolism because such changes were apparent even in Pt 3, whose severe cholestasis was not improved by the treatment.

The enzymes involved in lipoprotein metabolism have not been previously examined in PFIC patients. The results of this study demonstrate low LPL and HTGL activities in PFIC-1 patients and show that both activities were increased after the initiation of bezafibrate therapy. Lipoprotein lipase is an important enzyme for the hydrolysis of TGs in TG-rich lipoproteins, especially chylomicrons and very low-density lipoprotein. Patients with LPL deficiency typically present with prominent hypertriglyceridemia, together with low HDL-C levels [26,27]. Hepatic triglyceride lipase also hydrolyzes TGs in lipoproteins, especially intermediate-density lipoprotein; and HTGL deficiency leads to an accumulation of TG-rich LDL in plasma [28-31]. Based on a comparison of lipid and lipoprotein profiles before and after bezafibrate therapy, it seems likely that the increases in LPL and HTGL activities by bezafibrate therapy contributed

greatly to the increased levels of HDL-C, LDL-C, and TC and the decreased levels of TG in our patients.

The ability of bezafibrate to raise LPL and HTGL activities has been shown in earlier studies [12-15]. The present study confirmed that bezafibrate increased both lipase activities, even in PFIC-1 children. Unlike LPL and HTGL, the HDL regulators CETP and LCAT remained unchanged throughout the course of treatment [6,32-34].

The LDL in our patients was TG-enriched LDL, which displays poor affinity for the LDL receptor; therefore, the LDL in our patients was likely to accumulate in plasma [35,36]. Their high apo B levels before bezafibrate therapy probably reflect such accumulation of LDL because LDL has only 1 apo B per molecule. A decrease in the apo B levels, with a reciprocal increase in the LDL-C levels, after the initiation of this therapy may be explained by an increased affinity of the LDL for the LDL receptor resulting from the increased cholesterol ester content of the LDL at the expense of TG.

After the initiation of bezafibrate therapy, LpX, which was at normal levels before treatment, rose to levels greater than the reference range in all patients. It has been shown that bile acid regurgitation resulting from bile duct damage, together with a decrease in LCAT activity due to liver cell damage, is the main contributor to the formation of LpX [5,7-10,33,34,37,38]. Furthermore, Elferink and colleagues [39] demonstrated that Mdr2 (Abcb4) knockout mice failed to form LpX even during cholestasis; they suggested that the expression of MDR3 regulated the formation of LpX. An increase in MDR3 expression without a change in the LCAT activity was observed in our patients after bezafibrate therapy. In this context, we suggest that increased MDR3 expression by bezafibrate accounted for the increase in the LpX level in the PFIC-1 patients.

This study demonstrated beneficial effects of bezafibrate on cholestasis and dyslipidemia in patients with PFIC-1. The results lead us to consider the possibility that bezafibrate may be a useful therapeutic option for such patients.

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Sustaining hypercitrullinemia, hypercholesterolemia and augmented oxidative stress in Japanese children with aspartate/glutamate carrier isoform 2-citrin-deficiency even during the silent period

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ABSTRACT

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) shows diverse metabolic abnormalities such as urea cycle dysfunction together with citrullinemia, galactosemia, and suppressed gluconeogenesis. Such abnormalities apparently resolve during the first year of life. However, metabolic profiles of the silent period remain unknown. We analyzed oxidative stress markers and profiles of amino acids, carbohydrates, and lipids in 20 asymptomatic children with aspartate/glutamate carrier isoform 2-citrin-deficiency aged 1–10 years, for whom tests showed normal liver function. Despite normal plasma ammonia levels, the affected children showed higher blood levels of ornithine ($p < 0.001$) and citrulline ($p < 0.01$)—amino acids involved in the urea cycle—than healthy children. Blood levels of nitrite/nitrate, metabolites of nitric oxide (NO), and asymmetric dimethylarginine inhibiting NO production from arginine were not different between these two groups. Blood glucose, galactose, pyruvate, and lactate levels after 4–5 h fasting were not different between these groups, but the affected group showed a significantly higher lactate to pyruvate ratio. Low-density and high-density lipoprotein cholesterol levels in the affected group were 1.5 times higher than those in the controls. Plasma oxidized low-density lipoprotein apparently increased in the affected children; their levels of urinary oxidative stress markers such as 8-hydroxy-2′-deoxyguanosine and acrolein-lysine were significantly higher than those in the controls. Results of this study showed, even during the silent period, sustained hypercitrullinemia, hypercholesterolemia, and augmented oxidative stress in children with citrin deficiency.

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Introduction

Adult-onset type II citrullinemia (CTLN2, OMIM 603471), a disease caused by a mitochondrial transporter, is characterized by frequent bouts of hyperammonemia, liver steatosis, mental derangement, sudden unconsciousness, and ultimately death within a few years of onset [1–3]. In fact, CTLN2 results from mutations of the SLC25A13 gene on chromosome 7q21.3 encoding a calcium-binding mitochondrial protein: a liver-type aspartate/glutamate carrier isoform 2 (AGC2), so-called citrin [3–6]. In the malate-aspartate NADH shuttle and urea synthesis, AGC2 plays an important role [3,7,8]. Impairment of AGC2 function can engender an increased NADH/NAD$^+$ ratio in cytosol. Failure of the aspartate supply from the mitochondria to the cytoplasm for synthesis of argininosuccinate engenders hypercitrullinemia and hyperammonemia.

Clinical characteristics of citrin deficiency vary dramatically by age [1–6,8–12]. About half of the Japanese children diagnosed with citrin deficiency were found to have metabolic abnormalities such as hypergalactosemia, hyperphenylalaninemia, and hypermethioninemia

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by newborn mass screening (NMS) at the age of 5 days. The remaining children visited eligible hospitals to receive precise examinations for prolonged jaundice, acholic stool, and/or failure to thrive during early infancy [9–12]. These children present diverse clinical manifestations, namely neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) such as considerable liver dysfunction, along with cholestasis, citrullinemia, mild hyperammonemia, galactosemia, and hypoglycemia.

The clinical presentations of NICCD resolve from 6 months to one year of life. However, among patients who have manifested NICCD, only one-fifth of patients develop CTLN2 [8–12].

Prompted by the fact that onset of CTLN2 is fatal, we sought the metabolic profiles of affected children from the silent stage: before the onset of CTLN2. Correction of metabolic anomalies at this stage would be expected to prevent the onset of CTLN2.

For this study, we examined the profiles of amino acids, carbohydrates, and lipids. We also examined NO synthesis, which shares processes with the urea cycle [13,14]. Furthermore, the status of oxidative stress, which is related closely to the development of liver steatosis, was evaluated using biomarkers.

In this report, we describe hypercitrullinemia, dyslipidemia and augmented oxidative stress in the affected children. The metabolic abnormalities underlying the development of CTLN2 will be discussed.

Subjects and methods

Subjects and sample collection

This study enrolled 20 children with citrin deficiency (10 males and 10 females, 1 year 10 months – 10 years 5 months) and 32 age-matched healthy children (16 female and 16 males, 2 year 2 months – 9 years 5 months) as controls.

The affected children's blood levels of transaminase, gamma-glutamyl transpeptide, total bile acids, and total bilirubin at that time were entirely normal. Of the 20 affected children, 12 were found to have metabolic abnormalities (hypergalactosemia, $n = 7$; hyperphenylalaninemia, $n = 4$; hypermethioninemia, $n = 2$) by NMS performed at the age of 5 days (Table 1). Thereafter, they developed considerable liver dysfunction along with cholestasis manifesting hyperbilirubinemia, hypoproteinemia, and prolonged coagulation. Precise examination revealed that they had markedly elevated plasma citrulline levels accompanying higher plasma levels of arginine, threonine, tyrosine, and phenylalanine. The remaining eight patients developed hyperbilirubinemia and visited their respective hospitals at the ages of 1–4.5 months. Precise examination detected prominent citrullinemia accompanying higher plasma arginine, threonine, tyrosine, and phenylalanine levels.

They were diagnosed as having citrin deficiency at ages of 3 weeks – 2 years 2 months based on gene analyses for the SLC25A13 determining the genotypes as follows: [I] 851del4, [II] IVS11 + 1G > A, [III] 1638ins23, [IV] S225X, [V] IVS13 + 1G > A, [VI] 1800ins1, [VII] R605X, [VIII] E601X, [IX] E601 K, [X] IVS6 + 5-G > A, [XI] R184X and [XIV] IVS6 + 1G > C (Table 1) [4–6].

The liver function tests at the ages of 4–12 months yielded normal results. Their blood levels of transaminase, gamma-glutamyl transpeptidase, total bile acids, and total bilirubin at presentation were entirely normal.

Blood and urine samples were collected at 10:30–11:30 before lunch after 4–5 h fasting. The methods and purpose of the study were explained to each child's parents. Their informed consent was obtained before enrollment of the child. Approval of the project was obtained from the institutional medical ethics committee.

Methods

Estimation of amino acids metabolism in terms of urea cycle

The urea cycle is initiated by carbamoylphosphate synthesis from ammonia via carbamoylphosphate synthetase, a limiting enzyme in the urea cycle [13]. Carbamoylphosphate is subsequently transformed into citrulline, which is ultimately transformed into arginine via argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) under a supply of aspartate by citrin [7,8,13]. To estimate the urea cycle function, plasma levels of ammonia and amino acids—including citrulline, arginine and ornithine—were examined. Plasma amino acids were determined using routine ion-exchange chromatography with an auto-analyzer (L822; Hitachi High-Technologies Corp., Tokyo, Japan).

Estimation of NO pathway

Arginine and citrulline are also involved in the nitric oxide (NO) pathway [13,14]. In fact, NO is synthesized from arginine by NO

Table 1
Background and present liver functions in patients.

Pt	Age at present	M/F	Gene mutations	NMS	Age at diagnosis	Liver dysfunction	AST (IU/L)	ALT (IU/L)	Γ-GTP (IU/L)	TBA (μmol/L)
1	2y7m	F	II/II	Met	3w	3w–6m	31	13	12	3.3
2	5y3m	M	II/V	Gal	5m	1m–5m	42	23	14	4
3	5y4m	M	II/V	(–)	4.5m	4.5m–7.5m	35	22	14	7.6
4	6y7m	M	I/II	Phe	1m	1m–5.5m	34	30	16	3.4
5	4y10m	F	I/II	Phe	1m	1m–4.5m	36	15	11	4.9
6	1y8m	M	I/V	Gal	22d	3w–7m	48	20	21	3.2
7	10y5m	M	II/VIII	(–)	1m	1m–4m	26	16	10	4.9
8	5y9m	M	II/II	(–)	1y2m	1m–12m	29	16	13	16.2
9	4y1m	F	II/V	Gal	3m	1m–8m	33	23	15	3.1
10	3y3m	M	II/II	Gal	4m	3w–11m	19	27	9	6.5
11	5y6m	M	II/V	(–)	2m	2m–9m	40	35	11	7.1
12	4y9m	F	III/V	Phe	2m	1m–7m	29	29	18	5.2
13	3y7m	F	II/VI	(–)	3m	3m–11m	37	34	15	5.5
14	6y2m	M	I/I	Gal	3m	1m–6m	31	16	11	4.1
15	4y0m	M	I/VI	Met	4m	3w–10m	39	19	15	8.2
16	2y11m	F	I/II	(–)	5m	2m–7m	23	20	10	3.2
17	3y11m	F	II/II	Phe	1m	1m–4m	37	27	16	6.5
18	7y1m	M	V/XIX	(–)	2y2m	2m–11m	28	14	14	5.9
19	5y5m	F	I/II	Gal	5m	1m–7m	22	11	11	2.9
20	5y6m	M	I/I	Gal	4m	3w–8m	39	26	14	4.9

NMS, newborn mass screening at 5 days of age; AST, aspartate aminotransferase (normal range: 5–40 IU/L); ALT, alanine aminotransferase (5–40 IU/L); Γ-GTP, gamma-glutamyl-transpeptidase (5–60 IU/L); TBA, total bile acids (2–15 μmol/L); Gal, hypergalactosemia; Phe, hyperphenylalaninemia; Met, hypermethionemia.

synthase (NOS). The availability of arginine is a rate-limiting factor in cellular NO production. Citrulline, a by-product of the NOS reaction, is recycled to arginine through successive actions of ASS and ASL, forming the citrulline-NO cycle. Therefore, in this study, blood levels of nitrite/nitrate (NO_x^-) as stable metabolites of NO and asymmetric dimethylarginine (ADMA) as a putative inhibitor of NOS were determined to estimate the NO pathway activity [15].

Serum levels of (NO_x^-) and ADMA were measured using the Griess method (nitrate/nitrite colorimetric assay; Cayman Chemical, Ann Arbor, ML, USA) and a recently developed enzyme-linked-immunosorbent assay method (ADMA-ELISA; DLD Diagnostika GmbH, Hamburg, Germany) [16]. Competitive ADMA-ELISA uses the microtiter plate format. Briefly, serum samples, as well as standards and kit controls, are acetylated in 96-well plates. The acetylated samples, standards and kit controls are pipetted into the respective wells of the ADMA-coated microtiter strips and incubated with a polyclonal antibody (rabbit-anti-*N*-acetyl-ADMA). After incubation, the antiserum solution is discharged and the wells are washed with washing buffer. A peroxidase-conjugated secondary antibody is added; then all wells are washed and incubated with tetramethylbenzidine solution as the substrate for peroxidase. The enzymatic reaction is stopped using an acidic stop solution; the absorbance is then measured using a microplate reader at 450 nm. The amount of antibody bound to the solid-phase ADMA is inversely proportional to the ADMA of the sample concentration.

Estimation of carbohydrate metabolism

Carbohydrate metabolism was estimated indirectly using blood glucose, galactose, lactate, and pyruvate levels. Blood levels of glucose, lactate, and pyruvate were determined using their respective enzymatic methods. Blood galactose concentrations in dried blood spots were determined with microassay using a fluorometric microplate reader, as described by Yamaguchi and colleagues [17].

Estimation of lipid metabolism

Lipid metabolism was estimated according to the blood levels of free fatty acids (FFA), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), phospholipids (PL), triglycerides (TG), and apolipoprotein (apo) levels. Malondialdehyde-modified LDL (MDA-LDL) as oxidized LDL (Ox LDL) was also determined.

Serum levels of TC, PL, and TG were determined with commercial kits (Kyowa Medex Co. Ltd., Tokyo, Japan) using enzymatic methods. Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd., Osaka, Japan.) [18]. The serum FFA level was measured using enzymatic methods (NEFA-SS kit Eiken; Eiken Chemical Co. Ltd., Tokyo, Japan). Furthermore, LDL-C was measured using an enzyme immunoassay with a commercial kit (LDL-C Daiichi; Daiichi Pure Chemicals Co. Ltd.). Serum levels of apoA-I, apo-B, apo-CII, and apo-E were determined using turbidimetric immunoassay (Apo-AI, apo-B, apo-CII, and Apo-E Auto-N 'Daiichi'; Daiichi Pure Chemicals Co. Ltd.). A sensitive enzyme-linked immunosorbent assay for detection of MDA-LDL in serum was used for determination of oxidized LDL [19]. In this assay, a monoclonal antibody interacts with MDA-apo-B.

Western blot analyses of biopsy specimens

Liver samples were obtained from two affected children (patients 19 and 20) by percutaneous liver biopsy (Table 1). Liver expressions of three important lipoprotein regulators were examined: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase as a key enzyme of cholesterol synthesis, LDL-receptor and scavenger receptor B-I (SR-BI) as a major HDL receptor. As control livers, liver fragments obtained from two non-related donors for liver transplantation (1 male and 1 female) with ages of 28 and 33 years were used; they were entirely healthy. The donors had no history of smoking.

The frozen samples (50–100 mg) were divided into cytoplasmic and nuclear fractions using nuclear and cytoplasmic extraction reagent kits (NE-PER™; Pierce Biotechnology Inc., Rockford, IL). The former was used for analyses of SR-BI, LDL-receptor, and HMG-CoA reductase.

These samples were separated using 10% SDS–polyacrylamide gel electrophoresis. Then they were transferred to nitrocellulose membranes using a semi-dry transfer unit.

After blocking with Tris-buffered saline containing 10% non-fat dried milk, the membranes were reacted with primary antibodies and then with peroxidase-conjugated secondary antibodies. After vigorous washing, the membranes were incubated with an enhanced chemiluminescence reagent (ECL; GE Healthcare Life Sciences, Tokyo, Japan), and exposed to X-ray film. The following primary antibodies were purchased from two companies: HMG-CoA reductase (mouse, polyclonal; Abcam plc., Cambridge, UK), SR-BI (goat, polyclonal; Lifespan Biosciences Inc., Seattle, WA), and LDL-receptor (chicken, polyclonal; Abcam plc.).

Estimation of oxidative stress

As the marker for oxidative stress, urinary acrolein-lysine reflecting the amounts of lipid peroxidation products in plasma and urine and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) reflecting oxidative DNA damage were examined. Furthermore, vitamin E, functioning as an antioxidant in plasma and activities of anti-oxidative enzymes such as catalase and superoxide-dismutase (SOD) in erythrocytes, was examined.

Concentrations of urinary acrolein-lysine and 8-OHdG were determined, respectively, using competitive ELISA kits: ACR-Lysine Adduct ELISA (NOF Corp., Tokyo, Japan) and 8-OHdG Check (the Institute for the Control of Aging, Shizuoka, Japan) [20]. Plasma vitamin E levels were measured using high-performance liquid chromatography (HPLC), as described in a previous report [21].

The SOD activity was determined using spectrophotometry at 505 nm (RANSOD kit; Randox Laboratories Ltd.; Antrim, United Kingdom), as described in a previous report [22]. Catalase activity was determined using the method described by Aebi [23]. In brief, we monitored the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 M potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml.

Results

Effects on amino acids involved in the urea cycle and NO pathway

Among amino acids, despite the normal plasma ammonia level, ornithine and citrulline levels of the affected children were, respectively, 1.7 times ($p < 0.001$) and 1.4 times ($p < 0.01$) higher than those of the controls. Although their arginine level was 0.87 times as high as the controls' level, no significant difference was found between these two groups (Table 2).

The other amino acid levels of the affected patients were comparable to those of the control levels. Blood NO_x^- and ADMA levels were not different between the two groups, suggesting that the NO pathway in the affected children remained normal (Table 2).

Effects on carbohydrate metabolism

No significant difference was found between these two groups' blood glucose, galactose, lactate, or pyruvate levels at fasting

Table 2
Blood levels of amino acids involved in the urea cycle, NO_x and ADMA.

	Arginine (μmol/L)	Ornithine (μmol/L)	Citrulline** (μmol/L)	NO_x (μmol/L)	ADMA (μmol/L)	Ammonia (μg/dl)
20 patients	74.2(14.4)	105.1(24.2)	40.8(6.3)	31(5)	0.78(0.11)	35(14)
Ranges	45.4–137.8	65.0–193.4	25.3–56.4	22–49	0.60–1.12	20–91
32 controls	85.0(13.2)	61.3(13.6)	28.2(6.3)	30(9)	0.63(0.17)	31(9)
Ranges	52.8–106.8	40.1–90.0	14.4–41.4	22–49	0.42–0.97	18–49

NO_x, nitrite/nitrate; ADMA, asymmetric dimethylarginine.
Presented data are mean (SD) values and ranges.
* $p < 0.01$ versus controls.
** $p < 0.001$ versus controls.

Table 3
Blood levels of carbohydrate at 4–5 h fasting.

	Glucose (mg/dl)	Galactose (mg/dl)	Pyruvate (mg/dl)	Lactate (mg/dl)	*L/P*
Patients ($n = 20$)	84(5)	0.3(0.1)	0.8(0.3)	12(3)	15(2)
Ranges	72–95	0.1–0.6	0.2–1.7	6–26	9–18
Controls ($n = 32$)	85(5)	0.3(0.1)	0.8(0.2)	10(4)	11(1)
Ranges	76–99	0.1–0.5	0.3–1.1	7–19	7–13

L/P: ratio of lactate to pyruvate.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.

(Table 3). The *L/P* ratio in the affected children was significantly higher than that in the controls ($p < 0.05$), suggesting a high ratio of NADH to NAD^+ and/or suppressed mitochondrial functions in the affected children [24].

Effects on lipid metabolism

Serum LDL-C and HDL-C levels in the affected children were 1.5 times higher than those in the age-matched controls, resulting in high total cholesterol levels (Table 4). Triglycerides and FFA levels were not different between these two groups. The apo-AI and apo-B levels in the affected children were apparently higher than those in the controls, respectively, reflecting the higher LDL-C and HDL-C levels (Table 4). Surprisingly, oxidized LDL levels were much higher in the affected patients.

Western blot analyses showed that liver HMG-CoA reductase expression was elevated in the two affected children, although their liver LDL-receptor and SR-BI expressions were similar to those in the control subjects (Fig. 1).

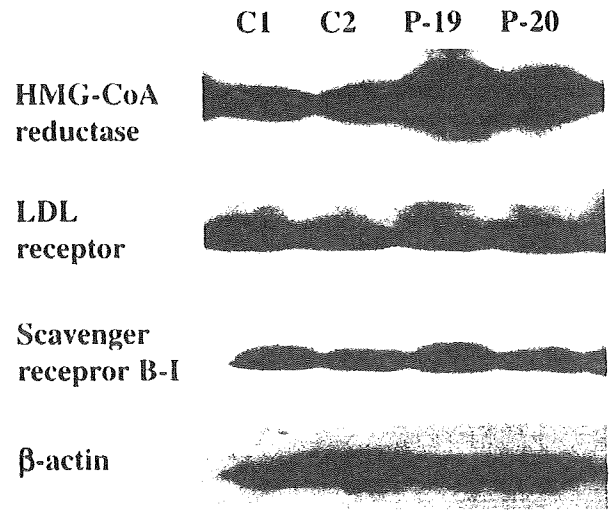

Fig. 1. Western blots of liver biopsy specimens against 3-hydroxy-3-methylglutaryl-coenzyme A reductase, low-density lipoprotein receptor and scavenger receptor B-I. HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL-receptor, low-density lipoprotein receptor. Liver biopsy specimens were obtained from healthy controls (C1 and C2) and two affected children (P19 and P20).

Effects of oxidative stress

Urinary acrolein-lysine and urinary 8-OHdG in the affected children were significantly higher than those in the age-matched con-

Table 4
Blood lipid and apolipoprotein levels at a fasting state.

Lipids	TC** (mg/dl)	LDL-C** (mg/dl)	HDL-C** (mg/dl)	PL** (mg/dl)	TG (mg/dl)	FFA (mmol/L)
Patients ($n = 20$)	213(32)	116(23)	79(7)	237(40)	77(19)	0.8(0.2)
Ranges	153–319	76–196	54–108	189–333	38–124	0.4–1.2
Controls ($n = 32$)	169(22)	85(13)	54(11)	193(25)	80(25)	0.9(0.2)
Ranges	111–207	42–106	39–77	123–239	35–139	0.4–1.5
Apoproteins & Ox LDL	Apo-AI** (mg/dl)	Apo-AII** (mg/dl)	Apo-B** (mg/dl)	Apo-CII* (mg/dl)	Apo-E* (mg/dl)	Ox LDL** (U/L)
Patients (n=20)	169(20)	36(5)	111(22)	3.5(1.1)	5.6(1.5)	82(24)
Ranges	133–213	30–50	79–192	1.5–7.5	3.1–9.4	39–156
Controls (n=32)	127(16)	30(4)	78(13)	3.1(1.1)	4.6(1.1)	25(7)
Ranges	88–165	20–42	48–109	0.8–5.5	1.9–7.5	5–50

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PL, phospholipids; TG, triglycerides; FFA, free fatty acids; Ox LDL, oxidized LDL.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.
** $p < 0.001$ versus controls.

Table 5
Levels of urinary biomarkers for oxidative stress, anti-oxidant enzyme activities in erythrocytes and blood vitamin E level.

	3-OHdG*** (ng/mg Cr)	Acrolein-lysine** (nmol/mg Cr)	SOD*	Cat*	Vitamin E** (mg/dl)
Patients (n = 13)	67(21)	481(125)	1.49(0.34)	3.60(0.52)	0.60(0.21)
Ranges	32–100	220–686	0.92–1.92	2.77–4.44	0.32–1.29
Controls (n = 32)	19(5)	272(90)	1.06(0.18)	2.96(0.21)	0.98(0.14)
Ranges	11–29	70–424	0.80–1.50	2.55–3.56	0.67–1.45

3-OHdG; urinary 8-hydroxy-2′-deoxyguanosine; SOD: superoxide-dismutase (U/mg protein); Cat, catalase (pmol/mg protein); Cr, creatinine.
Presented data are mean (SD) values and ranges.
* $p < 0.05$ versus controls.
** $p < 0.01$ versus controls.
*** $p < 0.001$ versus controls.

trols (acrolein-lysine, $p < 0.01$; 8-OHdG, $p < 0.001$). In contrast, blood vitamin E levels in the affected patients were significantly lower than those in the controls (Table 5). Erythrocyte SOD and catalase activities in the affected patients were significantly higher than those in the age-matched controls ($p < 0.05$) (Table 5).

These findings suggest that the affected children were substantially influenced by oxidative stress.

Discussion

Citrin deficiency manifests as NICCD during neonatal and infancy periods. Clinical manifestations of NICCD resolve at around 12 months of age, with no subsequent overt clinical presentations [1,3,6,8–12]. However, about one-fifth of affected subjects develop CTLN2 at ages of 11–79 years. It has been postulated that multiple factors involving diet can determine whether the affected patients develop CTLN2.

Results of recent studies indicate that the affected children prefer a low-carbohydrate high-fat/protein diet [25] and that a high-protein low-carbohydrate diet is effective for CTLN2 [26]. The diet patterns of the affected children enrolled in this study apparently differed from those of age-matched healthy children, although the total daily energy intake was not significantly different between these two groups. The former favored a low-carbohydrate high-lipid/protein diet: energies obtained from respective nutritional components to the total daily energy ratios were the following: carbohydrates, 35 ± 5% (control, 55 ± 3%); fat, 45 ± 4% (29 ± 2%); protein, 20 ± 3% (14 ± 2%). It is likely that their low-carbohydrate high-lipid/protein diets affected the metabolic profiles.

Results of this study suggest that the affected children at the silent stage differed from age-matched healthy children in many metabolic aspects.

Despite the high-protein intake, plasma ammonia levels in the affected children were comparable to those in healthy age-matched controls. However, plasma citrulline and ornithine levels were substantially higher in the affected children than in the healthy children. Citrulline is synthesized from ornithine and carbamoylphosphate by ornithine transcarbamylase. It is subsequently transformed by ASS into argininosuccinate under the supply of aspartate from the mitochondria by AGC2-citrin [3,7,8]. From this context, amino-profiles of affected children enabled us to assume that the supply of aspartate to cytosol from mitochondrial fraction remains at lower levels in them. Strikingly high plasma citrulline levels in NICCD and CTLN2 engender high synthesis of arginine by ASS and ASL in kidney or intestine, resulting in a high plasma arginine level [1,2,27]. As compared to NICCD and CTLN2, the increase in plasma citrulline level at the silent stage was too minute to increase plasma arginine levels.

The NO synthesis in the affected patients remained normal. Their NO_x^- and ADMA levels resembled those in the age-matched healthy controls. We recently reported that urea cycle defects exhibiting markedly abnormal arginine and citrulline levels show abnormal NO synthesis [28]. The citrulline level abnormalities in the affected children during the silent period might be too slight to affect NO synthesis.

The *L/P* ratio in the affected children was significantly higher than that in the controls, suggesting that their NADH to NAD^+ ratio remains high even during the silent period. The *L/P* ratio is determined by the NADH to NAD^+ ratio in cytosol [24]. It has been postulated that citrin plays a crucial role in the regulation of the NADH to NAD^+ ratio in the cytosol and that citrin deficiency presents a high NADH to NAD^+ ratio [3,7,8].

The affected children showed high levels of serum total cholesterol, LDL-C, and HDL-C. The mechanistic explanation for the hypercholesterolemia remains unclear. Affected patients favor a considerably high-lipid diet [25]. Accordingly, their hypercholesterolemia is expected to be at least partly attributable to such a dietary habit. Using Western blot analysis, we examined HMG-CoA reductase expressions in liver samples that had been obtained by percutaneous liver biopsies from a few affected patients. The results suggest that liver HMG-CoA reductase expression was increased in such patients, although expressions of LDL-receptor and HDL receptor such as SR-BI in their liver tissues were comparable to those in the controls (Fig. 1). For that reason, we now assume that hypercholesterolemia in the affected children was at least partly attributable to increased cholesterol synthesis.

On the other hand, their triglyceride levels remained at values comparable to those of the age-matched healthy children. As described above, our patients consumed a considerably low-carbohydrate diet during this study. Probably, such a diet prevented hypertriglyceridemia. A high-carbohydrate diet has been proven to promote production of NADH and thereby stimulate productions of triglycerides [1,8,29,30]. In particular, citrin-deficient individuals are apparently directed easily to hypertriglyceridemia [1,8,26]. We found in many cases that CTLN2 patients developed hypertriglyceridemia when consuming a high-carbohydrate diet. Furthermore, we are now following two citrin-deficit adults with postprandial hypertriglycemia but without overt liver dysfunction.

We inferred that the affected children were subjected persistently to considerable oxidative stress, although is difficult to judge the magnitude of the oxidative stress merely using oxidative stress biomarkers. Nevertheless, decreased blood vitamin E levels and increased erythrocyte anti-oxidant enzyme activities implied augmented oxidative stress in the affected patients. The increased SOD and catalase activities in erythrocytes can be interpreted as responses to increased plasma oxidants. The considerable increase in oxidized LDL supports this notion.

We speculate that the augmented oxidative stress might be partly attributable to the increased cytosolic NADH. Accumulation of cytosolic NADH has been shown to have some probability of causing oxidative stress [7,31,32].

Evidence that dyslipidemia and oxidative stress are closely related to development of liver steatosis or steatohepatitis has been accumulating [33,34]. Considering that citrin deficiency often

develops liver steatosis as a clinical presentation of CTLN2 in later life, it might be important to improve dyslipidemia and to reduce oxidative stress for management of citrin deficiency.

Results of this study show that metabolic abnormalities such as hypercitrullinemia and hypercholesterolemia were sustained in children with citrin deficiency, even during the silent stage. Results provide evidence that the affected children were subjected persistently to oxidative stress.

Further study is necessary to determine whether such sustained metabolic abnormalities might induce development of CTLN2.

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REGULAR ARTICLE

Glycine regulates proliferation and differentiation of salivary-gland-derived progenitor cells

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Abstract Amino acids have various physiological activities that influence processes such as intestinal regeneration, EGF secretion, protein synthesis, and cell growth. Salivary glands are exposed to nutrients that influence their proliferation and regeneration. Glycine is included in saliva in large quantities and reportedly has important roles in antibacterial activities and the inhibition of tumor growth and as a precursor of nucleotide synthesis in cell proliferation. We have investigated the effects of glycine on the proliferation and differentiation of salivary glands by using mouse salivary-gland-derived progenitor (mSGP) cells. In cultures of mSGP cells, cell proliferation is suppressed in the presence of glycine, whereas it is promoted by its removal. Glycine promotes three-dimensional formations of mSGP cells, which are negative for immature markers and positive for differentiation markers. In cell-cycle analysis, cell-cycle progression is delayed at the S-phase by glycine supplementation. Glycine also suppresses the phosphorylation of p42/p44MAPK. These results suggest that glycine suppresses the proliferation and promotes the differentiation of mSGP cells, and that it has inhibitory effects on growth factor signaling and cell-cycle progression. Glycine might therefore be a physiological activator that regulates the proliferation and differentiation of salivary glands.

Keywords Salivary glands · Glycine · Growth factor · Mouse salivary-gland-derived progenitor (mSGP) cells · Cytokeratin (CK) 19 · Cell cycle

Introduction

The major components of the gastrointestinal system are the oral cavity, salivary glands, esophagus, stomach, intestine, liver, and pancreas. Since the gastrointestinal tract is directly exposed to external nutrients, it is the component that is the most affected by them. For example, the intestinal epithelium contains subpopulations of cells that exhibit the most rapid turnover and synthesis of proteins (Drucker et al. 1996). The supply of most of the amino acids in the intestine is highly dependent on interstitial absorption. In particular, L-glutamate, L-glutamine, and L-aspartic acid are taken up as an energy source directly from the lumen. In addition, there are numerous reports of the role of L-glutamine as a biologically active factor in the salivary gland and the intestinal epithelium in which it stimulates epidermal growth factor (EGF) secretion, accelerates intestinal immunity by the secretion of EGF, and induces intestinal epithelial regeneration (Wilmore et al.

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1988). Therefore, these amino acids may regulate the functions of various organs.

Amino acids are known to have various physiological activities depending on the biological conditions. For example, branched-chain amino acids (BCAAs) such as L-valine, L-leucine, and L-isoleucine have physiological properties such as accelerating protein synthesis and inhibiting protein degradation in skeletal muscle (Goldberg and Chang 1978). BCAAs, which account for approximately 40% of all the free amino acids in serum, are useful energy sources during exercise and for maintaining blood-sugar levels, as mediated by the glucose-alanine cycle (Harper et al. 1984). L-arginine is an intermediate in the urea cycle and is also a precursor for the synthesis of nitric oxide and polyamines (Peranzoni et al. 2007; Rees et al. 1998; Reynolds et al. 1990). L-arginine is known to stimulate the secretion of various hormones (Ghigo et al. 1994). Moreover, L-arginine deprivation can cause a delay in cell-cycle progression in HeLa cells (Wheatley et al. 2000). A high-dose of L-alanine has been reported to improve liver function and the survival ratio in rats with acute liver failure caused by a lethal dose of D-galactosamine (Maezono et al. 1996). Several other amino acids are thought to be vital for cell proliferation and important for maintaining homeostasis as neurotransmitters and as a source of adenosine triphosphate. Glycine is a component of natural products and functions as an inhibitory neurotransmitter (Ghavanini et al. 2005). Glycine also exists in saliva, inhibiting the co-agglutination of oral bacteria (Amano et al. 1997). In addition, glycine prevents tumor growth in vivo (Rose et al. 1999a, 1999b).

In this study, we have analyzed the effect of glycine on cell proliferation and differentiation by using mouse salivary-gland-derived progenitor (mSGP) cells. The salivary glands originate from the endoderm and the ectoderm, both of which participate in organogenesis (Larsen et al. 2001; Denny et al. 1997). mSGP cells were originally isolated from c-kit$^+$/Sca-1$^+$ small epithelial ducts that had proliferated and been detected by a fluorescence-activated cell sorter (FACS) only after duct ligation (Hisatomi et al. 2004; Matsumoto et al. 2007; Okumura et al. 2003; Sato et al. 2007). Isolated immature mSGP cells, which differentiate into hepatic and pancreatic cells, are positive for c-kit, Sca-1, Thy-1, intracellular laminin, CD49f, and alpha-fetoprotein (AFP). These cells are reportedly negative for differentiation markers such as cytokeratin (CK) 19, insulin, and albumin, and they do not exhibit any of the characteristics of duct epithelial cells (Hisatomi et al. 2004). mSGP cells can form cell clusters autonomously on type I collagen and differentiate at the tips of the clusters, which exhibit intracellular-laminin disappearance and CK19 expression. Therefore, since the number of cell clusters, CK19-positive cells, and intracellular laminin-negative cells reflects differentiation, we have employed a three-dimensional (3-D) culture assay to screen and detect the effect of amino acids on the differentiation of mSGP cells.

Materials and methods

Cell isolation and culture

Cells were isolated and cultured as previously described (Hisatomi et al. 2004). Cells isolated from submandibular glands, named mSGP cells, were plated and cultured on type I collagen (Asahi Techno Glass, Tokyo, Japan) at a density of 1×10^6 cells/100-mm dish, in control culture medium. The medium was renewed every 3 days.

The control culture medium was Williams' medium E supplemented with 5% fetal bovine serum (Invitrogen), 20 ng/ml mouse EGF (Chemicon International), 10^{-6} mol/l dexamethasone (Sigma, St Louis, MO., USA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen), 1× insulin-transferrin-serenium-X (Invitrogen), and 10 mmol/l nicotinamide (Sigma).

Preparation of media with or without amino acids

Delta media (δ media) were provided by AJINOMOTO (Kawasaki, Japan): the 20 types of δ media consisted of the 20 kinds of amino acids with the exclusion of one amino acid (0 mM) each. The media were prepared based on an amino-acid-free medium of the same composition as Dulbecco's modified Eagle's medium (DMEM) except for the amino acids. The final concentration of amino acids in these media was based on that of DMEM as follows (in mM): 0.4 mM glycine, 0.4 mM L-alanine, 0.4 mM L-arginine, 0.4 mM L-asparagine, 0.4 mM L-aspartic acid, 0.2 mM L-cystine, 4.0 mM L-glutamine, 0.4 mM L-glutamic acid, 0.2 mM L-histidine, 0.8 mM L-isoleucine, 0.8 mM L-leucine, 0.8 mM L-lysine, 0.2 mM L-methionine, 0.4 mM L-phenylalanine, 0.4 mM L-proline, 0.4 mM L-serine, 0.8 mM L-threonine, 0.08 mM L-tryptophan, 0.4 mM L-tyrosine, and 0.8 mM L-valine.

Medium with the addition of glycine, L-alanine, or L-serine at a concentration of 10 mM was called plus (+) medium (−Gly, +Ala, or −Ser, respectively). All media were prepared 24 h before use and kept at 4°C.

In vitro cell proliferation studies

The number of proliferating cells present at 24 h and 48 h was determined by a modified 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT)-based assay (Kishida Reagents Chemicals, Osaka, Japan) and bromodeoxyuridine (BrdU) labeling as in the manufacturer's protocol.

In vitro cell differentiation studies

To induce cell differentiation, mSGP cells were cultured on type I collagen (Asahi Techno Glass) at a density of 2×10^5 cells/60-mm dish in +Gly, +Ala, or +Ser for 2 weeks. The medium was renewed every 3 days. After 2 weeks, the phenotypes of the differentiated cells cultured in these media were estimated by immunofluorescent stains and reverse-transcriptase polymerase chain reactions (RT-PCR).

In vitro cell signal inhibition studies

To inhibit cell proliferation signaling, mSGP cells were cultured in media supplemented with a 5 μM MEK inhibitor (U0126), 20 μM PI3K inhibitor (LY294112), 20 μM Akt inhibitor (Calbiochem Biochemicals, Darmstadt, Germany), or 5 nM rapamycin (Cell Signaling Technology, Danvers, Mass., USA) for 24 h. The cell phenotypes were then estimated, as was cell differentiation.

Analysis for DNA content, cyclin expression, and apoptosis

Cells were plated on type I collagen (Asahi Techno Glass) at a density of 2.5×10^5 cells/60-mm dish and treated with 10 mM glycine for 24 h. The DNA content, cyclin expression, and apoptosis of cells treated with glycine were analyzed by flow cytometry on a Becton Dickinson FACS Caliber (BD Bioscience), as in a previous study (Hisatomi et al. 2004).

Immunostaining of cultured cells

Cells on glass dishes were stained as previously described (Hisatomi et al. 2004). The primary antibodies used were anti-CD49f (BD Bioscience PharMingen), anti-AFP, anti-CK19, anti-E cadherin (Santa Cruz Biotechnology), anti-laminin, or anti-albumin (DAKO Cytomation) diluted at 1:100 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, for 1 h at 37°C. The secondary antibodies used were Alexa488-labeled anti-goat IgG, Alexa488-labeled anti-rabbit IgG, Alexa594-labeled anti-mouse IgG, Alexa594-labeled anti-goat IgG, Alexa594-labeled anti-rat IgG, Alexa594-labeled anti-rabbit IgG (Molecular Probes) diluted at 1:1,000 in PBS containing 1% bovine serum albumin, for 1 h at 37°C. Cells were viewed under a confocal laser-scanning microscope FV500 (Olympus Optical, Tokyo, Japan).

RT-PCR analysis

Total RNA isolation and complementary DNA preparation were as previously described (Hisatomi et al. 2004; Matsumoto et al. 2007; Sato et al. 2007). The resulting complementary DNA was amplified by using GeneAmp PCR 9700 (Perkin-Elmer, Norwalk, Conn., USA) with the following sets of primers: AFP: forward 5′-actcaccc caaccttcctgtc-3′, reverse 5′-cagcagtggctgataccagag-3′; albumin: forward 5′-catgacaccatgcctgctgat-3′, reverse 5′-ctctgatcttcaggaagtgtac-3′; CK19: forward 5′-gtcctacagattga caatgc-3′, reverse 5′-cacgctctggatctgtgacag-3′; GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase): forward 5-ccgccaccaccaactgctta-3′, reverse 5′-tcatgagccctccacaatg-3′.

Western blot analysis

Cells were grown in culture in the presence of 10 mM glycine or inhibitors or in control medium for the time periods indicated. The cells were lysed in complete RIPA buffer (150 mM NaCl, 50 mM TRIS-HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA) containing a protease inhibitor cocktail and phosphatase inhibitor cocktail (Nacalai Tesque). Aliquots containing 20 μg protein were loaded per lane onto 1.5-mm 10% SDS-polyacrylamide gels (ATTO, Tokyo, Japan) for electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane; the expression of each protein was confirmed as previously described (Lianguzova et al. 2007). The primary antibodies used were anti-phospho-p42/p44MAPK, anti-p42/p44MAPK (Cell Signaling Technology) diluted at 1:1,000, and the secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-biotin IgG (Cell Signaling Technology) diluted at 1:2,000.

Results

Short-term glycine addition inhibits cell proliferation

In order to investigate whether glycine affected cell proliferation in vitro, we utilized mSGP cells, i.e., tissue progenitor cells derived from duct-ligated submandibular glands and originating from small epithelia ducts; these cells were able to grow while maintaining their immaturity in the presence of EGF (Hisatomi et al. 2004; Matsumoto et al. 2007; Okumura et al. 2003; Sato et al. 2007). To investigate the effects of each amino acid on cell proliferation, we cultured mSGP cells in media supplemented with one of the 20 amino acids. Cell proliferation in the presence of glycine was reduced in comparison with that of the control, whereas cells treated with L-alanine or L-serine grew as well as the control population. Based on these data, we selected three amino acids, viz., glycine, L-alanine, and L-serine, in order to investigate whether these amino acids affected cell proliferation in vitro. We cultured mSGP cells for 48 h with glycine, L-alanine, or L-serine and observed