Pancreatic secretory trypsin inhibitor gene is highly expressed in the liver of adult-onset type II citrullinemia

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Abstract Deficiency of argininosuccinate synthetase (ASS) causes citrullinemia. Type II citrullinemia is found in most patients with adult-onset citrullinemia in Japan, and ASS is deficient specifically in the liver. Previous studies have shown that the decrease of hepatic ASS activity is caused by a decrease in enzyme protein with normal kinetic properties and that there are no apparent abnormalities in the amount, translational activity, and nucleotide sequence of hepatic ASS mRNA. Recent results of homozygosity testing indicate that the primary defect of type II citrullinemia is not within the ASS gene locus. In this present work, to understand the pathogenesis and pathophysiology of type II citrullinemia, we have characterized the alterations of gene expression in the liver of type II patients using the recently developed mRNA differential display method. Some cDNA bands expressed differently in type II citrullinemia patients and control were selected, cloned, and sequenced. Nucleotide sequence analysis and homology searching revealed an interesting clone which has 99% homology with the human pancreatic secretory trypsin inhibitor (hPSTI). Northern blot and RT-PCR analyses showed that the expression of hPSTI mRNA increased significantly in the liver of all type II patients tested. Furthermore, the concentration of hPSTI protein was found to be higher in the liver of type II citrullinemia than in control. These results suggest that hPSTI may be related to the primary defect of type II citrullinemia and may be useful as a diagnostic marker, although the detailed mechanism of the high expression of hPSTI mRNA in type II liver is not yet known.

Key words: Type II citrullinema; Argininosuccinate synthetase; Differential display; Pancreatic secretory trypsin inhibitor

1. Introduction

Citrullinemia is an autosomal recessive disease caused by deficiency of argininosuccinate synthetase (ASS; EC 6.3.4.5). The clinical, biochemical, and molecular aspects of citrullinemia and their heterogeneity have been reviewed elsewhere [1–4]. Saheki et al. [2] analyzed the enzyme abnormalities in Japanese citrullinemia patients and classified into three types. The classical form (type I and III) is found in most patients with neonatal/infantile-onset citrullinemia. The enzyme defect in the classical form is found in all tissues and/or cells where ASS is expressed. 21 mutations in ASS mRNA and 22 mutations in ASS gene have been identified in human classical citrullinemia ([5–11]; Kakinoki et al., manuscript in preparation). We can now diagnose 11 of 22 mutations in classical citrullinemia using genomic DNA by Southern blot analysis and by a combination of polymerase chain reaction (PCR) and restriction enzyme digestion ([6,7,10,11]; Kakinoki et al., manuscript in preparation).

We have also described a different type of citrullinemia, which occurs in association with decreased ASS activity and protein in the liver but normal ASS levels in other tissues, such as kidney, brain, and fibroblasts [2,12–16]. Most patients suffer from sudden disturbance of consciousness, such as disorientation, restlessness and coma. This unique citrullinemia was classified as type II [2], and most patients with adult-onset citrullinemia belonged to type II. The symptoms, such as high concentration of serum citrulline, slight increase of serum arginine, and hyperammonemia, resulted from the specific decrease in hepatic ASS level [2] and were corrected by liver transplantation in two patients ([17]; Kawamoto et al., manuscript in preparation). In adult-onset type II citrullinemia with liver-specific deficiency, the decreased hepatic ASS showed kinetic properties, heat stability and specific activity of ASS (acitivity/CRM) comparable to control [2,12–16,18–21]. The heterogeneous distribution of ASS in the liver of type II citrullinemia patients was found by immunohistochemical study [2,22,23]. Analysis of mRNA from type II citrullinemia patients revealed that hepatic ASS mRNAs have: (a) a translatable activity in vitro when a cell-free translation system is used; (b) no decrease in amount; (c) no structural abnormality detectable by Northern blotting and S1-nuclease digestion; and (d) no mutation detectable by nucleotide sequence analysis [2,24–27].

We have analyzed almost 100 patients with adult-onset type II citrullinemia, since Saheki et al. reported the first cases in 1981 [12], and the proportion of type II citrullinemia patients from consanguineous parents is approximately 20%. Homozygosity mapping analysis of the affected patients from consanguineous parents suggested that the abnormality is not within the ASS gene locus and that the primary defect is located in another unknown gene [27].

In order to understand the pathogenesis and the detailed pathophysiology and to identify changes in gene expression, we used a newly developed method called mRNA differential display [28] to select unique genes expressed differentially in the liver of patients with type II citrullinemia and other diseases.

2. Materials and methods

2.1. Patients

Biopsy or autopsy liver specimens included in the present paper were obtained from 11 patients with adult-onset type II citrullinemia. We diagnosed them as type II citrullinemia under criteria described previ-

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Abbreviations: ASS, argininosuccinate synthetase; PSTI, pancreatic secretory trypsin inhibitor; PCR, polymerase chain reaction; RT, reverse transcription.

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ously [2,27]; a high concentration of serum citrulline, a slight increase in serum arginine, an increase in the ratio of threonine to serine in serum, and a decrease in hepatic ASS activity with normal kinetic properties, together with no decrease in renal and/or fibroblast ASS activity. Biochemical data and molecular aspects of the patients (except AP-78 and AP-86) have already been reported [16,17,19–21,23–25,27]. The hepatic ASS activities of patients AP-78 and AP-86 were 3.3% and 6% of control, respectively. The serum citrulline levels of both patients were 400–500 nmol/ml (normal 20–40 nmol/ml). For patients AP-61 [17] and AP-86 (Kawamoto et al., manuscript in preparation), liver transplantation was performed as therapy immediately after diagnosis of type II citrullinemia. Patient AP-29 with type II citrullinemia was positive for hepatitis B-virus surface antigen, but other type II patients were negative for HB- and HC-virus. The histological findings of liver specimens from patients with type II citrullinemia were usually fatty infiltration, fibrosis, and no pathological signs [2,17,23], but some cases had cirrhosis (AP-29) and chronic hepatitis (AP-30).

Liver specimens used as controls in the present study were as follows: from a patient (AP-82) with type I citrullinemia of adult-onset [10,11], a patient (C-76) with angioma and positive for HB-virus, a patient (C-81) with rectal cancer, diabetes mellitus and old myocardial infarction, a patient (C-100) with intrahepatic calculus, and a patient (C-104) with gall bladder calculus. Non-tumor portions surrounding the hepatic carcinoma of a patient (C-201) with liver cirrhosis and of patients (C-82, C-83 and C-84) with metastatic hepatic cancer transferred from extrahepatic cancer were used as controls, too. To compare the level of human pancreatic secretory trypsin inhibitor (hPSTI) expression, tumor and non-tumor portions were obtained during surgery from 2 patients (C-1; negative for virus, C-2; positive for HC-virus) with hepatocellular carcinoma (HCC) and from a patient (C-3 or C-4) with metastatic hepatic cancer which originated from colorectal and gastric cancers, respectively.

2.2. Extraction of RNA and removal of DNA contamination from RNA

Total RNA was isolated from liver specimens as described by Chomczynski and Sacchi [29] and Kobayashi et al. [5–7,9–11,24–27]. The RNA (50 μg) was treated with 10 units of DNase I using MessageClean Kit (GenHunter Co.). After extraction with phenol/chloroform and ethanol precipitation, the RNA was redissolved in DEPC-treated water.

2.3. Differential display of mRNA

Differential display PCR was carried out on the type II patient and control RNAs using the method of Liang and Pardee [28] and the GenHunter Corporation mRNA differential display system (Brookline). The following oligonucleotide primers were used to analyze the RNAs present. Four oligo-d(T) primers, $(T)_{12}MN$ were used for reverse transcription and as the 3′-PCR primer. The 5′ primers for PCR were 20 kinds of AP-primer, AP-1 to AP-20, each constituted from 10mer. Polyacrylamide 6% gels (Long Ranger gel, AT Biochem) were run to separate the amplified bands labelled with [α-^{35}S]dATP (New England Nuclear). Re-amplification of the interest bands was carried out with the same primers as used in the initial reaction. Amplified PCR products were cloned into a pCR-II vector using the TA cloning system (Invitrogen) for further analysis. Double-stranded DNA was sequenced by the dideoxy chain-termination method using Sequenase (United States Biochemical) and ^{35}S-labelled dATP [6,7,9–11] and/or using the Hitachi model SQ-5500 DNA auto-sequencer by using *ΔTaq* fluorescent dye-primer cycle sequencing kit (Amersham) and Texas-red labelled primer.

2.4. Northern blotting and RT-PCR

Total RNA (20 μg) was analyzed by Northern blotting as previously described [5,25]. Specific probe for hPSTI was generated by labelling cloned cDNA fragments with [α-^{32}P]dCTP and random prime DNA labelling kit (Amersham). After hybridization at 42°C and high-stringency washing at 60°C in 0.3 M NaCl/0.03 M trisodium citrate/0.1% SDS, the blots were exposed to Fuji X-ray film for 6 days with intensifing screens.

For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 5 μg of total RNA using reverse transcriptase and oligo-d$(T)_{18}$ in 20 μl of final volume. Each 1 μl of first-strand cDNA sample was used for the amplification of the hPSTI and C-reactive protein (CRP) cDNA and 1 μl of 200-times diluted first-strand cDNA sample was used for the PCR of albumin mRNA. Oligonucleotides used as PCR-primers were 5′-CGTAGGATCCAT ATGAAGGTAACAGGCATCTTTC-3′ and 5′-CGTAGGATCCAGTCAGGCCTCGCGGTGA-3′ for hPSTI (underlined sequences are from hPSTI cDNA) [30], 5′-TGACCAGCCTCTCTCATGCT-3′ and 5′-GAGCAGGCCTGCAATGCATA-3′ for CRP [31], and 5′-TCTATCCGTGGTCCTGAACC-3′ and 5′-CTCATGGTAGGCTGAGATGC-3′ for albumin [32], respectively. Amplification of the cDNA was carried out for 25 cycles at 95°C for 1 min, 60°C for 0.5–1 min, and 72°C for 2–3 min as in previous reports [7,9–11]. The PCR-products were electrophoresed on a 2% agarose gel.

2.5. Determination of hepatic hPSTI protein

Tissue extracts were prepared by homogenizing specimens with an adequate volume of 0.15 M KCl containing 50 mM Tris-HCl, pH 7.5. Insoluble fractions were discarded by centrifugation. The concentration of hPSTI protein was measured by RIA system supplied from Shionogi Pharmaceutical Co. (Osaka) and the hPSTI content in tissues was estimated and expressed as μg/g wet weight.

3. Results

Total RNA was extracted from liver specimens of three pa-

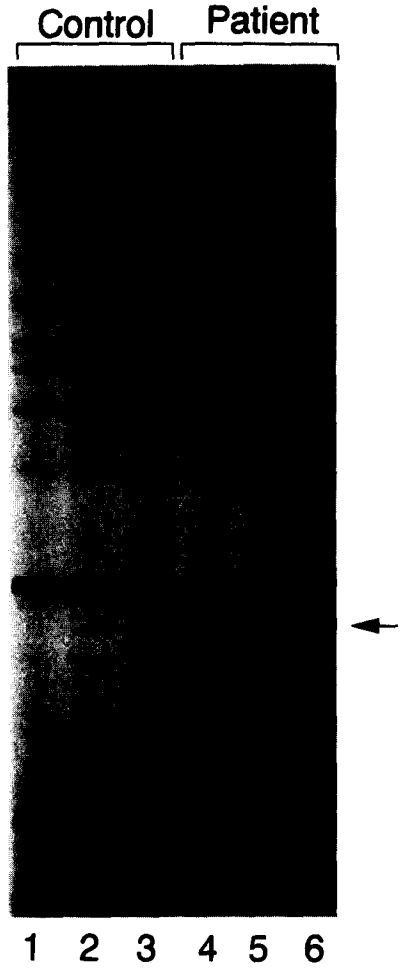

Fig. 1. Differential display comparing RNA from type II citrullinemia patients and controls. Total RNA was extracted from liver specimens and subjected to differential mRNA display analysis as described in section 2. Autoradiogram of amplified [α-^{35}S]dATP-labelled PCR products are shown. The primers used were AP-15, 5′-AGGGCCTGTT-3′ (5′-primer) and 5′-T_{12}MA-3′ (3′-primer). Candidate cDNA signal which demonstrated altered expression by differential display is marked by arrow. Lanes 1 to 3 are control, C-81, C-83 and C-84; lanes 4 to 6 are type II patients, AP-29, AP-30 and AP-38, respectively.

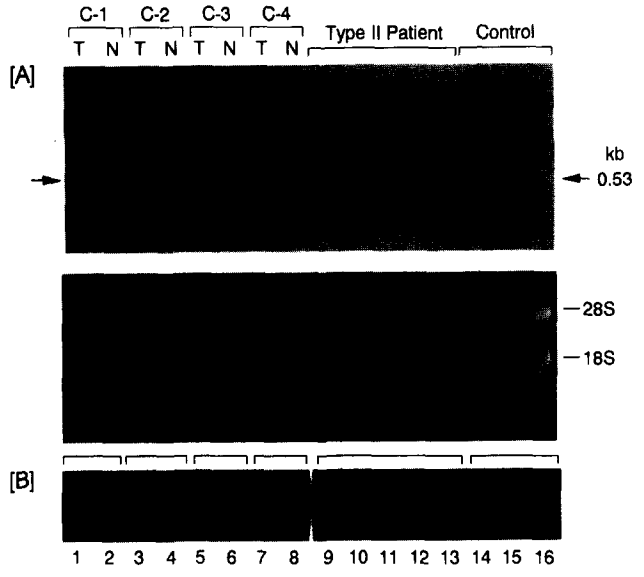

Fig 2. Confirmation of hPSTI mRNA expression by Northern blot hybridization (A) and RT-PCR (B) analyses. (A) Total RNA extraction and Northern blot were carried out as described in section 2. The upper panel shows the autoradiogram of hPSTI mRNA. Ethidium bromide-stained RNA is shown below column. (B) First-strand cDNA was synthesized from 5 μg of total RNA by using reverse transcriptase and oligo-d(T)$_{18}$. Amplification of hPSTI mRNA was carried out as described in section 2. T and N denote tumor and non-tumor portions of liver from patients C-1 to C-4 with hepatic cancer (lanes 1–8). Lanes 9 to 13 are type II citrullinemia patients AP-78, AP-86, AP-29, AP-30 and AP-38, and lanes 14 to 16 are control C-76, C-81 and C-83, respectively.

tients with adult-onset type II citrullinemia (AP-29, AP-30 and AP-38). The control RNA preparations used for differential display were from a liver specimen of a patient with rectal cancer but no hepatic metastasis (C-81) and noncancerous hepatic portions of two patients with metastatic hepatic cancer (C-83 and C-84). Several cDNA bands were found to be differentially expressed on the gel electrophoresis (data not shown). A cDNA fragment selectively expressed in type II citrullinemia was shown in Fig. 1. This cDNA fragment (A-15) was extracted from the gel, PCR-amplified using a set of AP-15 as 5′ primer and $T_{12}MA$ as 3′ primer, cloned into a pCR-II vector using the TA cloning system, and sequenced. From a homology search in GenBank, the nucleotide sequence from clone A-15 had 99% homology with the nucleotide sequence from 36 to 318 of human pancreatic secretory trypsin inhibitor (hPSTI) mRNA [30,33].

It has been reported that PSTI mRNA is expressed highly in hepatic cancer and also other cancer tissues [34–38]. Therefore, we tested tumor portions and non-tumor portions of liver specimens from patients with HCC and two patients with metastatic hepatic cancer (one from colon cancer and the other from stomach cancer). By Northern blotting and RT-PCR analysis as shown in Fig. 2, we confirmed that the hPSTI mRNA was expressed in the hepatic tumor portion regardless of origin. The hPSTI mRNA estimated to be about 0.53 kb on Northern blot as reported previously [30], was found clearly in the liver of adult-onset type II citrullinemia. However, hPSTI mRNA was not detected or was at a low level in the noncancerous hepatic portion of patients with hepatic cancer and liver specimens from other control patients (Figs. 2 and 3). On the other hand, the mRNA level of CRP which is a major acute phase reactant (APR) showed a slight tendency to be lower in type II patients than in controls but there was no significant difference between them. There was no significant difference in albumin mRNA level either, as shown in Fig. 3.

In order to investigate whether highly expressed hPSTI mRNA is translatable or not, we determined the concentration of hPSTI protein in liver and tumor specimens by RIA (Table 1). hPSTI protein was less than 0.01 μg/g wet tissue in the liver of control patients including noncancerous portions of liver from hepatoma patients and high level tumor portions from HCC, which agrees with previous reports [34,39]. As expected from the results of hPSTI mRNA analyses, the hPSTI protein level in the liver of most patients with type II citrullinemia tested was far higher but with a wider range than in cancerous tissues.

4. Discussion

PSTI was first isolated from bovine pancreas in 1948 by Kazal et al. [40] and has been thought to exist only in the pancreas. Its role has been considered to be a specific inhibitor that prevents autoactivation of trypsinogen in the pancreas and pancreatic juice [41]. Later studies showed that PSTI was also expressed in some extrapancreatic normal tissues [39,43–45] and in various cancers, including pancreatic [34], colorectal [35], gastric [36], lung [37], and hepatocellular [38] cancers. It was found that the serum concentration of immunoreactive PSTI is elevated after surgery, trauma and severe infection [45–47], suggesting that PSTI is an acute phase reactant. PSTI is expressed in the liver which secretes it into the blood in response to inflammatory cytokines, such as IL-6 [48,49]. These phenomena imply that PSTI has other physiological and pathological roles in addition to the inactivation of trypsin. Several papers suggest that tumor-derived PSTI acts as autocrine or paracrine growth factor [46,50–54], and is associated with

Table 1
The contents of hPSTI protein in liver specimens of type II citrullinemia and control patients and in tumor portions of liver from patients with hepatic cancer

Patients		hPSTI (μg/g wet weight)
Type II citrullinemia	AP-17	0.043
	AP-18	0.051
	AP-21	0.514, 0.377
	AP-27	0.659
	AP-30	<0.01
	AP-38	0.186
	AP-39	6.89, 5.98
	AP-61	0.385
	AP-78	0.063
	AP-86	0.025
Control	C-76	<0.01
	C-82	<0.01
	C-104	<0.01
	C-201	<0.01
	C-1	<0.01
	C-2	<0.01
	C-3	<0.01
Tumor portion	C-1	0.323, 1.19
	C-2	<0.01
	C-3	0.018

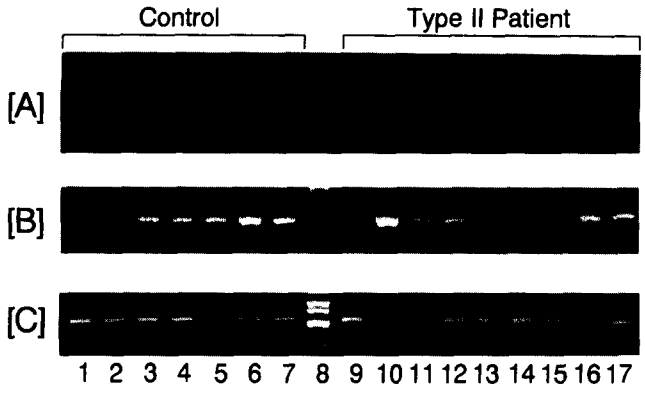

Fig. 3. Expression patterns of hPSTI (A), CRP (B) and albumin (C) mRNAs in the liver of type II citrullinemia and control patients. All procedures of cDNA synthesis and the amplification are shown in Fig. 2 and section 2. Lanes 1 to 7 are patients as control; AP-82 (adult-onset type I citrullinemia), C-76, C-81, C-82, C-83, C-84 and C-100, respectively. Lane 8 is DNA size marker. Lanes 9 to 17 are type II patients; AP-17, AP-18, AP-21, AP-27, AP-29, AP-30, AP-38, AP-39 and AP-61, respectively.

the tumor growth of stromal proliferation of fibrous tissues [36,37], although it has been reported recently that the evaluation of tumor-derived PSTI as a paracrine or autocrine growth factor is not definite and needs confirmation [55].

The hPSTI mRNA expression in type II citrullinemia patients was equivalent to the level in hepatic cancer portions (Figs. 2 and 3), although the liver specimens of most type II patients tested in the present paper did not show any histological appearance as cancer. It was suggested that the PSTI gene is regulated by the AP-1 binding site and IL-6 responsive element, and each region is active in hepatoma cells [38]. However, the biological implication of the high PSTI gene expression in hepatic cancer is not yet understood. We investigated the gene expression of CRP, a major acute phase reactant, by using RT-PCR method in the liver of type II patients although there is no sign for inflammation. The result showed that CRP mRNA is not increased in the liver of patients with type II citrullinemia (Fig. 3). This suggests that the high expression of hPSTI in the liver of type II citrullinemia is not related to acute phase reaction. The possibility can not be ruled out, however, that CRP levels may be increased in the serum of type II patients. It is known that the dramatic transcriptional induction of CRP by IL-6 is further enhanced by novel translational and post-translational mechanisms, which eventually lead to a negative-feedback loop in which CRP can downregulate its own levels during inflammatory responses [56]. In type II citrullinemia, the relationship between the decrease in hepatic ASS-protein levels and the increase in PSTI mRNA has not yet been explained. As shown in Table 1, the contents of immunoreactive hPSTI in the liver of patients with type II citrullinemia except one patient (AP-30) showed 20–3,000 fold increases over the normal level (0.002 ± 0.001 μg/g wet weight liver) [39].

Finally, we conclude that PSTI may be useful as a diagnostic marker for adult-onset type II citrullinemia, although further experiments will be required to verify this. We are now investigating the specificity, usefulness and meaning of high PSTI expression in type II citrullinemia patients by determining PSTI level in the serum and comparing it with the acute phase response, tumor and pancreatitis marker level.

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