A Variant Serum Cholinesterase and a Confirmed Point Mutation at Gly-365 to Arg Found in a Patient with Liver Cirrhosis

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A 64-year-old man was admitted to our hospital because of possible liver cirrhosis. His serum cholinesterase was anomalously low with a ΔpH of 0.1 (normal range; 0.8–1.1). His enzyme was more heat-labile than the normal controls. Km value of his enzyme for benzoylcholine was $1.1 \times 10^{-5}$ mol/l, while that for normal controls was $2.3 \times 10^{-6}$ mol/l. In addition, isozymic alteration of his enzyme was observed. Sequencing of the white blood cell DNA of the patient showed a point mutation at nucleotide 1093 (GGA to CGA), which changes codon 365 from glycine to arginine.

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Introduction

Serum cholinesterase (ChE: acylcholine acylhydrolase, EC 3.1.1.8, also called "pseudocholinesterase") has been recognized as an enzyme that hydrolyses choline esters. This enzyme is generally accepted as being synthesized in the liver and has been routinely measured as a test of liver function. Low serum ChE activity has been reported in acute hepatitis, chronic liver diseases and liver metastases (1).

Recently, complete amino acid and cDNA sequences of human serum ChE have been determined (2–4). ChE is a glycoprotein of 4 identical subunits. The protein contains 574 amino acids per subunit and nine carbohydrate chains attached to 9 asparagines; 1722 base pairs of coding sequence (exon 2–4) corresponding to the protein have been identified (4).

On the other hand, since the discovery of an individual having no detectable serum ChE activity was reported by Liddell et al. (5) in 1962, such individuals are said to have a “silent” gene for ChE and to date, more than one hundred subjects have been reported to be homogenous for “silent” ChE. However, the situation concerning silent ChE variants is rather complex and heterogeneity of “silent” ChE phenotypes has been reported (6). Recently, Nogueria et al. (7) described a frameshift mutation responsible for the silent phenotype of human serum ChE. The mutation causes a shift in the reading frame from Gly 117, where GGT (Gly)$\rightarrow$GGAG (Gly + 1 base) to a new stop codon created at position 129. We (8) also reported a family of acholinesterasemia. Genetic analysis was performed on the propositus’ DNA and revealed that exon 2 of the ChE gene was disrupted in both alleles by a 342bp insertion of Alu element. No other abnormalities in the nucleotide level responsible for the silent phenotypes have been described.

In the present study, we report a patient with liver cirrhosis whose serum ChE is anomalously low and has unique enzymic properties. A point mutation in the gene for serum ChE was identified in this patient at amino acid position 365 (Gly$\rightarrow$Arg).

Materials and Methods

Reagents

Dibucaine hydrochloride, and DL-propranolol were obtained from Sigma Chemical Co. (St. Louis, MO). Butyrylthiocholine iodide, sodium fluoride, other chemicals and the cholinesterase test kit were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Antibodies to human cholinesterase and erythrocyte membrane, produced in rabbits, were from DAKOPATT a/s.
positions 1131-1858 of the ChE cDNA, was amplified with oligo d(T) primer. A 728bp segment, corresponding to poly(A) + RNA with the sequence primers M4 and RV (Takara Shuzo Co., Kyoto, Japan). The mixture of serum enzyme and anti-ChE antibody or anti-erythrocyte membrane antibody, at an enzyme protein/antibody ratio of 20/1, was allowed to stand overnight at 4°C, followed by polyacrylamide gradient g gel electrophoresis. The first strand of CDNA was synthesized from HuH 7min. The primers used for PCR are shown in Table 1.

**Construction of a CDNA probe for ChE**

DNA amplification by polymerase chain reaction (PCR) DNA amplification was preformed, each round consisting of a denaturation step at 94°C for 1 min, an annealing step at 48°C for 2min and an extention step at 72°C for 5–7 min. The primers used for PCR are shown in Table 1.

**Construction of a cDNA probe for ChE**

The first strand of cDNA was synthesized from HuH-7 (human hepatoma cell line) (12) poly(A) + RNA with the sequence primers M4 and RV. A 728bp segment, corresponding to positions 1131–1858 of the ChE cDNA, was amplified by PCR with primers A and B (Table 1). After purifying, the 728bp PCR product was treated with T4 DNA polymerase to produce blunt ends and inserted into the Smal site of pUC 18. The resulting plasmid was termed pc728. This pc728 containing 3' region of cDNA was sequenced and confirmed to be identical to the cDNA reported previously (4).

**Electrophoresis of Polyacrylamide Gradient Gel**

Electrophoresis of polyacrylamide gradient (2–16%) gel was performed as described previously (10). After electrophoresis, the enzyme was stained by the method of Juul (11) with butyrylthiocholine as the substrate.

**Immunological Properties**

The mixture of serum enzyme and anti-ChE antibody or anti-erythrocyte membrane antibody, at an enzyme protein/antibody ratio of 20/1, was allowed to stand overnight at 4°C, followed by polyacrylamide gradient gel electrophoresis. The electrophoretic pattern was identical to that of normal controls.

**Case Report**

A 64-year-old man was admitted to our hospital because of possible liver cirrhosis. He was well until 5 years previously, when he went to another hospital because of fatigue, and laboratory studies revealed abnormalities in tests of liver function, suggesting the presence of chronic liver disease. From that time, he was followed up by his home doctor, but his serum aminotrans ferase (SGOT and SGPT) levels continued to fluctuate. There was no history of previous jaundice, liver disease in infancy, blood transfusion, exposure to patients with liver disease or alcohol consumption. There was no family history of hepatic, disease. The patient's parents...
were born in the same prefecture, but they were not consanguineous.

On examination he appeared well. His height was 165 cm and body weight 77 kg; his blood pressure was 150/90 mmHg. The lungs and the heart were normal. Examination of the abdomen was normal except that the liver was felt 3 cm below the right costal margin. Neither palmar erythema nor spider angiomas were found. The extremities were normal. On his 3rd hospital day, a liver biopsy was performed under ultrasonographic guidance. Microscopical examination of a biopsy specimen of the liver disclosed posthepatitic type of liver cirrhosis. His liver function tests on admission are briefly shown in Table 2. His serum ChE activity was a ΔpH of 0.1 (normal range: 0.8–1.1) which is anomalously low and has remained unchanged throughout the past 5 years.

Results

Electrophoretic Properties

As shown in Fig. 1, seven isozymes of serum ChE (I to VII numbered from the anodic side to the cathodic side) were usually observed in healthy controls. However, in the present patient bands II and III of his serum ChE isozymes were not observed.

Table 3. Enzymic Properties of Serum Cholinesterase of the Present Patient

<table>
<thead>
<tr>
<th>Controls</th>
<th>Present of original activity*</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibucaine</td>
<td>10^{-5} (mol/l)</td>
<td>16.0–19.5 (10)*</td>
</tr>
<tr>
<td>NaF</td>
<td>5 × 10^{-5} (mol/l)</td>
<td>30.0–36.0 (10)</td>
</tr>
<tr>
<td>Propranolol</td>
<td>10^{-5} (mol/l)</td>
<td>11.4–21.8 (10)</td>
</tr>
<tr>
<td>Activation by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butanol</td>
<td>10 (ml/l)</td>
<td>155–200 (10)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>100 (ml/l)</td>
<td>165–203 (10)</td>
</tr>
<tr>
<td>Km value for</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoylcholine</td>
<td>2.3 × 10^{-6}$mol/l$ (6)</td>
<td>1.1 × 10^{-5}$mol/l$</td>
</tr>
<tr>
<td>Heat stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(56°C)</td>
<td>1 min</td>
<td>58.0 ± 5.0¢ (3)</td>
</tr>
<tr>
<td></td>
<td>2 min</td>
<td>31.9 ± 4.4 (3)</td>
</tr>
<tr>
<td></td>
<td>4 min</td>
<td>16.5 ± 4.4 (3)</td>
</tr>
</tbody>
</table>

* Activity remaining is expressed as the percentage of value for untreated control.

* Numbers in parentheses denote the number of persons determined.

¢ The datum represents the mean ± S.D.
effect of n-butanol and ethanol on the enzyme activity at pH 7.4 was studied according to the method of Whittaker (16). The activity remaining was expressed as the percentage obtained to the untreated control. The present patient had dibucaine and fluoride numbers characteristic of the usual phenotype when measured with benzoylcholine as the substrate. The residual enzyme activity of the present patient examined with propranolol was within the normal range. On the other hand, n-butanol and ethanol activated the enzyme activity, but no significant difference was observed between the present patient and the normal individuals (Table 3).

Heat Stability
Inactivation of serum ChE at 56°C was examined after 1, 2, and 4 minute incubations. As shown in Table 3, the enzyme of the present patient was found to be more heat-labile than those of the normal controls.

Michaelis constant for benzoylcholine
The Km value of serum ChE of the present patient for benzoylcholine was $1.1 \times 10^{-5}$ mol/l, which is higher than that of normal controls, $2.3 \times 10^{-6}$ mol/l (Table 3).

Immunological Properties
After treatment of the patient’s serum with anti-ChE antibody, no bands with enzyme activity were visible at the proper position of the gel; the electrophoretic mobility of all isozymes was retarded (Fig. 1). But no electrophoretic retardation in isozymes of the patient’s enzyme was observed after treatment with anti-erythrocyte membrane antibody (which is known to contain anti-acetylcholinesterase antibody) (Fig. 1). This property of the present patient’s serum ChE was compatible with that of a pseudo-ChE.

Analysis of DNA
Sequencing of the WBC DNA of the patient showed a point mutation at nucleotide 1093 (GGA to CGA), which produces arginine in place of glycine at position 365 (Fig. 2). No other abnormalities were found in the whole coding region (exon 2–4). An unamplified genomic DNA library was screened with ChE cDNA probe, and the four positive clones obtained showed the same mutation at nucleotide 1093, suggesting that the present patient may be a homozygote for the mutation.

Discussion
The present patient had an anomalously low serum ChE activity which accounted for only 12.5% of the lower normal limit. The value of his enzyme activity remained constant for 5 years and his liver function, including the test showing the liver reserve function, was well preserved in spite of having liver cirrhosis. Therefore it could be postulated that the low activity of his enzyme was inherited, but not due to the liver cirrhosis. His father died of old age several years ago. His mother is still alive, but it was not possible to examine the enzymic properties of her serum ChE because of a personal reason. The patient had a younger sister but we were unable to make contact with her. The patient had no children. Thus, it could not be concluded whether this enzymic abnormality is congenital or acquired.

On the other hand, there are at least five recognized genes that participate in directing ChE biosynthesis. They are the usual (E$^+$), atypical (E$^+$), fluoride resistant (E$^+$), silent (E$^+$) and C$_5$ (E$_2$) genes. E$_2$, E$_4$, and E$_5$ are allelic to E$^+$ and these genes give rise to 10 genotypes, all of which have been recognized. E$_2$ is non-allelic to E$^+$. The genotypes determined by the genes E$^+$, E$^+$, and E$_5$ can be differentiated by use of certain inhibitors, but the genotype determined by the C$_5$ gene has been confirmed only by electrophoresis on starch gel. Several other genetically determined variants of ChE have also been found (6).

Regarding the silent gene, heterogeneity of “silent” ChE phenotypes has been reported (6). A rather large number of individuals with silent serum ChE has been
detected among the Eskimos of western Alaska (17). Among about 5,000 Eskimos investigated, 39 were reported to have no detectable enzyme activity, and 24 had 2–10% of the usual activity level when assayed with benzoylcholine. Two groups were recognized among these Eskimos, the group called type I, had about 1% of the usual activity and did not react with antibody against serum ChE. The other group called type II, had 3% of the usual activity and reacted with antibody against serum ChE. Rubinstein et al (18) stated that the type I enzyme has many similarities to acetylcholinesterase of erythrocyte ghosts and that the electrophoretic pattern of type II enzyme is similar to that of normal serum ChE. Scott (19) reported that type II enzyme is more heat stable. Another nearly silent variant has been described by Scott and Wright (20). This variant has less than 10% of the usual activity and a major band migrates slightly faster than the major band of usual serum ChE on starch gel electrophoresis. Some other "nearly silent" ChE variants have been reported, but it is difficult to compare these results with those of previous workers because of differences in experimental procedures (6).

Dibucaine and fluoride numbers of our patient were within the normal range. This indicates that this patient has neither the atypical (E\textsuperscript{1}) nor fluoride resistant (E\textsuperscript{2}) gene. Therefore, this patient seems to belong to the group possessing the "nearly silent" ChE variant. The enzyme of the present patient differed from the type I and II enzymes reported in the investigation of Eskimos in the reactivity with antibody to serum ChE and the electrophoretic pattern. The enzyme of the present patient was also different from the enzyme reported by Scott and Wright (20) in the electrophoretic properties.

The present patient lacked bands II and III of serum ChE isozymes. We (21) have previously reported that band II of serum ChE isozymes disappears or becomes very faint in about 55% of cirrhotic patients. A lack of band III was also observed in a few cirrhotic patients. Recently, we found another patient with hypocholinesterasemia who did not have any liver diseases but had the same isozymic alteration as the present patient (not shown). Thus, we can not conclude at present whether the isozymic alteration of the present patient's ChE may be characteristic of this patient or may be ascribed to the liver cirrhosis itself. The mechanism of the disappearance of bands II and III remains to be clarified.

We (10) previously reported that each ChE isozyme in normal individuals has the same heat stability and sensitivity to inhibitors. Therefore, the loss of bands II and III in the present patient's ChE isozymes does not seem to have any influence on the catalytic properties of the rest of the isozymes (I and IV to VII). In addition, enzymic properties of serum ChE in cirrhotic patients lacking band II were the same as those in normal controls (not shown). Therefore, the difference in the heat stability and the Km value observed in the present patient's enzyme may come from some modification of the catalytic site of the enzyme molecule. The specific activity of the patient's enzyme determined by the ChE test kit and enzyme-linked immunosorbent assay (ELISA), which we recently developed using monoclonal antibody to ChE, was 6.3 IU/mg protein, while that of the enzyme in normal controls was 12.7. This may indicate that the present patient's enzyme has a lower affinity to the substrate than the normal enzyme.

The sequencing of the WBC DNA of the present patient showed one point mutation at position Gly 365, which is far downstream from anionic (Asp-70) and catalytic (Ser-198) regions. Although the whole coding region, exon 2–4, was sequenced, no abnormalities other than the point mutation were found. Whether or not the point mutation may cause some steric effects on the enzymic properties of the present patient's ChE is not clear at present. Further studies are needed to clarify the meaning of the point mutation in relation to the changes of the enzymic properties of the patient's ChE.

From the above-mentioned results, the enzyme of the present patient seems to be a new 'nearly silent' ChE variant and has the following properties. First, isozymic alteration (lacking bands II and III of ChE isozymes) was found in this patient. Secondly, the enzyme of this patient is more heat-labile than the typical enzyme. Thirdly, the Km value of this patient's enzyme for benzoylcholine was higher than that of the typical enzyme. Finally, a point mutation at nucleotide 1093 produces arginine (CGA) instead of glycine (GGA) at position 365.

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References


