Biochemical and Molecular Genetic Analyses on Placental Aromatase (P-450_{AROM}) Deficiency*

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Biochemical and molecular genetic studies were made on a case of placental aromatase (P-450_{AROM}) deficiency. Of the enzymes participating in the electron transport system of placental microsomes, only aromatase activity was decreased specifically in the patient, being less than 0.3% of the normal activity. Northern and Western blotting analyses showed that the transcription of the aromatase gene and the translation of its mRNA proceeded normally in the placenta of the patient. However, aromatase cDNA isolated from a placental cDNA library of the patient was found to have an insert of 87 base pairs, encoding 29 amino acids in frame with no termination codon. The insert was located at the splicing point between exon 6 and intron 6 of the normal aromatase gene, and the extra DNA fragment was the first part of intron 6, except that its initial GT was altered to GC. These findings indicated that in this patient with aromatase deficiency, splicing between exon 6 and intron 6 did not occur at the normal position because of a point mutation in its consensus sequence and was forwarded to GT in the next cryptic consensus sequence 87 base pairs downstream according to the canonical GT/AG rule, resulting in translation of an abnormal protein molecule with 29 extra amino acids. During the transient expression in COS-7 cells, the aromatase cDNA of the patient was found to produce a protein with a trace of activity. This is the first report of a genetic defect for aromatase deficiency.

Liver (3), hair follicles (4), adipose tissue (5), and brain (6). These findings suggest that estrogen produced by this enzyme has physiological functions not only as a sex steroid hormone but also in growth or differentiation. Furthermore, it seems likely that few, if any, cases of deficiency of aromatase, which catalyzes the syntheses of such an essential estrogen, will be found. In fact, there are very few reports of cases of deficiencies of aromatase and estrogen receptor although lack of androgen receptor has been often reported and is known to cause the testicular feminization syndrome (7, 8).

In 1978, Mango et al. (9) reported the first case of primigravida showing low urinary estrogen excretion and demonstrated lack of placental aromatase activity by in vitro assays. Recently, Shozu et al. (10) reported a case of placental aromatase deficiency studied by biochemical procedures. However, there were obvious differences in clinical manifestations in the two cases, and in the latter case, maternal and fetal virilization and then infant female pseudhermaphroditism were observed.

In this paper we describe further biochemical analyses and molecular genetic studies of the case of placental aromatase deficiency reported by Shozu et al. (10). It has now been shown that placental aromatase is expressed only in parts of fetal origin and that this placental aromatase deficiency was caused by the expression of an abnormal aromatase protein molecule caused by a genetic defect in the fetus.

EXPERIMENTAL PROCEDURES

Placental Preparation—The placental tissues obtained at term from the primigravida with disease and healthy pregnant women as controls were stored at -80°C for preparation of the microsomal fraction or extraction of the poly(A)+ RNA fraction. All materials were obtained with consent from the donors.

Assay of Aromatase Activity—Microsomal fractions were prepared from placental homogenates by successive centrifugations (11). Aromatase activity was determined by two assay methods. One was an HPLC assay of reaction products (12). The other assay was a more sensitive method with [19,4-C]androst-4-ene-3,17-dione as substrate (13).

Northern Blot Analysis—The RNA fraction was obtained from the placenta by the method of Chirgwin et al. (14). Poly(A)+ RNA isolated from the RNA fraction by oligo(dT)-cellulose chromatography was separated by electrophoresis in 1% agarose gel and transferred to a nylon membrane (15). Full-length human placental aromatase cDNA (HA-24) prepared previously (16) was labeled with [α-32P]dCTP by the random primer labeling method (17). Then the membrane was hybridized with this 32P-labeled aromatase cDNA and autoradiographed after washing (15).

Isolation and Characterization of Placental Aromatase cDNA from the Patient—The double-stranded cDNA was synthesized using 1 μg...
of placent al poly(A') RNA from the patient by the procedure of
Gubler and Hoffman (18). A placental cDNA library of the patient
was constructed from the cDNA using ZAP II arms (Stratagene) as
described (15), and the aromatase cDNA of the patient was screened
with normal, 32P-labeled full-length aromatase cDNA (ha-24) as a
probe. Out of the 3 x 10^6 clones from the patient and five
controls, 31 positive clones were isolated, and cDNA inserts were
mapped with various restriction endonuclease.

The microsomal enzyme activities were measured three times in
the patient and five times in the five controls. The other two were
missense mutations from CGC to TGC and CAC to TAC at Val-218
and Gly-219, respectively, which don’t cause alterations of the encoded
amino acids (25, 26).

RESULTS

Aromatase Activity in Placental Microsomes of the Patient—
Aromatase is a terminal component of the microsomal elec-
tron transport system in placenta. To determine whether its
activity was lowered specifically in placental microsomes of
the patient, we measured the activities of other components
of the microsomal electron transport system, NADPH-cyto-
chrome P-450 reductase and NADH-cytochrome
reductase, which supplies electrons from
NADPH to aromatase, was found to be increased about 29%
in the patient. These results suggested that the loss of ara-
matase activity in the placenta of this patient was caused by
inactivation of the enzyme or decrease or absence of its
biosynthesis.

Western and Northern Blotting Analyses of Aromatase De-
ciency—The above possibilities were examined by Western
blotting analysis with anti-aromatase antibody. As shown in
Table I, microsomes from both the patient and a control gave
one major protein band of 51-53 kDa. Therefore, a protein
of about the size of aromatase which reacted with its antibody
seemed to be present in microsomes of the patient’s placenta.

Moreover, immunostaining of the band of the patient’s aro-
matase was weaker than that of the control, when the same
amounts of microsomal protein were applied to the gel.

Next, poly(A') RNA fractions prepared from placentas of
the patient and controls were analyzed by Northern blotting.
As shown in Fig. 1B, in both RNA fractions, two bands of
about 3.2 and 2.8 kilobases hybridized with 32P-labeled ara-
matase cDNA (ha-24), consistent with previous observations
(16, 25-27). Thus, transcription of the patient’s aromatase
gene seemed to proceed normally.

Characterization of the Patient’s Placental Aromatase
cDNA—When 31 clones giving positive reactions with control
aromatase cDNA (ha-24) used as a probe were isolated from
a placental cDNA library of the patient, all of them were
mapped on the same restriction map, as shown in Fig. 2. Most
of the restriction sites on the map corresponded to those on
normal cDNA (16), but BstPI digestion generated one larger
restriction fragment. Clone pha-3, carrying the longest insert
of the patient’s aromatase cDNA, was subjected to detailed
mapping, and the DNA sequence of this 3,056-bp clone was
compared with that of the normal aromatase cDNA, ha-24
(16).

The results showed four insertions of 1 bp and a deletion
of 1 bp in the 3’-noncoding region and four point mutations
and an insertion of 87 bp in the coding region. The changes
in the 3’-noncoding region may not affect the translation
and protein conformation of aromatase and be explained by hu-
man genetic polymorphism, as several discrepancies of DNA
sequences have been found in the 3’-noncoding region of
normal aromatase cDNAs (16, 25-27). Of the point mutations
in the coding region, two were changes from GTA to GTG
and GGT to GGG at Val-80 and Gly-219, respectively, which
do not cause alterations of the encoded amino acids (25, 26).

Table I

<table>
<thead>
<tr>
<th>Microsomal enzyme activities in placentas of the patient and controls</th>
<th>Control</th>
<th>Patient</th>
</tr>
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<tbody>
<tr>
<td>Aromatase (pmol/min/mg protein)</td>
<td>53.6 ± 6.7</td>
<td>0.142 ± 0.011</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (mmol/min/mg protein)</td>
<td>0.035 ± 0.006</td>
<td>0.045 ± 0.003</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase (mmol/min/mg protein)</td>
<td>0.164 ± 0.023</td>
<td>0.123 ± 0.009</td>
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</table>
and AGC to AAC, which result in encoding of Cys-264 and Asn-496 instead of arginine and serine, respectively (25-27). However, these changes were also found in the placental aromatase cDNA of healthy women (25-27) and may be human genetic polymorphisms.

The insertion of 87 bp in the coding region of aromatase cDNA, however, seemed to be significant as a cause of this aromatase deficiency. As shown in Fig. 3A, this 87-bp DNA fragment is inserted into the codon of Val-248 in the normal aromatase gene, and this position is also the junction between exon 6 and intron 6. This insertion fragment was derived from the first 87 bp of intron 6 in the normal aromatase gene (28, 29), except that the consensus splicing donor sequence (GT) at the 5' terminus of intron 6 was mutated to GC. Consequently, this mutation may have prevented splicing at this position by the canonical GT/AG rule and shifted the splicing point to 88 bp downstream with a cryptic consensus donor sequence (AG/CTAACC), as shown schematically in Fig. 3B. Since the resulting 87-bp insertion sequence encodes exactly 29 amino acids and contains no stop codon in frame, the aromatase gene of the patient was translated as a larger protein molecule with extra amino acids (Fig. 3A).

All the aromatase cDNAs prepared from placenta of the patient produced similar fragments of larger size (421 bp) than the control (334 bp) on digestion with BstPI, and these were separated by agarose gel electrophoresis, as shown in part in Fig. 4A. Furthermore, when the placental mRNAs were reverse transcribed and amplified by polymerase chain reaction, only 360- and 273-bp DNA fragments were produced from placental mRNAs of the patient and the control, respectively, as expected from the primers used (Fig. 4B). Therefore, this 87-bp fragment was not an artifact produced during preparation of the cDNA library, and the aromatase gene in placenta of the patient was found to be transcribed as an 87-bp larger mRNA molecule.

**Expressions of Aromatase cDNAs of Placentas of the Patient and a Control in COS-7 Cells**—The aromatase cDNAs from the placentas of the patient and a control were inserted into a eukaryotic expression vector in the right and reverse directions and transfected into COS-7 cells as described under “Experimental Procedures.” The results shown in Fig. 5 indicate that control aromatase cDNA inserted in the right direction expressed significant activity (28.9 pmol/min/mg of protein) but that its insertion in the reverse direction resulted in little activity and that insertion of the patient’s aromatase cDNA resulted in only a trace of activity.

**DISCUSSION**

In this work we examined the cause of placental aromatase deficiency in a patient by biochemical and molecular genetic studies. We confirmed that the aromatase activity of the patient was less than 0.3% of that of controls whereas the activities of other enzymes involved in the microsomal electron transport system were normal. Furthermore, Western and Northern blotting analyses showed that this decrease of the aromatase activity was not caused by abnormal translation or transcription of placental aromatase in the patient. However, in Western blotting analysis, the patient’s aromatase was immunostained more weakly than controls. There are three possible explanations for this weak staining: One is that aromatase of the patient was unstable and was rapidly degraded by proteolytic cleavage. Another possibility is that it might have partially lost the antigen recognition sites by conformational change caused by its mutation. The third possibility is that the rate of synthesis of aromatase might be reduced in the patient. Similarly, the RNA fraction from the patient gave a broad smear of less than 3.2 kilobases. This finding, together with the weak staining of protein bands described above, might suggest partial decomposition of mRNA and enzyme protein during storage for more than 2 years at -80 °C.

The aromatase cDNA isolated from the patient’s placenta was found to differ from that of the control in several regions. Most of these differences were thought to be human polymorphisms. But one possible cause of the patient’s aromatase deficiency seemed to be a point mutation detected in the consensus splicing donor sequence of the gene, resulting in a downstream shift of the splicing point and translation of a larger aromatase molecule with 29 extra amino acids. However, the insertion of these extra amino acids is estimated to elevate the molecular mass of about 3.5 kDa, and the difference of the molecular mass between the aromatases of the patient and a control observed by Western blotting analysis was unlikely so much. Similar observations were also obtained from the transient expression of the aromatases of the patient and a control in COS-7 cells (data not shown). Although the explanation for this disagreement has not been obtained, aromatase is known to be a glycoprotein (30) with three consensus glycosylation sites in a molecule (16), and these observations might reflect the decreased glycosylation in the patient’s aromatase caused by the extra peptide.
Placental Aromatase (P-450AROM) Deficiency

A) Patient’s cDNA

<table>
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<tr>
<th>Normal gene</th>
<th>Patient’s cDNA</th>
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<tbody>
<tr>
<td>AAGTCCTGGCAAGATAACCTTTGGAAGATTGTAGTAC</td>
<td>AAGTCCTGGCAAGATAATACCTTTGGAAGATTGTAGTAC</td>
</tr>
<tr>
<td>KSVQVQILKFKKIKY</td>
<td>AAGTCCTGGCAAGATAATACCTTTGGAAGATTGTAGTAC</td>
</tr>
<tr>
<td>Exon 6</td>
<td>Intron 6</td>
</tr>
</tbody>
</table>

B) Normal gene

COS-7 cells were transfected with expression vectors, pCH110 carrying β-galactosidase cDNA and pSVL carrying aromatase cDNAs of the patient and a control, and then cultured for 60 h. The microsomal fractions were prepared from COS-7 cells, and their aromatase activities were assayed as described under “Experimental Procedures.” At the same time, the β-galactosidase activities in the cell lysates were assayed for the correction of the transfection efficiency. These transfection experiments were repeated four times, and the aromatase activities were expressed by mean values with error bars in the figure. Columns C (+) and C (−) indicate values for cells transfected with pSVL carrying the control aromatase cDNA in the right direction and the reverse direction, respectively. Column P (+) indicates the value for cells transfected with pSVL carrying the patient’s aromatase cDNA in the right direction.

This patient diagnosed as a case of placental aromatase deficiency is of some other medical interests. The placenta is known to be derived from tissues of both the mother and fetus and to supply a major part of the estrogen required by the mother instead of the ovary after 9 or 10 weeks of gestation. However, in this case, no vital problems were found during pregnancy and delivery in spite of supply of estrogen to the mother at low level, and so placental aromatase may function.
mainly to protect the mother and fetus from exposure of adrenal androgen. Furthermore, judging from the normal function mediated by estrogens in the ovary of the mother, this placental deficiency was attributable to a genetic defect of the fetal aromatase gene, indicating that the placenta expresses aromatase in only parts of fetal origin.

The parents are consanguineous in their pedigree (fifth degree). To establish that this deficiency is a hereditary disease, it is necessary to analyze the aromatase genes among the family of the patient. These analyses are now in progress in this laboratory, and preliminary data indicate that the genotypes of the fetus and the parents are homozygous and heterozygous, respectively, as regards this genetic mutation.\(^2\) This is the first report of placental aromatase deficiency depending on a genetic defect of the fetus.

Acknowledgments—We thank Dr. Osamu Gotoh, Department of Biochemistry, Saitama Cancer Center Research Institute, for computer analyses of the patient's aromatase and helpful discussion.

REFERENCES

9. Mango, D., Montemurro, A., Scirpa, P., Bompiani, A., and Men-