EXPERIMENTAL STUDY

Aromatase and breast cancer: W39R, an inactive protein

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Abstract

Background: Aromatase (CYP19) catalyzes the conversion of androgens into estrogens. It is in particular involved in development, reproduction and breast cancer. One of its polymorphisms, W39R localized in the N-terminal region of CYP19, significantly decreases breast cancer risk among Japanese women and was chosen for this study. In this work, we studied the structure–function relationships between W39R polymorphism and CYP19 enzyme activity.

Objective: To examine the kinetic properties of the mutant W39R recombinant protein in transfected human cells devoid of steroidogenic activity.

Methods: Expression vectors for the wild-type or the mutated R39 aromatase were transiently transfected into E293 human embryonal kidney cells. The conversions of androstenedione to estrone and of testosterone and nortestosterone to 17\beta-estradiol were assayed by RIA. Expression of recombinant cDNAs was analyzed by semi-quantitative RT-PCR and immunoblotting.

Results: W39R recombinant protein was devoid of aromatase activity whatever the substrate used. This absence of activity was not due to the lack of expression of the recombinant enzyme since the mRNA and protein were detected.

Conclusion: Our present in vitro study shows that the R39 mutant is unable to synthesize estrogens. This work provides a novel observation, being consistent with the fact that Japanese women with the variant allele arg have significantly lower risk of developing a breast tumor.

Introduction

The human aromatase (CYP19), in concert with the ubiquitous flavoprotein NADPH-cytochrome P450 reductase, is a key enzymatic complex that catalyzes the conversion of androgens into estrogens. It is now well established that estrogens are involved in various physiological functions but that they also promote the growth of breast cancer cells (1, 2). This enzymatic complex belongs to the class of mammalian endoplasmic reticulum cytochrome P450, anchored with the N-terminal membrane domain extending from A20 to W39 (3, 4) (Throughout, the conventional single letter abbreviations for amino acids are used: A, alanine; C, cysteine; E, glutamic acid; Q, glutamine; R, arginine; W, tryptophan.) Moreover, Amarneh and Simpson (5) have reported that the deletion of the first 53 residues of the human aromatase protein abolishes its activity. In the last decade, several point mutations of CYP19 have been associated with a loss of the aromatase function (for review see (6)). On the other hand, one polymorphism, R264C, in the coding area of the gene was observed (7) but without affecting activity and without being correlated with breast cancer (8). However, recently, Miyoshi et al. (9) have identified two new polymorphisms. The first one, also described by Kristensen et al. (10), is a tetranucleotide repeat in intron 4 which could be associated with breast cancer susceptibility. The second polymorphism, chosen for our study, is localized in the N-terminal region of the CYP19 gene among Japanese women. Miyoshi et al. (9) observed that this single homozygous or heterozygous amino acid substitution from tryptophan to arginine at codon 39 (W39R), significantly decreased the breast cancer risk. The same authors speculated that this polymorphism may reduce the activity of CYP19 enzyme, resulting in the lower production of estrogens. In this work, we tested this hypothesis, i.e. the relationship between W39R polymorphism and CYP19 enzyme activity. Therefore in the present work, the W39R mutant was generated in order to determine its potential impact on the structure–function relationships of aromatase. A mammalian cell expression plasmid, pCMV-HA, was mutated or not, and transiently expressed in E293 human embryonic kidney cells in order to determine the kinetic properties of the recombinant proteins. E293 cells were used because they are an appropriate
cell line to study recombinant steroidogenic enzyme (11) since they are naturally devoid of aromatase, 5α-reductase and 17β-dehydrogenase enzyme activities (12). In order to investigate if this polymorphism at codon 39 affects the expression of aromatase or its cellular localization, we have performed a semi-quantitative RT-PCR analysis of mRNA from transfected E293 cells and a CYP19 protein analysis in microsomal fractions by Western blot. According to the aromatase sequence, one possible structural modification induced by R39 could result from its ionic interaction with the i+3 residue, E42, leading to a modification of the hinge region (13). In order to check this hypothesis, we have performed the double mutation W39R-E42A. In such a protein, alanine will be unable to form an electrostatic interaction with R39 and, thus, could restore the aromatase activity.

In this study, we have shown that the W39R recombinant protein is devoid of aromatase activity whatever the substrate used and that the double mutation W39R-E42A is unable to restore the activity.

Materials and methods

pCMV-human aromatase cDNA construction (pCMV-HA)

A 2920 bp fragment of human aromatase cDNA EcoR1-EcoR1 was subcloned into the pCMV EcoRI site (14). Orientation was then checked by sequencing. The pCMV-HA was purified from the transformed JM109 bacterial strain by using the Qiagen Plasmid Mega kit (Qiagen, Courtaboeuf, France). The length, the concentration and the purity of the plasmid-cDNA construction were verified by 1% agarose electrophoresis and ethidium bromide staining.

Site-directed mutagenesis

The introduction of specific base changes in the aromatase cDNA was accomplished by using the Quickchange site directed mutagenesis kit from Stratagene (France). Oligonucleotide primers used for the mutagenesis experiments were synthesized at Eurobio (Les Ulis, France). Sequences of oligonucleotides were 5'-CCT TTT TCT CTG GGT GAG GAA TTA TG-3' and 5'-CAT AAT TCC TCA CCA AGA GAA AAA GG-3' for W39R and 5'-CCT GGT GAG GAA TTA TGC GGG CAC ATC C-3' and 5'-GGA TGT GCC CGC ATA ATT CCT CAC CAA G-3' for W39R-E42A. All mutated cDNAs were sequenced by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction from Biosystems (Warrington, UK) to confirm that there were no other mutations except those designed.

E293 cell culture and transient transfection assay

The stable reductase-transfected human embryonal kidney cell line E293 (a gift from Dr Van Luu-The, CHUL, Quebec, Canada) was maintained at 37°C (5% CO₂, 95% air) in EMEM phenol red-free medium containing 2 mmol/l glucose, 100 U/ml antibiotics (penicillin, streptomycin and Fungizone), 10% heat-inactivated fetal calf serum (supreme serum), and 1% non-essential amino acids (BioWhittaker, Gagny, France). Forty-eight hours prior to transfection, cells were grown up to 50% confluence on 24-well cell culture plates, washed with medium and supplemented with 500 μl EMEM-free serum. Cell transfection was as reported by Bousis et al. (15). Briefly, 2 μg plasmid and 54 nmol polyethylenimine (PEI) aqueous solution (pH 7) were diluted separately with 50 μl NaCl (150 mmol/l), incubated 10 min at room temperature and mixed together. After a further 10 min incubation, PEI–DNA mix was added to each well. Then, after 3–4 h incubation at 37°C, cells were supplemented with 500 μl EMEM-10% serum. Aromatase activity ‘in cell’ was evaluated 48 h post-transfection. Transfection efficiency was evaluated by ELISA of the wild-type and several mutated proteins without showing differences in recombinant aromatase expressions (16).

Aromatase activity ‘in cell’

The conversion of androstenedione to estrone and of testosterone and nortestosterone to 17β-estradiol was measured in E293 cell supernatants byRIA according to Auvray et al. (14). Briefly, transfected cells were washed with EMEM and incubated with increasing concentrations of androstenedione (0–800 nmol/l) or 200 nmol/l testosterone or 20 nmol/l nortestosterone for 45 min at 37°C under a 5% CO₂ atmosphere. After incubation, cells were placed for 5 min on ice and medium was extracted by 10 volumes diethyl ether. Anti-estrone and anti-estradiol rabbit antibodies (Ref PF1247 and Ref 1287, P.A.R.I.S., Compiègne, France) were prepared according to the manufacturer’s instruction. The extraction efficiency was 80±5% and the sensitivity of this RIA was 10 pg/ml. Intra- and inter-assay coefficients of variations were approximately 3 and 7% respectively. Results are the mean of at least triplicate experiments±S.D.

RNA extraction and semi-quantitative RT-PCR

Total cellular RNA was isolated from E293 cells, alone or transfected with pCMV-HA (used as positive control) or pCMV-R39 and pCMV-R39-A42, using the TRIzol reagent (Gibco BRL, Cergy Pontoise, France). Total RNA (3 μg) was treated for 10 min at 37°C with 2 units RNase-free DNase (Promega, Charbonnières, France) and reverse transcribed with 200U I M-MLV
RT (Promega) and 0.2 μg oligo dT primer (12–18 bases), in RT buffer (Promega) containing 500 μmol/l dNTPs and 24 IU Rnasin for 1 h at 37 °C, in a total volume of 10 μl. PCR amplification of 5 μl of reversed transcribed sample was carried out in a final volume of 50 μl PCR buffer supplemented with 200 μmol/l dNTP, 50 pmol of each primer, 1.5 mmol/l MgCl₂ and 1.5 IU Taq DNA polymerase from Promega. Amplification was performed on a Stratagene Robocycler through 30 PCR cycles with the profile: 94 °C (1 min), 60 °C (1 min) and 72 °C (2 min) and an extra cycle of 72 °C (5 min). The sequences of sense and antisense primers were 5'-AAA GGA AAT CCA GAC TGTT ATT GGT GAG-3' (exon IX, 1116 – 1143) and 5'-GTA TCT TCT GTA TGC TCT CAA CAC ACT GTG-3' (exon X, 1511 – 1540) respectively. The resulting PCR product of 424 bp was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

The internal control (GAPDH) was amplified for 23 cycles, in the conditions described above. The sequences of sense and antisense primers used to detect the PCR identification of the GAPDH mRNAs were 5'-TGA ACG GGA AGC TCA CTG GCA TGG CTT T-3' (731 – 758) and 5'-GTG TGG TGG GGG ACT GAG TGT GGC AGG GAC-3' (1132 – 1162) respectively. The resulting PCR product of 431 bp was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Twelve microliter aliquots of the PCR reaction were size separated on 1.5% agarose equilibrated in 0.04 mol/l Tris–acetate, 0.001 mol/l EDTA. Gels were stained with ethidium bromide (1 mg/ml), photographed using Polaroid film under UV light and analyzed using an AGFA Snap-Scan 1200® Scanner, Adobe Photoshop (version 4) software and the NIH image computer program (http://rsb.info.nih.gov/nih-image).

Subcellular fractionation and Western analysis

Transfected cells from confluent T75 tissue culture flasks were scraped into 20 mmol/l Tris–HCl (pH 7.5), 20% glycerol, 15 mmol/l β-mercapto-ethanol, 50 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l dithiothreitol containing 4 mg/ml polymethylsulfonyl fluoride, and a mixture of antiproteases (benzamidine, aprotinin, leupeptin, pepstatin A and antipain at 1 μg/ml each). Then cell lysates were centrifuged at 20 000 g for 15 min at 4 °C and the supernatant was transferred to a fresh tube and centrifuged at 100 000 g for 1 h at 4 °C to obtain the microsomal fraction. Each fraction was mixed with an equal volume of twofold concentrated Laemmli sample buffer (17) and denatured by heating in boiling water for 2 min. Proteins (100 μg) were resolved on a 10% SDS-PAGE using Tris base (0.25 mol/l), glycine (1.92 mol/l), SDS (1%) running buffer and then electrotransferred onto PVDF membrane (Appligen, Illkirch, France). Proteins were detected by immunoblotting with the P450 aromatase antiserum (1:1000) produced in our laboratory (18), followed by an incubation with horseradish peroxidase-linked anti-rabbit IgG at 1:3000 (BioRad, Ivry sur Seine, France) and addition of Sigma Fast 3,3'-diaminobenzidine (Tablet Sets; Sigma, Saint Quentin Fallavier, France).

Results

The aim of our study was to examine the enzyme kinetic properties of the mutants W39R and W39R-E42A in transfected E293 cells and to determine the importance of these residues in protein activity.

Figure 1 Aromatase activity and mutations. (A) Enzymatic activity of pCMV-HA (upper curve), and of R39 and R39-A42 (lower two curves) transfected in E293 cells, incubated for 45 min with increasing doses of androstenedione (0–400 nmol/l). Results are expressed as pg estrone (E1) formed/min per mg protein and are the mean of triplicate values ± S.E.M. Insert: apparent Kₘ value determination of the wild-type enzyme by a Lineweaver–Burk plot (results are representative of one from three experiments showing similar profiles). (B, C) Enzymatic activity of pCMV, pCMV-HA, R39 and R39-A42 transfected E293 cells, incubated for 45 min with 200 nmol/l testosterone (B) or nortestosterone (C). Results are expressed as pg estradiol (E2) formed/min per mg protein and are the mean of triplicate values ± S.E.M.
Cells were transfected with either the wild-type plasmid (pCMV-HA) or with the mutated plasmids (W39R and W39R-E42A) or with pCMV plasmid, used as control. Forty-eight hours after transfection, cells were incubated for 45 min with increasing concentrations of androstenedione (0-800 nmol/l) or with 200 nmol/l testosterone or nortestosterone. As illustrated in Fig. 1, the mutation of W39R and the double mutation induced a total activity loss with androstenedione (Fig. 1A). With 200 nmol/l testosterone as substrate, both mutations decreased strongly the aromatase activity (activities below 6 and 3% relative to the wild-type for R39 and R39-A42 respectively) (Fig. 1B). In the same manner, a lower activity with 200 nmol/l nortestosterone was observed for the R39 and R39-A42 (41 and 27% of the wild-type respectively) (Fig. 1C). Moreover, Fig. 1A shows a typical result of kinetic analysis of the wild-type aromatase. From these results, the apparent $K_m$ value of the wild-type aromatase expressed in E293 was 76.6±14.1 nmol/l for androstenedione. To study the effects of the point mutation T/A on the CYP19 expression, aromatase mRNA from the E293 transfected cells was analyzed by RT-PCR. As illustrated in Fig. 2A, the corresponding 424 bp RT-PCR product of aromatase mRNA was absent in untransfected E293 cells and present in E293 cells transfected with pCMV-HA, pCMV-HA-W39R and pCMV-HA-W39R-E42A, and in granulosa cells used as a positive control. As illustrated in Fig. 2C, the semi-quantitative RT-PCR analysis of CYP19 mRNA levels, by using GAPDH mRNA as standard (Fig. 2B), showed a 34% decrease in mRNA coding for R39 and a 56% decrease in mRNA coding for R39-A42 in comparison with mRNA coding for the wild-type enzyme. The Western blot analysis of the wild-type protein and the recombinant R39 and R39-A42 proteins on subcellular fractions, illustrated in Fig. 3, reveals that the proteins are seen only in the microsomal fraction and not in the cytosol.

**Discussion**

Recent studies of 204 Japanese women have shown that the single amino acid substitution in aromatase of tryptophan by arginine at codon 39 was significantly associated with a decrease in breast cancer risk (9). Those authors speculated that this polymorphism may reduce the activity of the CYP19 enzyme, consequently resulting in a lower production of estrogens. We tested this hypothesis.

In our study, aromatase activity was measured in cell and allowed us to determine an apparent $K_m$ value for the wild-type protein for androstenedione (76 nmol/l) in the same range as values previously reported by others (19). However, different estimates of apparent $K_m$ and $V_m$ values can be obtained from microsomal and intact cell methods. According to Zhou et al. (20), similar $K_m$ values were obtained with both approaches but $V_m$ values were lower by using microsomes. The authors suggested instability of the microsomal preparation. Moreover, Kadohama et al. (21) have suggested that the cytochrome P450 reductase/aromatase ratio may be involved in differences in the two protocols. In such a way, we have used stable reductase transfected E293 cells. Our results show

![Figure 2](image-url)
that mutation of residue 39 abolishes or reduces considerably the protein activity, whatever the substrate tested, and consequently suggest that this position appears to be important for aromatase activity. Slight differences according to the substrates is in agreement with our previous results. According to multiple sequence analysis, W39 is well conserved in most P450 aromatase enzymes (human (22), horse (23), pig (24) and rabbit (25)) except in rat (26), as well as in other P450s involved in steroidogenesis (human P450scc (27), steroid 17α-hydroxylase/lyase (CYP17A) (28)) or other P450s such as P450 2E1 (29) and P450 2C2 (30). Tryptophan is a hydrophobic amino acid, in contrast to arginine, which is a positively charged residue, and consequently could be involved in an ionic interaction with E42 according to structural models (31). Moreover, several ionic interactions between arginine and glutamic acid have been described in P450 cytochromes which could favor secondary structures of the protein. Carani et al. (32) have identified a homozygous mutation, R365Q on the CYP19 gene, which is associated with a very low activity of the enzyme. According to the computer model of Graham-Lorence et al. (31), R365 may form a salt-bridge with E362 and may play a major role in the stabilization of the general structure of the aromatase protein. Moreover, glutamic acid is likely to play a role in aromatase structure and function by affecting enzyme activity and modulating substrate binding affinities (33). We speculated that this W39R mutation could destabilize the structure of aromatase by forming a salt-bridge with E42, leading to its inactivity. Thus, to check this possibility, we analyzed the double-mutant pCMV-HA-R39-A42. In such a case, alanine will be unable to form an electrostatic interaction with R39 and, thus, could restore the aromatase activity. In the present study, the double mutant R39-A42 was devoid of activity whatever the substrate tested. This lack of activity may result from a lower expression rate of the double mutant, since corresponding mRNA levels were 56% lower than these of the wild-type. Another hypothesis to explain this lack of activity may be that the mutation W39R could prevent the interaction between the cytochrome P450 aromatase and the reductase. For instance, Lee-Robichaud et al. (34) have described the importance of arginine residues which are involved in protein–protein interaction between cytochrome b5 and human CYP17 and consequently affect the lyase activity of the enzyme required for androgen formation. Moreover, Muller-Enoch & Gruler (35) have recently reported that the N-terminal binding domain of cytochrome P450 enzymes determines the complexation process of the binary P450 reductase system. We also hypothesized that R39 may form a salt-bridge with another glutamic acid residue located far away in the sequence, as has been described in CYP19 between R9 and E296 (36), and consequently could prevent the interaction between P450 aromatase and NADPH reductase. Further studies will be necessary to explore this hypothesis.

Genetic polymorphism of the gene CYP19 may be involved in mechanisms other than in the protein structure, such as mRNA stability, modulation of transcription or post-translational regulation of expression. Recently, Kristensen et al. (37) have observed that the C/T polymorphism in exon 10 of the CYP19 gene is associated with variations in mRNA levels. They reported that, according to the computer model of Walter et al. (38), the C/T change may influence mRNA stability. In addition, it was shown that the introduction of a polar residue in the hydrophobic anchor domain of cytochromes P450 2E1 and 2B4 changes their cellular location from membrane to cytosol (39). Because a point mutation could modify the expression of the protein (37), or its cell trafficking (39), we have further analyzed this mutant at the RNA and subcellular levels. To study the effects of the single nucleotide mutation T/A on CYP19 gene expression, aromatase mRNA from the E293 transfected cells were analyzed by RT-PCR. The results show that the corresponding product of aromatase mRNA was detected in E293 cells transfected with R39. However, the semi-quantitative RT-PCR analysis of mRNA levels have shown that they were significantly affected by the mutation. In fact, a 34% decrease in HA-R39 mRNA levels was observed in comparison with HA-W39 mRNA levels. The new mRNA structures may affect their half-lives but this hypothesis needs further investigation.

In order to determine whether the enzyme localization was modified by the W39R mutation, an immunoblot analysis was performed on subcellular fractions. As illustrated in Fig. 3, aromatase was detected only in the
microsomal fraction and not in the cytosol as was observed for P450 2E1 and 2B4 (39). However, the mutants are completely inactive whatever the substrate used. This inactivity may partly result from a decrease in the protein expression but also from a structural modification of the enzyme.

In conclusion, our present study provides a novel observation, being consistent with the fact that Japanese women with the variant allele arg have a significantly lower risk than the non-carrier of a decrease in the protein expression but also from a structural modification of the enzyme.

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