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P450arom Gene Expression in Peripheral Blood Lymphocytes: Identification of a Cryptic Splice Site for Exon-1 After Epstein–Barr Virus Transformation

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The human aromatase gene (P450arom) is widely expressed, albeit in a tissue-specific manner. In the present study, we measured aromatase activity and investigated the transcribed and translated products of the P450arom gene before and after Epstein–Barr virus (EBV) transformation in peripheral blood lymphocytes (PBLs) from normal individuals. Aromatase activity was determined by [^{3}H]-Δ_4-androstenedione (A) to [^{3}H]-estrone (E1) conversion. Cellular total RNA and protein lysates were subjected to RT-PCR and Western analysis, respectively. Rapid amplification of cDNA ends (RACE) was used for the detection of novel 5′-untranslated ends of the P450arom mRNA, which were subsequently sequenced and compared to the known transcripts of this gene. In untransformed PBLs, two known variants of exon 1 of the P450arom gene were expressed, corresponding to promoters PI.3 and PII, or 1c and 1d, respectively. In EBV-transformed PBLs, a cryptic splice site was revealed and a new 5′-untranslated product was found. RNase protection assay confirmed that this splice variant is not a RACE artifact. The 53 K P450arom protein was detectable in PBLs both before and after EBV transformation. We conclude that (i) the P450arom mRNA is present in human PBLs and (ii) EBV transformation of the latter leads to novel alternative splicing of the 5′ end of this gene. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Human aromatase, also called cytochrome P450arom or estrogen synthetase, is the product of the CYP 19 gene, a member of the cytochrome P450 gene superfamily. It is associated with the flavoprotein NADPH-P450 reductase and is responsible for carbon-19 (C19) to C18 steroid conversion in both gonadal and extragonadal tissues [1]. P450arom mRNA is expressed in the placenta, ovary, testis, brain, skin fibroblasts, adipocytes and in the fetal liver and intestine [2]. The wide distribution of P450arom mRNA suggests that local estrogen production may be involved in autocrine and/or paracrine activity in a number of tissues; this is supported by the significant role of estrogens in a wide array of cellular functions, including growth and proliferation [1, 3].

Tissue-specific expression of the P450arom gene is mediated by alternative splicing of exon 1 and part of exon 2 driven by at least five major promoters located in the 5′-untranslated end of the gene [1–11]. Sequences with both silencing and enhancing function have been shown to be present in these regions and a variable pattern of P450arom promoter usage has been demonstrated in disorders with inappropriate aromatase activity, including neoplasms [1]. Accordingly, aromatase activity has been demonstrated in human endometrial cancer but not in the corresponding normal tissue [4] and in several other

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tumors [5, 6], including breast and adrenocortical carcinomas [7, 8].

Aromatase expression with alternative promoter use has been found in human myeloid leukemia cells [12], however, the pattern of expression of the P450arom gene in normal peripheral blood lymphocytes (PBLs) has not been studied. Berstein *et al.* suggested the presence of 'pseudoaromatase' in PBLs, i.e. apparent aromatase activity in the absence of P450arom gene transcripts [13]. PBLs provide a tissue which is readily accessible for the investigation of aromatase gene expression in estrogen-dependent cancer or other conditions with evidence for peripheral aromatization; transformation of PBLs by Epstein–Barr virus (EBV) is a common way of establishing permanent lymphoblastoid cell lines. In the present study, the expression of P450arom gene in PBLs from normal subjects before and after EBV transformation was investigated.

MATERIALS AND METHODS

Subjects and cell lines

Five normal controls (2 males and 3 females) were studied. 20 ml of blood were collected from all subjects and peripheral blood lymphocytes (PBLs) were extracted by the Ficoll method as previously described [14]. Approximately 5×10^6 of these cells were immortalized by transformation with EBV by an indirect method employing a permanently infected marmoset cell line (B-95), as previously described [14]; the remaining cells were frozen in liquid nitrogen and stored at −70°C, or used directly for RNA extraction prior to transformation. Transformed cells were cultured in RPMI medium supplemented with 1% penicillin/streptomycin, 1% fungizone, 1% L-glutamine and 10% fetal bovine serum (GIBCO-BRL Life Technologies, Gaithersburg, MD).

Aromatase activity

The aromatase activity of three cell lines before and after EBV transformation was measured by Δ_4-androstenedione (A)-to-E1 conversion and expressed in fmol estrogen per ml of protein per h (fmol/mg/h), as previously described [13]. The assays were performed twice and the mean was calculated. Briefly, cell cultures were washed with Hank's solution before addition of 1 ml of assay medium containing approximately 0.3 μCi of 1β-[^{3}H]-A and unlabeled substrate. The culture plates were then placed in an incubator (5% CO_2) at 37°C. After 2 h, the medium was transferred to a test tube and 2 ml chloroform were added. The unconverted substrate and steroid products were extracted into the organic phase. An aliquot of 0.7 ml of the aqueous phase was treated with 2.5% activated, dextran-coated charcoal suspension to remove residual steroids. Tritiated water (^3H_2O) formed during the aromatization reaction was measured by counting the radioactivity in the supernatant.

Western analysis

Western blot analysis was performed with proteins extracted from PBLs before and after EBV transformation, as previously described [15, 16]. Protein content of each sample was determined by BCA Protein Assay Reagent (PIERCE, Rockford, IL); bovine serum albumin (BSA) was used as standard. Proteins lysates (100 μg) were resolved by electrophoresis on an 8% SDS-PAGE, transferred to nitrocellulose membrane (Novex, San Diego, CA) and incubated with rabbit polyclonal antiserum, which was raised against human placental aromatase, purified and characterized by Bellino *et al.* [17]. This antibody recognizes a single protein band with M_r 53,000, the molecular weight of P450arom. Proteins were detected using the ECL detection system (Amersham Little Chalfon, Buckingamshire, England).

DNA extraction, RNA isolation, cDNA cloning and RNase protection assay

Isolation of total RNA was performed by the single-step liquid phase separation (TRI Reagent Kit, Molecular Research Center, Cincinnati, OH) and cDNA synthesis was accomplished with the antisense primer 24 [9] and the reverse transcriptase-PCR kit (Boehringer-Mannheim, Mannheim, Germany), as previously described [15, 16]. The sense primers 1a, 1b, 1c, 1d and the antisense primer 2d (sequences listed in Ref. [3]) were used for the subsequent PCR amplification of the cDNA synthesized from PBL and EBV-transformed cell lines of normal subjects as previously described [3].

Cloning of the 5′ termini of P450arom transcripts expressed in PBLs was performed using the rapid amplification of cDNA ends (RACE) procedure, according to the Marathon™ cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). PCR-amplified cDNA fragments were ligated into the PCR™-II vector using the TA-cloning kit (Invitrogen Corporation, San Diego, CA) and subsequently sequenced with the use of the ABI PRISM dye terminator reaction kit (Perkin-Elmer, Norwalk, CT) and primers shown in Table 1.

The RNase protection assay was performed using total RNA extracted from EBVtLs and a probe transcribed and labelled *in vitro* from a plasmid (TA-cloning method, Invitrogen Corporation, San Diego, CA) that harbored the most proximal part of exon 2 up to the end of exon 1c of the P450arom gene (as per Ref. [3]). The products of the reaction were run on a 6% polyacrylamide gel (Promega Corp, Madison, MI) which was dried and used for autoradiography.

Table 1. Primers used in sequencing of P450arom 5′-end

Primer sequences, exon 1d	DNA location
5′-AAA ACC ATC TTG TGT TCC TT-3′	113 (antisense) (Ref. [20])
5′-AAT GTA TCG GGT TCA GCA TT-3′	136 (antisense) (Ref. [20])

DNA sequence location is given relative to the transcriptional initiation site.

Table 2. Aromatase activity in human lymphocytes

	Peripheral lymphocytes (untransformed)	Following transformation by EBV
Female cell line 1,	<2 fmol/mg/h	<2 fmol/mg/h
Male cell line 2,	<2 fmol/mg/h	2.96 fmol/mg/h
Female cell line 3,	<2 fmol/mg/h	2.9 fmol/mg/h

RESULTS

Aromatase activity

Background aromatase activity in our assay was 1–2 fmol/mg/h; this was not exceeded by any of the nontransformed lymphocytes (Table 2), whereas in two of the cell lines (established from a male and a female, respectively), activity was just above the detection limit following transformation.

RT-PCR analysis and Western blots

RT-PCR analysis using primers from the known transcripts of the P450arom gene demonstrated that the proximal promoters 1c and 1d [3] or PI.3 and PII [1] were expressed in the cell lines established from normal individuals before and after EBV transformation; promoters a and b or PI.1 and PI.4 [1, 3, 7] were not present in either type of cell (Fig. 1).

A 53 K immunoreactive band corresponding to the P450arom molecular weight was detected in PBLs both prior to and after transformation, although the relative amount of P450arom protein was increased in immortalized cells (data not shown).

Identification of novel P450arom gene transcripts

RACE was used for the identification of the P450arom transcripts in PBLs before and after transformation with EBV. The cloned cDNA pieces were sequenced and compared to the known 5′-variants of the gene [2–4, 6, 7, 18–20]. Exons PI.3 and PII (or 1c and 1d), respectively, were present; in addition, however, to these known sequences of P450arom transcripts, a novel 5′-untranslated product was ident-

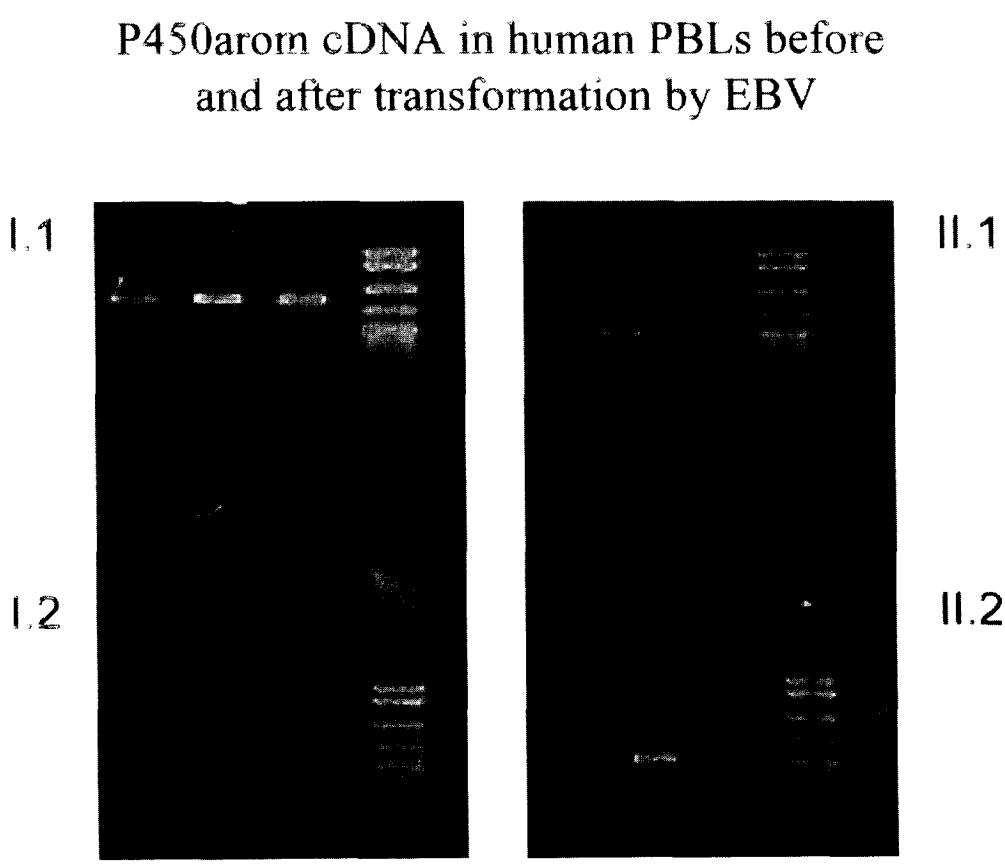
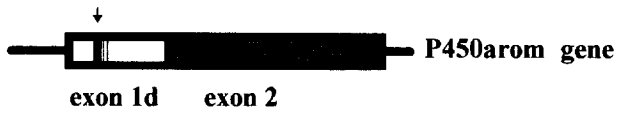


Fig. 1. Transcripts of the P450arom gene in human PBLs before and after transformation by EBV; amplification with primers from the known exon 1 for this gene. Exon 1c[3] before (I.1) and after (I.2) transformation; the size is 374 bp. Exon 1d[3] before (II.1) and after (II.2) transformation; the size is 248 bp.



Exons 1d and 2:

5'-tactgtacaGCACCCTCTGAAG**CAACAGGAGCTATAGATGACCTT**
TTAGGGGATTCTGTAATTTTTCTGTCCCTTTGATTTCCACAGG
ACTCTAAATTGCCCCCTCTGAGGTCAAGGAACACAAGATGGTTTT
GGAAATGCTGAACCCGATACATTATAACATCACCAGCATCGTGCC
TGAAGCCATGCCTGCTGCCACCATGCCAGTCCTGCTCCTCACTGGC
CTTTTTCTCTTGGTGTGGAATTATGAGGGCACATCCTCAATACCAG
gtaagtca-3'

Fig. 2. Diagram and nucleotide sequence of exons 1d and 2 of the aromatase P450 gene. Exon 2 is underlined and the sequence of the new exon is indicated in bold letters; untranscribed sequences are indicated by the straight lines in the diagram and lower case letters in the sequence. The arrow points to the new cryptic splice site in use by the P450arom gene in human EBV-transformed lymphocytes.

ified. Its sequence unravelled a new cryptic splice site located in the 5'-end of exon 1d (Fig. 2). RNase protection assay confirmed the initiation of transcription at this new site (data not shown).

DISCUSSION

P450arom transcripts are products of a complex process of alternative splicing of the 5'-end of this gene and are present in several different tissues [1]. In a human myeloid leukemia cell line P450arom gene splice variants are different from the ones previously described and that of the present report [12]. Aromatase activity was previously reported in human PBLs with absence of the P450arom mRNA [13]. However, repeated measurements in human non-transformed lymphocytes by our laboratory has shown very little aromatase activity in this tissue (this study, and Brodie *et al.* unpublished data). The present investigation confirmed the presence of P450arom mRNA and protein in these cells and demonstrated that the proximal promoters c and d (PI.3 and PII) [1, 3] were utilized for this gene's expression. The variable results on lymphocytic aromatase activity (in nontransformed cells) may be due to differences in culture conditions [1] and other factors, such as the performance of the assay in recently extracted [13] vs previously frozen cells (this study). Interestingly, there does not appear to be a gender-dependent difference in aromatase expression in human lymphocytes, but this has to be confirmed by additional studies.

Although aromatase activity in PBLs is significantly lower than that of other tissues, the ability of these cells to produce estrogen is of particular significance, since estrogen appears to participate in the development of the immune system [21, 22]. In breast cancer, a condition often associated with increased aromatase activity in the neoplastic tissue [23], the response of the peripheral white cells to mitogens decreases as the tumor load increases [24]. Also, interleukin-6 (IL-6), a potent inflammatory cytokine, induces P450arom gene expression [25, 26] in adipose tissue, which is responsible for a large proportion of extragonadal aromatization. Several other transcription factors, with a *bona fide* role in immune system development and function, appear to influence P450arom gene expression in various tissues [1, 12, 19].

Estrogens produced by PBLs may not only have an auto- or paracrine function. It is possible that under certain circumstances, such as those characterized by increased activity of the enzyme or the presence of excessive substrate, lymphocyte-derived estrogens may have an endocrine effect. Thus, in the syndrome of familial aromatase excess, increased estradiol and estrone levels and clinically significant feminization are due to excessive and inappropriate P450arom gene expression by various tissues, including PBLs [27]. Furthermore, increased androgen levels due to androgen-producing tumors and/or precocious puberty may lead to extragonadal aromatization, gynecomastia and/or skeletal age advancement [28–30].

In vitro expression of the P450arom gene is dependent on culture conditions and other factors [1, 31]. Transformation of human PBLs by EBV is one of the most commonly used techniques for immortalization of cells. The present study shows that human EBV-

transformed cells express P450arom, being, hence, appropriate for the study of P450arom gene expression in human disorders. In addition, aromatase activity is enhanced in these cells by transformation, which is a phenomenon observed in a number of other cell lines and for a variety of genes [32–34]. Our laboratory has published similar findings in PBL and EBV-tL cell lines expressing the glucocorticoid receptor gene established from patients with glucocorticoid resistance and normal controls [15]. The mechanism behind the induction of expression of several genes by EBV has not been fully elucidated but interaction with the Notch signalling system [32], regulation of the retinoblastoma protein and, thus, of the cell cycle [33] and integration of EBV genomic regulatory sites in the promoter regions of target genes [34] have been proposed as likely reasons. Interference of EBV with the normal regulatory mechanisms of P450arom expression in human lymphocytes, is supported by the findings of this study: In addition to P450arom exon 1 untranslated sequences used in nontransformed PBLs, a novel exon 1 sequence is present in transcripts of this gene in transformed cells. The novel cryptic splice site used in transformed cells was more proximal than the two sites employed in untransformed PBLs. This is consistent with the report on P450arom transcripts in breast cancer tissues, in which more proximal splice sites are favored by the P450arom gene over distal sites as the expression of the gene increases [31].

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