Differential effects of \( n \)-3 and \( n \)-6 polyunsaturated fatty acids on \( BRCA1 \) and \( BRCA2 \) gene expression in breast cell lines

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Current evidence strongly supports a role for the breast tumour suppressor genes, \( BRCA1 \) and \( BRCA2 \), in both normal development and carcinogenesis. In vitro observations reported that \( BRCA1 \) and \( BRCA2 \) are expressed in a cell cycle-dependent manner. Interestingly, differences in the actions of \( n \)-3 and \( n \)-6 polyunsaturated fatty acids have been observed: while the \( n \)-3 polyunsaturated fatty acids have been described to reduce pathological cell growth, the \( n \)-6 polyunsaturated fatty acids have been found to induce tumour proliferation. Here, we examined the expression of \( BRCA1 \) and \( BRCA2 \) in breast cell lines after treatment with polyunsaturated fatty acids. Real-time quantitative polymerase chain reaction determinations conclusively demonstrated increases in \( BRCA1 \) and \( BRCA2 \) mRNA expressions in MCF7 and MDA-MB 231 tumour cell lines after treatment with \( n \)-3 polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid), but no variation was noticed with the \( n \)-6 polyunsaturated fatty acid (arachidonic acid). On the other hand, no variation of the expression of \( BRCA1 \) and \( BRCA2 \) mRNA was detected in MCF10a normal breast cell line treated by polyunsaturated fatty acids. The level of \( BRCA1 \) and \( BRCA2 \) proteins quantified by affinity chromatography remained unchanged in tumour (MCF7, MDA-MB 231) and normal (MCF10a) breast cell lines. We suggest the presence of a possible transcriptional or post-transcriptional regulation of \( BRCA1 \) and \( BRCA2 \) after \( n \)-3 polyunsaturated fatty acid treatment in breast tumour cells.

\( BRCA1 \): \( BRCA2 \): Polyunsaturated fatty acids: Mammary cell lines: Real-time quantitative polymerase chain reaction

Dietary \( n \)-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA; 20:5\(n\)-3) and docosahexaenoic acid (DHA; 20:6\(n\)-3) have been found to reduce pathological cell growth (Rose & Connolly, 1990; Tisdale & Dhesi, 1990; Shiina et al. 1993; Welsch et al. 1993). In contrast the \( n \)-6 PUFA arachidonic acid (AA; 20:4\(n\)-6) has been found to exert mainly mitogenic activity (Rose & Connolly, 1989, 1991; Sellmayer et al. 1991; Brouard & Pascaud, 1993). Recent research on the functional effects of \( n \)-6 PUFA has shown that, in cooperation with the \( n \)-3 PUFA, they directly and indirectly contribute to modulate functional parameters at the cellular level, such as receptor function, ion channels, and gene expression (Danesch et al. 1996; Galli & Marangoni, 1997).

To study the underlying molecular mechanisms, we evaluated the effects of the \( n \)-3 PUFA EPA and DHA, as compared with the \( n \)-6 PUFA AA on \( BRCA1 \) and \( BRCA2 \) tumour suppressor gene expressions. As a control, we used the monounsaturated fatty acid oleic acid (OA; 18:1\(n\)-9).

Germline mutations of \( BRCA1 \) and \( BRCA2 \) have been implicated in inherited predisposition to female breast cancer (Miki et al. 1994; Wooster et al. 1995). On the other hand, in sporadic breast cancer no mutation has been found so far, but a decrease in \( BRCA1 \) expression has been found (Thompson et al. 1995), which was associated with inactivation of the promoter by methylation in some cases (Dobrovic & Simpfendorfer, 1997; Magdinier et al. 1998; Mancini et al. 1998; Rice et al. 1998). Recently, it has been shown that \( BRCA2 \) is significantly expressed in breast cancer tissues.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; OA, oleic acid; PUFA, polyunsaturated fatty acid.

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overexpressed in many sporadic breast cancers (Bieche et al. 1999). The important biological roles for BRCA1 and BRCA2 interacting with other proteins are to maintain genomic integrity and to repress tumour formation (Deng & Brodie, 2000).

Therefore, we investigated the effects of the n-3 PUFA (EPA and DHA), the n-6 PUFA (AA) and OA as a control, on the transcription and the translation of BRCA1 and BRCA2 genes in two breast cancer cell lines (MCF7 and MDA-MB 231) and in a normal breast cell line (MCF10a).

Materials and methods

Human cell lines

Breast epithelial normal cells MCF10a and breast cancer cells MCF7 and MDA-MB 231 were purchased from American Type Culture Collection (Soule et al. 1973, 1990; Caillec et al. 1974) (Rockville, MD, USA). MCF10a were cultured in DMEM/Ham’s F-12 with 2% horse serum. MCF7 and MDA-MB 231 were grown in RPMI 1640 and L-15 media respectively with 2% fetal bovine serum. All cultures were supplemented with glutamine 2 mM and 1% gentamycin. All reagents were obtained from Life Technologies SARL (Cergy Pontoise Cedex, France). All cells were grown at 37°C, in a humidified atmosphere of 5% CO2 (except MDA-MB 231).

Fatty acids

The PUFA, EPA (20 : 5n-3), DHA (20 : 6n-3), AA (20 : 4n-6) and a monounsaturated fatty acid, OA (18 : 1n-9) were used as methyl esters (Sigma Chimie, St Quentin Fallavier, France) dissolved in ethanol.

Proliferation experiments

Cytotoxicity was evaluated using the SulfoRhodamine B colorimetric test assay (Skehan et al. 1990). Cells seeded in ninety-six-well plates (3000 cells in 200 µl media supplemented with fatty acids at different concentrations from 0, 1·5, 3, 9, 90 to 300 nM in 1% final ethanol for 96 h). After treatment, cells were fixed by adding 50 µl cold 50% trichloroacetic acid (Prolabo, Briare, France) for 1 h at 4°C. The wells were washed three times with water. Trichloroacetic acid-fixed cells were stained for 15 min with 50 µl 0·4% Sulforhodamine B dissolved in 1% acetic acid. The wells were washed in 1% acetic acid. Bound dye was solubilized with 200 µl 10 mM Tris-HCl for 20 min on a shaker. Optical density was read at 540 nm in a Titertrek Multiscan MCC340 microtitre plate (Labsystems, Cergy-Pontoise, France).

RNA extraction

Cells were plated in T75 cm2 flasks at a density of 3 × 106 cells. At 80% confluency, cells were washed twice with phosphate-buffered saline. Total RNA was isolated using 7 ml TRIZOL® (Life Technologies) according to the manufacturer’s protocol. Total RNA samples were dissolved in diethylpyrocarbonate-treated water and their concentrations determined by A260 measurements using a Hitachi spectrophotometer U-2000 (Hitachi Ltd, Tokyo, Japan).

cDNA synthesis

Total RNA (1 µg) was used for the synthesis of first strand cDNA using the First Strand cDNA Synthesis kit (American Pharmacia Biotech, Bucks, UK) following the manufacturer’s instructions.

Determination of BRCA1 and BRCA2 mRNA using real-time quantitative polymerase chain reaction

Polymerase chain reaction amplification. BRCA1 and BRCA2 probes and primers have been chosen using the Primer Express software (Applied Biosystems, Courtaboeuf Cedex, France) so that they overlapped splice junctions (exons 11–12 for BRCA1 Ex11 probe, exons 23–24 for BRCA1 Ex11 probe, exons 26–27 for BRCA2 probe), thereby avoiding the potential amplification of genomic DNA (Table 1). BRCA1- and BRCA2-synthesized probes and primers, TaqMan universal polymerase chain reaction Master Mix and the 18S rRNA doubly-labelled TaqMan® probe and primers were obtained from Applied Biosystems.

Multiplex polymerase chain reaction was performed in ninety-six-well plates on cDNA equivalent to 10 ng total RNA. A typical 25 µl reaction sample contained 12·5 µl TaqMan universal polymerase chain reaction Master Mix (2X) (containing TaqMan buffer, 200 µM dATP, dCTP, dGTP, and 400 µM dUTP, 5 mM MgCl2, 1·25 AmpliTaq-Gold, 0·5 units of Amplerase uracil-N-glycosylase), 200 nM BRCA1 or BRCA2 primers and 50 nM 18S rRNA primers, 200 nM BRCA1 or BRCA2 TaqMan® probes and 50 nM 18S rRNA TaqMan® probe. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed with forty cycles at 95°C for 15 s and 60°C for 1 min. Data were collected using the ABI PRISM 7700 SDS analytical thermal cycler (Applied Biosystems). Each sample was assayed in triplicate.

Relative determinations of BRCA1 and BRCA2 gene expression. Determination, assuming the same amplification efficiency for target and reference gene, was achieved using the comparative Ct method (Fink et al. 1998), which consists of the normalization of the number of target gene copies to an endogenous reference gene (i.e. 18S rRNA), designated as the calibrator. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of dual-labelled probe (TaqMan® methodology) is detectable by the sequence detector (ABI Prism 7700 SDS analytical thermal cycler).

The level of BRCA1 or BRCA2 mRNA expression in each treated cell line was then normalized to the result obtained in the untreated cells. The amount of target, normalized to the 18S rRNA endogenous reference is given by the formula: 2−ΔΔCt, where ΔΔCt = ΔCt treated cells − ΔCt untreated cells and ΔCt = ΔCt target − ΔCt 18S rRNA.

To ensure the reproducibility of a determination of mRNA in each special treatment of cell line, two indepen-
dent total RNA extractions were done. With one RNA extraction, two independent reverse transcriptions were done. With the second RNA extraction, only one reverse transcription was performed. On the three reverse transcriptions, one determination was done with the $\Delta C_T$ formula. In addition, each determination was done in triplicate and expressed in mean values and standard deviations (Favy et al. 2000).

BRCA1 and BRCA2 protein quantification

Cells were grown in a 75 cm$^2$ flask in culture medium supplemented with 100 $\mu$Ci $[^{35}S]$methionine (1000 Ci/mM; Amersham Pharmacia Biotech). The incubation was carried out for 20 h at 37°C in a 5% CO$_2$ atmosphere (without for MDA-MB 231 cell line). Metabolic radiolabelling was stopped by the addition of 10 ml cold PBS and cells were washed twice with PBS at 4°C. $10^{-10^6}$ labelled cells were solubilized in 5 ml per flask of 0.1 M Tris-HCl pH 7.1 containing 0.5% Nonidet P40 (Roche Diagnostics, Indianapolis, IN, USA) at 4°C for 30 min. The lysates were ultracentrifuged at 30 000 g for 30 min and the supernatant fraction yielded 1–2 mg labelled proteins per $10^7$ cells, measured with the Bio-Rad protein assay. The supernatant fractions were poured onto a POROS 20 HE (heparin) media column (4.6 mm diameter × 50 mm length; Applied Biosystems) of a Biocad Sprint HPLC system (Applied Biosystems) equipped with a fraction collector (Gilson Inc, Middelton, WI, USA). Labelled DNA-binding proteins bound to the gel were eluted with a gradient of NaCl from 0.1 to 1 M in 20 mM (2-[N-Morpholino]ethanesulfonic acid) MES pH 5.5. The flow rate was 5 ml/min. Detection of proteins was performed at 280 nm. The 0.5 ml fractions containing DNA-binding proteins were collected and pooled. Then the radioactivity was measured in a sample of 10 ml in 5 ml scintillation cocktail (Packard Ready Safe; Downers Grove, IL, USA). Radio-labelled BRCA1 or BRCA2 proteins were immunoprecipitated during 30 min at 37°C, by addition of 8 $\mu$g anti-BRCA1 polyclonal antibodies (K-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which recognized epitopes between amino acids 70–89 of human BRCA1 or 8 $\mu$g anti-BRCA2 polyclonal antibodies (N-19; Santa Cruz Biotechnology), which recognized epitopes between amino acids 20–38 of human BRCA2. The immune complex was isolated after fixation onto a POROS A column (4.6 mm diameter × 50 mm length; Applied Biosystems) containing protein A media. Elution was performed with 0.1% (v/v) 12 mM HCl–0.15 mM NaCl, pH 2 (flow rate, 5 ml/min with a Biocad Sprint HPLC system). Detection of the immune complex was performed at 280 nm. The radioactivity of each fraction was measured as described previously. The protein A affinity chromatography gives the amount of DNA-binding proteins that bind specifically to anti-BRCA1 or anti-BRCA2 antibodies, and a ratio is calculated as follows: (activity (disintegrations per minute) of BRCA1 or BRCA2 DNA-binding proteins that bind specifically to the anti-BRCA1 or BRCA2 antibodies: activity (disintegrations per minute) of labelled DNA-binding proteins eluted from heparin column).

<table>
<thead>
<tr>
<th>Oligonucleotide primer pairs and TaqMan® probes</th>
<th>Probes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td>BRCA1</td>
<td>Ex 11</td>
<td>5'-CAAGGAGACATGCTCCGTGCTCAAA-3'</td>
<td>5'-AAAAATAATCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-4157AAGAGGAACGGGCTTGGAA-3'</td>
<td>5'-4175-4177AAAATAATCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-4236CACACCCAGATGCTGCTTCA-3'</td>
<td></td>
</tr>
<tr>
<td>BRCA2</td>
<td>3'</td>
<td>5'-CTACACTGTCCAACACCCACTCTC-3'</td>
<td>5'-4157AAGAGGAACGGGCTTGGAA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-9794 CCAAGTGGTCCACCCCAAC-3'</td>
<td>5'-9812-9818 ACTGTACTTCAGGGCCGTACACTGCTCAAA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-9895 CACAATTAGGAGAAGACATCAGAAGC-3'</td>
<td>5'-9870-9895 CACAATTAGGAGAAGACATCAGAAGC-3'</td>
</tr>
<tr>
<td>rRNA 18S</td>
<td></td>
<td>5'-CGGCTACCACATCCAAGGAA-3'</td>
<td>5'-TGCTGGCACCAGACTTGCCCTC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GCTGGAATTACCGCGGCT-3'</td>
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Table 1. Summary of oligonucleotide primer pairs and TaqMan® probes

Superscript numbers indicate the nucleotide position of oligonucleotides in the BRCA1 or BRCA2 cDNA sequence (GenBank Accession No. U14680 for BRCA1 and No. U43746 for BRCA2).
×100. All data are mean values and standard deviations of four assays (Hizel et al. 1999; Vissac et al. 2001).

Results

Treatment with fatty acids: determination of the IC_{50} values

Table 2 reports the half maximum inhibitory concentration (IC_{50}) values calculated after 96 h exposure to the different fatty acids. In MCF7 cells, 30 μM was chosen for EPA, DHA, AA and OA was used as a control. The tumour MDA-MB 231 cell line was more sensitive to each of

Table 2. (IC_{50}) values of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA) and oleic acid (OA) in MCF7, MDA-MB 231 and MCF10a breast cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>EPA</th>
<th>DHA</th>
<th>AA</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MDA-MB 231</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>MCF10a</td>
<td>95</td>
<td>145</td>
<td>125</td>
<td>165</td>
</tr>
</tbody>
</table>
the fatty acids and was applied, 9 μM for EPA, 3 μM for DHA, 6 μM for AA and 10 μM for OA. The normal MCF10a cell line was the less sensitive: determined IC50 values were 95 μM for EPA, 145 μM for DHA, 125 μM for AA and 165 μM for OA.

Analysis of the impact of n-3 and n-6 polyunsaturated fatty acids on BRCA1 and BRCA2 mRNA levels and BRCA1 and BRCA2 protein synthese

We compared the level of expression of BRCA1 and BRCA2 mRNA and proteins in MCF7 and MDA-MB 231 tumour cell lines and MCF10a normal breast cell line after treatment with n-3 and n-6PUFA.

To study BRCA1 mRNA expression, we designated two different BRCA1 Taqman probes. The BRCA1 3’ Taqman probe was used to mainly quantify the expression of all BRCA1 mRNA species together because no alternative splicing of exon 23 has been described (Wilson et al. 1997) (Table 1). The BRCA1 Ex 11 Taqman probe was used to estimate the level of BRCA1 mRNA containing exon 11 because Thakur et al. (1997) previously described...
the isolation and expression of two cDNA of BRCA1, one of them being a spliced variant generated by exclusion of exon 11 by in-frame splicing and producing a 4·6 Kb mRNA. They finally concluded that there is a complex tissue-specific pattern of multiple spliced forms of BRCA1 and suggested that splicing may play a role in the regulation of BRCA1 function.

To evaluate the BRCA2 mRNA expression, we designed the BRCA2 3' Taqman probe in exons 26–27 because no alternative splicing has been observed so far.

All results with different treatment of fatty acids are reported in Figs. 1, 2, 3 and 4. In each cell line, expressions of BRCA1 and BRCA2 in treated cells were normalized to BRCA1 and BRCA2 expression levels in untreated cells, normalized at 1. As shown in Fig. 1, the expressions of each BRCA1 mRNA species and BRCA2 mRNA were increased in tumour cell lines (MCF7 and MDA-MB 231) after treatment with EPA (n-3) when compared with the untreated cells, corresponding to value 1. In contrast, BRCA1 and BRCA2 protein expressions were not modified.
by treatment with EPA. They remained at the basal line attributed to 1. Using normal mammary MCF10a cell line treated with EPA, we report no variation in BRCA1 and BRCA2 mRNA and protein expressions in treated and untreated cells.

Results obtained with DHA (n-3) are reported in Fig. 2. We observed an increase of BRCA1 and BRCA2 mRNA expressions in treated tumour breast cell lines (MCF7 and MDA-MB 231), similar to the EPA (n-3) effect. On the contrary, no variation of BRCA1 and BRCA2 mRNA expressions was noted in MCF10a normal cell line in the presence of DHA. No change in BRCA1 and BRCA2 protein expression was detected in tumour or normal breast cell lines. They stayed around the value 1 attributed to untreated cells in each cell line.

Results obtained with AA (n-6) are reported in Fig. 3, demonstrating no variation in BRCA1 and BRCA2 mRNA and protein expressions in tumour and normal breast cell lines. Values remained around 1. These results are comparable to OA treatment (Fig. 4). OA, a monounsaturated fatty acid, was used as a control because it was without effect on cell proliferation (Grammatikos et al. 1994).

Fig. 4. Expression of BRCA1 and BRCA2 mRNA and protein expressions in (a) MDA-MB 231, (b) MCF7 and (c) MCF10a breast cells after oleic acid treatment. Untreated cells corresponded to the arbitrary value of 1 and treated cell expression was normalized to untreated cells. Whole mRNA of BRCA1 and BRCA2 species were quantified with a BRCA1 3' probe and BRCA2 3' probe respectively. BRCA1 exon 11 mRNA corresponded to BRCA1 unspliced variant transcripts. Mean values for treated cells were significantly different from untreated cells: *P<0.05, **P<0.005, ***P<0.0005.
All mRNA and protein determinations were expressed in mean values and standard deviations, with a Student’s t test, to ensure the reproducibility of the methods.

Discussion
The n-3 PUFA EPA and DHA exert an anti-cancer activity in vitro and in animal models of experimental cancer although the n-6 PUFA are stimulators of development and progression of tumours (Rose & Connolly, 1989, 1990).

Our quantitative method demonstrated that the amount of BRCA1 and BRCA2 mRNA increased upon EPA and DHA treatment of breast cancer cell lines (MCF7 and MDA-MB 231), as compared with the normal breast cell line MCF10a. The level of BRCA1 and BRCA2 proteins after treatment with EPA and DHA remained unchanged. This result suggests that EPA and DHA stimulate the transcription of both genes but a post-transcriptional regulation is also possible with effects on the translational activity as well as the stability of BRCA1 and BRCA2 mRNA (Wickens et al. 1997). To discriminate between these two hypotheses, cycloheximide and actinomycin D assays will be necessary. The differences in the level of RNA between the two cancer cell lines may be due to their different backgrounds resulting in different levels in the proteins required for the regulation of the transcription and/or degradation.

AA (n-6 PUFA), as for the control OA, has no effect upon the level of BRCA1 and BRCA2 mRNA. Therefore, it is possible to postulate that EPA and DHA (n-3 PUFA) exert their activity, at the transcription level, via a BRCA1- and BRCA2-dependent pathway. In contrast, n-6 PUFA would follow a BRCA1- and BRCA2-independent pathway.

It is noteworthy that the amounts of two major variants of BRCA1 mRNA, with and without the exon 11 which harbours the nuclear localisation signal are not equivalent in the MDA-MB 231 cell line. It is also possible that some differences occur between the other spliced variants involving the first exons.

The protein quantification does not show any difference, whatever the fatty acid and the cell line, confirming our previous results by affinity chromatography in MCF7 and MCF10a cell lines with other n-3 PUFA (α-linolenic acid, 18 : 3n-3) and n-6 PUFA (α-linoleic acid, 18 : 2n-6) on BRCA1 protein expression (Bernard-Gallon et al. 1998).

EPA has been shown to decrease cachexia by reducing the protein turnover (Beck et al. 1991). Lately, Whitehouse et al. (2001) demonstrated that EPA antagonizes loss of skeletal muscle proteins in cancer cachexia by down-regulation of proteasome expression (Whitehouse et al. 2001). However, this mechanism looks unlikely in the case of BRCA1 and BRCA2 proteins in the studied cell lines because the amount of mRNA increased, while the amount of the proteins remained unchanged. Moreover, it has been suggested that BRCA1 degradation is due to cathpsin-like protease and not to the proteasome (Blagosklonny et al. 1999).

Cachexia is studied as a general phenomenon and it is possible that different tissues and cell lines would have a different reaction towards the treatment. Therefore a post-translational regulation might be suggested and it would be interesting to check the phosphorylation status of BRCA1 and BRCA2 proteins, as well as other post-translational modifications such as acetylation or ubiquitination level. As a matter of fact, DHA treatment results in decreased pRb phosphorylation in melanoma cells (Albino et al. 2000).

A post-transcription regulation might maintain the steady-state level of BRCA1 and BRCA2 proteins in cancer cells. This might explain the paradoxical lack of BRCA1 protein induction after treatment by n-3 fatty acids which are known to have protective effects in cancer.

In conclusion, n-3 PUFA (EPA and DHA) have obvious effects on BRCA1 and BRCA2 mRNA although n-6 PUFA (AA) has no effect on breast tumour cell lines. No variation in BRCA1 and BRCA2 protein levels are observed. Further experimental data are needed to precisely identify the relationships between PUFA metabolism and the pathways involving the BRCA1 and BRCA2 genes.

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