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Quantification by affinity perfusion chromatography of phosphorylated BRCAl and BRCA2 proteins from tumor cells after lycopene treatment

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Abstract

A new procedure for the quantification of phosphorylated BRCA1 (P-BRCA1) and BRCA2 (P-BRCA2) proteins in breast cell lines after different treatments was carried out. Cells were cultivated with [^{35}S]-methionine and extracts subjected to three perfusion chromatographies. First heparin affinity chromatography purified cellular DNA-binding proteins. Subsequent specific immunoprecipitation of BRCA1 and BRCA2 proteins was performed with antibodies raised against BRCA1 or BRCA2. The immune complexes were isolated by protein A affinity chromatography. Phosphorylated BRCA1 or BRCA2 proteins were then purified with a Poros 20 AL column where anti-phosphothreonine and anti-phosphoserine antibodies were previously bound. The percentage of phosphorylated BRCA1 or BRCA2 proteins was calculated as follows: 100 × dpm of P-BRCA1 or P-BRCA2 eluted from the POROS® 20AL column/total dpm eluted from POROS® 20AL column. Treatment with 10 μM lycopene increased P-BRCA1 and P-BRCA2 in the breast tumor cell line MCF7 but not in MDA-MB-231 or MCF-10a, breast tumor or fibrocystic cell lines, respectively.

Keywords: Breast cancer; *BRCA1*; *BRCA2*; Lycopene; Affinity chromatography

1. Introduction

BRCA1 and *BRCA2* are the two major genes involved in hereditary breast and ovarian cancers. The breast cancer tumor suppressor protein BRCA1 is a nuclear phosphoprotein with 1863 amino acids [1] and BRCA2 a 3148 amino acid nuclear phosphoprotein [2]. These two proteins are central components of the DNA damage response mechanism and are involved in cell division [3–8]. Phosphorylation of BRCA1 and BRCA2 is cell cycle-dependent, with hyperphosphorylation during late G_1 and S phase and dephosphorylation in M phase [9–11]. The phosphorylation of BRCA1 and BRCA2 are also regulated in response to DNA damage, via interactions with other proteins such as ATM, ATR, Chk2 and Rad51 [12–16]. Several studies have identified the most common BRCA1 phosphorylation sites: Ser-988 by Chk2 [13]; Ser-1423 and Ser-1524 by both ATM and ATR [17]. In addition, Ser-1387 is specifically phosphorylated after treatment with infra red irradiation, while Ser-1457 is phosphorylated predominantly after ultra-violet treatment [18]. BRCA2 has been reported to be phosphorylated in its N-terminal region [11]. More recently, it has been showed that BRCA2 undergoes phosphorylation by the Polo-like kinase Plkl [19], a key regulator of mitotic progression [20–22].

To accurately determine the expression level of BRCA1 or BRCA2 proteins in breast, a quantitative method previously developed by our laboratory to assay the expression of HLA-DR glycoproteins [23] was adapted to BRCA1 glycoproteins [24], late a faster method using perfusion chromatography

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was developed [25,26]. To measure the percentage of phosphorylated BRCA1 and BRCA2 compared with total proteins, three successive affinity chromatographies were used, starting with a heparin affinity column for initial isolation of DNA-binding proteins, because BRCA1 protein has a Zn finger at its amino terminus and BRCA2 has 8 BRC repeats, both characteristics of DNA-binding proteins. The second chromatography was performed on a protein A affinity column to purify BRCA1 or BRCA2 immunoprecipitated from the eluate of the heparin column. Then a POROS 20 AL column linked to anti-phosphothreonine and anti-phosphoserine antibodies, was used to purify phosphorylated-BRCAl (P-BRCA1) and phosphorylated-BRCA2 (P-BRCA2). Quantification of the proteins bound to these antibodies might give the amount of P-BRCA1 and P-BRCA2 proteins expressed in cells. Validation of this new procedure was performed with three human breast cell lines treated or not with lycopene.

2. Experimental

2.1. Cell cultures

MCF7 is a cell line originating from a pleural effusion containing tumor cells from a human mammary carcinoma [27]. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 20 μg/mL gentamycin and 0.04 U/mL insulin. 10^6 cells in 15 mL of medium were cultured in T-75 flasks at 37 °C in a humidified atmosphere of 5% CO_2. When the cells at 80% confluence, they were passed using 0.1% trypsin and 1.6 mM EDTA. All reagents came from Invitrogen SARL, 95613 Cergy Pontoise, France.

The MDA-MB-231 cell line originated from a pleural effusion of a patient with invasive breast carcinoma [28]. Cells were grown in T-75 flasks in growth medium composed of L-15 Leibovitz (Invitrogen SARL, 95613 Cergy Pontoise, France) supplemented with 2 mM L-glutamine, gentamycin (20 μg/mL) and 15% heat-inactivated FBS. Cells were grown in a humidified incubator without carbon dioxide at 37 °C.

The MCF-10a cell line was established from mammary tissue from a patient with fibrocystic breast disease [29]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Invitrogen SARL, 95613 Cergy Pontoise, France) with 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin and 500 ng/mL hydrocortisone and 15% heat-inactivated horse serum. Cells were grown in a 37 °C humidified atmosphere with 5% CO_2.

2.2. Lycopene treatment

Cells were trypsinized and reseeded in medium for control cells or in medium supplemented for 48H with l0 μM lycopene in 0.25 g/L THF-BHT (both from Sigma Chimie, St. Quentin Fallavier, France) for treated cells. This concentration was determined previously by flow cytometry [30] to induce a G_1/S-phase cell cycle arrest with an increase in late-G_1 phase cell number.

2.3. Metabolic protein labeling

Metabolic labeling with [^{35}S]-methionine was performed in T-75 flasks when the cultures were at 80% confluence. After removing the medium, cells received 5 mL of medium supplemented with 100 μCi [^{35}S]-methionine (1000 Ci/mM; Amersham International plc, UK). The incubation was performed 24 h at 37 °C in a 5% CO_2 atmosphere for MCF-7 cells and MCF-l0a, and without CO_2 atmosphere for MDA-MB-231. Metabolic radiolabeling was stopped after 24 h by adding 10 mL cold PBS, and cells were gently washed twice with PBS at 4 °C. $8 \times l0^6$ labeled cells were solubilized in 5 mL of 0.1 M Tris–HCl pH 7.1 containing 0.5% Nonidet P40 (Boehringer Mannheim) at 4 °C for 30 min. The insoluble material was removed by ultracentrifugation at 40,000 × *g* for 30 min.

2.4. Purification of DNA-binding proteins by affinity chromatography

The NP 40 cell lysates were loaded onto a POROS 20 HE (heparin) media column (4.6 mmD/50 mmL, PerSeptive Biosystems, Framingham, MA, USA). Labeled DNA-binding proteins specifically bound to the gel were eluted with a gradient of NaCl from 0.1 to 1 M in 20 mM MES pH 5.5. Elution was performed with a BioCAD Sprint high-performance liquid chromatography system (PerSeptive Biosystems) equipped with a fraction collector (Gilson, Middleton, WI, USA) at a flow rate of 5 mL/min. Detection of proteins was performed at 280 nm. 0.5 mL fractions containing DNA-binding proteins were collected and pooled. Then the radioactivity was measured by adding 10 μL of eluate to 5 mL of scintillation cocktail (Packard Ready Safe) and counted in a Wallac 1414 liquid scintillator (Fig. 1).

2.5. Immunoprecipitation and purification of immune complex by affinity chromatography

Radiolabeled BRCA1 or BRCA2 DNA-binding proteins were specifically immunoprecipitated by addition of 20 μL anti-BRCAl polyclonal antibodies (556441 BD PharMingen, San Diego, CA) which specificity have been previously demonstrated in our laboratory [31] or 200 μL anti-BRCA2 polyclonal antibodies (C-19, Tebu) [32] for 30 min at 37 °C.

The immunoprecipitate was isolated after fixation on POROS A column (4.6 mmD/50 mmL, Perseptive Biosystems, Framingham, MA, USA) containing Protein A media, and elution was performed with 0.1% (v/v) (12 mM) HCl/0.15 M NaCl (flow rate 5 mL/min). Detection of the immune complex was performed at 280 nm. Radioactivity was measured by adding 750 μL of the collected fractions to 5 mL

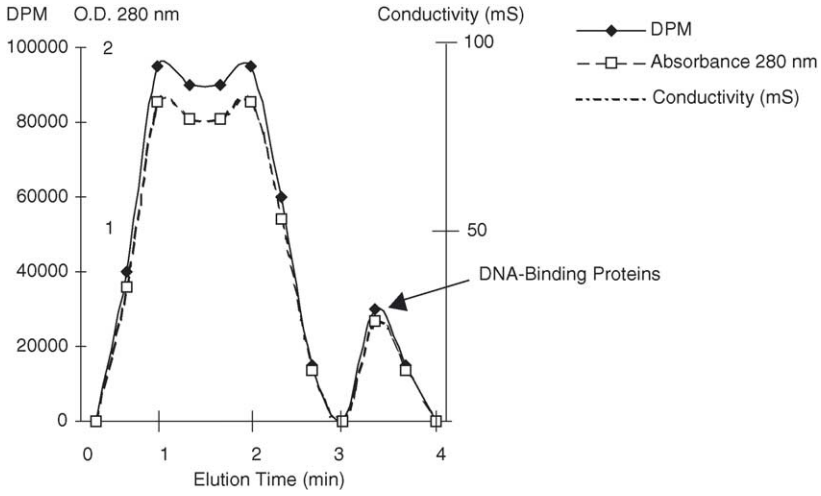

Fig. 1. Example of elution of ^{35}S-labeled DNA-binding proteins from MCF-7 cells onto POROS 20 HE (heparin) media affinity column. Radioactive labeled DNA-binding proteins specifically bound to the gel were eluted with a gradient of 0.1–1 M NaCl in 20 mM MES pH 5.5. Flow rate, 5 mL/min. Detection of proteins was performed at 280 nm.

of scintillation cocktail and counting as previously described (Fig. 2).

2.6. Quantification of P-BRCA1 and P-BRCA2 proteins

The immune complex eluates were neutralized with 100 μL of 1 M KH_2PO_4 and passed onto a POROS® 20AL column (2.1 mmD/30 mmL; PerSeptive Biosystems, Framingham, MA, USA) linked to with anti-phosphothreonine (P-3555, Sigma) and anti-phosphoserine (P-3430, Sigma) mouse monoclonal antibodies. P-BRCA1 and P-BRCA2 proteins were eluted by reducing the pH to 2 with 150 mM NaCl/HCl (v/v) (flow rate 2 mL/min). Radioactivity was measured by adding 5 mL of scintillation cocktail to each 1 mL fraction. The percentage of phosphorylated BRCA1 or BRCA2 proteins was calculated as follows: 100 × dpm of P-BRCA1 or P-BRCA2 eluted from the POROS® 20AL column/total dpm eluted from POROS® 20AL column (Fig. 3).

3. Results and discussion

3.1. Characteristics of the treatment

After exposure to 10 μM lycopene for 48 H, MCF7, MDA-MB-231 and MCF-10a cells were blocked in G_1 phase and decreased in S phase. We also chose this concentration to determine the effects of induction of P-BRCAl and P-BRCA2 proteins by lycopene, because higher concentrations [30 μM] were toxic for these cells [30].

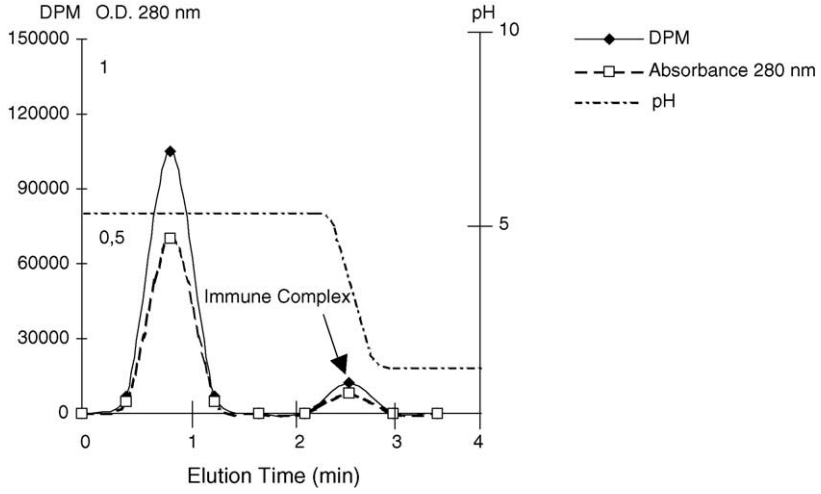

Fig. 2. Example of elution of ^{35}S-labeled immune complex from MCF-7 cells on POROS A (Protein A) affinity column. After immunoprecipitation with 20 μL anti-BRCAl polyclonal antibodies (556441 BD Pharmingen), the immune complex was eluted with 0.1% (v/v) 12 mM HCl/0.15 M NaCl. Flow rate, 5 mL/min. Detection of proteins was performed at 280 nm.

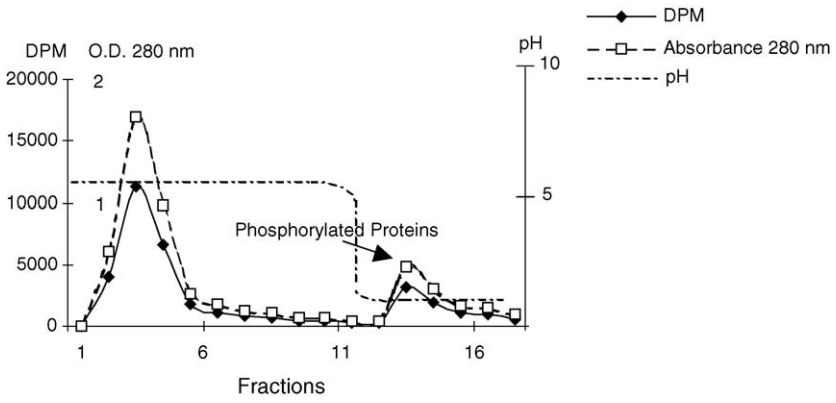

Fig. 3. Example of elution of BRCA1-P proteins from MCF-7 cells on POROS® 20AL. The phosphorylated proteins were eluted by a fall of pH to 2 with NaCl 150 mM buffer. Flow rate, 2 mL/min. Detection of proteins was performed at 280 nm.

3.2. Precision and sensitivity of the method

We purified P-BRCAl and P-BRCA2 from cell extracts by affinity perfusion chromatography. Accurate approaches such as the Western blotting can be used but this qualitative technique only detects the difference of migration between the phosphorylated antigen and the non-phosphorylated antigens. We therefore developed a quantitative assay to measure the phosphorylation state of BRCAl and BRCA2 in order to better understand their biological functions in tumor cell lines after lycopene treatment.

3.3. Quantification of P-BRCAl and P-BRCA2 proteins

Cells were internally labeled with [^{35}S]-methionine, proteins were solubilized with a detergent, and DNA-binding proteins isolated by heparin affinity chromatography on POROS 20 HE using an ionic strength gradient (Fig. 1). Specific immunoprecipitation was then performed and the immune complex constituted of BRCAl or BRCA2 proteins labeled with [^{35}S]-methionine bound to antibodies was isolated with a pH gradient by protein A affinity chromatography on POROS A. An example elution profile is shown in Fig. 2. Finally, to isolate P-BRCAl and P-BRCA2, we used a POROS 20AL column linked to anti-phosphoserine and anti-phosphothreonine (Fig. 3). The amount of P-BRCAl and P-BRCA2 was expressed as a percentage (100 × dpm of P-BRCAl or P-BRCA2 eluted from the POROS® 20AL column/total dpm eluted from POROS® 20AL column) (Table 1). For untreated MCF-7 cells, 13.3% of BRCAl and 10.1% of BRCA2 was phosphorylated. After lycopene treatment of MCF-7 cells, the expression of P-BRCA1 increased significantly to 19.9% ($p < 0.01$) and 14.4% ($p < 0.05$) for P-BRCA2 (Student's *t*-test). For MDA-MB-231, 22.5% of P-BRCA1 and 26.1% of P-BRCA2 proteins bound the POROS 20AL column before treatment, and 24.6% P-BRCA1 and 27.5% P-BRCA2 after lycopene treatment. For MCF-10a cells, 9.0% of P-BRCA1 and 11.5% of P-BRCA2 were eluted and 9.9 and 11.9%, respectively after treatment. These results show that P-BRCA1 and P-BRCA2 expression was considerably increased in MCF-7 cells after lycopene treatment. In contrast, no significant variation in phosphorylation of BRCAl or BRCA2 were observed in MDA-MB-231 and MCF-l0a.

3.4. ER and RAR interaction

This difference might result from an interaction between estrogen receptors (ER) and lycopene. We reported that lycopene may act through the ER status of cells [30]. Indeed, MCF-7 is an ER-positive cell line and MDA-MB-231 and MCF-10a ER-negative cell lines. Our previous work showed an increase in *BRCA1* and *BRCA2* mRNA expression in

Table 1
Amount of BRCA1-P and BRCA2-P proteins expressed by two human tumor breast cell lines (MCF-7 and MDA-MB-231) and one human fibrocystic breast cell line (MCF-l0a) after 10 μM lycopene treatment and time of exposure of 48 h

Cell lines	Percentage of phosphorylated proteins	
	BRCA1-P	BRCA2-P
MCF-7		
Untreated	13.3 ± 2.6	10.1 ± 1.5
10 μM lycopene	19.9 ± 2.9**	14.4 ± 1.7*
MDA-MB-231		
Untreated	22.5 ± 1.7	26.1 ± 1.1
10 μM lycopene	24.6 ± 2.1	27.5 ± 1.9
MCF-10a		
Untreated	9.0 ± 1.0	11.5 ± 1.9
10 μM lycopene	9.9 ± 0.8	11.9 ± 0.4

Quantification of BRCA1-P and BRCA2-P proteins are obtained with DNA-binding protein purification, specific immunoprecipitation with anti-BRCA1 or anti-BRCA2 antibodies, protein A affinity chromatography and POROS® 20AL chromatography successively. The amount of BRCA1-P and BRCA2-P proteins was expressed in percentage, calculated as follows: 100 × dpm of P-BRCA1 or P-BRCA2 eluted from the POROS® 20AL column/total dpm eluted from POROS® 20AL column. All data are expressed as means ± S.D. of three assays.

* $p < 0.05$ when compared to control (Student's *t*-test).

** $p < 0.01$ when compared to control (Student's *t*-test).

MCF-7 cells and a decrease in MDA-MB-231 cells, in response to lycopene whereas no difference was observed in the fibrocystic breast cell line MCF-10a. These results were explained by an interaction between ER and retinoic acid receptors after lycopene treatment. These results suggested that the action of lycopene on mammary carcinogenesis may be in part mediated by its effect on *BRCA1* and *BRCA2* gene expression. It appears that lycopene may exercise a translational or post-translational regulation of *BRCA1* and *BRCA2*. In MCF-7 cells, despite the *BRCA1* and *BRCA2* mRNA increase observed after lycopene treatment, no variation was found in BRCAl and BRCA2 protein expression, which suggested translational regulation of BRCAl and BRCA2. Moreover, the increase of P-BRCA1 and P-BRCA2 expression corroborates the suggestion of a post-translational effect of lycopene on BRCA1 and BRCA2.

3.5. ER and BRCA relation

BRCA1 and BRCA2 proteins are reported to stably associate with each other in meiotic and mitotic cells [33]. The carboxyl terminus of BRCA1 contains two BRCT (BRCA1 C-terminal) domains that interact indirectly with RAD51 to form a stable complex with BRCA2 during DNA damage repair [34]. Moreover, BRCA1 was found to inhibit estrogen-induced signaling by blocking the transcription activation function of estrogen receptor α (ERα). This inhibition is consistent with a negative role for BRCA1 in tumor progression, because ERα signals play an important role in breast tumor progression. These data could explain our results because MCF-7 is an ERα+/ERβ+ cell line whereas MDA-MB-231 is ERα−/ERβ+ and MCF-10a is ERα−/ERβ−. We hypothesized that lycopene bound to RAR receptors may act on BRCA1 expression by an activation of its phosphorylation which enhance inhibition of ERα and tumor progression. Finally, post-translational modification by phosphorylation is required for normal *BRCA1* and *BRCA2* function. Whereas it has been known for sometimes that *BRCA1* is regulated by phosphorylation, it now appears that *BRCA2* is phosphorylated as well. The C-terminus of *BRCA2* is phosphorylated by *hBUR1*, a mitotic checkpoint gene [35]. In addition the activation domain of *BRCA2* has recently been shown to possess a binding site for a kinase [36]. It is not yet clear how phosphorylation of *BRCA2* is critical to its function.

3.6. Other possible post-translational regulations

Further research on lycopene should be undertaken to determine other post-translational regulation of BRCA1 and BRCA2, such as ubiquitination and methylation. It has been established that BRCA1 has endogenous ubiquitin ligase activity through its ring domain [37].

It has also been suggested that the ubiquitin protein ligase activity of BRCA1/BARD1 contributes to many of the biologic functions of BRCA1, including its breast and ovarian cancer suppressor activity [38]. Furthermore, aberrations in DNA methylation patterns are currently recognized as a hallmark of human cancer. One of the most characteristic changes is the hypermethylation of CpG islands of the *BRCA1* gene associated with transcriptional silencing [39]. The *BRCA2* promoter does not appear to be methylated in normal tissues or in breast or ovarian tumors [40]. It will be interesting to study these other post-translational modifications to determine the molecular effects of lycopene on breast cancer and the possible links between these mechanisms.

In conclusion, the method described in this paper allows the quantification of P-BRCA1 and P-BRCA2 from cell extracts with a BioCAD Sprint HPLC system following heparin purification, isolation of the immune complex BRCAl-anti-BRCAl or BRCA2-anti-BRCA2 with a protein A chromatography and a final purification of BRCA1-P or BRCA2-P proteins with a POROS® 20AL chromatography. The internal labeling of cells allowed quantification of the P-BRCA1 and P-BRCA2. This technique was set up with breast cancer cell lines treated with lycopene, but it will be extended to other micronutrients, such as resveratrol [32], genistein and daidzein [41,42] or $n-3$ and $n-6$ polyunsaturated fatty acids [43] studied other there in our laboratory.

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