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Isolation, purification and quantification of BRCA1 protein from tumour cells by affinity perfusion chromatography

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Abstract

A new procedure for the isolation, purification and quantification of the product of the oncosuppressor gene *brca1* in breast tissues, was carried out. It involves internal cell protein [^{35}S]methionine labelling followed by two perfusion chromatographies. The first one is heparin affinity chromatography, to purify all of the cell DNA-binding proteins. A subsequent specific immunoprecipitation of BRCA1 protein was performed with an antibody raised against BRCA1. The immune complex was isolated using the second chromatographic step, Protein A affinity chromatography. The amount of BRCA1 expressed by cells was expressed as a ratio, in percent, calculated as follows: 100× amount of labelled DNA-binding proteins (dpm) that bound specifically to the anti-BRCA1 polyclonal antibodies (K-18)/amount of whole labelled DNA-binding protein (dpm) purified on a heparin column. Applications to MCF7 and T-47D human breast tumour cell lines, which were treated or not using 2 m*M* sodium butyrate demonstrated an increase in BRCA1 protein expression.
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Keywords: BRCA1 protein; Affinity perfusion chromatography; Sodium butyrate; Brest tumour cell lines

1. Introduction

brca1 was the first major cloned gene involved in familial breast and ovarian cancers [1]. However, somatic mutations in the *brca1* coding sequence have not been detected in sporadic breast cancers, indicating that these alterations do not play a significant role in their development [2]. Comparison of *brca1* messenger RNA levels in a series of normal and sporadic breast cancer tissues demonstrated an apparent decrease in *brca1* gene expression in tumour cells [3]. At the BRCA1 protein level, Chen et al. [4,5] showed that BRCA1 was localized in the cytoplasm in 80% of sporadic breast cancer tissues, whereas it was localized in the nuclei of HBL100 cells, which were considered as a normal cell line. This immunoenzymatic technique (immunoperoxidase) used to demonstrate the expression of BRCA1 proteins in tumour breast tissues on sections only allowed a qualitative evaluation of the presence of BRCA1 proteins in the tissues studied. To accurately determine the expression level of BRCA1 proteins in

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breast, a quantitative method previously that had been developed by our laboratory to assay the HLA-DR glycoproteins expressed by cells [6] was adapted to BRCA1 glycoproteins [7]. In this work, we describe a new, faster method using perfusion chromatography. Two successive affinity chromatographies were used: (1) a heparin affinity column was used first for the purification of all DNA-binding proteins from the cell extract, because BRCA1 protein has a Zn finger at its amino terminus and seems to be a DNA-binding protein, (2) a protein A affinity column was used, just after immunoprecipitation with an anti-BRCA1 antibody to isolate specifically BRCA1 protein from all of the purified DNA-binding proteins that had been obtained previously. Thus, quantification of the labelled immune complex could be realized to give the amount of BRCA1 protein expressed in cells. Validation of this new procedure was performed with two human mammary carcinoma cell lines that were either treated or not with sodium butyrate (NaB) and the differences in the BRCA1 protein levels were investigated.

2. Experimental

2.1. Cell cultures

MCF7 is a cell line originating from a pleural effusion containing tumour cells from a human mammary carcinoma [8]. Cells were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) and were supplemented with sodium bicarbonate (2 g/l), 5% heat-inactivated fetal bovine serum (FBS; Life Technologies), 2 m*M* L-glutamine and 20 μg/ml gentamycin (Schering-Plough, Levallois-Perret, France), and 0.04 U/ml insulin. Cells (10^6 in 30 ml of medium) were cultured in 75 cm^2 flasks (Falcon, Lincoln Park, NJ, USA) at 37°C in a humidified atmosphere of 5% CO_2. When the cells were grown to 80% confluence, they were treated with 0.05% trypsin and 0.02% EDTA.

The T-47D cell line originated from a pleural effusion of a patient with invasive breast carcinoma [9]. Cells were grown in closed plastic T-75 flasks in growth medium composed of RPMI 1640 buffered with hydrogen carbonate (2 g/l) and supplemented with 2 m*M* L-glutamine, gentamycin (20 μg/ml) and 10% heat-inactivated FBS. Cells were grown in a humidified incubator with 5% CO_2 at 37°C.

2.2. Sodium butyrate treatment of cells

MCF7 or T-47D cells were trypsinized and reseeded in maintaining medium for control cells or in maintaining medium supplemented with NaB for treated cells. Media were respectively supplemented with NaB at 2, 5, 7, 10, 15 and 20 m*M* (Sigma Chimie, St. Quentin Fallavier, France) in ethanol. Ethanol alone was added to controls. Treated and untreated cells were collected after 24, 48 and 72 h by trypsinization and the DNA content was assessed by flow cytometric analysis. Each experiment was performed in triplicate.

2.3. Flow cytometric analysis

Cell cycle analysis for the determination of the cell DNA content was undertaken after propidium iodide labelling of cells. Briefly, 100 μg of cell sample at 10^7 cells/ml were suspended in 50 μg/ml of propidium iodide (PI; Sigma Chimie) and 500 μg/ml of ribonuclease A (Sigma Chimie). The cells were stained for 30 min at 4°C in the dark, then filtered through a 40-μm nylon mesh just before analysis. The flow cytometric analysis of cell DNA content was performed using an Epics XL (Coulter, Hialeah, FL, USA). Fluorescence attributable to PI was determined using excitation by an argon laser operating at 488 nm and at a power output of 15 mW. A minimum of 15 000 events was acquired in list mode for each sample. For each DNA histogram, the cell cycle distribution was calculated using the Multicycle Software program (Phoenix, Flow Systems, San Diego, CA, USA).

2.4. Protein cell labelling with radioactivity

Protein cell labelling with [^{35}S]methionine was performed in 75 cm^2 flasks when the cultures were at 80% confluence. After removing the medium, the cells received by flask, 5 ml of new sterile culture medium as described previously, supplemented with 100 μCi [^{35}S]methionine (1000 Ci/mM; Amersham, Bucks, UK). The incubation was performed for 20 h

at 37°C in a 5% CO_2 atmosphere. Then, metabolic radiolabelling was stopped by adding 10 ml of cold phosphate-buffered saline (PBS) and the cells were gently washed twice with PBS at 4°C.

2.5. Preparation of cell lysates

Washed labelled cells (8×10^6) were solubilized in 0.1 *M* Tris–HCl, pH 7.1, containing 0.5% Nonidet P40 (NP 40; Boehringer Mannheim) (750 μl per flask) and incubated at 4°C for 15 min. The insoluble material was removed by ultracentrifugation at 30 000 *g* for 30 min.

2.6. Purification of DNA-binding proteins by affinity chromatography

The NP 40 cell lysates were loaded onto a POROS 20 HE (heparin) media column (50×4.6 mm I.D.; PerSeptive Biosystems, Framingham, MA, USA). Labelled DNA-binding proteins that specifically bound to the gel were eluted with a gradient of NaCl from 0.1 to 1 *M* in 20 m*M* MES pH 5.5. The flow-rate was 5 ml/min, using a BioCAD Sprint high-performance liquid chromatography system (PerSeptive Biosystems) equipped with a fraction collector (Gilson, Middleton, WI, USA). Detection of proteins was performed at 280 nm. Fractions (0.5 ml) containing DNA-binding proteins were collected and pooled. Then the radioactivity was measured by adding 10 μl of the collected proteins to 5 ml of scintillation cocktail (Packard Ready Safe) and counting.

2.7. Immunoprecipitation

Radiolabelled BRCA1 DNA-binding proteins were specifically immunoprecipitated by the addition of 16 μg of anti-BRCA1 polyclonal antibody (K-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to the previously collected fractions, with a 30-min incubation time at 37°C. The specificity of the polyclonal antibody against BRCA1 (K-18) has been demonstrated elsewhere [10,11]. It was ascertained by Western blotting and the 220-kDa band corresponding to BRCA1 was detected in both HBL100 and MCF7 breast cell lines. Moreover, other major bands appeared at around 100-kDa in the two cell lines, they may correspond to different variants to BRCA1 [11]. Recently, Thakur et al. [12] found three protein species with apparent molecular masses of 220, 100 and 97 kDa in HBL100 cellular extract. One of this three BRCA1 isoforms lacks exon 11 and corresponds to a splicing form of BRCA1.

2.8. Isolation of immune complex by affinity chromatography

The immunoprecipitate was isolated after fixation on a POROS A column (50×4.6 mm I.D.; PerSeptive Biosystems) containing Protein A media (PerSeptive Biosystems), and elution was performed with 0.1% (v/v) (12 m*M*) HCl/0.15 *M* NaCl (flow-rate, 5 ml/min using a BioCAD Sprint HPLC system). Detection of the immune complex was performed at 280 nm. The radioactivity of each 1 ml fraction was measured as described previously.

Protein A affinity chromatography after elution of the immune complex gave the amount of DNA-binding protein that bound specifically to the anti-BRCA1 polyclonal antibody, and a ratio was calculated as follows: 100×[disintegrations per minute (dpm) of BRCA1 DNA-binding protein that bound specifically to the polyclonal antibody raised against BRCA1 protein/amount of whole labelled DNA-binding protein (dpm) purified on the heparin column). All data were expressed as means±SD of three assays.

3. Results and discussion

NaB treatment with different concentrations (2 to 20 m*M*) were studied by flow cytometry after different exposure times (24, 48 and 72 h). At 72 h, the number of cells surviving was too weak to permit analysis (data not shown) (Fig. 1). Representative flow cytograms demonstrating the effect of NaB on MCF7 and T-47D cells are represented in Fig. 2. After exposure to NaB, the population of treated MCF7 cells as well as the T-47D cells were blocked in G1 phase and decreased in S phase. The percentage of cells in S phase was considerably decreased after treatment with NaB, whereas the percentage of cells in the G1 phase was highly increased. So, experiments were performed in triplicate

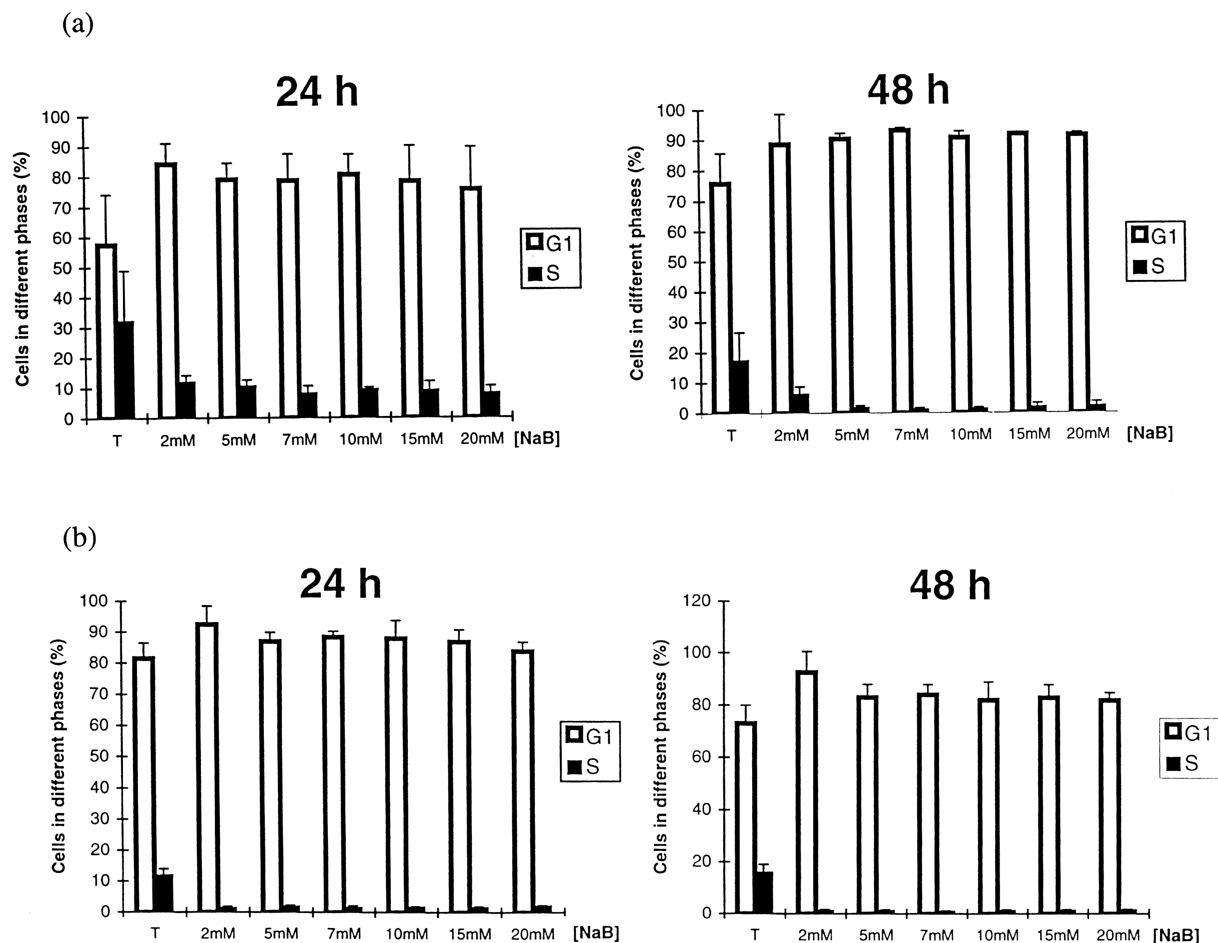


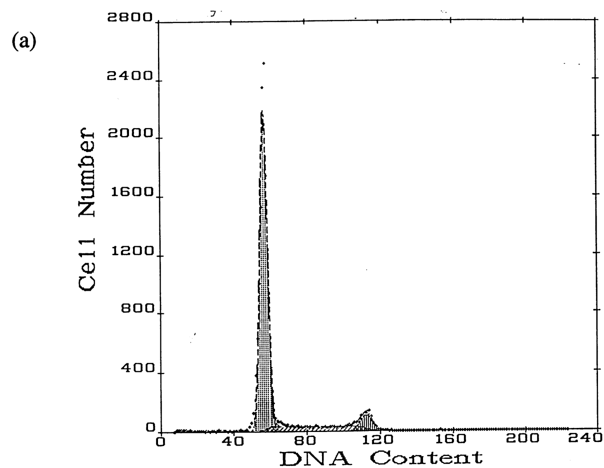
Fig. 1. Results of using different concentrations of NaB on MCF7 (a) and T-47D (b) cells by flow cytometry in the G1 and S phases. Cells were cultured with NaB (2 to 20 m*M*) or in normal medium (T) and harvested after the indicated times (24 and 48 h) and prepared for flow cytometry. (Each value given for the G1 and S phases corresponds to mean±SD of three assays for each group corresponding to different concentrations of NaB inside the different times of exposure.)

for each selected time of exposure and each concentration of NaB and analyzed by flow cytometry, and all data were represented as mean values±SD for G1 and S phases (Fig. 1). We have thus chosen a time of exposure of 48 h to determine the effects of induction of BRCA1 protein by NaB and a concentration of 2 m*M*, because higher concentrations of NaB completely reduced the S phase and were too toxic for MCF7 and T-47D cells. It is well known that NaB treatment causes an accumulation of cells in the G1 phase [13].

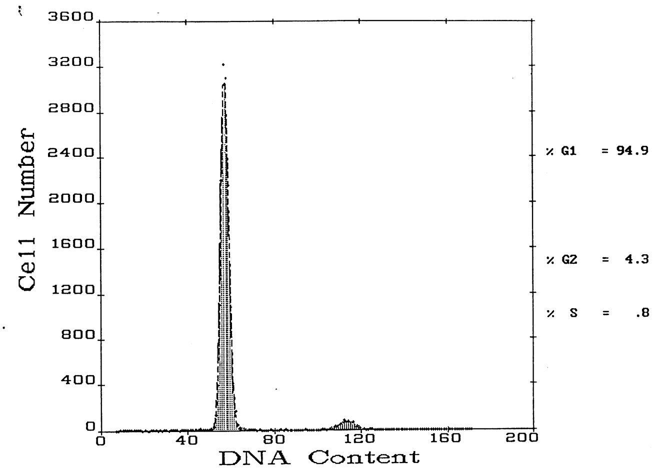
We have carried out the purification of BRCA1 protein from cell extract by affinity perfusion chromatography. More frequently, immunoenzymatic techniques have been used to demonstrate the expression of different antigens in tumour breast tissues and cell lines (immunoperoxidase), as has immunofluorescence on cryostat sections. However, these methods allow only a qualitative evaluation of the presence or absence of antigens on the studied tissue. Accurate approaches, such as the Western blotting, can be used but this technique only allows the antigen to be identified and characterized. So, we developed a quantitative assay of BRCA1 protein in order to better understand its biological function in cells. This method can be applied to breast cancer by

MCF7

(a)

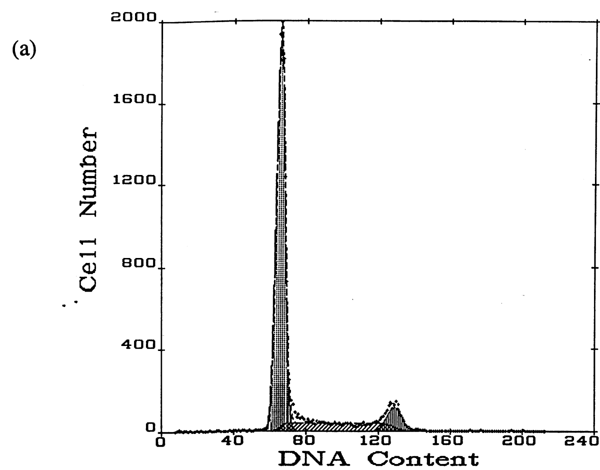


(b)



T47D

(a)



(b)

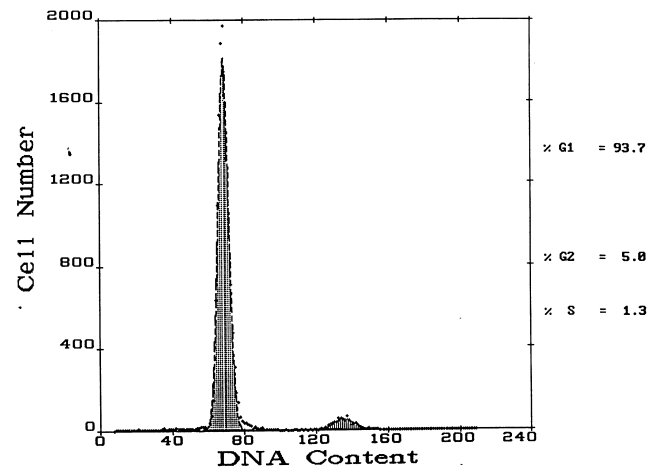


Fig. 2. Examples of cytometric analysis of propidium iodide-stained samples from MCF7 and T-47D cell lines. (a) Untreated cells, (b) cells were cultured with 2 m*M* NaB for 48 h, and were arrested in G1.

comparison of BRCA1 expression in normal breast cells and tumour breast cells, after iodine labelling of proteins in the presence of lactoperoxidase and glucose oxidase. Then, the isolation and the purification of the immune complex were also performed by successive affinity chromatographic step [14].

Here, cells were internally labelled with [^{35}S]methionine. All proteins were solubilized with a detergent and DNA-binding proteins were isolated by heparin affinity chromatography on Poros 20 HE according to ionic strength (Fig. 3). We hypothesized that BRCA1, which includes a zinc finger [15], could be eluted with all nucleic acid-binding proteins. Specific immunoprecipitation was then performed with anti-BRCA1 polyclonal antibody (K-18) and the immune complex, which consisted of BRCA1 proteins labelled with ^{35}S bound to antibodies, was isolated by protein A affinity chromatography on Poros A using a pH gradient. The elution profile on protein A is shown in Fig. 4. A small labelled peak corresponding to ^{35}S-labelled BRCA1 proteins bound to anti-BRCA1 polyclonal antibody was obtained after an elution time of 2.5-min, when the pH started to decrease. The immune complex BRCA1/ anti-BRCA1 polyclonal antibodies was also followed by counting the radioactivity in the collected fractions.

Final quantification of BRCA1 proteins was performed. The amount of BRCA1 proteins was expressed as a percentage, meaning the ratio: amount of labelled DNA-binding proteins that bound specifically to the anti-BRCA1 polyclonal antibodies (K-18)/amount of whole labelled DNA-binding protein purified by heparin chromatography (Table 1). For the MCF7 cells used as controls, 1.36% of the purified DNA-binding proteins bound to anti-BRCA1 polyclonal antibodies (K-18). After NaB treatment, 2.10% of BRCA1 proteins bound specifically to anti-BRCA1 polyclonal antibodies. For T-47D controls, 1.43% of the DNA-binding proteins bound to anti-BRCA1 polyclonal antibodies and after NaB treatment, the expression of BRCA1 proteins increased to 2.68%. These results show that BRCA1 protein expression by MCF7 and T-47D cell lines was considerably increased after NaB treatment. In the two cell lines, the differences found in BRCA1 expression after NaB treatment are significant, $p<0.05$, when compared to controls (Wilcoxon's test). These data may be explained by the fact that *brca1* reached a maximal level in late G1 and S phases, in normal and tumour-derived breast epithelial cells. Vaughn et al. [16] reported that the induction of *brca1* was shown to occur before the onset of DNA synthesis at the G1–S boundary and levels of

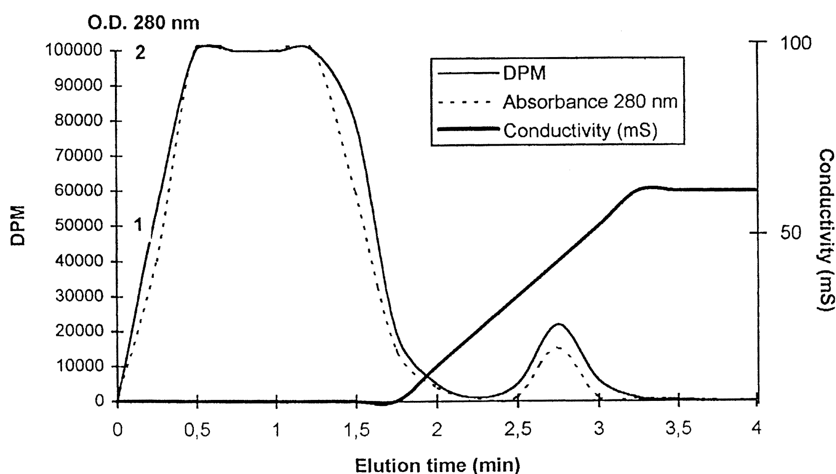


Fig. 3. Elution pattern on Poros 20 HE media for heparin affinity chromatography of ^{35}S-labelled DNA-binding proteins from T-47D cells. Flow-rate, 5 ml/min.

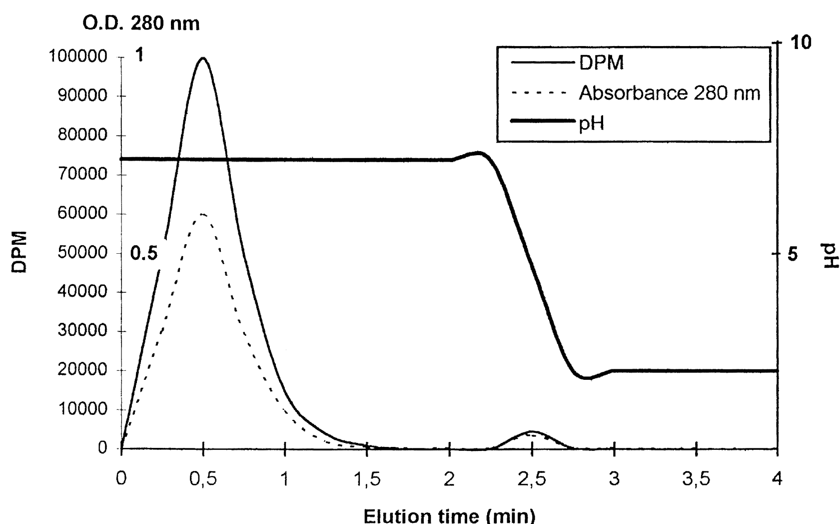


Fig. 4. Elution pattern on Poros A for Protein A affinity chromatography of ^{35}S-labelled immune complex from T-47D cells. Flow-rate, 5 ml/min.

BRCA1 protein were regulated in a similar manner. However, these results hypothesize that the action of NaB on mammary carcinogenesis may be in part mediated by its effect on *brca1* gene expression. It appears that NaB may exercise transcriptional or post-transcriptional regulation of *brca1*. Further research on NaB should be undertaken, such as quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), to determine any possible differences between the regulation of *brca1* mRNAs and BRCA1 protein expression by NaB.

Table 1
Amount of BRCA1 protein expressed by two human breast cell lines (MCF7 and T-47D) after treatment with 2 m*M* NaB and an exposure time of 48 h

	Cell lines	BRCA1 proteins assayed (%)[a]
MCF-7	Controls	1.36±0.49
	2 m*M* NaB	2.10±0.17[b]
T-47D	Controls	1.43±0.30
	2 m*M* NaB	2.68±0.39[b]

[a] Quantification of BRCA1 protein was obtained after DNA-binding protein purification, specific immunoprecipitation with anti-BRCA1 antibodies and protein A affinity chromatography, successively.
The amount of BRCA1 was expressed as a percentage, which was calculated as follows: 100× amount of labelled DNA-binding protein (dpm) that bound specifically to antibodies raised against BRCA1/amount of whole labelled DNA-binding protein (dpm) purified by heparin affinity chromatography.
All data are expressed as means±SD of three assays.
[b] $p<0.05$, when compared to control (Wilcoxon's test).

4. Conclusions

We carried out the purification of BRCA1 protein from cell extracts using a BioCAD Sprint HPLC system, following heparin purification and isolation of the immune complex BRCA–anti-BRCA1 by protein A chromatography. The internal labelling of cells allowed us to quantify the BRCA1 proteins. Here, this technique was set up with breast cancer cell lines that were treated with NaB, but it will be extended to the quantification of BRCA1 expression by comparison with normal breast and tumour breast tissues after iodine labelling of proteins.

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