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Presence of BRCA1 and BRCA2 proteins in human milk fat globules after delivery

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Abstract

We evaluated BRCA1 and BRCA2 oncosuppressor protein expression in 26 milk samples in women just after delivery. The quantification of BRCA1 and BRCA2 proteins was performed in isolated milk fat globules using an affinity chromatography strategy. The amounts of BRCA1 and BRCA2 proteins were found to be similar. We explained the presence of BRCA1 and BRCA2 proteins in human milk fat globules by the fact that they are formed by exocytosis of lipids from epithelial cells of the mammary gland and are enveloped by plasma membrane from the apical part of the milk-secreting cells. This raises the possibility that BRCA1 and BRCA2 proteins are a protective response to proliferation and play a possible role in newborn nutrition.

Keywords: Oncosuppressor; BRCA1; BRCA2; Quantification of protein; Human milk fat globule; Delivery; Affinity perfusion chromatography

1. Introduction

Current evidence strongly supports a role for the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, in both normal development and carcinogenesis. A main characteristic of the in vivo pattern of *BRCA1* and *BRCA2* expression is that these tumor suppressor genes are expressed at maximal levels in rapidly proliferating cells. This is well illustrated during mammary gland development wherein the expression of *BRCA1* and *BRCA2* is induced in rapidly proliferating cellular compartments undergoing differentiation, such as terminal end buds during puberty and developing alveoli during pregnancy [1]. In situ hybridization performed on the mammary glands of mice revealed that *Brca1* and *Brca2* mRNA levels are both markedly upregulated early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli

Abbreviations: HMFG, human milk fat globule(s)

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[2–5]. This upregulation of *Brca1* and *Brca2* expression occurs preferentially in developing alveoli as compared with adjacent epithelial ducts, consistent with patterns of proliferation [3,6]. These authors have also reported in mice, that *Brca1* and *Brca2* expression levels decline late in pregnancy, reaching their lowest point during lactation and early postlactational regression, though expression levels remain above background even during these stages of development [1–5]. Following these results, we investigated the quantification of BRCA1 and BRCA2 expression in milk fat globules in women just after delivery.

2. Materials and methods

2.1. Patients

Twenty-six women were delivered of live-born infants of 37–42 completed weeks' gestation between 3 April 2000 and 29 June 2000. Milk samples (10 ml) ranging from day 2 to day 9 after delivery were collected with a breast pump.

2.2. Isolation of human milk fat globules

Human milk fat globules (HMFG) were isolated according to modified methods [7,8]. Fresh human milk was centrifuged at $10\,000\times g$ for 30 min. The cream, which floated to the top of the tube, was recovered and suspended in 0.01 M Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$ and 0.28 M sucrose. After homogenization, this suspension was recentrifuged at $10\,000\times g$ for 30 min, and the washed cream (i.e. the HMFG) was resuspended in 0.05 M Tris–HCl buffer, pH 7.5 for 1 h at room temperature. A centrifugation at $10\,000\times g$ for 30 min was effected. HMFG were suspended in ice-cold 0.02 M Tris–HCl buffer, pH 8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Nonidet P-40. The sample was sonicated for 30 s. After 1 h incubation in ice, a centrifugation was done at $20\,000\times g$ for 45 min at 4°C. The supernatant containing the solubilized HMFG proteins was removed and clarified again with a centrifugation at $20\,000\times g$ for 30 min at 4°C to eliminate the remaining triglycerides.

2.3. Quantification of BRCA1 and BRCA2 proteins

Proteins were radioiodinated with ^{125}I according to the lactoperoxidase procedure [9]. To the removed particulate matter (1 ml) were added lactoperoxidase (50 μg/ml), glucose oxidase (25 μg/ml) and 0.5 mCi of ^{125}I. The reaction was set off by adding D-glucose (250 μg/ml). After 10 min at room temperature, the reaction was stopped by adding tyrosine (20 μg/ml). The labeled proteins of the supernatant were separated from the nonreactant iodide by gel filtration on a Sephadex G-25 column (Pharmacia Biotech). Elution was performed with phosphate buffered saline (PBS). The radioactivity of each eluted fraction (0.5 ml) was measured in a crystal γ-counter (Packard, Meriden, CT, USA). Eluted fractions with the highest radioactivity obtained in the front of the elution were pooled for subsequent quantification of BRCA1 or BRCA2 oncosuppressor proteins.

Then, the previously pooled fractions were poured onto a POROS 20 HE (heparin) column (PerSeptive Biosystems, Framingham, MA, USA) which is known to have a great affinity for DNA-binding proteins and *BRCA1* and *BRCA2* oncosuppressors belong to them. 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. The flow rate was 5 ml/min with a Biocad Sprint high-performance liquid chromatography system (PerSeptive Biosystems) equipped with a fraction collector (Gilson, Middelton, WI, USA). Detection of proteins was performed at 280 nm. The 0.5 ml fractions containing DNA-binding proteins were collected, the radioactivity was measured in a crystal γ-counter and pooled. The collected sample was divided into two equal parts. In one part, radiolabeled BRCA2 proteins were immunoprecipitated with a 30 min incubation at 37°C, by the addition of 5 μg of anti-BRCA2 polyclonal antibodies (66076E, Pharmingen, San Diego, CA, USA), which recognizes epitopes between amino acids 2586 and 2600 of human BRCA2. In the other part, radiolabeled BRCA1 proteins were immunoprecipitated with anti-BRCA1 polyclonal antibodies (66036E, Pharmingen), which recognize epitopes between amino acids 2 and 20 of human BRCA1.

The two immune complexes (BRCA1/anti-BRCA1 antibodies and BRCA2/anti-BRCA2 antibodies)

were isolated separately by chromatography onto POROS A containing protein A medium. Elution was performed with 0.1% (v/v) 12 mM HCl/0.15 M 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 below. Protein A affinity chromatography by means of immune complex elution gave the amount of DNA-binding proteins that bind specifically to anti-BRCA1 or anti-BRCA2 antibodies, and the ratio was calculated as follows: (activity (dpm) of BRCA1 or BRCA2 DNA-binding proteins that bound specifically to the anti-BRCA1 or anti-BRCA2 antibodies/activity (dpm) of whole labeled DNA-binding proteins eluted from heparin column) $\times$ 100 [10].

2.4. Statistical analysis

All data are the means $\pm$ S.D. of all experiments. All statistical analyses were performed using Student's *t*-test. One-tailed *P* values < 0.05 were considered statistically significant.

2.5. Western blot analysis

HBL 100 cells (1×10^7) were lysed in 1 ml of lysis buffer (20 mM Tris–HCl, pH 8, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, leupeptin 10 μg/ml, aprotinin 10 μg/ml, 1 mM NaF), sonicated for 30 s and kept at 4°C for 30 min, then centrifugation was performed at 12 000 $\times g$ for 3 min and the supernatant was collected. Protein assay was performed (Bio-Rad, Munich, Germany). One hundred and fifty micrograms of lysate were denatured for 10 min in 200 μl of Laemmli's buffer 2$\times$ (100 mM Tris–HCl pH 6.8, 200 mM dithiothreitol, 0.4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol) at 55°C for BRCA2 [11] and 90°C for BRCA1, separated by 5% SDS–PAGE.

For Western experiments, the electrophoresed proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), 2 h at room temperature. After incubation for 1 h in blocking buffer (PBS, 0.1% Tween, 5% dry milk), the membranes were probed for 2 h by the addition of the anti-BRCA1 (66036E) (0.5 μg/ml) or anti-BRCA2 (66076E) (4 μg/ml) primary antibody, followed by three washings of 10 min in PBS, 0.1% Tween. The membrane was then incubated for 1 h with peroxidase-conjugated second antibody (anti-IgG-HRP, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) (0.2 μg/ml) in PBS, 0.1% Tween, 1% dry milk, washed three times for 15 min in PBS, 0.1% Tween, followed by developing with an enhanced chemiluminescence mixture (Western blotting luminol reagent, sc-2048; Santa Cruz Biotechnologies) and exposed to Amersham Hyperfilm ECL film.

2.6. Competition with the relevant antigenic peptide

The competition level was also evaluated by POROS affinity chromatography, as described previously for BRCA1 and BRCA2 protein determination. After isolation of DNA-binding proteins by affinity chromatography on a POROS 20 HE column, radioactive fractions were separated into two equal parts. To one part were added 5 μg of anti-BRCA1 polyclonal antibodies (66036E), raised against amino acids 2–20 of human BRCA1, or anti-BRCA2 polyclonal antibodies (66076E) elicited against amino acids 2586–2600 of human BRCA2, and after incubation, the immune complex was isolated on a POROS A column. To the other part were respectively added anti-BRCA1 polyclonal antibodies previously incubated for 1 h with 10 μg of a synthetic peptide corresponding to amino acids 1–100 of human BRCA1 (sc:4255; Santa Cruz Biotechnologies) or anti-BRCA2 polyclonal antibodies previously incubated for 1 h with 5 mg of the relevant peptide corresponding to amino acids 2586–2600 of human BRCA2 protein (Genosphere Biotechnologies, Paris, France). Quantification of the immune complex was performed. Elution profiles obtained in the presence or absence of peptide for these two antibodies were plotted on a chart to evaluate the displacement of the equilibrium.

3. Results

3.1. Characterization of anti-BRCA1 and anti-BRCA2 antibodies in HBL 100 by Western blot analysis

To examine the specificity of the used anti-BRCA1 and anti-BRCA2 antibodies, HBL 100 protein extracts were used for the determination of the human BRCA1 and BRCA2 proteins by Western blotting (Fig. 1). The rabbit polyclonal antibodies (66036E) which may predominantly recognize the amino-terminus of human BRCA1 protein were used to probe the blots and they were able to recognize the 220 kDa BRCA1 form in a breast carcinoma cell line (HBL 100). The rabbit polyclonal antibodies (66076E) which may recognize the central portion of BRCA2 were used to probe the blots with a mammary carcinoma cell lines (HBL 100) and the human BRCA2 protein was detected at 384 kDa.

Nevertheless, for these two polyclonal antibodies no cross-reaction was seen with other proteins.

3.2. Quantification of BRCA1 and BRCA2 proteins in milk fat globules

Using a quantitative method, we detected in HMFG among 26 fresh milk samples an amount of BRCA1 protein of $1.87 \pm 0.93\%$ and an amount of BRCA2 of $1.65 \pm 0.88\%$. These results are expressed in Fig. 2. The difference between the BRCA1 and BRCA2 levels was not significant ($P = 0.4$).

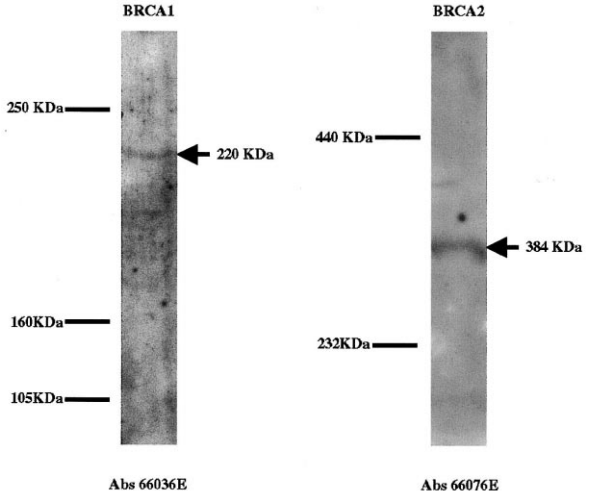

Fig. 1. Expression of BRCA1 and BRCA2 proteins in a breast tumor cell line (HBL 100). One hundred and fifty micrograms of proteins were analyzed by SDS–PAGE, transfered and probed respectively with antibodies (Abs) against BRCA1 (66036E) and BRCA2 (66076E) as primary antibody and subsequently with HRP-labeled secondary antibody.

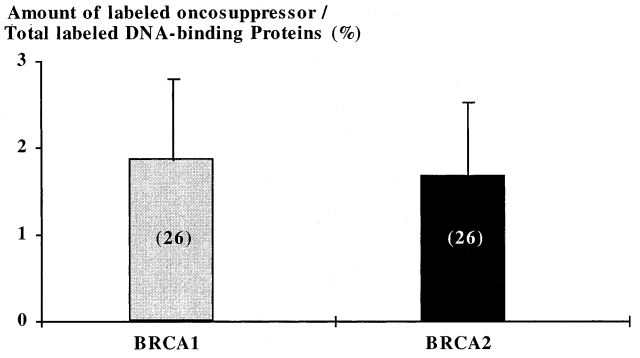

Fig. 2. Amount of BRCA1 and BRCA2 proteins found in human milk fat globules isolated from 26 women's fresh milk collected from day 2 to day 9 after delivery. On the *y*-axis, the amounts of BRCA1 and BRCA2 obtained after a protein A POROS column are expressed as a percentage, i.e. the ratio of the amount of labeled DNA-binding proteins that bound specifically to the polyclonal antibodies raised against BRCA1 or BRCA2 to the amount of total labeled DNA-binding proteins purified on a heparin POROS column. The difference between the BRCA1 and BRCA2 levels was not statistically significant ($P = 0.4$, Student's *t*-test).

3.3. Displacement of the equilibrium with the relevant antigenic peptide

By displacement of the equilibrium with the relevant antigenic peptide during immune complex formation, we demonstrated the specificity of the reac-

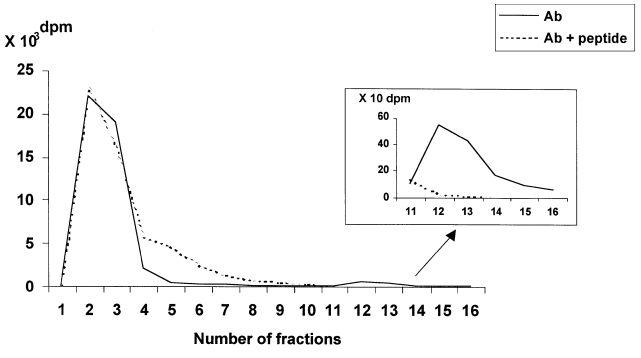

Fig. 3. Equilibrium displacement of the immune complex by a synthetic peptide (amino acids 1–100 of BRCA1) with anti-BRCA1 (66036E) antibodies elicited against amino acids 2–20 of BRCA1.

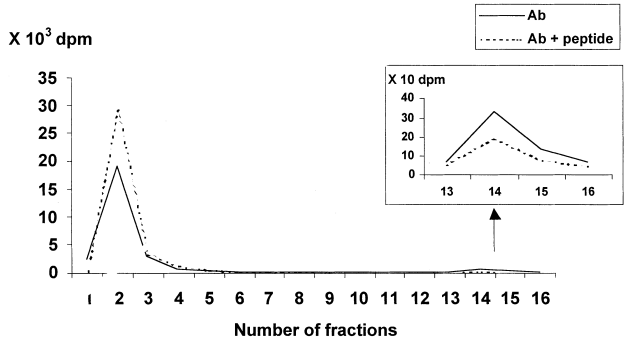

Fig. 4. Equilibrium displacement of the immune complex by a synthetic peptide (amino acids 2586–2600 of BRCA2) used to produce anti-BRCA2 (66076E) antibodies.

tion due to the anti-BRCA1 polyclonal antibody 66036E by competition assay with the synthetic peptide corresponding to amino acids 1–100 of human BRCA1 (Fig. 3). In Fig. 4, we demonstrate the displacement of the equilibrium corresponding to BRCA2 antigens and anti-BRCA2 polyclonal antibody 66076E by the synthetic peptide corresponding to epitopes between 2586 and 2600 of human BRCA2 used as immunogen [12].

4. Discussion

The staining specificities of 66036E antibodies to BRCA1 and 66076E antibodies to BRCA2 were ascertained by Western blotting, a 220 kDa band and a 384 kDa band, respectively, being detected for the HBL 100 breast cell line.

We have carried out the quantification of BRCA1 or BRCA2 proteins from cell extract by a strategy of affinity chromatography. The linearity and reproducibility of this quantification have been previously tested for measuring class II HLA antigen amounts [13,14]. This quantification was lately adapted to BRCA1 glycoproteins [15,16]. Now, we used a new faster method with perfusion chromatography. Two successive affinity chromatographies were used: (i) a heparin affinity column for the first purification of all DNA-binding proteins from cell extract because BRCA1 and BRCA2 proteins possess a ring finger and are implicated in transcription [17,18], (ii) a protein A affinity column, just after immunoprecipitation with anti-BRCA1 or anti-BRCA2 antibodies to quantify specifically BRCA1 or BRCA2 proteins from all the purified DNA-binding proteins previously obtained [10,19].

Furthermore, the specificity of this procedure for BRCA1 and BRCA2 oncosuppressor protein purification has been verified here by using respective relevant antigenic peptides added during the formation of the immune complex (^{35}S-BRCA1 proteins/anti-BRCA1 antibodies (66036E) or ^{35}S-BRCA2 proteins/anti-BRCA2 antibodies (66076E)) in MCF7 cells. So, we demonstrated the displacement of the respective immune complex equilibrium during elution on a protein A POROS column.

Therefore, the patterns of BRCA1 and BRCA2 expression are similar. This can be explained by the fact that these proteins may function in a common pathway of tumor suppression [20].

In addition, we previously reported the presence of BRCA1 and BRCA2 proteins by immunohistochemistry in HMFG [21]. These analyses were performed on two mammary gland samples collected from children who respectively died suddenly at the age of 4 weeks, when the mammary gland is active after birth and may continue to grow and secretes milk, and at the age of 5 months, when newborn breast development and milk secretion begin to regress [22], and in a lactating hamartoma of the breast from an 8 month pregnant woman. Using several antibodies elicited against BRCA1 and BRCA2, we reported that ducts of lobules in the mammary gland of the young child of 4 weeks contained milk secretion with BRCA1 and BRCA2 expression in the cytoplasm of HMFG as well as the lactating hamartoma of the breast. In contrast, tissue sections of the older child's mammary gland, at the age of 5 months when milk secretion has stopped, were devoid of HMFG expressing BRCA1 and BRCA2 proteins.

In women, we explained the presence of BRCA1 and BRCA2 proteins in these HMFG by the fact that they are formed by exocytosis of lipids from epithelial cells of the mammary gland, and are enveloped by plasma membrane from the apical part of the milk-secreting cells [23]. Similarly, this hypothesis might explain the presence of BRCA1 and BRCA2 proteins in the HMFG of the secreted milk. Thus, HMFG provide an excellent source of easily accessible material, including BRCA1 and BRCA2 proteins, from the mammary gland epithelial cells.

These interesting observations are in agreement

with our previous results and demonstrated the presence of BRCA1 and BRCA2 proteins in the human milk collected from women 2–9 days after delivery. This raises the possibility that the induction of BRCA1 and BRCA2 expression is a protective response to proliferation and addresses the question of the possible role of BRCA1 and BRCA2 in newborn nutrition.

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