Short Communication

Resveratrol increases BRCA1 and BRCA2 mRNA expression in breast tumour cell lines

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The phytochemical resveratrol, found in grapes, berries and peanuts, has been found to possess cancer chemopreventive effects by inhibiting diverse cellular events associated with tumour initiation, promotion and progression. Resveratrol is also a phyto-oestrogen, binds to and activates oestrogen receptors that regulate the transcription of oestrogen-responsive target genes such as the breast cancer susceptibility genes BRCA1 and BRCA2. We investigated the effects of resveratrol on BRCA1 and BRCA2 expression in human breast cancer cell lines (MCF7, HBL 100 and MDA-MB 231) using quantitative real-time RT–PCR, and by perfusion chromatography of the proteins. All cell lines were treated with 30 μM resveratrol. The expressions of BRCA1 and BRCA2 mRNAs were increased although no change in the expression of the proteins were found. These data indicate that resveratrol at 30 μM can increase expression of genes involved in the aggressiveness of human breast tumour cell lines.

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Resveratrol is a natural phytoalexin compound found in grapes and other food products. It has been found to possess oncopreventive activity by inhibiting ribonucleotide reductase (Fontecave et al, 1998) and cellular events associated with cell proliferation and tumour initiation, promotion and progression (Jang et al, 1997; Mbonyebyi et al, 1998). Resveratrol is a phyto-oestrogen, binding to and activating the oestrogen receptors that regulate the transcription of oestrogen-responsive target genes (Gehm et al, 1997) by either binding directly to DNA, at oestrogen response element (EREs), or by interacting with other transcription factors, for example, Sp1 (Sun et al, 1998), bound to their cognate sites on DNA (Bowers et al, 2000). Others have shown that steady-state BRCA1 mRNA levels are elevated in response to oestrogens in human breast cancer cells, and that BRCA2 expression is also regulated by oestrogens in human breast cancer cell lines (Gudas et al, 1995; Spillman and Bowcock, 1996).

BRCA1 and BRCA2 are breast cancer susceptibility genes: inheritance of one defective copy of either of the two genes predisposes individuals to breast, ovarian and other cancers. The contribution of these genes to the pathogenesis of breast cancer is still unclear. No sporadic breast tumours have been shown to harbour mutations in the coding sequence of BRCA1 or BRCA2 (Miki et al, 1994; Foster et al, 1996). In contrast to normal breast epithelial cells, BRCA1 mRNA levels in tumours appeared to be downregulated by methylation (Dobrovic and Simpfendorfer, 1997), while BRCA2 showed significant overexpression in sporadic breast cancers (Bieche and Liderrea, 1999).

Here, we studied the effects of resveratrol on the expression of BRCA1 and BRCA2 in human breast cancer cell lines at the transcription level using quantitative real-time reverse transcription (RT)–PCR, and at the translation level by perfusion chromatography of the proteins.

MATERIALS AND METHODS

Cell cultures

MCF7 (Soule et al, 1973), MDA-MB 231 (Cailleau et al, 1974) and HBL 100 (Ziche and Gullino, 1982) cell lines were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured, respectively, in RPMI 1640, Leibovitz’s L15 and McCoy’s 5a medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FBS. Cells were grown in a humidified incubator with 5% carbon dioxide (except for MDA-MB 231 without CO₂) at 37°C. Insulin (0.04 U ml⁻¹) was added for MCF7 culture medium.

The ER status was checked in cell lines by immunocytochemistry with Centre Jean Perrin’s anatomopathologist. MCF7 were found ER +/−β+/+, HBL 100 ER −/−β+ and MDA-MB-231 ER −/−β+.

Resveratrol treatment of cells and flow cytometry analysis

MCF7, MDA-MB 231 and HBL 100 cells were maintained in medium supplemented with 10, 30 or 50 μM trans-resveratrol (Sigma Chimie, St Quentin Fallavier, France) in DMSO for treated cells. A cell control was performed with DMSO. Cells were collected after 24, 48 or 72 h by trypsinisation and the DNA content was assessed by flow cytometry according to Krishan’s (1975) method. Each experiment was performed in triplicate.

Radiolabelling of cellular proteins

For cellular protein labelling, the cells were fed with 5 ml of medium supplemented with 100 μCi [³⁵S] methionine (1000 Ci ml⁻¹; Amersham International, Bucks, UK) and incubated for 20 h at
37°C in a 5% CO₂ atmosphere. Metabolic radiolabelling was stopped by adding 10 ml cold PBS and cells were gently washed twice with PBS at 4°C. Labelled cells were solubilised in 750 μl of 0.1 M Tris-HCl pH 7.1 containing 0.5% Nonidet P40 (NP 40; Boehringer Mannheim, Germany), sonicated for 2 min in ice and incubated at 4°C for 30 min. The insoluble material was removed by ultracentrifugation at 30 000 g for 30 min.

**Purification of DNA-binding proteins by affinity chromatography**

The NP 40 cell lysates were loaded onto a POROS 20 HE (heparin) media column (PerSeptive Biosystems, Framingham, MA, USA). 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⁻¹ with a BioCAD Sprint high-performance liquid chromatography system (PerSeptive Biosystems, Framingham, MA, USA) equipped with a fraction collector (Gilson, Middleton, WI, USA). Fractions (0.5 ml) containing DNA-binding proteins were collected and pooled. Radioactivity was measured by adding 10 μl of the collected fractions to 5 ml of scintillation cocktail (Packard Ready Safe) and counting.

**Isolation of BRCA1 and BRCA2 by affinity chromatography**

BRCA1 or BRCA2 were immunoprecipitated from the previous eluate by addition of 16 μg anti-BRCA1 polyclonal antibodies (556445; GeneTex, San Antonio, TX, USA) or anti-BRCA2 polyclonal antibodies (C-19; Santa Cruz Biotechnology) with a 5 M NaCl from 0.1 to 1 M in 20 M MES pH 5.5. The flow rate was 5 ml min⁻¹ with a BioCAD Sprint high-performance liquid chromatography system (PerSeptive Biosystems, Framingham, MA, USA) equipped with a fraction collector (Gilson, Middleton, WI, USA). Fractions (0.5 ml) containing DNA-binding proteins were collected and pooled. Radioactivity was measured by adding 10 μl of the collected fractions to 5 ml of scintillation cocktail (Packard Ready Safe) and counting.

**RNA extraction and cDNA synthesis**

Total RNA was isolated using TRIZOL® (Gibco BRL, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA (1 μg) was used for the synthesis of first strand cDNA using the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer’s instructions.

**Determination of BRCA1 and BRCA2 mRNA using real-time quantitative RT-PCR**

For BRCA1 and BRCA2 expression analysis, probes and primers were designed so that they overlapped splice junction, thereby avoiding the potential amplification of genomic DNA. The sequence of forward primers, TaqMan® probes and reverse primers were, respectively, for BRCA1-exons (ex) 23/24 amplification: 5’-55646CAGAGAGAAGGCTGTCCATC5’-3’, 5’-5588AATTGAAGCCAAGTGCTGCCATCTC5’-3’, 5’-55646CAGACAGGCACACCTG5’-3’; for BRCA1-ex 11/12 amplification: 5’-4173AAGGAGGAAGAGCTTGAAG18S rRNA probe, primers plus TaqMan® probe. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed with 40 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, Foster City, CA, USA). Relative gene expression was determined using the comparative Ct (threshold cycle) method, which consists of the normalisation of the amount of target gene copies to an endogenous reference gene (18S rRNA), designated as the calibrator (Fink et al., 1998). The level of BRCA1-ex 23/24, BRCA1-ex 11/12, BRCA2-ex 12/13 or BRCA2-ex 26/27 mRNA expression in each treated cell line was then normalised to the result obtained in the untreated cells. The amount of target, normalised to the 18S rRNA endogenous reference is given by the formula: 2⁻ΔΔCt. To guarantee the reproducibility of mRNA determination, two independent total RNA extractions were performed. Two independent RTs were carried out for one RNA extraction, while only one was performed for the second extraction. Each RT was analysed in triplicate and expressed as a mean ± s.d. (Favy et al., 2000).

**RESULTS**

Cell proliferation by DNA content analysis

Treatments with 10, 30 and 50 μM resveratrol were studied by flow cytometry after different times of exposure (24, 48 and 72 h) in MCF7, MDA-MB 231 and HBL 100 breast tumour cell lines. After exposure, all three cell lines were blocked in S phase. At 48 h, the percentage of cells in S phase was considerably increased after treatment with 30 μM resveratrol, whereas the percentage of cells in G1 phase was decreased. It is well known that resveratrol treatment causes an accumulation of cells in S phase (Ragione et al., 1998).

**Analysis of the impact of resveratrol on BRCA1 and BRCA2 mRNA level**

We compared the levels of BRCA1 mRNA after treatment with 30 μM resveratrol using two different BRCA1 Taqman probes. The probe, BRCA1-ex 23/24, was used to quantify all BRCA1 mRNA species together because no alternative splicing of ex 23 has been described (Wilson et al., 1997). The BRCA1-ex 11/12 probe was used to estimate the level of mRNA containing ex 11, because Thakur et al. (1997) described the isolation and expression of two BRCA1 cDNAs, one of them is a splicing variant generated by alternative splicing of ex 23, because no alternative splicing has been observed so far, at this site. This BRCA1-ex 12/13 probe was used to estimate the level of mRNA containing ex 12, because Bicéhe and Lidereau (1999) identified an alternatively spliced BRCA2 transcript that was widely expressed in all normal tissues examined. This A12-BRCA2 transcript was found to be overexpressed in steroid receptor-negative breast tumour tissues, suggesting that dysregulation of the A12-BRCA2 isoform may contribute to progression in human breast cancer.
Expression of BRCA1 and BRCA2 mRNA in resveratrol-treated cells was normalised to their expression levels in untreated cells, normalised to 1. As shown in Figure 1, the expression of each BRCA1 mRNA species (BRCA1-ex 23/24 and BRCA1-ex 11/12) was increased in all three cell lines after treatment.

We also observed an increase of BRCA2-ex 26/27 and BRCA2-ex 12/13 mRNA in all three cell lines (Figure 2). All mRNA determinations with treated cells were expressed as mean ± s.d. with a student’s t-test.

Analysis of the impact of resveratrol on BRCA1 and BRCA2 protein synthesis

The amount of BRCA1 or BRCA2 protein was expressed as the following ratio: d.p.m. of labelled DNA-binding proteins bound specifically by the antibodies to BRCA1 or BRCA2/d.p.m. of total DNA-binding proteins purified by heparin chromatography (Table 1). BRCA1 and BRCA2 protein expression was not modified 48 h after treatment with 30 μM resveratrol.

DISCUSSION

We studied the effect of resveratrol, a natural polyphenolic compound found especially in black grapes, peanuts, berries and Itadori tea (Burns et al, 2002), on the expression of the BRCA1 and BRCA2 genes in the human breast cancer cell lines MCF7, MDA-MB 231 and HBL 100. We chose 48 h exposure to 30 μM resveratrol because this treatment was shown to increase significantly the number of cells blocked in S phase (Park et al, 2001). It is well known that BRCA1 and BRCA2 reach their maximal levels in late G1 and S phases in normal and tumour-derived breast epithelial cells (Vaughn et al, 1996; Bertwistle et al, 1997).

Moreover, the effects of 30 μM resveratrol correlated with results from others also studying resveratrol in cell lines (Ragione et al, 1998; Hsieh et al, 1999; Igura et al, 2001). In addition, we used a lower dose of 10 μM resveratrol in the three cell lines (data not shown), but that did not show any accumulation of cells in S phase and consequently no significant alteration was found for BRCA1, BRCA2 mRNA and BRCA1, BRCA2 proteins.

Then, the quantification of BRCA1 and BRCA2 mRNA was performed with real-time quantitative RT–PCR. This method allowed us to compare the effect of resveratrol by comparison with untreated cells, which were normalised to one. In MCF7, BRCA1 mRNA increased 2.5-fold and BRCA2 mRNA four-fold after 48 h in the presence of 30 μM resveratrol. Similarly, in MDA-MB 231, BRCA1 mRNA increased three-fold and BRCA2 mRNA two-fold while in HBL 100, BRCA1 mRNA increased 2.6-fold and BRCA2 mRNA 1.9-fold.

The effect of resveratrol on BRCA1 and BRCA2 mRNA in human breast cancer cell lines could be explained by its different properties. First, it is structurally similar to the synthetic
oestrogen, diethylstilbestrol, which exhibits oestrogenic activity. Gehm et al (1997) reported that resveratrol inhibited the binding of labelled oestradiol to the oestrogen receptor and activated transcription of oestrogen-responsive reporter genes transfected into human breast cancer cells. This transcriptional activation was oestrogen receptor-dependent, required an ERE in the reporter gene, and was inhibited by specific oestrogen antagonists. Moreover, resveratrol showed oestrogen agonist activity in MCF7 cells by activating the expression of two oestrogen-responsive genes, such as progesterone receptor (PR) and p52 genes (Jang and Pezzuto, 1999). And, we also found elsewhere an increase in BRCA1 and BRCA2 proteins quantified using two successive perfusion affinity chromatographies. Resveratrol had no effect on the level of either BRCA1 or BRCA2. We displayed the specificity of the anti-BRCA1 and anti-BRCA2 polyclonal antibodies by competition with the synthetic peptides used to generate the antibodies. A complete displacement of the equilibrium was obtained in each case demonstrating the specificity of the antibodies (data not shown) (Hizel et al, 1999; Vissac et al, 2001, 2002).

We found an increase in BRCA1 and BRCA2 mRNA after treatment with resveratrol in breast cancer cell lines but no effect at the protein level. These result suggest an uncoupling between mRNA and protein levels under these conditions. A similar uncoupling of BRCA1 mRNA and protein levels was detected in synchronised populations of immortalised MCF10 and 184B5 cells proliferation. In these two cell lines, BRCA1 mRNA level was tightly regulated during the cell cycle while BRCA1 protein level remained constant. Thus, it has been shown that BRCA1 mRNA is highly expressed in late G1 phase of the cell cycle, whereas conditions that lead to cell cycle exit downregulate the BRCA1 mRNA (Gudas et al, 1995, 1996; Jin et al, 1997). There are several possible explanations for discrepancies between mRNA and protein level under different physiological conditions. BRCA1 and BRCA2 might be post-transcriptionally regulated with effects on the translational activity as well as the stability of BRCA1 and BRCA2 mRNA (Wickens et al, 1997). Alternatively, the level of BRCA1 mRNA in cells may be translationally regulated by other cellular proteins or antisense RNA transcripts. Precedents for both mechanisms of regulation exist for other genes. Interestingly, many developmentally regulated genes exhibit regulation at the level of mRNA translation (Hentze, 1995). More recently, Blagosklonny et al (1999) demonstrated a substantial role for proteolysis in regulating BRCA1 steady-state protein levels in several cell lines. Degradation by a cathepsin-like protease in fine balance with BRCA1 transcription is responsible for maintaining the low steady-state level of BRCA1 protein seen in many cancer cells. At the opposite of oestrogen, which increased the level of mRNAs and proteins of the two oncosuppressors BRCA1 and BRCA2, the resveratrol seems to play a role in one of the different pathways of previous mechanisms.

To better understand the effects of resveratrol on BRCA1 and BRCA2 oncosuppressor genes in mammary gland, we will use cDNA microarrays to study gene-expression profiles of proteins interacting with BRCA1 and BRCA2 after phytochemical treatment and it would be helpful to study proteomics.

In conclusion, the present study demonstrates that 30 μM resveratrol can increase expression of the BRCA1 and BRCA2 oncosuppressors, involved in the aggressiveness of human breast cancer cell lines.

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Table 1 Amount of BRCA1 and BRCA2 proteins expressed by MCF-7, MDA-MB 231 and HBL 100 cells after treatment with 30 μM resveratrol and 48 h exposure.

| Cell lines          | BRCA1 (%) | BRCA2 (%) |
|---------------------|-----------|-----------|
| MCF-7 Untreated controls | 0.58±0.01 | 1.00±0.03 |
| 30 μM resveratrol   | 0.51±0.02* | 0.80±0.16* |
| MDA-MB231 Untreated controls | 1.65±0.34 | 0.48±0.12 |
| 30 μM resveratrol   | 1.80±0.32  | 0.41±0.02  |
| HBL100 Untreated controls | 0.95±0.04 | 0.90±0.09  |
| 30 μM resveratrol   | 1.15±0.18  | 1.00±0.03  |

BRCA1 and BRCA2 proteins were obtained by DNA-binding protein purification, specific immunoprecipitation with anti-BRCA1 (SS6445) or anti-BRCA2 (C-19) antibodies and protein A affinity chromatography and expressed in percentage calculated as follows: 100 x d.p.m. DNA-binding proteins bound specifically to antibodies raised against BRCA1 or BRCA2/d.p.m. of total DNA-binding proteins purified on heparin affinity chromatography. All data are expressed as mean±s.e. of three assays (*), P<0.05, vs untreated controls (Student’s t-test).

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