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Resveratrol induces senescence-like growth inhibition of U-2 OS cells associated with the instability of telomeric DNA and upregulation of BRCA1

Marek Rusin *, Artur Zajkowicz, Dorota Butkiewicz

Department of Tumor Biology, Maria Skłodowska – Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, 44-101 Gliwice, Poland

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ABSTRACT

Resveratrol decreases cancer risk and improves health of laboratory animals. However, it can also promote genomic instability. Part of the beneficial activity of resveratrol may result from the activation of SIRT1 deacetylase. We examined how resveratrol influenced the growth of human cancer cell lines of different origin: osteosarcoma (U-2 OS) and lung adenocarcinoma (A549) and how it modulated the expression as well as the localization of key proteins, involved in DNA repair and cell cycle regulation. Resveratrol-induced growth arrest was associated with signs of stress-induced senescence. Differential expression of BRCA1, cyclin B1, pRb and p21 in U-2 OS and A549 cells indicates that resveratrol can engage various molecular mechanisms to arrest cell cycle progression. In subset of U-2 OS cells, the upregulated BRCA1 formed foci closely associated with WRN and the telomeric protein (TRF1). Moreover, resveratrol induced telomeric instability in U-2 OS cells and the activation of DNA damage signaling in both cell lines, manifested as the phosphorylation of histone H2AX at serine 139 and of p53 at serines 15 and 37. Our data are consistent with the hypothesis that resveratrol inhibits cell growth and induces senescence by altering DNA metabolism.

1. Introduction

The resveratrol (*trans*-3,5,4′-trihydroxystilbene) has been intensely studied since the discovery of its cancer protective activity (Jang et al., 1997). Its anti-cancer role was initially associated with the prevention of oxidative damage to DNA. Later, it was observed that resveratrol could inhibit the growth of cancer cell lines arresting them at various stages of the cell cycle. The stage apparently depended on the identity of the cell line (Sgambato et al., 2001; Delmas et al., 2006 and refs. therein).

The understanding of the mechanisms by which resveratrol induces the cell cycle arrest and protects against cancer is far from complete. The resveratrol-induced cell cycle inhibition was associated with the upregulation of the well-known, cell cycle arresting proteins, e.g., p53, p21 (reviewed by Signorelli and Ghidoni, 2005) suggesting that resveratrol generated cell stress by, e.g., direct or indirect DNA damage induction. The chronic treatment of p53-positive cancer cell lines in culture with resveratrol resulted in ATM-dependent senescence that was associated with the redox stress (Heiss et al., 2007). The comet assay showed that long treatment with resveratrol induced slight DNA damage in a dose and time-dependant manner (Quincozes-Santos et al., 2007). On the other hand, resveratrol did not generate point or frameshift mutations in *S. typhimurium* reverse mutation assay (Matsuoka et al., 2001), however, it induced genetic instability that was manifested by increased frequency of micronuclei (Schmitt et al., 2002) and sister chromatid exchanges (Matsuoka et al., 2001). These observations appear paradoxical considering the initial report of chemo-preventive role of resveratrol against cancer (Jang et al., 1997). Moreover, recent experiments performed on mice indicated that resveratrol improved health and increased survival of the animals kept on high-calorie diet (Baur et al., 2006). These results were consistent with conclusions of previous studies showing that resveratrol extended the lifespan of unrelated species (*S. cerevisiae*, *C. elegens*, and *D. melanogaster*). This extension depended on the presence of a Sir2 protein that deacetylates histones and other proteins (Wood et al., 2004). Thus, part of the protective role of resveratrol may result from its ability to enhance the activity of the human homolog of Sir2 protein–SIRT1 (Howitz et al., 2003). Previously, we showed that SIRT1 overexpression induced the relocalization of the fluorescent-labeled WRN protein from nucleoli to the nucleoplasm (Vaitiekunaite et al., 2007). WRN protects against cancer and slows the aging in humans. Individuals having no

* Corresponding author at: Department of Tumor Biology, Maria Skłodowska – Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, ul. Wybrzeże Armii Krajowej 15, 44-101 Gliwice, Poland. Tel.: +48 32 278 96 38; fax: +48 32 231 35 12.
E-mail address: rusinm@rocketmail.com (M. Rusin).

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functional WRN protein, due to the gene mutations, show premature aging symptoms starting from adolescence and increased risk for sarcomas and thyroid carcinomas, what results in early death (Kudlow et al., 2007).

The aforementioned observations prompted us to explore the biological and molecular consequences of the treatment of cancer cells with resveratrol. Considering our recent findings (Vaitiekunaite et al., 2007), we focused our attention on processes and molecules that are functionally related with the WRN protein.

2. Materials and methods

2.1. Cell culture, treatment, analysis of DNA content and clonogenic assay

The U-2 OS (human osteosarcoma, ATCC) and A549 (human lung adenocarcinoma, ATCC) cells were grown at 37 °C/5% CO_2 in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MI) supplemented with 10% FBS (fetal bovine serum, Gibco-Invitrogen, Carlsbad, CA) and penicillin–streptomycin solution (Sigma–Aldrich, St. Louis, MI).

Unless otherwise mentioned, the cells were treated with 50 μM resveratrol for the indicated number of hours. The 200 mM stock solution of resveratrol (Sigma–Aldrich, St. Louis, MI) was prepared in 95% ethanol. The control cells were treated with the relevant concentration (no more than 0.024%) of ethanol in culture medium. If the recovery was needed, the resveratrol containing medium was removed, the cells were washed in resveratrol-free medium and the cells were incubated in fresh medium for the indicated number of hours.

The cell cycle profile was obtained by FACS analysis (FACSCanto flow cytometer, Becton Dickinson, Franklin Lakes, NJ) of the attached cells after trypsinization, ethanol fixation, RNase treatment and propidium iodide (PI) staining.

The clonogenic assay was performed for the two cell lines (U-2 OS, A549) used in our study. The cells were seeded into 6-cm plates at 3% or 35% confluency (for control and resveratrol treatment, respectively). The pilot experiments showed that this difference in cell number was required due to active growth of control cells and quick growth inhibition of resveratrol treated cells. At the end of treatment we needed to have the comparable number of cells. Starting the next day, the cells on experimental plate were treated with 50 μM resveratrol for 96 h; the control cells were mock treated. Subsequently, the control and treated cells were trypsynized. Twenty five percent of control cells (to avoid overgrowing) and all of the treated cells (which stopped division and did not overgrow the dishes) were seeded into new plates and grown in fresh medium for 48 h. The cells were trypsynized, counted and 1000 cells were seeded into each of 6-cm plates containing 10 ml of fresh medium. After growing for 14 days, the cells were fixed with methanol: acetone and visualized with Giemsa stain. For each data point the large colonies on three plates were counted.

2.2. SA-β-gal staining and fluorescent in situ hybridization (FISH) of telomeric DNA

Senescence-associated β-galactosidase (SA-β-gal) staining of cells on glass slides (Lab-Tek II, NUNC, Roskilde, Denmark) was performed according to the method of Dimri et al. (1995). The fluorescent *in situ* hybridization (FISH) staining of telomeric DNA with the fluorescein-labeled PNA probe was performed using the kit and protocol provided by DAKO A/S (Glostrup, Denmark).

2.3. Telomeric DNA detection by Southern blotting

The DNA was isolated by genomic DNA purification kit (Fermentas, Vilnius, Lithuania) using triple chloroform extraction and ethanol precipitation. The quality of DNA was checked by agarose gel electrophoresis and was found to be very similar in control and in the treated cells. The telomeric DNA was detected by Telo*TAGGG* Telomere Length Assay kit (Roche Diagnostics, Mannheim, Germany), which uses chemiluminescent visualization of telomere-specific, digoxigenin-labeled hybridization probe. The procedure was performed using manufacturer's protocol. In short, 1 μg of DNA was digested with HinfI and RsaI restriction enzymes, separated on 0.8% agarose gel containing 0.9 μg/ml ethidium bromide in TAE buffer (pH 8.0) with molecular weight marker provided by manufacturer. After electrophoresis, the equal loading of DNA was check by UV-transilluminator. DNA was moved to the positively charged nylon membrane by capillary transfer in 20× SSC buffer and was bound to the membrane by UV-crosslinking. After hybridization, washing and detection, the bound telomeric probe was visualized using various exposures of the blot to the X-ray film.

2.4. Immunofluorescent staining

The cells growing on glass slides (Lab-Tek II, NUNC, Roskilde, Denmark) were washed with PBS, fixed in 3.7% formalin for 2 min at room temperature (Sigma–Aldrich, St. Louis, MI), washed with PBS and permeabilized by 0.5% Triton X-100 (Sigma–Aldrich, St. Louis, MI) in PBS for 10 min. After PBS washing, the cells were blocked in 5% BSA and 0.15% glycine in PBS at room temperature for 30 min and incubated for 2 h 15 min at room temperature with the relevant antibody diluted in a blocking solution. The following antibodies were used: rabbit polyclonal anti-WRN antibody (diluted 1:900, NB100-140; Novus Biologicals, Littleton, CO, USA or the equivalent antibody Ab-200, Abcam, Cambridge, UK), mouse monoclonal anti-BRCA1 antibody (D-9, diluted 1:65; SCB), goat polyclonal anti-TRF1 antibody (N-19, diluted 1:60, SCB), mouse monoclonal anti-phospho-Ser139 histone H2AX antibody (JBW301, diluted 1:500, Upstate-Millipore, Billerica, MA). After washing, the bound antibody was detected with fluorescein-conjugated anti-rabbit IgG or Texas Red-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA), diluted 1:400–1:300 and incubated at room temperature for 1 h. When the simultaneous staining of TRF1, BRCA1 and WRN proteins was performed, the following combination of secondary antibodies was used: anti-goat AMCA-conjugated IgG made in donkey (Chemicon-Millipore, Billerica, MA), anti-rabbit fluorescein-conjugated IgG made in donkey (Chemicon-Millipore), anti-mouse Texas Red-conjugated IgG made in horse (Vector Laboratories, Burlingame, CA). After the second antibody incubation, the cells were washed again in PBS and embedded in Vectashield with DAPI (Vector Laboratories). DAPI was not added when the anti-goat AMCA-conjugated secondary antibody was used. The cells were observed with the Nikon Eclipse E800 fluorescent microscope through either 20× or 100× fluorescent microscope objective (numerical aperture, NA = 1.3, immersion oil). The pictures were captured using Hamamatsu Color Chilled 3CCD camera. The following filter settings were used to observe the fluorophores: DAPI and AMCA—EX 330–380 nm, DM 400 nm, BA 420 nm; fluorescein—EX 450–490 nm, DM 505 nm, BA 520 nm; Texas Red—EX 510–560 nm, DM 575 nm, BA 590 nm.

2.5. Western blotting

The cells growing on 10-cm plates (NUNC, Roskilde, Denmark), treated with resveratrol or mock treated, were harvested by trypsinization. After PBS washing and centrifugation, the cell pellet was treated with ice-cold EC buffer (20 mM Tris pH 7.6, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 0.5% NP40, 2.5% glycerol) supplemented with a protease inhibitor cocktail: PMSF, pepstatin A, aprotinin and leupeptin. The suspension was incubated on ice for 10 min. Subsequently the samples were centrifuged at 310 × *g*, 4 °C for 10 min. The cytoplasmic fraction in the supernatant was discarded and the pellets enriched in cell nuclei were frozen at −70 °C. After thawing on ice, the pellets were lysed on ice for 20 min with RIPA buffer (0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) supplemented with the protease inhibitors. The lysates that were used to detect the phosphorylated forms of BRCA1 were prepared with buffers containing the phosphatase inhibitor cocktail 2 (Sigma–Aldrich). The lysates were cleared by centrifugation (14 000 rpm, 4 °C for 20 min), denatured and stored at −70 °C. Subsequently, 5–30 μg of protein lysate was separated on the 6% SDS-PAGE and electrotransferred onto the nitrocellulose membrane. To detect histone H2AX (total or phosphorylated at serine 139), the cells were lysed in IP buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl, 0.5% NP-40) with protease and phosphatase inhibitors. Subsequently, the aliquots of lysates for one gel lane were mixed (2:1) with loading buffer containing 6% SDS. After boiling, the lysated were loaded into wells of 12% SDS–polyacrylamide gel.

The following proteins were detected on the membranes with the antibodies indicated in parentheses: p53 (DO-1, SCB), p53-Ser15, p53-Ser37 (Cell Signaling Technology), WRN (Ab-200, Novus Biologicals), BRCA1 (D-9, SCB), cyclin B1 (D-11, SCB), PARP1 (F-2, SCB), pRb (mAB245, Millipore), $p21^{WAF1}$ (F-5, SCB), H2AX (rabbit antiserum, Upstate-Millipore), H2AX-Ser139 (JBW301, Upstate-Millipore). HSC70 loading control was detected by the B-6 antibody (SCB). The antibodies recognizing BRCA1 phosphorylated at serines 1423 (NB 100-226), 1457 (NB 100-227) or 1524 (NB 100-200) were from Novus Biologicals. All incubations with primary antibodies were performed overnight at 4 °C in a 5% skim milk solution in PBS with 0.1% Tween-20. The secondary antibodies were HRP-conjugated and detected by chemiluminescence.

3. Results

The study was started with assessing the biological consequences of resveratrol treatment of two cancer cell lines, U-2 OS and A549. The results of the dose–response experiment measuring resveratrol's influence on cell growth are shown in Fig. 1. At 50 μM concentration resveratrol significantly inhibited the growth of both cell lines. We used this concentration in subsequent experiments. Next, we explored the cell cycle distribution of resveratrol treated cells (Fig. 2). After 24-h treatment, the peak of cells with G2/M DNA content disappeared in both cell lines. Starting from 48 h, the majority of cells of both lines were in S-phase. Apparently, resveratrol slows the progression of the cells through the S-phase of the cell cycle, what suggests that it interferes with some aspects of DNA metabolism.

Subsequently, we observed the fate of resveratrol treated cells (50 μM, 96 h) that recovered in the resveratrol-free medium for 48 h. Based on the results of Heiss et al. (2007), we suspected that