Effects of resveratrol on topoisomerase II-α activity: induction of micronuclei and inhibition of chromosome segregation in CHO-K1 cells

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In recent years, a great interest has emerged in resveratrol (RSV) activity in the prevention of various pathologies including cancer. We recently showed that RSV is able to interfere with topoisomerase II-α (TOPO2) activity in cancer cells, thus inducing a delay in S-phase progression with concomitant phosphorylation of the histone H2AX. TOPO2 is mainly active in proliferating cells and is involved in the resolution of supercoiled DNA and chromosome segregation during mitosis. Here, we studied the effects of RSV in CHO-K1 cells concerning to chromosome damage and segregation as a consequence of TOPO2 inhibition. We show an increase in micronuclei and in polyploid and endoreduplicated cells due to incorrect chromosome segregation. Furthermore, since incomplete segregation can also affect the normal distribution of mitotic figures, we checked mitosis progression showing an increase in metaphase in relation to anaphase-telophase after RSV treatment. On the whole, our data show that RSV affects chromosome stability and segregation in proliferating cells, probably interfering with TOPO2 activity.

Introduction

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) (RSV) is a polyphenol found in grapes (Vitis vinifera), berries and peanuts and in medicinal plants such as Japanese knotweed (Polygonum cuspidatum) (1).

Scientific interest in RSV has continually grown since 1997, when it was first demonstrated to prevent carcinogenesis in mice (2). In recent years, this molecule has received considerable attention for its anti-inflammatory, anti-tumorigenic and antioxidant properties, as well as its ability to increase lifespan in lower organisms and improve general health in mammals (3). Particular attention has grown relatively to RSV ability in interfering with carcinogenic process, thus suggesting the potential use both as chemopreventive and therapeutic agent. In particular, many in vitro studies have been focused on anti-proliferative and pro-apoptotic effects through the activation of many intracellular targets such as tumour suppressors and cell cycle regulators (4). These actions together with the ability in activating DNA damage response via ATM/ATR are in line with the concept of RSV as a cancer chemopreventive agent (5,6). However, despite the large amount of data present in literature, several aspects of its mechanism of action remain unclear. Some clinical trials of either supplemented or dietary (e.g. grapes, grape juice, peanut butter) RSV are currently at various stages of completion. These trials are focused on resveratrol’s ability to produce beneficial effects on health and also on cancer patients as target population (7).

We have previously reported that RSV treatment of human glioblastoma cells induces a delay in cell cycle progression during S phase associated with an increase in histone H2AX phosphorylation (γH2AX) (8) that is a hallmark of DNA double strand breaks (DSBs) (9). Furthermore, with an in vitro assay of topoisomerase II-α (TOPO2) catalytic activity we showed that RSV is able to inhibit the ability of recombinant human TOPO2 to decatenate kDNA, so that it could be considered a TOPO2 inhibitor (8). Previously, Jo et al. (10) showed that polyphenolic fractions from grape cell culture are potent inhibitor of human TOPO2, utilising the same assay.

Recently, through a molecular modelling we showed that RSV binds at the TOPO2/DNA interface establishing several hydrogen bonds and as a result of this interaction it is able to stabilise cleavable complexes between DNA and TOPO2. We also found an increase of DSBs as measured through micronuclei analysis confirmed by an increase in the expression of phosphorylated form of ATM, Chk2 and H2AX (11).

TOPO2 is an essential enzyme maximally expressed in G2 and M phases of the cell cycle because it is involved in the final stages of DNA replication to facilitate chromosome untangling, condensation and mitotic segregation (12,13). Thus, TOPO2 can separate knotted and intertwined DNA molecules and its activity is required for chromosome condensation, decatenation of intertwined daughter DNA duplexes and centromere resolution (14,15). DNA and TOPO2 form a reversible, covalent complex, often referred to as the cleavage complex (13,16).

The levels of TOPO2 protein are particularly high in cancer cells due to their high proliferative rate and this behaviour has made TOPO2 the primary cellular target for a number of widely used antineoplastic drugs. In general, TOPO2 inhibitors are divided into two classes: poisons and catalytic inhibitors. TOPO2 poisons stabilise the cleavage complex, which may block DNA replication forks or transcriptional machinery and create DSBs (17). Catalytic inhibitors are a heterogeneous group of compounds that might interfere with the binding between DNA and the enzyme, stabilise non-covalent DNA-TOP2 complexes or inhibit ATP binding (18).

Studies using TOP2 catalytic inhibitors suggest that G2 cells monitor the catenation state of interwoven sister chromatids following DNA replication and actively delay progression into mitosis pending sufficient chromatid decatenation (19).

In this study, we analysed the effects of RSV treatment on the maintenance of genomic stability and chromosome segregation in Chinese Hamster Ovary (CHO) K1 cells. We performed the cytokinesis-block micronucleus assay (CBMN) accompanied by anti-kinetochore antibody staining (CREST staining) in order to investigate the induction of micronuclei containing or not containing a centromere (20). Furthermore, as it has been demonstrated that the clastogenic effect of a topo-poison could be reduced by the concomitant action of a topoisomerase inhibitor (21), we analysed the effect of combined treatment...
RSV plus ethidium bromide. Then we analysed the effects on chromosome segregation by detecting the presence of polyploid (POL) or endoreduplicated (ENDO) metaphases. Endoreduplication consists of two successive rounds of DNA replication without an intervening mitosis. The visible mitotic manifestation of previous endoreduplication is the presence of diplochromosomes, made up of four chromatids held together, instead of the two normally observed in metaphase chromosomes (22). Finally, we checked the presence of anaphase bridges during mitosis as a consequence of an incomplete decatenation (23).

Materials and methods

Cell culture and treatments

CHO-K1 cells were maintained in Ham’s F-10 medium supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin solution, 1% l-glutamine in a 37°C humidified incubator with 5% CO2. All reagents were purchased from Euroclone (Milan, Italy). Further, 10^5 cells were seeded on coverslips in 35-mm Petri dishes the day before the experiment for CBMN, mitosis analysis, immunofluorescence and anti-kinetochore antibody staining (CREST). Next, 3 x 10^6 cells were seeded in 60-mm Petri dishes for 4 h in CBMN and metaphase preparation experiments, followed by a recovery time of 22 h. Furthermore, in CBMN experiments, we also performed the combined treatments RSV + 50 μM ethidium bromide (EtBr). In CREST experiments, we utilised the same doses of RSV but treatments lasted 2 h followed by a recovery time of 4 h in presence of cytochalasin B.

The same RSV doses were utilised in mitosis and immunofluorescence experiments and treatments lasted 1 or 3 h without any recovery time.

Cytokinesis-block micronucleus assay

The recovery time after treatment was carried out in presence of 2 μg/ml cytochalasin B (Sigma, St Louis, MO, USA) in order to obtain binucleated cells. Cells were fixed in cold methanol for 10 min, allowed to air dry and stained with the conventional Giemsa method. For each experimental point, at least 1000 binucleated cells (BN) were analysed and those with one or more micronuclei (MN) were recorded. The results are expressed as total MN on 1000 BN (MN%). Cell proliferation was evaluated through the nuclear division index (NDI) according to the formula: NDI = (1 x M1 + 2 x M2 + 3 x M3 + 4 x M4)/N, where M1 through M4 represent the number of cells with one to four nuclei and N is the total number of cells scored.

Metaphase preparation

In order to obtain a sufficient number of metaphase spreads, colcemid (Sigma, St Louis, MO, USA; 0.1 μg/ml) was added to each culture 2 h before fixing. After hypotonic treatment (0.075M KCl) lasting 10 min at 37°C, the cells were treated three times with freshly prepared methanol, glacial acetic acid 3:1 fixative. The fixed cells were dropped onto glass slides, allowed to air dry and stained with conventional Giemsa method. The frequency of POL and ENDO cells was analysed in at least 1000 metaphases containing not less than 21 ± 1 chromosomes for each experimental point (POL% and ENDO%). We considered as POL cells those owning a triploid (3n) or higher karyotype and as ENDO cells those showing all chromosomes as diplochromosomes. The images were captured and analysed using a Leica TCS SP5 laser scanning confocal microscope controlled by the LAS AF software.

Analysis of mitotic progression

After treatment, cells were treated two times in methanol/glacial acetic acid 3:1 fixative for 10 min and stained with conventional Giemsa method. For each experimental point, at least 200 mitosis were analysed and the frequency of prophase, prometa/metaphases and ana-telophases was scored. The frequency of anaphase bridges was detected in at least 100 anaphases.

Immunofluorescence and anti-kinetochore antibody staining (CREST staining)

Cells were rinsed in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2) fixed for 10 min with 3.7% formaldehyde in PHEM and permeabilised 5 min in 0.2% Triton X-100. Cells were blocked in phosphate-buffered saline containing 20% goat serum for 30 min at 37°C, before being processed for immunofluorescence. Antibody dilution was as follows: human anti-KT serum (CREST, Antibodies Inc., Davis, CA, USA; 1:50) and FITC-anti-tubulin (Sigma, St Louis, MO, USA; 1:100). Secondary antibody conjugated to Rhodamine-RedX (Jackson Laboratories, Suffolk, UK) was chosen as appropriate and used as recommended by the supplier. Where indicated, DNA was counterstained with 0.05 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO, USA). Coverslips were sealed in antifade solution (Vector, Burlingame, CA, USA). Preparations were examined under an Olympus AX70 microscope using a 100x/1.35NA objective. Images were acquired using a SPOT charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI, USA) controlled by ISO 2000 software (DeltaSistemi, Verona, Italy).

Statistical analysis

All data were obtained through at least three independent experiments and expressed as means ± standard deviations. We applied the unpaired t-test with Welch correction in order to assess the significance of RSV treatments effects. Values of P < 0.05 were considered statistically significant. Statistical analysis of data was performed using Graph Pad software Instat version 3.02 (Graph Pad Software, San Diego, CA).

Results

Induction of polyploidy, DNA damage and chromosome lost

The effects on chromosome segregation and micronuclei induction exerted by RSV are shown in Figures 1 and 2 and Table 1. Figure 1 shows a CHO metaphase cell with diplochromosomes made up of four chromatids (a), a polyploid metaphase (b) and a binucleated cell with a micronucleus (c), as a consequence of RSV treatment. In Figure 2, the induction of both POL and ENDO cells after the treatment with RSV are shown. The increase became significant at doses of 80 and 120 μM.

As far as micronuclei induction, it followed a dose-dependent trend with a significant increase after 80 and 120 μM treatment (Table 1). The NDI decreased with increasing dose being always significantly lower in treated versus control samples. In order to determine if the MN induction by RSV could be antagonised by concomitant treatment with a topoisomerase

Fig. 1. ENDO cell with diplochromosomes (a) POL cell (b) and binucleated cell with one micronucleus (arrow) (c). Scale bar: 10 μm.
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Inhibitor, we performed combined treatments RSV + EtBr. As shown in Table I, we found a significant decrease of MN when the two drugs were provided simultaneously in comparison with single RSV treatment. In particular, the MN induced by 80 and 120 μM RSV were significantly reduced (P < 0.01 and P < 0.05, respectively) when EtBr concomitant treatment was performed. Furthermore, the loss of a whole chromosome as a consequence of an improper chromosome segregation induced by RSV treatment was analysed through CREST staining of MN. We found a significant increase of MN with centromere (CREST+) after 2 h of treatment with 80 and 120 μM RSV (Table I).

**Effects on mitotic progression**

In order to check if a defective chromosome segregation could affect mitosis progression, we monitored the capacity of RSV treatment to modify the distribution of cells in the different phases of mitosis. In Figure 3, we show that RSV treatment caused an increase in the prometa/metaphase percentage with a concomitant decrease in the other mitotic stages. This effect is already evident at 80 and 120 μM RSV after 1 h of treatment; the increase of metaphases and the reduction of both prophases and ana-telophases reached the significance (P < 0.01 and P < 0.05, respectively) just after 120 μM dose. After 3 h of treatment all RSV doses induced a significant increase in prometa/metaphase percentage (P < 0.01). These results demonstrate that RSV-treated cells spend longer time in prometa/metaphase, possibly by trying to decatenate replicated DNA threads.

Incomplete decatenation of sister chromatids during mitosis can originate anaphases showing DNA bridges. In Figure 4 (upper part) is reported an immunofluorescence image showing the presence of DNA bridges in anaphase and ana-telophase. Immunostaining, with CREST, anti-tubulin antibodies and the DNA dye DAPI, demonstrates that DNA threads remain connecting the two groups of migrated chromosomes, as visualised by the CREST signals at the two poles. In the lower part of Figure 3, the frequency of anaphase bridges after RSV treatments is reported. Although the frequency of anaphase bridges is low (the highest value is 15%), the effect is evident at the highest dose (120 μM) both after 1 and 3 h treatment, reaching a significant value just after the longer treatment period (P < 0.05).

**Discussion**

In recent decades, the natural compound RSV has excited great interest for its chemopreventive activity and beneficial

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**Table I. Micronuclei (MN‰) induced by RSV in CHO-K1 cells**

| RSV treatment: 6 h |  | RSV treatment: 2 h |  |
|--------------------|  | Recovering: 4 h cytochalasin B |  |
|                    |  | CREST− |  | CREST+ |  |
| RSV treatment: 6 h |  | Recovering: 22 h cytochalasin B |  |
|                    |  | −EtBr | +EtBr | NDI | CREST− | CREST+ |  |
| C                  |  | 18.6 (11.1) | 17.0 (2) | 1.9 (0.04)* | 15.8 (3.3) | 5.9 (1.8) |  |
| RSV 40 μM          |  | 32.1 (11.4) | 22.3 (7) | 1.76 (0.04)* | 47.5 (18.1) | 28.6 (8.9)* |  |
| RSV 80 μM          |  | 57.3 (13.5)** | 34 (5.3)* | 1.58 (0.04)** | 56.3 (17.6)* | 51.8 (12.6)** |  |
| RSV 120 μM         |  | 97.8 (19.9)** | 63.7 (15)* | 1.47 (0.07)** | 66.6 (22.5)* | 39.5 (3.4)** |  |

The inhibitory action of EtBr on the clastogenic effect of RSV is shown when the two drugs are provided at the same time. The CREST staining highlighted the aneugenic effect of RSV. The NDI is reported in order to show the delayed proliferation after treatment with RSV. Values are mean with SD in bracket.

−EtBr: cells treated with RSV; +EtBr, cells treated with RSV and EtBr at the same time; CREST−: MN lacking centromere; CREST+: MN containing centromere.

*P < 0.05, **P < 0.01 performed by t-test treated cells versus control ones.

†P < 0.05, ††P < 0.01 performed by t-test +EtBr versus −EtBr.
effects against various diseases. There is a great amount of data collected both in vitro and in vivo showing that RSV is able to modulate various cell pathways in different cells. In particular as far as the induction of DNA damage, the data present in literature seem to converge on the claim that RSV could act on proliferating cells, principally cancer cells, inducing the expression of markers of DNA damage, such as γ-H2AX. DNA damage is one of the targets of chemoprevention and a

Fig. 3. Distribution of mitotic figures in CHO-K1 cells after RSV treatment lasting 1 and 3 h. *P < 0.05, and **P < 0.01 (unpaired t-test) when comparing treated samples with control ones.

Fig. 4. Upper part: Immunofluorescence detection of anaphase bridges. Normal anaphase with a correct segregation of chromatids (a). Anaphase with a DNA bridge (b). Ana-telophase with a DNA bridge (c). The red signals represent kinetochores, the blue ones represent DNA and the green ones represent α tubulin. Scale bar: 5 µm. Lower part: percentage of anaphase bridges in CHO-K1 cells after treatment with different doses of RSV. *P < 0.05 at unpaired t-test.
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desirable outcome is the induction of apoptosis and/or the arrest of cell proliferation. Thus, the ability of RSV in inducing DNA damage might also explain the activation of the checkpoints responsible for cell cycle arrest and, in some cases, apoptosis (4). A possible explanation for this behaviour could be also the inhibition of topoisomerases activity exerted by RSV. This will combine the delay in the S-phase progression with the increase in DNA damage. The results from our group showing the increase in γ-H2AX after RSV treatment in glioma cells associated with a delay in the S phase of the cell cycle are consistent with this hypothesis (11).

On the other hand, controversial data are present in the literature about the influence of RSV treatment on the redox status of the cells. In fact, although its antioxidant power in certain conditions and concentrations is known, an enhancing number of data are now present in literature dealing with its pro-oxidant ability exerted in particular condition (for a review see (24)).

Therefore, it is conceivable that RSV can exert some genotoxic action mainly in actively proliferating cells that express high levels of TOPO2. In this study, we investigated the consequences of TOPO2 inhibition by RSV not only as micronuclei induction but also in respect to DNA decatenation and chromosome segregation, utilising the non-cancer cell line CHO-K1. CHO cells are established cells routinely employed in cytogenetic experiments with a substantially stable karyotype and a low spontaneous background of micronucleated cells (25). In this respect they can be a good surrogate of normal cells.

TOPO2 is an essential mammalian enzyme that topologically modifies DNA through the production of transient DSBs and is required for chromosome segregation during mitosis. TOPO2 is the target of widely used anti-cancer agents such as etoposide, anthracyclines (doxorubicin and daunorubicin) and mitoxantrone. These agents act by trapping DNA and TOPO2 in the so called ‘cleavable complex’ (26). Previous researches also suggest that inhibition of TOPO2 decatenatory activity triggers a G2 checkpoint response, which delays the entry into mitosis due to insufficient decatenation of sister chromatids (27–29).

In this study, we show that RSV treatment induces micronuclei at the two highest doses as previously shown in human tumour cells (11). Indeed, one of the consequence of TOPO2 poisoning is the induction of DSBs that may produce MN, tumour cells (11). Indeed, one of the consequence of TOPO2 inhibition by RSV not only as micronuclei induction but also in respect to DNA decatenation and chromosome segregation, utilising the non-cancer cell line CHO-K1. CHO cells are established cells routinely employed in cytogenetic experiments with a substantially stable karyotype and a low spontaneous background of micronucleated cells (25). In this respect they can be a good surrogate of normal cells.

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In this study, we show that RSV treatment induces micronuclei at the two highest doses as previously shown in human tumour cells (11). Indeed, one of the consequence of TOPO2 poisoning is the induction of DSBs that may produce MN, whereas the catalytic inhibition does not, except for some evidences reporting that treatment with ICRF-193 induced G2 arrest and DNA damage (30). This statement is strongly supported by the reduction in micronucleated cells we obtained when combined treatments (RSV plus ethidium bromide) were performed. In fact catalytic inhibitors and intercalating agents, such as ethidium bromide, prevent the formation of TOPO2-induced DNA DSBs (31). We found a dose-dependent significant increase of micronuclei concomitant with a NDI decreases, indicating that the induction of DNA damage goes with a reduction of cell proliferation. This last result is in agreement with what is widely known on the ability of RSV to delay cell cycle progression especially in cancer cells, thus leading cells to death (8,32).

In this study, we also report that RSV causes an increase in polyploidy and endoreduplication, two phenomenon that occur when DNA replicates without a cell division (33). In fact endoreduplication is a different manifestation of polyploidy, which has to be considered as a proof of the prevention of decatenation of fully replicated chromosomes following TOPO2 inhibition. This event will lead to the failure of correct segregation at mitosis (34). Data are present in literature about the ability of different flavonoids to induce endoreduplication in CHO cells (35,36) at doses very close to those used in this article and these compounds are all able to interfere with TOPO2 activity.

The analysis of mitotic figures after treatment with RSV highlights a delay of mitotic progression with an accumulation of cells in the prometa/metaphase stage. This is in agreement with a reduction of TOPO2 activity respect to the resolution of intertwined chromatids, that is a critical step of the transition from metaphase to anaphase (15).

The outcome of a defective DNA decatenation is shown by the presence of DNA bridges during the ana-telophase stage, that could evolve in chromosome breaks. We found an increase of DNA bridges in the ana-telophase after treatment with RSV. TOPO2 is an enzyme active in proliferating cells that through the same mechanism of action is involved in DNA synthesis and catenated DNA resolution and its action is performed during a large part of the cell cycle, from the S phase to the G2/M phases. Our data show that RSV can affect TOPO2 activity at different stages of the cell cycle, during the DNA replication (through the induction of DSBs) and during the DNA segregation (through the induction of ENDO and POL cells). In this article, this 2-fold effect has been demonstrated through the presence of micronuclei CREST+ that represent acentric chromosome fragments originated by DSBs and micronuclei CREST− that represent whole chromosomes originated by the failure of chromatid segregation.

On the whole, these results reinforce the statement of the role of RSV as a TOPO-poison and strongly suggest to explore more thoroughly its involvement in new therapeutic strategies.

References


