Resveratrol protects mouse embryonic stem cells from ionizing radiation by accelerating recovery from DNA strand breakage

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Resveratrol has elicited many provocative anticancer effects in laboratory animals and cultured cells, including reduced levels of oxidative DNA damage, inhibition of tumor initiation and progression, and induction of apoptosis in tumor cells. Use of resveratrol as a cancer-preventive agent in humans will require that its anticancer effects not be accompanied by damage to normal tissue stem or progenitor cells. In mouse embryonic stem cells (mESC) or early mouse embryos exposed to ethanol, resveratrol has been shown to suppress apoptosis and promote survival. However, in cells exposed to genotoxic stress, survival may come at the expense of genome stability. To learn whether resveratrol can protect stem cells from DNA damage and to study its effects on genomic integrity, we exposed mESC pretreated with resveratrol to ionizing radiation (IR). Forty-eight hours pretreatment with a comparatively low concentration of resveratrol (10 μM) improved survival of mESC 2-fold after exposure to 5 Gy of X-rays. Cells pretreated with resveratrol sustained the same levels of reactive oxygen species and DNA strand breakage after IR as mock-treated controls, but repaired DNA damage more rapidly and resumed cell division sooner. Frequencies of IR-induced mutation at a chromosomal reporter locus were not increased in cells pretreated with resveratrol as compared with controls, indicating that resveratrol can improve viability in mESC after DNA damage without compromising genomic integrity.

Introduction

Resveratrol has exhibited a broad range of chemopreventive and therapeutic properties in experimental settings. In animal models, resveratrol prevented atherosclerosis by preventing leukocyte recruitment, inhibiting production of vascular cell adhesion molecules and suppressing proliferation of vascular smooth muscle cells (1). In mice on a fat-rich diet, resveratrol significantly improved glucose homeostasis and prevented development of insulin resistance and hyperglycemia (1). Resveratrol has suppressed carcinogenesis in a variety of animal models (2) and chemopreventive effects of resveratrol with regard to cellular transformation in culture have been documented for several primary mammalian cell types (3–7). At the molecular level, putative targets of resveratrol have been identified (8). Resveratrol can exert antioxidant properties by upregulating expression of reactive oxygen species (ROS) scavengers and phase II enzymes such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, selenophosphate synthase 2, thioredoxin reductase and NAD(P)H:quinone oxidoreductase-1 (9–13). Antitumor effects of resveratrol have been attributed to abilities to induce cell cycle arrest, apoptosis or terminal differentiation in tumor cells, to inhibit angiogenesis and inflammation, and to reduce cellular adhesion that promotes invasion and metastasis (14). However, the usefulness of resveratrol as a cancer-preventive agent will depend upon its ability to exert these effects selectively, such that normal stem or progenitor cells of adult tissues are spared. We have explored the effects of resveratrol on mouse embryonic stem cells (mESC) as a model for the stem/progenitor cell compartment of normal tissues.

Embryonic stem cells are distinct in their ability to divide indefinitely in culture and to differentiate in vivo into cells of all lineages. Maintenance of genomic stability is a priority for embryonic stem cells because mutations would be propagated into all somatic tissue derivatives, increasing the likelihood of tumorigenesis, and possibly into the germ line, compromising fertility and development. In mESC, frequencies of spontaneous mutation measured at reporter loci were found to be two to three orders of magnitude lower in than in isogenic fibroblast cells (15,16). The existence of highly sensitive surveillance systems that remove mESC with damaged DNA from the population via apoptosis or differentiation is well supported (17,18), although the molecular mechanisms underlying this phenomenon remain obscure. mESC are also distinctive in their responses to DNA damage caused by exogenous agents, differing with respect to the mechanisms of DNA repair used and the regulation of cell cycle progression after damage. After DNA damage induced by ionizing radiation (IR), mESC do not activate the G1/S cell cycle checkpoint as somatic cells typically do, and a significantly larger fraction of irradiated mESC becomes apoptotic as compared with isogenic primary fibroblasts. The surviving fraction of mESC relies predominantly on homologous recombination for repair of DNA double-strand breaks (DSB) (16,19,20), whereas somatic cells in general rely more heavily on non-homologous end joining (21).

Exposure to ethanol induces DNA damage in addition to other forms of cellular injury. Pretreatment with resveratrol was shown to reduce ethanol-induced apoptosis and promote survival of mESC in culture and to reduce ethanol-induced injury to blastocyst-stage mouse embryos in vivo (22). However, morphological assessment revealed that blastocysts rescued by resveratrol harbored fewer cells in their inner cell mass than controls and more frequently failed to continue development. Resveratrol has also been reported to have negative effects on genomic stability (23). Apoptosis plays a major role in maintaining the genomic stability of mESC populations (21,24), and it has been unclear whether resveratrol truly protects mESC from DNA damage or merely prevents removal of cells harboring damaged DNA by apoptosis and by doing so compromises genomic stability.

In this study, we examined the effects of resveratrol pretreatment on mESC subjected to DNA damage by IR. Our results indicate that a low concentration of resveratrol introduced well in advance of IR exposure can improve the survival of mESC without jeopardizing their genomic integrity.

Materials and methods

mESC culture and X irradiation

The mESC lines 3C4 and 2B5 have been described previously (15). They carry one wild-type Aprot allele and one allele disrupted by insertion of a Neo marker through gene targeting. mESC were propagated without feeder cells, using synthetic N2B27 medium supplemented with lymphocyte inhibitory factor and bone morphogenic protein 4 (25). Detachment and dissociation of mESC for passage or assays was done with Accutase (Innovative Cell Technologies, #AT104). Resveratrol (Sigma–Aldrich, #R5010) was prepared as a fresh 10 mM solution in dimethyl sulfoxide (DMSO; Sigma–Aldrich, #D2650) and stored for no more than 48 h at room temperature in the dark. For survival assays, mESC grown in the presence of 10 μM resveratrol or an equivalent...
concentration of vehicle only (0.1% DMSO), or untreated, were plated at 1000 cells per 100 mm gelatinized dish. Sixteen hours after initial plating, cells were exposed to 5 Gy of X-rays using a Toren X-ray cabinet (Faxitron X-ray Corporation). Immediately after irradiation, cultures were refed with fresh medium and returned to the incubator for colony formation. Ten days later, plates were stained and colonies counted.

**MTS cell proliferation assays**

MTS cell proliferation assays were carried out using a CellTitre 96 Aqueous one-solution cell proliferation assay kit (Promega, #G3580) according to the manufacturer’s instructions. mESC were plated at 5000 cells per well into a 96-well plate in medium with resveratrol or DMSO only added or left untreated. After incubation for 48 h, the culture medium was replaced with 100 μl of fresh medium per well and 20 μl of MTS tetrazolium solution was added directly to each well. Plates were incubated for a further 2 h and then absorbance at 490 nm was recorded using a plate reader. Measured values were corrected for the absorbance of control wells with no cells. At least four replicates were measured for each treatment condition.

**ROS assay**

5-/6-Chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, #C6827; Invitrogen) was used to measure ROS levels in mESC (26). CM-H₂DCFDA was dissolved in DMSO. mESC cultures were washed once with phosphate-buffered saline (PBS), detached from culture dishes with Accutase, counted and collected by centrifugation. Approximately 1 million mESC were incubated with 20 μM CM-H₂DCFDA for 30 min, washed, irradiated and stored on ice until analyzed by flow cytometry.

**Cell cycle analysis**

Cells were irradiated or mock-irradiated 24 h after plating. After irradiation, cells were detached with Accutase at indicated timepoints and washed once with PBS, fixed in 70% ethanol and stained with propidium iodide in the presence of RNase A and then analyzed for DNA content using a Becton–Dickinson FaxCaliber flow cytometer.

**Measurement of Aprt mutation frequency**

mESC cultures treated with resveratrol or vehicle for 48 h, or not treated, were irradiated and then immediately refed with fresh medium and allowed to recover for 30 h. After recovery from irradiation, cells were replated at 1 million/100 mm dish. Twenty-four hours later, 2-fluoro-adenine (FA) (Sigma–Aldrich, #F5024) was added to a final concentration of 2 μg/ml, to select for growth of Aprt-deficient cells (27). Selection was carried out for 10 days, during which cells were refed with fresh medium supplemented with FA every 3 days. Colony-forming efficiency was measured in parallel platelets without FA at 1000 cells/100 mm dish. Mutation frequency was calculated as a ratio of the number of FA-resistant colonies divided by the total number of cells plated for selection, corrected for colony-forming efficiency.

**DNA strand breakage assays**

DNA strand breakage assays were carried out using the PicoGreen ‘Fast Micro-Method’ as described by Schroder et al. (28). mESC treated with resveratrol, or vehicle only, or left untreated were plated and irradiated 48 h later. Cells were then detached immediately (zero time point) or refed and allowed to recover in a cell culture incubator for the indicated periods of time. Cell suspensions were counted with a ViCell counter (Beckman/Coulter), and the volumes were adjusted with calcium/magnesium-free PBS to a density of 200 000 viable cells/ml. Twenty-five microliters of cell suspension per well (5000 viable cells) was distributed into 96-well plates already containing 25 μl of lysis solution (9.0 M urea, 0.1% sodium dodecyl sulfate, 0.2 M EDTA pH 10.00) supplemented with PicoGreen reagent in DMSO (Invitrogen, # P11495), 2% vol/vol per well. Each cell treatment was prepared in at least four replicate wells. Plates were incubated for 1 h at room temperature in the dark for cell lysis and dye intercalation to occur and then 250 μl of DNA denaturing solution (100 mM NaOH, 20 mM EDTA pH 12.4) was added and fluorescence was measured every 5 min over a 30 min course at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Measured fluorescence values were corrected for background by subtracting values measured in wells containing PBS only. Results were expressed as the fraction of double-stranded DNA remaining in the cell population.

**Results**

**Sensitivity of mESC to X-rays and to resveratrol**

To clarify the effects of resveratrol on viability after DNA damage and genotoxic stability in mESC, we have taken advantage of the previously established mouse ESC strain 3C4, which is heterozygous for the adenine phosphoribosyl-transferase (Aprt) gene (15). The remaining wild-type Aprt allele can be inactivated by a variety of mechanisms, including mitotic recombination, partial or complete chromosome loss, microdeletions, point mutations or (rarely) epigenetic silencing. Cells deficient for APRT protein can be selected by supplementing growth medium with FA, which is converted into a toxic nucleoside analog by APRT. Hence, the frequency of Aprt mutants can be measured as a broad indicator of genome stability, reflecting multiple classes of genomic events. We previously determined the frequency of spontaneous Aprt mutants in untreated 3C4 mESC to be ~7 × 10⁻⁷ (15,16).

We used colony formation assays to measure the viability of mESC after exposure to a range of X-ray doses and observed the expected inverse correlation between survival (cell colony-forming fraction) and X-ray dose (Figure 1A). As a working X-ray dose for assessing effects of resveratrol in mESC, we chose 5 Gy, which killed ~70% of the cell population.

Biological effects of resveratrol have been reported over a wide range of concentrations, but from a dietary or pharmacological perspective, only concentrations in a low micromolar range are relevant (29–32). Evidence for toxicity of resveratrol at higher concentrations has been documented in a variety of systems (9,33,34). We used the MTS cell proliferation assay to evaluate the effects of resveratrol on growth of mESC. The results indicated that mESC tolerate 10 μM resveratrol well, but concentrations as low as 30 μM significantly inhibited their proliferation in comparison with control cultures treated with vehicle (DMSO) only (Figure 1B). We chose a working resveratrol concentration of 10 μM for the remainder of this study.

10 μM resveratrol protects the viability of mESC exposed to IR

In a previous study, oral administration of resveratrol to mice for 2 days prior to whole-body irradiation reduced the frequencies of chromosomal aberrations in bone marrow cells (35), which implies...
amelioration of DNA damage. We hypothesized that resveratrol might be radioprotective for mESC in culture as well.

mESC were pretreated with 10 μM resveratrol or vehicle for 48 h and then exposed to 5 Gy of X-rays. Afterward, relative viability was assessed by colony formation assays. The ability of resveratrol-treated mESC to give rise to colonies was improved >2-fold over untreated controls (Figure 2A). Forty-eight hours pretreatment with vehicle (DMSO) alone gave no statistically significant improvement. To confirm that this effect was not peculiar to mESC line 3C4, the experiment was repeated with another clonal mESC line, 2B5, with similar results (Supplementary Figure 1 is available at Carcinogenesis Online). A shorter pretreatment with 10 μM resveratrol, for only 40 min prior to IR, did not improve viability (Figure 2B). Resveratrol is a small hydrophilic molecule, presumed to cross the cellular membrane rapidly. The lack of effect after 40 min implies that mere presence of intracellular resveratrol at the time of irradiation is not sufficient to protect. Having established that resveratrol can promote survival of mouse ESC exposed to IR, we sought to explore possible mechanisms for this protection.

Pretreatment with resveratrol does not prevent increased ROS levels after irradiation of mESC

Resveratrol has been shown to protect a variety of somatic cell lines in culture from deleterious effects of oxidative damage (5,11). A large component of the DNA damage induced by IR is mediated by formation of ROS (26). Resveratrol has been proposed to act as a direct ROS scavenger (36) and can induce expression of cellular proteins that scavenge ROS in a number of cell types (9–13), though this has not been confirmed in mouse ESC.

To assess ROS levels in mESC exposed to IR, we used the cell permeable dye CM-H2DCFDA. Upon crossing the cell membrane, CM-H2DCFDA is rapidly hydrolyzed by endogenous esterases to a non-fluorescent form that, in turn, is readily oxidized to the fluorescent form dichlorofluorescein (37). Flow cytometry was used to measure intracellular ROS levels in populations of mESC (26). Prior to IR exposure, mESC treated for 48 h with 10 μM resveratrol showed no decrease in ROS levels relative to vehicle-only or non-treated controls (Figure 3). After exposure to 5 Gy of X-ray, ROS levels were significantly and similarly increased in all treatment groups including resveratrol-treated cells (Figure 3). Pretreatment with resveratrol for only 40 min did not alter ROS levels in non-irradiated or irradiated cells. We concluded that 10 μM resveratrol has little if any influence on ROS levels in mESC and that radioprotection of mESC by resveratrol is independent of antioxidant properties, direct or indirect.

mESC pretreated with resveratrol resume cell division more rapidly after X-irradiation

Resveratrol has been shown to induce transient cell cycle arrest in a number of cell types other than mESC (9,33). mESC do not arrest at the G1/S boundary after DNA damage, as most somatic cell types do (24). Hypothetically, if resveratrol activated the G1 checkpoint in mESC, this would delay replication of damaged DNA and reduce the formation of DSBs. We examined the cell cycle phase distributions of mESC pretreated with resveratrol or vehicle only, and untreated cells, using flow cytometry. In the absence of IR exposure, the phase distributions were identical (Figure 4A). The proportions of cells in G1 phase and G2/M phase each comprised 20–25%, whereas the proportion in S phase was over 50%. This is typical of mouse ESC cultures, and in accord with MTS assays showing that growth was not slowed by 10 μM resveratrol (Figure 1B). Exposure of mESC to 5 Gy of IR caused a reduction in the G1 cell fraction and accumulation of cells in the S and G2/M fractions. This dynamic was expected because, although mESC lack the G1 cell cycle checkpoint, they have a fully competent G2 checkpoint, which causes transient G2 phase arrest after IR exposure (24). The G1 fraction reached a minimum at ~3.5 h after irradiation, when only 5–7% of all cells had 2N DNA content, whereas the S + G2/M fractions reached a maximum of 90–92% (Figure 4B). All treatment groups followed the same dynamics, and there was no sign of a G1 arrest attributable to resveratrol. By 7.5 h after irradiation, passage out of S phase had resumed, but the G1 fractions of control groups had risen only slightly and cells continued to accumulate in G2 (Figure 4C). In contrast, the G1 fraction of the resveratrol-treated population had increased >2-fold, indicating resumption of transit through M phase. The G1/G2/M ratio recovered to a greater extent in resveratrol-treated cells than in control populations, reflecting more transit through M (Figure 4C). By 12–18 h postirradiation, the initial cell cycle phase proportions were re-established in

![Fig. 2. Effects of resveratrol on viability of mESC after X-ray exposure. (A) mESC pretreated for 48 h with 10 μM resveratrol or vehicle (DMSO) only, or not treated, were exposed to 5 Gy of X-rays, and subsequent cellular survival was measured by colony formation assay. Relative survival is expressed as the mean numbers of colonies formed in irradiated cultures as a fraction of those in untreated non-irradiated cultures. (B) ESC were pretreated for only 40 min with 10 μM resveratrol, or vehicle-only, or not treated and then irradiated and assayed for relative survival as for panel A. Values graphed are means of three independent experiments, each performed in triplicate. Error bars represent standard error of the mean. Asterisk (*) indicates P < 0.05, t-test.](image)

![Fig. 3. ROS levels in X-irradiated mouse ESC and their non-irradiated counterparts. Cells pretreated for 48 h with 10 μM resveratrol or vehicle only, or not treated, were loaded with CM-H2DCFDA and irradiated (open bars) or mock-irradiated (solid black bars). Dichlorofluorescein fluorescence was measured in arbitrary units by FACS analysis. Values graphed are means of three experiments, each done in duplicate. Error bars represent standard error of the mean. Asterisks indicate P < 0.05 (*) or P < 0.01 (**), by t-test.](image)
Fig. 4. Cell cycle distributions of mESC after 5 Gy of X-irradiation. FACS analysis was carried for a non-irradiated cell population (panel A), and in cells exposed to 5 Gy of X-rays at 3.5 h (panel B) and 7.5 h (panel C) after exposure. Solid gray bars represent untreated cells, white bars, DMSO-treated cells and solid black bars, cells treated with 10 μM resveratrol for 48 h. Values graphed are means (±SEM) of three independent experiments. Asterisk (*) indicates P < 0.05, t-test.

Fig. 5. Frequencies of APRT-deficient mutants in X-irradiated mouse ESC pretreated with resveratrol or vehicle only and in non-treated cells. Mutant frequency was measured by selection of APRT-deficient clones in colony formation assays, as described in Materials and Methods. Each diamond symbol represents an independent frequency measurement; the short horizontal bars represent the median of all frequencies associated with each treatment group. The vertical scale is linear, rather than logarithmic and expanded to display the small differences in mutation frequency detected.

surviving cells of all three treatment groups (data not shown). Thus, all groups ultimately recovered and re-entered the cell cycle, but resveratrol-treated cells re-entered sooner. In principle, this could mean either that resveratrol facilitates DNA repair or that resveratrol overrides the G2 checkpoint and allows cell division despite incomplete repair of DNA damage. The latter alternative would be expected to compromise genome stability.

**mESC pretreated with resveratrol maintain genomic stability after irradiation**

mESC have demonstrated significantly lower mutation frequencies at chromosomal reporter loci than isogenic somatic cells, indicating greater genomic stability (15,16,24). We reasoned that inactivation of the G2 checkpoint machinery should negatively affect genomic stability in mESC and lead to increased mutation load in the cell population. We measured Aprt mutant frequencies after an X-ray exposure of 5 Gy in populations of mESC pretreated with resveratrol, or vehicle only, and their untreated counterparts (Figure 5). Over six independent selection experiments, resveratrol-mediated rescue of viability was not associated with increased Aprt mutation in the surviving populations of cells. Despite a 2-fold increase in survival of resveratrol-treated cells, Aprt mutant frequencies, when adjusted for survival, remained at approximately the same level as in cells treated with vehicle (DMSO) only. Interestingly, in either population, the median Aprt mutant frequency was ~2-fold lower than in the untreated control cells, and these differences were statistically significant (t-test, P < 0.05). The latter result implies that DMSO alone can reduce the overall frequency of radiation-induced mutation in surviving mESC, even though it does not improve overall survival. Because resveratrol treatment increased mESC survival without increasing mutation frequency, it is unlikely that resveratrol treatment attenuates the G2 checkpoint. This result indicated that resveratrol does not promote mESC survival at the expense of genomic integrity but raised a new question: If resveratrol does not reduce the initial surge of ROS caused by X-irradiation (or by implication, the load of DNA damage) and does not attenuate the G2 checkpoint, then how does it allow mESC to re-enter the cell cycle earlier?

Even though ROS levels after irradiation were not reduced by resveratrol, we sought to test the possibility that resveratrol nonetheless mitigated IR-induced DNA damage by some other mechanism. IR causes multiple forms of DNA damage, including damaged nucleotides that are processed into DNA single-strand breaks as an intermediate step of base excision repair. IR also rapidly induces DNA DSBs both directly, by hydrolysis of phosphodiester bonds and indirectly when single-strand breaks are encountered by a replication fork (38). To more directly assess levels of DNA strand breakage, we used a variation of the PicoGreen Fast Micro-Method (28). PicoGreen is a dye, similar to Sybr-Green, that becomes highly fluorescent upon intercalating into double-stranded DNA (39). DNA denaturation quenches PicoGreen fluorescence. Denaturation of genomic DNA at alkaline pH is accelerated by the presence of single-strand breaks and/or DSBs, so that the extent of PicoGreen quenching at intermediate timepoints after the initiation of denaturation reflects the degree of strand breakage initially present.

In genomic DNA of non-irradiated mESC, PicoGreen fluorescence was initially high but fell off rapidly over time after the beginning of alkaline denaturation. No significant differences in baseline levels of endogenous strand breakage were detected between resveratrol-treated, vehicle-only and untreated populations of mESC (Figure 6A). mESC pretreated with resveratrol or vehicle, or untreated, were then exposed to 5 Gy of X-ray and assayed for DNA strand breakage immediately afterward. Results of three independent experiments indicated that the degree of DNA breakage after IR was similar in mESC pretreated with resveratrol, as compared with their vehicle-only or untreated counterparts (Figure 6B). Evidently, pretreatment with 10 μM resveratrol does not promote survival of mESC after IR by reducing the load of DNA strand breaks they initially sustain.

We then used the PicoGreen assay to compare recovery from DNA strand breakage in mESC pretreated with resveratrol or with DMSO.
only, or not treated. After irradiation, cells were refed with fresh medium and allowed an interval of time to recover and then assayed for strand breakage. At 15 min or 1 h after IR exposure, levels of genomic DNA strand breakage remained similar in all three cell populations (data not shown). However, at 3.5 h after exposure, a difference became apparent (Figure 6C and Supplementary Figure 2, available at Carcinogenesis Online). Although vehicle-only cells harbored about the same amount of DNA damage as untreated cells, DNA from the cells pretreated with 10 μM resveratrol for 48 h had significantly less strand breakage. Lower levels of DNA strand breakage in a cell population could potentially occur in two ways: by selective loss of the most damaged cells or by repair of strand breaks. However, differential loss of damaged cells between treatment groups was not detected. Equal numbers of cells were plated for all groups 48 h in advance of irradiation. After IR exposure and recovery, cells were counted again, and approximately equal numbers of cells were recovered from each group. The cell counter used (Beckman/Coulter ViCell) reports the fraction of cells that have lost viability (fail to exclude dye), and this did not differ between groups (data not shown). The breakage assays thus indicate that DNA repair started sooner after irradiation and/or proceeded more rapidly in the cells pretreated with resveratrol.

**Discussion**

Previous studies with resveratrol have produced differing and sometimes discordant results. Resveratrol can itself be cytotoxic under some conditions (12) but can also protect mESC and embryos from ethanol toxicity (22). Resveratrol has been found to reduce ROS levels and/or oxidative DNA damage in some experimental settings (9,10,40,41) but also to induce ROS in others (12,13). In different cell culture systems, resveratrol has been reported either to induce apoptosis (13) or to suppress induction of apoptosis by other agents (42). Similarly, resveratrol has been reported to cause DNA damage (43,44) and to induce genomic instability (23) but also to prevent chromosomal aberration in cells of irradiated mice (35). Some of the apparent discrepancies between previous reports may arise from genuine biological differences between the cell types studied or between the specific assays applied, but it also seems likely that at least some of the inconsistencies among cell culture studies stem from the...
wide differences in resveratrol concentrations used, which have ranged from <5 to >100 μM (45). In this study, we confined our attention to mESC and the influence of a comparatively low concentration of resveratrol on their response to a single form of cellular stress: DNA damage induced by IR. The 10 μM resveratrol concentration used here was near the low end of the range used in previous cell culture studies yet within the upper bounds of transient blood plasma concentrations reported in rodents fed resveratrol (31). We found that 48 h pretreatment with 10 μM resveratrol protected mESC from exposure to 5 Gy of X-irradiation, in that viability was improved ~2-fold over controls. This is a significant result, statistically and in the qualitative sense that, while many agents can reduce the resistance of cells to acute radiation damage, few are known to increase it by ≥2-fold.

Pretreatment of mESC with 10 μM resveratrol did not prevent the increased levels of intracellular ROS typically seen after exposure to IR (Figure 3). This was surprising in view of previous reports that resveratrol can reduce ROS levels, directly by acting as a ROS scavenger and indirectly by upregulating proteins that act to control ROS levels (9–13,41). Furthermore, pretreatment with 10 μM resveratrol did not reduce the initial load of DNA strand breakage sustained by mESC after exposure to 5 Gy of X-rays (Figure 6B). These two results militate against what we initially considered the most likely explanation for the radioprotective effect of resveratrol: that pretreated mESC experience less DNA damage from ROS induced by IR.

Induction of cell cycle arrest is an important component of cellular response to DNA damage that serves to prevent further replication or segregation of chromosomes before repair has been accomplished. mESC delay transit through the S and G2 phases after IR exposure, even though they do not show the pronounced G1 phase arrest seen in most somatic cell types (21,24). Pretreatment with resveratrol did not alter the initial cell cycle response of mESC after 5 Gy X-ray exposure (Figure 4). The distribution of ESC across cell cycle phases was identical in resveratrol-treated, mock-treated or untreated populations prior to irradiation, and accumulation of cells in S and G2 phases occurred equally across the three groups by 3.5 h after damage. There was no indication of G1 checkpoint reactivation. These results rule out the possibility that resveratrol promotes mESC viability after IR by prolonging damage-induced cell cycle arrest and thereby allowing more time for DNA repair.

Rather than augmenting cell cycle arrest, resveratrol pretreatment promoted earlier re-entry into the cell cycle after IR, such that pretreated mESC resumed transit through G1, S and G2 by 7.5 h post-irradiation, whereas controls did not (Figure 4). We tentatively interpreted this as attenuation of the G2 checkpoint. Assuming that G2 checkpoint attenuation would compromise genome stability, we anticipated that the higher viability of resveratrol-pretreated cells after irradiation would be accompanied by higher frequencies of mutation at a reporter locus, the Aprt gene on chromosome 8. Instead, Aprt mutant frequency was modestly reduced among cells pretreated with resveratrol as compared with untreated cell populations (Figure 5). A similar decrease in mutant frequency was also measured in irradiated cells pretreated with DMSO alone, which suggests that this effect is attributable to the vehicle, rather than resveratrol. The mechanism(s) by which DMSO might reduce mutant frequency is unclear. Although we did not detect a reduction of ROS in cells treated with the vehicle, DMSO has been reported to reduce cellular levels of other kinds of reactive species and DNA damage after IR exposure (46,47). Nonetheless, it is clear that resveratrol did not increase mutation frequency, and this argues against attenuation of the G2 checkpoint. We conclude that resveratrol promotes earlier resumption of cell cycle transit after irradiation without compromising genomic integrity.

Recovery from DNA strand breakage was detectable sooner after irradiation in cells pretreated with 10 μM resveratrol than in DMSO-treated or non-treated controls (Figure 6 and Supplementary Figure 2, available at Carcinogenesis Online). This result, together with their improved survival and unaltered mutant frequency, indicates that resveratrol-treated cells repair DNA damage more rapidly after IR exposure and do so without sacrificing fidelity of repair. Our cell culture results complement an in vivo study by Carsten et al. (35), who fed resveratrol to mice for a period of 48 h in advance of whole-body gamma irradiation. After irradiation, the mice were killed and metaphase chromosome spreads were prepared from bone marrow cells. Cells collected from resveratrol-treated mice 1 day after irradiation had significantly fewer chromosome breaks than those of control mice, suggesting that prior ingestion of resveratrol can protect cells of irradiated mice from chromosomal defects arising through failed repair or misrepair of DNA DSBs. This is logically consistent with our results. More rapid, high-fidelity repair of DSBs after IR would favor correct restitution of ends, thus reducing the likelihood of chromosome aberrations such as interstitial deletions, translocations or dicentric chromosome fusions. The latter in particular can initiate ongoing cycles of chromosome breakage and fusion in subsequent rounds of cell division.

We have demonstrated a radioprotective effect of resveratrol in mESC, and taken a first step toward understanding the mechanistic basis of this effect, by showing that it correlates with more rapid DNA repair, rather than reduced DNA damage or delayed cell cycle progression after exposure. The next level of mechanism, how resveratrol promotes rapid non-mutagenic repair of DNA strand breaks in mESC, remains to be determined. In principle, resveratrol might speed repair by facilitating a critical step in one repair pathway or, alternately, it might enhance cellular sensing of DNA damage, such that all downstream repair pathways are more rapidly activated in response to IR exposure. Several possible modes of action by which resveratrol might alter cellular response to DNA damage have been suggested (45). These include modulation of the expression or activity of specific target enzymes that modify DNA or chromatin (e.g., SIRT1) or direct physical interactions with DNA that alter the conformation of chromatin or contacts by structure-responsive proteins of the DNA damage-sensing pathways. The upshot of such effects would be pre-activation of DNA damage response by direct or indirect enhancement of DNA damage signaling. It may be that 48 h treatment of ESC with 10 μM resveratrol causes a low level of DNA damage signaling, sufficient to upregulate DNA damage responses, yet not severe enough to induce cell cycle arrest or apoptosis. If resveratrol effects require changes in transcription or other biosynthetic steps, this might explain why pretreatment for only 40 min failed to protect. If resveratrol induces DNA damage signaling in a concentration-dependent fashion, this might explain why 48 h exposure to 30 or 70 μM resveratrol retarded growth of ESC in this study, but 10 μM resveratrol did not. More generally, a concentration-dependent induction of DNA damage signaling by resveratrol could help to explain why previous reports on its effects have sometimes appeared contradictory. Induction of low-level DNA damage response by low concentrations of resveratrol could account for reported beneficial effects such as protection from apoptosis, chromosomal damage and loss of viability after genotoxic challenge, whereas high levels of DNA damage signaling induced by resveratrol at higher concentrations might underlie its documented abilities to induce apoptosis, genomic instability or general toxicity in some cell culture systems.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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