Chronic Treatment with Resveratrol Induces Redox Stress- and Ataxia Telangiectasia-mutated (ATM)-dependent Senescence in p53-positive Cancer Cells*

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The induction of senescence, an irreversible growth arrest, in cancer cells is regarded as a mean to halt tumor progression. The phytoalexin resveratrol (RV) is known to possess a variety of cancer-preventive, -therapeutic, and -chemosensitizing properties. We report here that chronic treatment with RV in a subapoptotic concentration induces senescence-like growth arrest in tumor cells. In contrast to the widely accepted antioxidant property of RV, we demonstrate that one causative stimulus for senescence induction by chronic RV is an increased level of reactive oxygen species (ROS). The ROS formed upon RV exposure include hydrogen peroxide and superoxide and originate largely from mitochondria. Consistently, co-incubation with the antioxidant N-acetyl cysteine interfered with RV-mediated reactivation of the senescence program. Molecular mediators on the way from increased ROS levels to the observed growth arrest include p38 MAPK, p53, and p21. Moreover, we provide evidence that RV-initiated replication stress, apparent by activation of the ataxia telangiectasia-mutated kinase pathway, is associated with increased ROS levels and senescence induction. This is the first report linking cell cycle effects with a pro-oxidant and pro-senescence effect of RV in cancer cells.

Normal cells possess a finite mitotically active life span. After an inherent number of cell divisions, the so-called Hayflick number, they enter the state of senescence, an irreversible proliferation arrest despite intact metabolic activity (1). There are several forms and triggers of senescence. Replicative senescence is caused by redox stress and/or telomere erosion below a certain threshold (2–5). A second form of senescence is triggered by DNA-damaging agents. Both forms are reported to depend on the DNA damage checkpoint kinases ataxia telangiectasia-mutated (ATM) and Chk (checkpoint kinase) (6–8). ATM is a protein that is well known as a central mediator of responses to DNA double strand breaks and subsequent replication stress. Oncogene activation has been identified as a third trigger for senescence (9, 10) and is also associated with signs of replicative stress (11–13). Downstream mediators of senescence include the tumor suppressor networks around p53 and retinoblastoma protein/p16 (9, 14, 15). If the senescence program fails in transformed cells, mostly due to inactivation of tumor suppressor genes, the cells continue to divide and may proceed to malignancy. Reactivation of senescence in these cells could therefore represent an attractive complementary way to prevent or halt cancer progression (16, 17).

Resveratrol (RV), a polyphenol found in berries, nuts, and red wine, has been assigned a variety of cancer chemopreventive activities including anti-inflammatory, pro-apoptotic, anti-angiogenic, and chemosensitizing properties, in a variety of cell culture and in vivo systems (18–21). As a polyphenol, RV is redox-active and has been claimed to be an antioxidant (22, 23). However, there are also reports showing a pro-oxidant capacity of RV (24). Its anti-proliferative actions in a variety of cell systems are primarily explained by a reversible delay or an irreversible arrest of cells in the S-phase of the cell cycle (25, 26), presumably via inhibition of DNA polymerase and ribonucleotide reductase activity (27, 28). Notably, one study found the DNA damage checkpoint pathways including ATM/ATR (ATM and Rad3-related) and Chk1 kinases to be activated by RV (26).

Outgoing from these data, we asked whether RV can trigger reactivation of senescence in cancer cells, and if so, whether reactive oxygen species (ROS) and/or activation of ATM are involved. In the course of our studies, we observed that RV, chronically administered in a subapoptotic concentration, caused a senescence-like growth arrest in HCT 116 colon carcinoma (and other cancer) cells that was partially reliant on ROS-dependent activation of p38 MAPK, p53, and the induction of p21. Moreover, we gained evidence that also activation of the DNA damage checkpoint kinase ATM contributes to the reactivation of senescence in cancer cells upon RV exposure and that there is an interdependence between redox and cell cycle effects of RV.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Antibodies, Reagents, and Cell Lines**—All chemicals and reagents including resveratrol were purchased from...
Resveratrol Triggers Stress-dependent Senescence

Sigma unless stated otherwise. MitoQ and decyl-tetraphenylphosphonium (decy-TPP) were generous gifts from M. Murphy (Medical Research Council (MRC) Dunn and Antipodean Pharmaceuticals). Anti-p21 antibody was obtained from BD Biosciences, and anti-tubulin and anti-p53 antibodies were from Santa Cruz Biotechnology. Anti-p38-MAPK (phospho (Thr-180/Tyr-182) and total), anti-ERK 1/2 (p (Thr-202/Tyr-204) and total) as well as anti-phospho-(Ser-15) p53 and anti-phospho-Chk1 (Ser-345) antibodies were obtained from Cell Signaling.

Cell Culture, Long Term Cultivation of HCT Cells in the Presence of RV, and Determination of Population Doublings—HCT 116 cells (wild type (WT) and isogenic p53/p21 knock-out clones, kindly provided by B. Vogelstein, The Johns Hopkins, University, Baltimore, MD), were grown in McCoy’s medium (Cambrex) supplemented with 10% serum and 1% penicillin/streptomycin at 37 °C/5% CO2 in a humidified incubator. For determination of a cumulative population doubling curve, cells were plated at each passage in 6-well plates at a density of 200 – 300,000 cells/well and exposed to Me2SO or different concentrations of RV with regular renewal every 48 h. Confluent cells were passaged, and population doublings were determined according to PD = log N (t) – log (N(t0))/log 2, where N(t) is the number of cells per well at time of passage, and N(t0) is the number of cells seeded at the previous passage. The sum of PDs was taken as a measure of cellular superoxide levels.

The fluorescence of each treatment group (corrected for autofluorescence) was then plotted against time of culture. The sum of PDs was then plotted against time of culture.

SA-β-gal Staining—Senescence-associated β-galactosidase (SA-β-gal) staining was performed as described previously (29).

Immunoblotting—After the indicated treatment, cells were lysed and subjected to PAGE and Western blot analysis as described previously (25). Blots were analyzed by a CCD camera (Fuji LAS 3000), and densitometry was performed employing AIDA image analyzer 4.06 software (raytest).

Flow-cytometric Methods for Determination of Total ROS and Superoxide—For determination of total ROS levels, cells were grown in 6-well plates and treated as indicated. Cells were then washed once with 0.2% bovine serum albumin/phosphate-buffered saline and incubated with 20 μM 2’,7’-di-chlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probes/Invitrogen), which is cleaved by intracellular esterases and transformed to a fluorescent dye when oxidized at 37 °C for 30 min prior to immediate analysis by flow cytometry (FACSCalibur, BD Biosciences). The mean fluorescence of 10,000 analyzed cells (corrected for autofluorescence) of each treatment group was taken as a measure for the total ROS load. For determination of superoxide levels, cells were grown in 6-well plates, treated as indicated, and exposed to 20 ng/ml dihydroethidium, a dye specifically oxidized by superoxide, in phosphate-buffered saline, 0.2% bovine serum albumin for the last hour of incubation. After a wash in phosphate-buffered saline, 0.2% bovine serum albumin, cells were immediately analyzed by flow cytometry (FACSCalibur). The mean fluorescence of each treatment group (corrected for autofluorescence) was taken as a measure of cellular superoxide levels.

Determination of H2O2 Levels—Extracellular H2O2 levels were determined with the Amplex Red assay (Molecular Probes/Invitrogen) according to the manufacturer’s instructions and normalized to the analyzed number of cells.

Statistical Analysis—All data are expressed as mean ± S.D. One-way analysis of variance with Dunnett’s multiple comparison test was used to reveal significant differences between control and treatment groups. Differences with p < 0.05 were considered significant. Analyses of the data were performed using the software GraphPad PRISM, Version 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Resveratrol Induces a Senescence-like Growth Arrest in Cancer Cells—To assess a potential pro-senescent activity of RV, we cultivated WT HCT 116 colon carcinoma cells in the presence of different concentrations (1–100 μM) of RV and determined population doublings over time. Although 0–10 μM RV did not affect proliferation rate of HCT 116 cells, 100 μM induced rapid cell death. 30 μM RV slowed down proliferation until cells finally came to a complete growth stop after 30–40 days of treatment (Fig. 1A). The chronic (repetitive) administration of 30 μM RV did not elicit any obvious signs of apoptosis (absence of floating cells or DNA fragmentation throughout the duration of the experiment; data not shown). Cells that were growth-arrested for at least 10 days were stained for SA-β-gal expression and showed up to 60% positive staining, indicating that 30 μM RV induces a senescent phenotype in HCT 116 colon carcinoma cells (Fig. 1B). RV (30 μM)-treated cells displayed a flattened and enlarged morphology and an abrogated bromodeoxyuridine-labeling index (<5%) (data not shown). Moreover, RV growth-arrested cells showed a decreased response to growth factors (Fig. 1C); control cells (no RV treatment) and RV growth-arrested HCT cells were serum-starved for 16 h and stimulated with EGF for 5 min. pERK activation was taken as readout for growth factor responsiveness. Although control cells showed a prominent activation of ERK, chronically RV-treated cells failed to activate ERK upon EGF stimulation. In contrast, short term treatment of RV did not negatively interfere with EGF-mediated mitogenic signaling. These results indicate that repetitive treatment with 30 μM RV over time can trigger senescence-like growth arrest in HCT 116 cells. To rule out that the pro-senescent effect of RV is restricted to HCT 116 cells, we chronically treated the breast carcinoma cell line MCF-7 as well as the epidermoid carcinoma cell line A431 with the appropriate subapoptotic concentration of RV. We observed growth retardation and subsequent proliferation stop as well as SA-β-gal expression in both RV-exposed cell lines, suggesting a general and not tumor cell type-specific pro-senescent effect of RV (supplemental Fig. 1).
growth arrest. For this, we cultivated a p53 or a p21 knock-out HCT cell line (knock-out status confirmed by immunoblotting, supplemental Fig. 2A) that was isogenic to WT clones and of comparable passage number, respectively, were cultivated in the continuous presence of 30 μM RV until cells reached a growth stop. Cells that were growth-arrested for at least 10 days were stained for SA-β-gal and photographed (only knockout cells are shown). The bar graph depicts the counting of SA-β-gal-positive cells of at least five fields of two independent experiments for the indicated clone. DMSO, Me2SO. B, HCT 116 WT and p53 knock-out cells were treated with Me2SO (D) or 30 μM RV for 72 h before their lysates were subjected to a Western blot analysis for p21 and tubulin. C, WT cells were treated with Me2SO or 30 μM RV for 24 and 48 h before their lysates were subjected to a Western blot analysis for phospho-p53 (p-p53 (Ser-15)) and total p53. Representative blots out of three experiments with consistent results are shown. The numbers below the blots depict values of densitometric evaluation (ratio p21/tubulin and p-p53/total p53, respectively, normalized to Me2SO control).

The knock-out lines (Fig. 2A). For unknown reasons, the p53 clones of the HCT line displayed an overall weaker expression of SA-β-gal (up to 60% positive cells in p21 WT versus up to 25% cells in p53 WT cells after the same RV treatment protocol). Furthermore, the growth arrest in p53 and p21 knock-out cells was reversible after withdrawal of RV, whereas halted WT cells were not able to resume proliferation upon RV removal (supplemental Fig. 2B). These data suggest that p53 and p21 are essential mediators for maintenance of an irreversible senescence-like growth arrest triggered by RV. In addition, there is a minor, but reproducible, p53/p21-independent senescence induction by RV that will not further be discussed in the following sections. To exclude HCT 116-specific effects of RV, we also included the p53-negative breast carcinoma cell line MDA-MB 231. Continuous treatment with RV led to a growth arrest that was not accompanied by a significant increase of SA-β-gal staining (in contrast to the p53-positive MCF-7 line) underlining the importance of a functional p53 for senescence induction by RV (supplemental Fig. 2C).
Induction of p21—Since p21 is one of the most prominent downstream targets of p53, and since p53 as well as p21 appeared to be crucial for RV-mediated irreversible growth arrest, we tested whether RV induces p21 in a p53-dependent manner. For this, we examined p21 levels in WT and p53 knock-out HCT 116 cells upon RV treatment. 30 μM RV led to an increase of p21 levels in WT cells as early as 48–72 h after treatment, which could not be observed in the p53 knock-out cells (Fig. 2B), indicating that RV induces p21-mediated senescence-like growth arrest in a p53-dependent manner. Consistently with this hypothesis, 30 μM RV was found to induce phosphorylation of p53 at serine 15 without changing total levels of p53 (Fig. 2C).

Next we addressed the underlying mechanism by which RV activates p53. p53 is known to be a transcription factor highly responsive to changes in the cellular redox state, and RV has been reported to exert both pro-oxidant and antioxidant effects (24). Moreover, redox stress has been implicated in the onset of cellular senescence. We therefore examined whether repetitive treatment with RV could elicit chronic redox changes in HCT cells. Employing the redox-sensitive fluorescent dye H2DCF-DA (detecting all intracellular ROS, namely H2O2, superoxide, and peroxynitrite) and subsequent flow cytometric analysis, we observed a time-dependent increase of total ROS with a significant elevation as early as 12 h after RV exposure (Fig. 3A) that lasted up to 48 h after a single pulse of 30 μM RV. The rise in intracellular ROS load did not require p53 nor p21 since both knock-out clones responded in a comparable fashion to RV exposure (data not shown).

To rule out a mere accidental coincidence of ROS accumulation and senescence induction by RV, we employed the antioxidant N-acetylcysteine (NAC). We observed that incubation with 10 mM NAC was able to significantly suppress the number of senescent cells that occurred after a long term incubation with RV (Fig. 3B). We furthermore showed that NAC interfered with RV-triggered p53 phosphorylation (Ser-15) and p21 induction (Fig. 3, C and D). Consistent results were obtained with two additional antioxidants, namely 4 mM tempol, a superoxide scavenger, or 2000 units/ml polyethylene glycol-catalase (data not shown). We therefore conclude that elevated ROS levels contribute to senescence induction by RV.

In accordance with the elevated ROS levels, the redox-sensitive protein kinase MAPK-p38 was found to be significantly hyperphosphorylated 24 h after treatment with RV without changes in its total levels. This effect could be overcome by preincubation of cells with 10 mM NAC (Fig. 3E), suggesting that activation of p38 MAPK by RV depends on elevation of ROS. p38 MAPK has been reported to be a positive regulator of senescence (30–32). We could observe that p38 also contributes to activation of p53 and p21 induction in our system since the p38-specific inhibitor SB 203580 overcame the RV-trig-
triggered induction of p21 (Fig. 3F, upper panel). SB 203580 interfered with RV-mediated p53 phosphorylation (Fig. 3F, lower panel), although to a lesser extent than with p21 induction, which implies a p53-independent induction of p21 downstream of p38 (33). We conclude that RV can induce senescence-like growth arrest in p53-positive cancer cells, at least partly, by the sequel: RV → increase of ROS → activation of p53 (via MAPK-p38) → the induction of p21 → growth arrest.

RV-triggered ROS Include Superoxide and Hydrogen Peroxide and Originate from Mitochondria—Since ROS turned out to be one major trigger for RV-induced senescence, we next aimed to elucidate the nature and source of ROS produced upon RV exposure. Using dyes specific for H2O2 (Amplex Red) (Fig. 4A) and superoxide (dihydroethidium) (Fig. 4B), respectively, we observed an increase in both H2O2 and superoxide in HCT cells after 24 h with RV. Preincubation of the cells with the superoxide scavenger tempol (4 mM) substantially reduced both the RV-mediated increase of dihydroethidium fluorescence and the Amplex Red signal, suggesting that RV-induced H2O2 levels are directly derived from superoxide.

To investigate the source of ROS upon RV treatment, we employed the following inhibitors: diphenyliodonium (inhibitor of all flavoproteins including NAD(P)H-dependent oxygenases (NOXes), nitric oxide synthases (NOSes), and mitochondrial respiratory complexes), apocynin (selective inhibitor of NOXes), L-N(G)-nitro-L-arginine-methylester (NOS inhibitor), allopurinol (xanthine oxidase inhibitor), mitoQ (targeted mitochondrial antioxidant) decyl-TPP (negative control for mitoQ to exclude effects due to mere mitochondrial accumulation), and AA861 (selective inhibitor of 5-lipoxygenase) (Fig. 4C). Since mitoQ and diphenyliodonium next to the general antioxidants NAC and catalase but not selective NOX, NOS, xanthine oxidase, nor 5-lipoxygenase inhibitors were able to significantly overcome RV-mediated ROS production, we conclude that mitochondria are the main source of ROS during RV treatment in our setup. Consistently, we were also able to observe a decrease in the MMP upon RV exposure (Fig. 4D). Consistent results were obtained in the breast carcinoma cell line MCF-7 (supplemental Fig. 3).

Activation of the DNA Damage Checkpoint Kinase ATM Contributes to Senescence Induction by RV and Is Associated with Elevation of ROS—After having defined a role for ROS, we next focused on the potential contribution of ATM kinase to senescence induction by RV. During the cultivation of cancer cells in the presence of RV (Fig. 1A), we observed a retarded growth rate until the cells finally entered senescence– like growth arrest. We observed that 30 μM RV hereby induced a transient retardation of cells in S-phase that is accompanied by foci of phosphorylated γ-histone 2AX as well as an activation of ATM/Chk1 (but not Chk2)/cdc2 pathway (supplemental Fig. 3, A and B). This agrees with the report of Tyagi et al. (26) in ovarian cancer cells and suggests the occurrence of DNA damage or replication stress (34) upon RV exposure. To decipher the role of ATM kinase activity for senescence reactivation by RV, we employed two ATM inhibitors, namely caffeine (5 mM) and KU55993 (10 μM). We could demonstrate that both inhibitors overcame RV-induced H2O2 increase related to the respective Me 2SO control (p < 0.05 RV versus Me2SO, #, p < 0.05 time point RV (no tempol) versus RV (plus tempol)). C, HCT cells were exposed to Me2SO or RV (30 μM) for 22–24 h prior to a 2-h incubation with the inhibitors/antioxidants as indicated. Total ROS levels were then determined by H2DCF-DA as described under “Experimental Procedures.” RV-triggered elevation of ROS is depicted as -fold Me2SO control (n = 3) (*, p < 0.05; RV versus Me2SO; #, p < 0.05 RV (no tempol) versus RV (plus tempol)). D, HCT 116 cells were treated with 30 μM RV for the indicated periods of time before MMP was determined by JC-1. Compiled data of three independent experiments are depicted as -fold Me2SO control (p < 0.05 time point X versus time point 0).
We next were interested in whether there is an association between elevated ROS and replication stress in RV-treated cells. For this, we explored (a) the influence of the antioxidant NAC on ATM activation and signaling and (b) the influence of KU 55993 and caffeine on ROS levels. 10 mM NAC was able to reduce ATM activity measured by Chk-1 phosphorylation, suggesting that ROS are implied in RV-mediated replication stress (Fig. 5C). On the other hand, inhibition of ATM kinase activity by caffeine or KU55993 was capable of reducing the elevation of ROS upon RV exposure, indicating that replication stress *vice versa* influences ROS production (Fig. 5D).

These data suggest that the RV-mediated effects on redox milieu and cell cycle progression are interconnected and that both contribute to the induction of senescence upon repetitive exposure to RV. Fig. 6 depicts our findings schematically.

**DISCUSSION**

We showed that chronic administration of subapoptotic concentrations of RV induces senescence-like growth arrest in cancer cells. This process occurs to be dependent on p53 and p21. The potential causative trigger for senescence induction, apart from the already described inhibition of telomerase at high concentrations (35, 36), could be confined to chronically increased ROS production as well as activation of the DNA damage checkpoint kinase ATM upon RV exposure. The source of RV-induced elevation of ROS, including H2O2 and superoxide, could be largely assigned to mitochondria in our system. Moreover, we observed mutual cross-talk between increased ROS levels and activation of ATM after RV exposure. This is to the best of our knowledge the first report that investigates the long term effects of RV on cancer cells, reveals ROS as a major signaling intermediate in the action of RV, and thereby elucidates causal interrelationships between so far seemingly isolated actions of RV, namely redox activity, cell cycle effects, and putative anti-cancer activity including the novel pro-senescent effect.

Elevated ROS levels have been widely accepted as a causative trigger for senescence-like growth arrest (2, 3) and have here been demonstrated to be involved in senescence induction by RV. RV has so far been mostly described as an antioxidant. A potential explanation for this apparent contradiction may lie in the readouts used. When looking at the induction of antioxidant defense enzymes such as manganese superoxide dismutase or glutathione S-transferase (22, 37), RV may appear as an antioxidant since intracellular ROS, possibly caused by RV itself,
Resveratrol Triggers Stress-dependent Senescence

**FIGURE 6. Proposed mechanism of senescence induction by RV-mediated redox and replication stress.** Chronic RV triggers the induction of senescence by activation of p53 and the induction of p21. Upstream events include elevation of ROS levels (from mitochondria (mito)) and replication stress (presumably by inhibition of DNA replication) that merge into the transcriptional activation of p53 by phosphorylation at Ser-15 by activated p38-MAPK and ATM kinases, respectively. There is an apparent cross-talk between redox and replication stress with so far unidentified molecular transducers.

The tumor suppressor p53 turned out to be an essential downstream mediator and integrator between ROS or ATM and senescence-like growth arrest in RV-treated tumor cells. RV induces p21 expression and subsequent growth arrest by phosphorylation of p53 at serine 15 via activation of both ATM and p38 kinases. RV-mediated effects on other phosphorylation or acetylation sites of p53 cannot be excluded and need further investigation. The same applies for the role of Rb in RV-induced senescence. Since HCT 116 cells lack a functional p16 due to promoter methylation and RV could not be shown to change p16 expression in our cells, RV-triggered senescence induction appears to occur independently of p16 (40).

We further observed a positive and mutual feedback between ATM activation and increased ROS levels upon RV exposure (Fig. 5). Our data are compatible with reports that indicate an association of replication stress in S-phase with subsequently elevated ROS (41, 42) as well as studies that identify activation of ATM mainly as a response to oxidative stress and subsequent DNA damage (34, 43, 44). The issue of how RV-triggered redox and replication stress exactly are temporally and causally related remains unresolved and warrants further investigation. In this context, it will be interesting to examine the possibility of oxidative DNA lesions upon RV exposure as done for the cancer chemopreventive compound epigallocatechin-gallate (45, 46) or to unambiguously show RV-mediated inhibition of DNA polymerase or ribonucleotide reductase activity in vivo. Moreover, it is still unclear what immediate target RV is hitting to elicit its effects. Immediate binding partners and targets of RV that have been described include the histone deacetylase SirT1 (47) and the α/βV-integrin (48). However, neither of them seems to be involved in the effects of RV that are reported here. In preliminary experiments, we employed the SirT1 inhibitors sirtinol and nicotinamide as well as the integrin-blocking peptide RGD. None of them were able to abrogate replicative or oxidative stress responses upon RV exposure, although the integrin signalosome including NOXes and other redox-sensitive proteins as well as SirT1 influencing mitochondrial biosynthesis seemed promising candidates to explain the effects of RV.

Overall, our studies add a novel facet to the cancer chemopreventive action of RV and set so far isolated actions of RV in a causal context. Chronically administered in a sub-apoptotic concentration, RV elicits redox and replication stress that both merge, via activation of ATM and p38 MAPK, into p53/p21-dependent senescent-like growth arrest in cancer cells. For a potential application of RV as a cancer chemopreventive agent or adjuvant in chemotherapy, however, thorough studies focusing on bioavailability, the half-life of RV and the efficacy of its metabolites are needed since senescence induction might require the continuous presence or regular pulses of the active principle in a sufficiently high concentration.
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Addendum—During the preparation of this manuscript, Boocock et al. (49) published the results of a phase I dose escalation pharmacokinetic study with RV in healthy volunteers. Examining blood and urine after oral incorporation of RV, they found up to eight times higher systemic levels of RV metabolites (sulfate and monoglucuronides) than of the parent compound. These findings warrant the investigation of the pharmacological profile of RV metabolites. Moreover, and as for almost all chemotherapeutic agents, a selective targeting of RV to cancer cells seems desirable since a pro-oxidative and/or pro-senescent effect in untransformed cells cannot be excluded at this point.

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