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Original Contribution

Resveratrol delays replicative senescence of human mesothelial cells via mobilization of antioxidative and DNA repair mechanisms ☆

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ABSTRACT

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; RVT) is a natural phytoestrogen known to modulate the rate of senescence in cultured cells. The mechanism by which RVT affects this process is still elusive. In this paper we used human peritoneal mesothelial cells (HPMCs) to examine the effect of RVT (0.5 and 10 μM) on their growth and senescence, with particular emphasis paid to parameters associated with oxidative stress. The results showed that RVT used at a concentration of 0.5 μM (but not at 10 μM) markedly improved HPMC growth capacity, as evidenced by elevated expression of PCNA antigen, augmented fraction of cells in the S phase of the cell cycle, and increased number of divisions achieved before senescence. These effects coincided with diminished expression and activity of senescence-associated β-galactosidase but were not associated with changes in the telomere length and an incidence of apoptosis. Moreover cells exposed to 0.5 μM RVT were characterized by increased release of reactive oxygen species, which was accompanied by up-regulated biogenesis of mitochondria and collapsed mitochondrial membrane potential. At the same time, they displayed increased activity of superoxide dismutase and reduced DNA damage (8-OH-dG and γ-H2A.X level). The efficiency of 8-OH-dG repair was increased which could be related to increased activity of DNA glycosylase I (hOgg1). As shown using RT-PCR, expression of hOgg1 mRNA in these cells was markedly elevated. Collectively, our results indicate that delayed senescence of HPMCs exposed to RVT may be associated with mobilization of antioxidative and DNA repair mechanisms.

Introduction

Replicative senescence is a phenomenon that limits growth of normal somatic cells in vitro and also, probably, in vivo [1]. There is broad agreement that senescence is a cell response to extensive and usually irreparable injury to telomeric and/or nontelomeric regions of the genome [2,3]. Although numerous internal and external stimuli have been recognized to elicit cellular senescence [4], the oxidative stress—especially in terms of a deleterious activity of reactive oxygen species (ROS)—has been found to be the major one [5]. Since a key intracellular source of ROS are mitochondria, dysfunctional metabolism of these structures has been identified as a central element driving the development of the oxidative stress in cells undergoing replicative senescence [6,7].

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; RVT) is a naturally occurring polyphenolic phytoestrogen produced by a wide

Abbreviations: 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; BSA, bovine serum albumin; CPD, cumulative number of population doublings; CPM, counts per minute; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; hOgg1, DNA glycosylase I; HPMCs, human peritoneal mesothelial cells; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; HUVEC, human umbilical vein endothelial cells; NAO, *N*-nonyl acridine orange; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; RVT, resveratrol; SA-β-Gal, senescence-associated β-galactosidase; SOD, superoxide dismutase; TCA, trichloroacetic acid

☆ The authors declare no conflict of interest.

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range of plants, including grapes, peanuts, and mulberries. This compound is acknowledged for its cardioprotective, anti-inflammatory, and anticancer properties (see [8] for review). In recent years RVT has attracted a great deal of attention also due to its antiaging activity. It has been observed that it is able to extend the lifespan of multiple model organisms, including *Saccharomyces cerevisiae* [9], *Drosophila melanogaster* [10], and *Caenorhabditis elegans* [11]. There is evidence that this effect may be associated with the activation of sirtuins which belong to the family of NAD(+)-dependent histone deacetylases and play an important role in the regulation of energy homeostasis, maintenance of genetic stability, and stress response [9,12]. In contrast to well established life-prolonging effects of RVT in vivo, the results obtained from the in vitro studies are sparse and ambiguous. It has been found that RVT improves the proliferative capacity of mesenchymal stem cells [13] and endothelial progenitor cells [14], whereas in keratinocytes it exerts the opposite effect [15]. The conflicting results have also been shown with respect to its impact on replicative cell lifespan. Namely, in fibroblasts RVT increased the number of population doublings achieved before entry into senescence [16], while in endothelial cells (HUVECs), the cell lifespan was significantly diminished [17].

Interestingly enough, there is an ongoing debate on whether RVT displays antioxidative or prooxidative activity. In a classic view, RVT is considered as a strong antioxidant due to its ability to reduce the production of ROS [18] and up-regulate the efficacy of the antioxidative systems, including reduced glutathione [19] and superoxide dismutase [20]. This picture has been blurred, however, by the plethora of studies on cancer cells in which RVT was found to disrupt cellular antioxidant activities leading to the enhanced oxidative stress and concomitant growth inhibition and/or apoptosis [21,22]. Also in endothelial cells, their premature senescence in response to RVT has been found to proceed with an increased generation of ROS [17]. Because of this "oxidative duality," the issue of whether and how RVT modulates cellular oxidative stress during senescence is still poorly understood.

Taking the aforementioned into account we designed a project to deeply elucidate both the ambiguities concerning the activity of RVT in vitro, namely its effect on cell growth/senescence, and the oxidative cell balance. To this end we employed human peritoneal mesothelial cells (HPMCs)—the major cell population within the peritoneal cavity with a well-characterized mechanism of senescence [23–25]—and examined parameters such as the efficiency of DNA synthesis, the number of population doublings achieved, the expression of senescence marker (SA-β-Gal), the length of telomeres, the occurrence of apoptosis, the generation of ROS, the activity of antioxidants (superoxide dismutase; SOD), the magnitude of DNA damage (8-OH-dG, γ-H2A.X), the activity of DNA repair mechanism (DNA glycosylase I; hOgg1), and the metabolism of mitochondria (biogenesis, inner membrane potential).

Methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The tissue culture plastics were from Nunc (Roskilde, Denmark).

Approximately 99% pure RVT was obtained from Sigma-Aldrich Corp. A stock solution was prepared in dimethyl sulfoxide (DMSO) and diluted in a culture medium to desired final concentration. The concentration of DMSO was always 0.05% (v/v). During the experiments RVT was used at concentrations up to 10 μM which, as shown in the preliminary studies, did not affect HPMC viability. Fresh culture media were prepared from the stock solutions every 3 day. A chemical stability of RVT in culture media within this time period was confirmed using high performance liquid chromatography (HPLC), as described in detail in [26].

Cell cultures

Primary cultures of human peritoneal mesothelial cells were isolated from the pieces of omentum, as described elsewhere [27]. Briefly, the tissue fragments were obtained from consenting patients undergoing elective abdominal surgery. The study was approved by the institutional ethics committee and all the patients gave their informed consent. All cultures were established from individuals with no evidence of peritonitis and no overt diabetes, uremia, and peritoneal malignancy. The age of the donors ranged from 24 to 30 years. The cells were propagated in medium M199 supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.4 μg/ml), and 10% (v/v) fetal bovine serum (FBS) (Gibco, Invitrogen, Karlsruhe, Germany). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

HPMCs were forced to senescence by the serial passaging at 7-day intervals with a fixed seeding density of 3×10^4 cells/cm^2. The cells from passages 1–2 were treated as "young," while those that failed to increase in number during 4 weeks and stained $>70\%$ for SA-β-Gal were considered as "senescent" [25]. During the experiments, HPMCs were continuously exposed to standard growth medium (control group) and to the medium supplemented with 0.5 and 10 μM RVT. The culture media were exchanged every 3 day.

Measurement of cell proliferation using MTT assay

HPMCs were seeded into 96-well plates at a low density of 1×10^3 cells/cm^2 and allowed to attach for 12 h. Then the cells were incubated in medium containing 1.25 mg/ml of the MTT salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 24 h at 37 °C. The formazan product generated was solubilized by the addition of 20% sodium dodecyl sulfate and 50% *N,N*-dimethylformamide. Absorbance of the converted dye was recorded at 595 nm with a reference wavelength of 690 nm.

Measurement of cell proliferation using [^{3}H]thymidine uptake assay

HPMCs were plated onto 48-well clusters at a density of 2.5×10^4/cm^2 and allowed to attach for 4 h. The subconfluent cultures were then exposed to the control medium or medium with RVT for 24 h at 37 °C. The mixture was labeled with 1 μCi/ml [*methyl*-^{3}H]thymidine (Institute of Radioisotopes, Prague, Czech Republic) and supplemented with 1% FBS to stimulate HPMC proliferation. After incubation, cells were harvested in trypsin–EDTA solution and precipitated with 20% (w/v) trichloroacetic acid (TCA) overnight at 4 °C. The precipitate was washed with 10% TCA and dissolved in 0.1 N NaOH. The radioactivity released was measured in a beta liquid scintillation counter (LKB Wallac, Turku, Finland). Results were expressed as the counts per minute (cpm).

Detection of senescence-associated β-galactosidase (SA-β-Gal)

The presence of SA-β-Gal was detected according to Dimri et al. [28]. Briefly, HPMCs were grown on Lab-Tek Chamber Slides, fixed with 3% formaldehyde, washed, and exposed for 6 h at 37 °C to a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-