Chronic Resveratrol Treatment Ameliorates Cell Adhesion and Mitigates the Inflammatory Phenotype in Senescent Human Fibroblasts

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We evaluated the effect of resveratrol on the senescence-associated secretory phenotype (SASP) and on adhesion-related processes in cultured human MRC5 fibroblasts. Presenescent cultures were chronically treated with or without 5 µM resveratrol. The development of SASP in MRC5 fibroblasts approaching senescence was significantly attenuated by resveratrol treatment, which reduced both gene expression and release of proinflammatory cytokines. Although to a lesser extent, 1 µM resveratrol proved to be effective on cytokine gene expression. Cell spreading capacity and plating efficiency were strikingly increased and accompanied by recovery of type I collagen expression to presenescent levels. As p16INK4a protein expression was not significantly modified, and based on our previous data, we propose that resveratrol does not affect fibroblast replicative senescence, but improves tissue maintenance and repair during normal cellular aging. Considering these low concentrations proved effective in vitro, translation of these data to human research on inflammation-related pathologies can be envisaged.

Key Words: Resveratrol—Aging—Natural antioxidants—SASP—Cell adhesion.

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CELLULAR senescence is a complex biological process causing modifications in the protein expression profile of the cell and leading to replicative arrest and changes in metabolism, adhesion efficiency and secretion phenotype (1). Some of these modifications have tumor-suppressive effects because they reduce the proliferation capacity of mutated cells and as such are beneficial to tissues and organs. However, senescent cells are characterized by a strong increase in the secretion of growth factors, inflammatory cytokines, and proteolytic enzymes, termed the “senescence-associated secretory phenotype” (SASP), that can exert the opposite activity by creating a tumor-favoring tissue microenvironment (2,3).

Fibroblasts produce and organize the components of the extracellular matrix (ECM) and play a major role in intercellular communication and regulation of tissue homeostasis. It has been repeatedly shown that senescent, but not presenescent, breast fibroblasts are able to transform cell lines in a mouse xenograft model, to promote progression of tumour cell lines (4), and to stimulate the growth of breast cancer (5) and of preneoplastic and neoplastic prostate epithelium in vitro (6). These activities are thought to be strictly related to their SASP. In fact, in senescent fibroblasts, interleukin-6 (IL-6) and interleukin-8 (IL-8) are the SASP cytokines most robustly released (7), and they have been shown to play an important role in the regulation of growth, survival, and invasive behavior of cancer cells (8,9). In addition, both IL-6 and IL8 exhibit potent effects on vascular endothelial cells and consequently on tumor angiogenesis (10,11). Another proangiogenic SASP component is vascular endothelial growth factor (VEGF). Conditioned medium from senescent fibroblasts, rather than from presenescent cells, was shown to stimulate cultured human umbilical vein endothelial cells to invade a basement membrane (12).

Fibroblasts participate in wound healing and tissue ECM remodeling, and these functions are modified by senescence. In fact, senescent fibroblasts have a poor migration capacity and they attach to and detach from the culture substrate less readily than young cells. Senescent tendon fibroblasts exhibited low motility, a poorly organized actin cytoskeleton, and a different localization of key focal adhesion proteins as compared with young cells (13). Human fibroblasts from skin and fetal lung at senescence

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showed changes in cytoskeletal and focal adhesion proteins expression and subcellular localization (14). Also, a reduced type I collagen expression has been reported in senescent fibroblasts (15,16). The expression of type I collagen was associated with better tissue remodeling and repair in rat tendon fibroblasts (17). Therefore, it is feasible that these changes in adhesion properties and ECM molecules production occurring during the senescence process are associated with the reduced healing capacity and increased frailty affecting senescent tissues.

Resveratrol (trans-3,4’,5-trihydroxystilbene), a polyphenolic phytoalexin found in several fruits, mainly in red grape skin, has recently attracted considerable interest for its many biological and pharmacological activities. Among them, antiaging effects have been reported in complex organisms, from Nematodes to mammals (18), and in cultured endothelial cells (19,20). We have previously shown that resveratrol chronically administered to human MRC5 fibroblasts until senescence was able to counteract senescence-associated changes, such as DNA damage and nuclear rearrangements, and to increase the number of completed population doublings (PDL) before replicative arrest (21).

The aim of the present work was to further investigate the antiaging activity of resveratrol in vitro. Preliminary results (data not shown) of gene expression profiling in senescent MRC5 fibroblasts indicated that inflammatory pathways and ECM remodeling were modulated by the long-term treatment with resveratrol. Thus, we focused our research on the effects of resveratrol on collagen expression, cell adhesion and spreading, and expression and secretion of SASP cytokines in MRC5 fibroblasts treated until the onset of the senescent phenotype.

**METHODS**

**Cell Cultures**

MRC5 cell line is a normal human fibroblast cell line derived from a fetal lung tissue, purchased from National Institute of Aging (NIA) Aging Cell Repository (Coriell Institute, Camden, NJ, USA). MRC5 cells were cultured in high-glucose (4,500 mg/L) Dulbecco’s Modified Eagle’s Medium (BioWhittaker, Lonza, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (BioWhittaker, Lonza), and penicillin-streptomycin (Sigma–Aldrich Chemicals Co., St. Louis, MO, USA), at 37°C in 5% CO2 humidified atmosphere. When at confluence, cultures were propagated by trypsinization and splitting. The experiments were conducted starting from the so-called presenescent cells, defined as having undergone more than 44 and fewer than 55 PDL. After 4–5 weeks of propagation in culture, these cells became senescent, as already described (21). Senescent cultures were defined as having completed more than 55 PDL and having more than 80% SA-β-gal positive cells (assayed by using the Senescence β-Galactosidase Staining Kit, Cell Signaling, Danvers, MA, USA). Fibroblast cultures were treated with 5 µM resveratrol (Sigma–Aldrich, St. Louis, MO, USA) from the beginning of the experiments till senescence. The culture medium was replaced every 2 days to maintain the resveratrol concentration relatively constant over time (21).

**Proinflammatory Cytokine Gene Expression Assessed by Quantitative Real-Time Polymerase Chain Reaction**

Presenescent fibroblasts were maintained for 3 or 5 weeks with and without 5 µM resveratrol. In dose-effect experiments, cultures were maintained for 5 weeks with and without both 1 and 5 µM resveratrol concentrations. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Then mRNA expression of proinflammatory cytokine genes were assayed by quantitative real-time polymerase chain reaction (PCR) using the primers listed in Table 1. Fifty nanograms of cDNA was amplified using the following conditions: 50°C for 2 minutes, 95°C for 15 seconds and 60°C for 1 minute. For each target gene analysis was performed according to the 2−ΔΔCT method, using GAPDH as the reference gene.

**Table 1. Primer Sequences used for Real-Time PCR Evaluation of the Indicated Genes Expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>5'-AGTAGGAAACCAAGCCGGAAAGG-3'</td>
<td>5'-TGTTGGTGTCTCCTATCTTG-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-ACAGATGAAGTGCTCCTTCCA-3'</td>
<td>5'-GTCGGAGATTGCTAGTGATG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GGTATCCCTGACGACCTCAT-3'</td>
<td>5'-GTGCCCTTGGCTGATTAC-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-ATGACTTCCAAGCTGCGGTTG-3'</td>
<td>5'-CTCTAGGCCTTCCTTCAAACCT-3'</td>
</tr>
<tr>
<td>GROα</td>
<td>5'-GGTCTCAACATTTTCTCATG-3'</td>
<td>5'-CTAAGCCAGAACAACCTGGT-3'</td>
</tr>
<tr>
<td>MMP3</td>
<td>5'-AGCAAGGACTCGTTTATTCT-3'</td>
<td>5'-GTCATCGAGAGCCTGGTTCA-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>5'-GAATGATGTGAAAATTCATGCTCAT-3'</td>
<td>5'-TCTCGACCCCTTTCCAACCTT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CTCCTCTGACTTCAACA-3'</td>
<td>5'-GTTGCCAAACTTGTTGTCA-3'</td>
</tr>
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*Notes: GROα = growth-related oncogene-α; IL = interleukin; MMP3 = matrix metalloproteinase 3; VEGF = vascular endothelial growth factor.*
Presenescent fibroblasts (2 × 10^4 cells per well) were seeded into 96-well plates and cultured in the presence and in the absence of 5 μM resveratrol. At different time points (48 hours, 1 week, 3 weeks, and 5 weeks), 48-hour conditioned media were collected, centrifuged at 1000g per 10 minutes, and frozen at −20°C. Simultaneous assessment of the secretion of IL-6, IL-8, interleukin-1 (IL-1)α, IL-1β, growth-related oncoenzyme-α (GROα), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and VEGF proteins was performed on the thawed conditioned media by using Millipore MilliPlex Human Cytokine and Chemokine Kit (Millipore, Billerica, MA, USA) and Bio-Plex 200 System apparatus (Biorad Laboratories), according to the manufacturer’s recommendations. Cell density was determined for each sample with a Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA). Briefly, 20 μL of a combined solution of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; inner salt), and an electron coupling reagent, phenazine methosulfate, was added to each well. The absorbance at 490 nm (optical density 490 nm) was recorded using an enzyme-linked immunosorbent assay plate reader, after a 60-minute incubation at 37°C in humidified 5% CO2 atmosphere. Cytokine and chemokine concentrations were normalized to corresponding cell density and reported as pg/mL of secreted protein per cell.

**Western Blot Analysis**

Presenescent fibroblasts were cultured for 4 weeks with and without 5 μM resveratrol, and cell aliquots were collected at both the beginning and the end of the treatment for western blot analysis. Harvested cells were lysed in radio-immunoprecipitation assay buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with 1% protease and phosphatase inhibitor Cocktail (Sigma–Aldrich Chemicals Co.) and disrupted by sonication (Microson XL-2000; Misonix, Farmingdale, NY, USA). Lysates were clarified by centrifugation and supernatants collected at −20°C to perform western blot analysis. Protein content was estimated by using the Bio-Rad DC protein assay kit (Bio-Rad, Segrate, Milan, Italy). Thirty micrograms of protein aliquots was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation ([SDS–PAGE] NuPAGE, Novex; Invitrogen, San Giuliano Milanese, Italy) and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Total amounts of type I collagen and GAPDH protein (used as loading control) were determined by immunostaining with specific primary rabbit polyclonal antibodies (anti-Collagen I, ab34710, Abcam, Cambridge, MA, USA; anti-GAPDH, 14C10, Cell Signaling Technology, Danvers, MA, USA) and suitable peroxidase-conjugated secondary antibodies (Sigma–Aldrich Chemicals Co.). Proteins were visualized using the enhanced chemiluminescence procedure on Immobilon Horseradish Peroxidase Substrate (Millipore) and immune-reactive bands were quantified using the Quantity-One software (Bio-Rad Laboratories) by densitometric analysis. Each density measure was normalized by using the corresponding GAPDH level as internal control.

**Cell Adhesion and Spreading Assay**

About 10^5 presenescent fibroblasts were cultured for 4 weeks in the presence and in the absence of 5 μM resveratrol. At the end of treatment, cells were serum starved for 24 hours before detaching with 0.25% trypsin for 1 minute. Trypsin was blocked with 0.2 mg/mL soybean trypsin inhibitor, and cells were resuspended in 2 mL of fresh medium per 10-cm dish and directly seeded onto dishes precoated overnight with 10 μg/mL human fibronectin. Cells were observed and photographed at different time points for a period 3 hours, using a phase-contrast microscope (Nikon Instruments Inc., Melville, NY, USA). At the end of the experiment, cells were fixed with methanol–acetic acid (3:1) solution and then stained with 0.5% crystal violet in 20% MetOH. After solubilization in 200 μL per well sodium citrate 0.1 M, pH 4.2, density of cells adhering to dish was quantified at 595 nm by a spectrophotometer.

**Statistical Analysis**

Unless specifically indicated, all data were analyzed using one-way analysis of variance. Data were expressed as mean ± SEM. Statistical significance was defined as p < .05.

**Results**

**Gene Expression and Secretion of Proinflammatory Factors**

We assayed the gene expression levels and release of some cytokines whose production was reported to increase during cell senescence (3), namely, IL-1α and β, IL-6, IL-8, TNF-α, VEGF, GM-CSF, and GRO, and the gene expression of matrix metalloproteinase 3 (MMP3). Presenescent MRC5 fibroblast cultures were maintained in the presence or absence of 5 μM resveratrol for different times up to senescence (corresponding to 5 weeks in culture) and then cells and 48-hour conditioned media were collected.

Real-time PCR experiments showed that in fibroblasts approaching replicative senescence, the gene expression levels of IL-1α and β strongly increased, like those of IL-6, IL-8, GROα and VEGF (Figure 1, panel A). The treatment with resveratrol significantly reduced these increases both after 3 weeks and after 5 weeks. MMP3 expression level did not change between 3 and 5 weeks in control cultures, nor did resveratrol treatment modify it.
Dose-effect experiments were performed following 5 weeks of treatment, and showed that the 1 µM concentration of resveratrol also proved to be effective, although to a lesser extent (Figure 1, panel B).

Cytokine release was assayed in MRC5 conditioned media collected as reported earlier. Under these conditions, IL-1α and β and TNFα were not detectable. As shown in Figure 2, in untreated cells, the secretion of all the assayed cytokines increased with the progression of senescence. At 5 weeks, a tendency toward a release reduction was observed for all the assayed cytokines, less pronounced for VEGF that was detectable after 3 weeks and remained almost constant around 250 pg/mL/10^5 cells. Instead, the secreted level of GM-CSF increased from 13.86 pg/mL at
48 hours to 51.99 pg/mL at 3 weeks and remained constant at 5 weeks (53.32 pg/mL). The treatment with resveratrol induced a statistically significant reduction in the release of IL-6, IL-8, GROα and VEGF, compared with untreated cells, at 3 but not at 5 weeks of treatment. GM-CSF release instead was not modified by the treatment (data not shown).

Collagen Expression
Type I collagen protein produced by senescent fibroblasts was measured by western blot analysis. The results shown in Figure 3 indicate that during senescence, the expression of this protein decreases and the treatment for 4 weeks with 5 μM resveratrol restores its expression level to values similar to those found in younger cells, although with increased variability.

Cell Spreading and Adhesion
Cell spreading on a fibronectin-coated substrate was impaired in senescent fibroblasts in comparison to young cells. In fact, at 180 minutes after seeding, the cells appeared to have not completed the attachment to the dish, contrary to young cells, which at that time were already attached and exhibited their normal morphology (data not shown). The treatment with 5 μM resveratrol for 4 weeks significantly improved adhesion capacity of senescent cells, as shown by both morphological analysis (Figure 4, panel A) and counting of attached cells, which resulted to be about six times higher than that of untreated cells (Figure 4, panel B).

p16ink4a Protein Expression
p16ink4a protein expression was measured by western blot analysis. The results show an increase in p16 protein levels from 1 to 5 weeks in culture in the control cells progressing towards senescence; this increase was not significantly affected by resveratrol (Figure 5).

Discussion
Chronic low-grade inflammation has been associated with numerous age-related diseases and with aging itself (22). The present data confirmed the development of SASP in human fetal lung MRC5 fibroblasts approaching cell senescence. We observed that the amount of released inflammatory cytokines was lower when replicative senescence was fully attained, possibly due to the miR-146a and/or b-mediated negative feedback loop limiting cytokine release (23). We also showed that the long-term treatment with resveratrol significantly attenuated the

Figure 2. Effect of resveratrol treatment on the release of four proinflammatory cytokines.
Presenescent MRC5 fibroblasts were maintained with and without 5 μM resveratrol for 48 hours and for 1, 3, or 5 weeks. Cytokine assays were performed at different time points (48 hours, 1, 3, and 5 weeks) on 48-hour conditioned media. Results are displayed from control (white dots) and treated (grey dots) culture conditions. Data are expressed as pg/mL/10^5 cells (mean ± SEM, n = 3–5). *p < .05 vs control (Student’s t test for paired data).
development of SASP in senescent fibroblasts, by reducing the release of proinflammatory cytokines both at presenes-
cence and at senescence.

Resveratrol is a potent anti-inflammatory agent. One of the mechanisms of this activity is the inhibition of the nuclear transcription factor NF-kB, whose activation triggers the expression of proinflammatory cytokines like TNF, IL-1, and IL-8. Resveratrol has been shown to be an inhibitor of NF-kB activation and NF-kB-dependent gene expression, through its ability to inhibit IkB kinase activity, the key regulator in NF-kB activation (24). However, the release of the major SASP proteins IL-6 and IL-8 has been reported to be reduced by resveratrol in different experimental conditions, and not always with concomitant NF-kB inhibition. For example, resveratrol inhibited IL-6 gene expression and/or protein secretion in rat cortical mixed glial cells under hypoxic and hypoglycaemic conditions followed by reoxygenation (25) and in stimulated peritoneal murine macrophages (26). Shen and coworkers reported that resveratrol suppressed phorbol ester-induced IL-8 gene transcription in human monocytic cells, with a concomitant small effect on NF-kB activation (27).

Figure 3. Western blot analysis of type I collagen protein expression. Presenescent MRC5 fibroblasts were cultured for 4 weeks without (control) and with (resv) 5 µM resveratrol. At week 1 and 4 cell cultures were collected for total protein extraction. 30 µg protein aliquots were submitted to 12% SDS-PAGE, and after western blotting type I collagen protein bands were immuno-detected and quantified by densitometric analyses. GAPDH protein was used as loading control. Data are expressed as mean ± SEM of the integrated intensity of specific bands normalized to GAPDH (n = 3). * p < .05 vs control (Student’s t test for paired data). Representative blots are shown in Panel B.
Our data are in agreement with the results by Csiszar and coworkers (28), showing that 1 µM resveratrol reverts the SASP phenotype in vascular smooth muscle cells (VSMC) derived from aged monkeys to levels found in cells from young monkeys upon 48-hour treatment. At this time point, we did not observe any effect on the analyzed cytokines, even though the concentration employed by us was higher. This discrepancy might be due to the difference in model (aging in vivo vs in vitro) or due to cell type-specific mechanisms, although the age-induced SASP
profiles of VSMC and human fibroblast were correlated (28). The same authors also reported that resveratrol concomitantly counteracted the age-induced increase of NF-kB transcriptional activity and of mitochondrial-free radical production, and activated the antioxidant response (Nrf2). It is possible that the antioxidant activity of resveratrol plays a role in these effects. In agreement, under our experimental conditions the long-term treatment with resveratrol completely reverted the senescence-associated increase of DNA oxidative damage in human fibroblasts (21). However, although oxidative stress plays an important role in aging, its reduction does not always ameliorate all age-associated changes: for example, the antioxidant activity of resveratrol in skeletal muscle in vivo was not associated with a reduction of age-induce sarcopenia in mice (29).

Recently, Orjalo and coworkers showed that IL-6 and IL-8 secretion was regulated by IL-1α and IL-1R signaling pathway in senescent human fibroblasts (30). These authors reported that IL-1α-deficient senescent fibroblasts secreted much less IL-6 and IL-8 in the extracellular medium, and that this conditioned medium supported invasiveness of cancer cells to a much lesser extent than that from normal senescent fibroblasts. Furthermore, IL-1α deficiency was

Figure 5. Western blot analysis of p16INK4a protein expression. Presenescent MRC5 fibroblasts were cultured for 5 weeks without (control) and with 5 µM resveratrol (resv 5). At week 1 and 5 cell cultures were collected for total protein extraction. 30 µg protein aliquots were subjected to 12% SDS-PAGE, and after western blotting, p16INK4a protein bands were immuno-detected and quantified by densitometric analysis. GAPDH protein was used as loading control. Panel A. Data are expressed as mean ± SEM of the integrated intensity of specific bands normalized to GAPDH (n = 3). * p < .05 vs control (Student’s t test for paired data). Representative blots are shown in Panel B.
associated with a reduced NF-κB DNA-binding efficiency. These findings indicated IL-1α as a crucial regulator of tissue microenvironment for senescent cells. In addition, IL-1α and IL-1β were released in very little amounts from senescent cells although their expression levels showed a 6- to 7-fold increase upon bleomycin-induced senescence (30). In agreement with these findings, we found undetectable levels of both IL-1α and IL-1β proteins secreted in senescent fibroblasts—conditioned medium, but a strong increase (50- and 10-fold, respectively) in mRNA expression for both proteins in senescent compared with presenescent cells. It has been found that cell surface–bound IL-1α can act in a juxtacrine manner (31), and this mechanism appeared to be active also in senescent fibroblasts (30). Chronic treatment with 5 μM resveratrol (for 3 or 5 weeks) was able to reduce to 30% and 65%, respectively, the expression of IL-1α and to a lesser extent that of IL-1β. This decrease in IL-1α expression might explain the reduced release of both IL-6 and IL-8 that we observed in senescent fibroblasts upon chronic resveratrol treatment. The reduction of IL-6, IL-8, and GROα release was at least in part due to transcriptional downregulation, as indicated by the real-time PCR data. This effect of resveratrol was dose dependent as the 1 μM concentration was also effective in reducing cytokine gene expression, although to a lesser extent.

We did not observe any effect of resveratrol on another inflammation and cancer-related protein, the ECM-degrading enzyme MMP3, whose expression has been reported to increase with senescence (32). Angiogenesis plays an essential role in tumor growth and wound healing. In this context, several studies reported antiangiogenic effects of resveratrol. Among the mechanisms involved, the inhibition of proliferation of endothelial cells has been shown (33,34). VEGF stimulates the proliferation, migration, and tube formation of endothelial cells, as well as ECM degradation. Inhibition of VEGF expression by resveratrol has been reported in renal cancer cells (35), in hepatocellular carcinoma cells (36), and in melanoma-endothelial cell cocultures (37). Our data in senescent nontumor cells indicate that resveratrol exerts an antiangiogenic and antitumor activity in this context as well through a downregulation of VEGF gene expression.

Finally, we reported a striking effect of resveratrol on the recovery of plating efficiency and spreading capacity in senescent fibroblasts. Treated senescent cells exhibited a 5-fold increased ability to adhere to the plate compared with untreated cells, and this effect was accompanied by a recovery of type I collagen expression to presenescent levels. Thus, resveratrol ameliorated a series of functions related to cell migration, tissue repair, and remodeling. The reported effects of resveratrol on adhesion are various, and mostly related to short-term exposures. In some cases, inhibition of cell attachment to a substrate has been found: for example, Srivastava and coworkers (2005) reported inhibition of Jurkat cells attachment to fibronectin after exposure to a high concentration (100 μM) of resveratrol (38). Conversely, Chan (2005) showed that the treatment with concentrations up to 20 μM resveratrol prevents the decrease in attachment ability to collagen I induced by high glucose in K562 cells (39). The ability of resveratrol to increase cell adhesion and spreading was also shown in human erythroleukemic and breast cancer cell lines: this effect was associated with increased expression of tensin, a cytoskeletal actin-binding protein associated with focal adhesion and spreading of fibroblasts on fibronectin (40). In cancer cells, this activity of resveratrol contributes to its tumor-suppressing effect by reducing cell motility and invasiveness, whereas in senescent cells it may favor the transition from a proinflammatory to a resting state.

As a whole, these data indicate that resveratrol ameliorates some functional parameters altered during aging and relevant for age-related diseases. These effects are most likely not related to lifespan extension: we have previously shown, in the same model of human fibroblasts, that long-term exposure to resveratrol (from PDL 37 to 55) does not modify the time at which cells undergo the senescence-associated replicative arrest, and only modestly affects fibroblast proliferation capacity, increasing the number of PDL at arrest from 55 to 57 (21). In agreement with these observations, it has been recently shown in vivo experiments that rapamycin, but not resveratrol, can extend mouse lifespan (41), indicating that the mechanisms that regulate lifetime are independent from those that have the potential to improve life quality during aging, such as reduction of oxidative stress and inflammation.

In the present study, we found detectable levels of p16ink4a protein in our presenescent cells at the beginning of the experiment, indicating that the replicative activity was already approaching arrest at that point. These levels increased over the course of the experiment as cells were progressing towards senescence, but the treatment with resveratrol did not significantly modify this increase. This confirms that the mechanism by which resveratrol ameliorated the investigated functions was not a rejuvenation, that is, resveratrol did not set back the timing of replicative arrest making the fibroblasts less senescent.

Among the potentially important mechanisms of the observed effects of resveratrol, sirtuin-1 (SIRT-1) activation has been shown to mediate protection from senescence via increased resistance to oxidative stress (20) and telomerase induction (42). We have shown decreased acetylation of SIRT-1 substrates such as H3 and H4 histones and p53 upon long-term resveratrol exposure of human fibroblasts (21). However, considering that we have not observed prevention or delay of the onset of replicative senescence, it is unlikely that telomere length was changed by the treatment in our conditions. Many data indicate that oxidative stress can affect cell adhesion. Among these, oxidative stress induced by high glucose has been shown to induce deterioration of adhesion in various types of fibroblasts, and this...
might be relevant for deficient wound healing observed in diabetic subjects (43). Selenoprotein T knock down alters cell adhesion and induces expression of genes involved in redox regulation in murine fibroblasts (44). Thus, it can be hypothesized that resveratrol modulation of oxidative stress via SIRT-1- and/or Nrf2-mediated mechanisms is responsible for the effects on cellular adhesion that we report.

The effects of resveratrol described in this work take place at micromolar doses. The 1 µM concentration proved to be effective, although to a lesser extent than 5 µM. In humans, increased maximal plasma concentration upon repeated administration has been shown by Almeida and coworkers (45) and Brown and coworkers (46). The latter Authors showed that a Cmax of 4.24 µM was attainable by administering 5 g of resveratrol for 29 days, whereas the same dose in a single administration yielded 2.4 µM plasma Cmax (47). Furthermore, the concentration of resveratrol in specific tissues may be significantly higher than that in plasma: Patel and coworkers (48) have reported that the administration of resveratrol (0.5 or 1 g per day) to colon cancer patients for 8 days yielded maximal concentrations of 18.6 and 674 nmol/g, respectively, in normal colonic tissue.

In conclusion, these results indicate that resveratrol, at low concentrations that can be attained in vivo, has the potential to improve tissue structure maintenance and repair during aging and to reduce the incidence of inflammation-related tissue pathologies, including cancer.

**References**


