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Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells

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Abstract Resveratrol (RSV) exerts anti-proliferative and pro-apoptotic actions in different cell lines. Hepatic stellate cells (HSCs) are major fibrogenic cell types that contribute to collagen accumulation during chronic liver disease. In the present study, the inhibitory effects of RSV on cell proliferation, cell cycle, and apoptosis were evaluated in the mouse hepatic stellate cell line GRX. Cells treated with 1 nM–1 μM of RSV demonstrated a decrease in cell growth of about 35% after 5 days. GRX cells, treated with RSV (100 nM or 1 μM), were analyzed by flow cytometry; RSV induced an increase in the number of GRX cells in the S- and sub-G1 phases. The increase in sub-G1 phase cells and the nuclear condensation and fragmentation shown by DAPI staining identified a possible pro-apoptotic effect of RSV on GRX cells. Furthermore, the RSV anti-proliferative effects could be explained by an S-phase accumulation caused by a decrease in the progression through the cell cycle or an inhibition of S or G2 phase transition. It is notable that these RSV actions are mediated at nanomolar levels, compatible with the concentrations of free RSV in biological fluids after ingestion of polyphenol-rich foods, suggesting a possible effect of these foods as an adjuvant treatment in chronical liver diseases.

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Introduction

Liver fibrosis is a dynamic and sophisticated regulated wound healing response to chronic hepatocellular damage that represents a major medical problem with significant morbidity and mortality. This fibrotic process results from the accumulation of extracellular matrix (ECM) proteins including collagens, proteoglycans, and glycoproteins [1]. Hepatic stellate cells (HSCs) (previously known as Ito cells, lipocytes, or fat storing cells) are liver-specific pericytes which were identified as a major source of collagen in pathologic fibrosis following their activation to miofibroblast-like cells [2].

Indeed, much of the knowledge on the cell and molecular biology of liver fibrosis has been gained through animal models and from in vitro models employing culture activated HSC isolated from rat, mouse, or human liver. From these, in vitro models grew a large body of information characterizing stellate cell activation, cytokine signaling, intracellular pathways regulating liver fibrogenesis, production of extracellular matrix proteins, and development of antifibrotic drugs [3].

In this study, we used the GRX cell line, established from hepatic fibrogranulomatous reactions, which mobilizes adjacent stellate cells. Under standard conditions, these cells express a transitional myofibroblast phenotype and present morphological and biochemical aspects of hepatic connective tissue [4–6]. It has been previously demonstrated that the GRX cell line can be induced, in vitro, to express alternative phenotypes. The lipocyte

phenotype can be induced by retinol, indomethacin, or β-carotene treatment, with a decrease in proliferation and collagen production and progressive accumulation of fat droplets, corresponding to the frequently termed quiescent fat storing phenotype, in vivo [7–9]. These cells are also responsive to cytokines, such as TNFα, increasing oxidative stress levels, and ECM production [10–12].

Resveratrol (3,5,4′,-trihydroxystilbene—RSV) is a polyphenolic compound found in the skins of red fruits. It is thought that the presence of RSV in red wine explains, in part, the "French paradox" and may be responsible for many of the health benefits ascribed to the consumption of red wine. Resveratrol has been proposed to exhibit anti-oxidative, anti-proliferative, and anti-inflammatory properties [13, 14]. This harmless compound exhibits chemopreventive activities in multiple animal and in vitro models with less defined molecular mechanisms [15, 16].

Accumulating data from many cell line studies indicate that RSV possesses strong anti-proliferative and apoptosis-inducing properties. S-phase or G1-phase arrest was mainly observed during RSV-induced apoptosis, but the apoptosis inducing effects of RSV appeared diverse in different cells. These conflicting data regarding RSV-induced biological effects may be due to the specific cell type and cellular environment [17].

The purpose of the present investigation was to study the role of RSV with respect to the modulation of cell proliferation, cell cycle, and stimulation of apoptosis in activated HSCs.

Material and methods

Cell culture and treatments

The murine HSC cell line, GRX, was established by Borojevic [5] and kindly provided by the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). Cells were routinely maintained in Dulbecco's Modified Minimum Essential Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS, Cultilab, Campinas, Br) and 2 g/l HEPES buffer, pH 7.4 under 37°C and 5% CO_2 conditions. The cells were plated (5×10^4/ml) in 12, 24, or 96-well plates and cultured for 24 h to reach 60–70% of confluence before RSV treatment. Resveratrol (Sigma Inc., Saint Louis, MO, USA) was dissolved in ethanol (Merck, Darmstadt, Germany) to a stock concentration of 100 mM and diluted in culture medium to final concentrations of 1, 10, 100 nM, and 1 μM, just before use. The GRX cells were treated with the above-mentioned RSV concentrations for 5 days and the treatment medium was daily changed. Each concentration group included four wells. The routinely cultured cells were used as normal controls.

Cell growth assays

Bromodeoxyuridine incorporation into DNA

Cell proliferation was measured 5 days after RSV treatment by the extent of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA. BrdU, a thymidine analog that is incorporated into proliferating cells mainly during the S-phase of DNA synthesis is detected by peroxidase-conjugated mouse monoclonal anti-BrdU antibody, followed by the addition of *o*-phenylenediamine substrate. The optical density (OD) was measured using a spectrophotometric microtiter plate reader (Spectra Max 190, Molecular Devices), at 492 nm wavelength [18].

Tritiated thymidine incorporation assay

GRX cells treated, or not, for 5 days with RSV (at the above described concentrations) were incubated (24 h, 1 μCi/ml) with [6-^{3}H] thymidine ([3H]dT) (specific activity 23.0 Ci/nmol, Amersham Biosciences, Hillerod, Denmark). Subsequently, the medium was removed, and 10% of trichloroacetic acid (TCA) was added to each well. The cell pellet was then dissolved in 200 μl of 0.1 N NaOH, the incorporated DNA radioactivity was determined by scintillation counting [19]. The protein content was measured according to Peterson [20].

Cell viability assays

Colorimetric MTT assay

MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Inc., Saint Louis, MO, USA) is a yellow tetrazolium salt that is reduced to a blue formazan. The MTT assay assesses cell viability by measuring cellular redox environment [21, 22]. Pre-confluent GRX cells were incubated with variable concentrations of RSV for 5 days. Cells were then incubated with 1 mg/ml MTT for 2 h at 37°C. Blue crystals were dissolved in dimethylsulfoxide (DMSO, Sigma Inc., Saint Louis, MO, USA). OD was measured using a spectrophotometric microtiter plate reader (Spectra Max 190, Molecular Devices) at 570 nm and 630 nm.

Lactate dehydrogenase (LDH) assay

The cytotoxicity of RSV was also evaluated by determination of LDH activity in the culture medium. LDH activity was measured by a colorimetric assay (Kit from Doles, Brazil). Cells were treated or not with RSV (100 nM–1 μM) for 5 days. Subsequently, the medium was removed after centrifugation, LDH in the supernatant of the conditioned

media was determined as medium LDH. Attached cells were lysed completely by 1 ml of 5% FBS/DMEM with Triton X-100 (0.1%). After centrifugation, LDH in the supernatant was determined as cellular LDH. LDH in DMEM with 5% FBS was determined as contamination arising from FBS and subtracted from medium LDH values. Results were shown as the percentage of total LDH, i.e., [medium LDH/(medium LDH + cellular LDH)] × 100 [23].

Cell cycle analysis by flow cytometry

Flow cytometry analysis [24] was used to assess the cell cycle phase distribution and the percentage of fragmented nuclei. Briefly, after treatment with 100 nM or 1 μM of RSV for 5 days, cells were harvested by trypsinization and counted. Samples of 1×10^6 cells were fixed in 70% ethanol at 4°C overnight. After centrifugation, the cell pellet was washed in ice-cold PBS, resuspended in hypotonic propidium iodide solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100, 0.1 mg/ml RNAse A) and incubated in the dark at room temperature for 30 min. The DNA content was then analyzed using a FACScan Calibur flow cytometer equipped with a Modfit 2.0 software (Beckton Dickinson, San Jose, USA). The number of cells having a subdiploid DNA content was taken as a measurement of apoptotic cells.

Identification of apoptotic cells after DAPI nuclei staining

The nuclear morphology of cells was studied using the cell-permeable DNA dye DAPI. GRX cells grown on coverslips were treated with 1 μM RSV for 5 days, fixed in 4% (v/v) paraformaldeyde for 30 min, washed in PBS, and permeabilized with 0.5% Triton X-100 in PBS 3 times for 10 min. The DAPI nuclear dye was added at 0.1 μg/ml in 0.9% NaCl for 5 min. Coverslips were mounted with Fluorsave (Calbiochem, San Diego,CA, USA). Cells were analyzed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Apoptotic cells were morphologically defined by nuclear shrinkage and chromatin condensation or fragmentation.

Statistical analysis

Data were expressed as mean ± standard error. $P < 0.05$ was considered significant. Statistical comparisons were performed using ANOVA. For post-hoc testing, the Duncan test was used. All analyses were performed with the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Resveratrol inhibits cell growth and interferes with cell viability in GRX cells

The anti-proliferative effects of RSV were assessed by incubating pre-confluent GRX cells with RSV at indicated concentrations for 5 days. Proliferation of GRX cells measured by BrdUrd assay was significantly decreased by about 35% at all concentrations of RSV, compared to control (Table 1). This effect was confirmed by a thymidine incorporation assay, where the decrease in GRX cell proliferation was dose dependent (Table 1).

In addition, the effects of RSV on cell viability were determined by MTT assay and by measuring LDH activity in extracellular medium. The results show that, only concentrations above 10 μM significantly decreased cell viability (Data not shown). In view of these results, in subsequent experiments, we used the concentrations of 100 nM and 1 μM.

Resveratrol induces S-phase cell cycle arrest in GRX cells

The effect of RSV on cell proliferation could be due to its actions on cell cycle and the initiation of programmed cell death. In order to explore this possibility, we treated exponentially growing GRX cells with RSV and assayed its effects on cell cycle. The untreated cells showed the expected pattern for continuously growing cells, whereas the cells treated with 100 nM or 1 μM RSV for 5 days showed a 50% increase in the number of GRX cells in S-phase (Fig. 1).

Table 1 Effect of RSV on GRX cell growth. GRX cells were treated with variable concentrations of RSV for 5 days and the proliferation measured by BrdUrd or [3H]dT incorporation assay

RSV [μM]	Control	0.001	0.01	0.1	1
BrdU-DNA (Immunoactivity)	0.57 ± 0.020^a	0.41 ± 0.024^b	0.36 ± 0.023^b	0.39 ± 0.017^b	0.36 ± 0.011^b
^{3}H Thymidine (cpm/μg protein)	16.9 ± 0.94^a	15.1 ± 0.33^a	13.7 ± 1.17^b	12.0 ± 1.01^b	ND

Results are expressed as the means ± S.E.M. of three independent experiments. Different letters indicate statistically significant differences between groups $P < 0.02$. ND, not determined

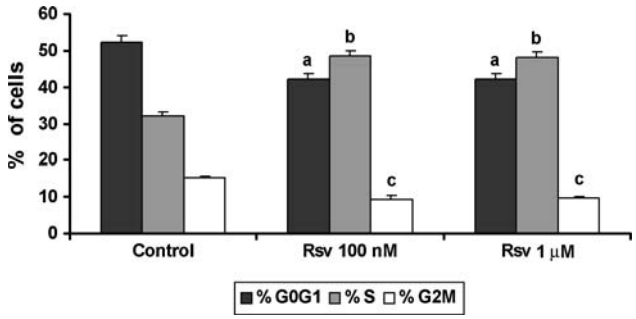

Fig. 1 Effect of RSV on GRX cell cycle. Exponentially growing GRX cells were treated with RSV for 5 days at indicated concentrations and their effects on cell cycle were assayed as described in the "Material and methods" section. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. (**a**), (**b**), and (**c**) significantly different from control G0/G1, S, and G2 + M, respectively, $P < 0.02$

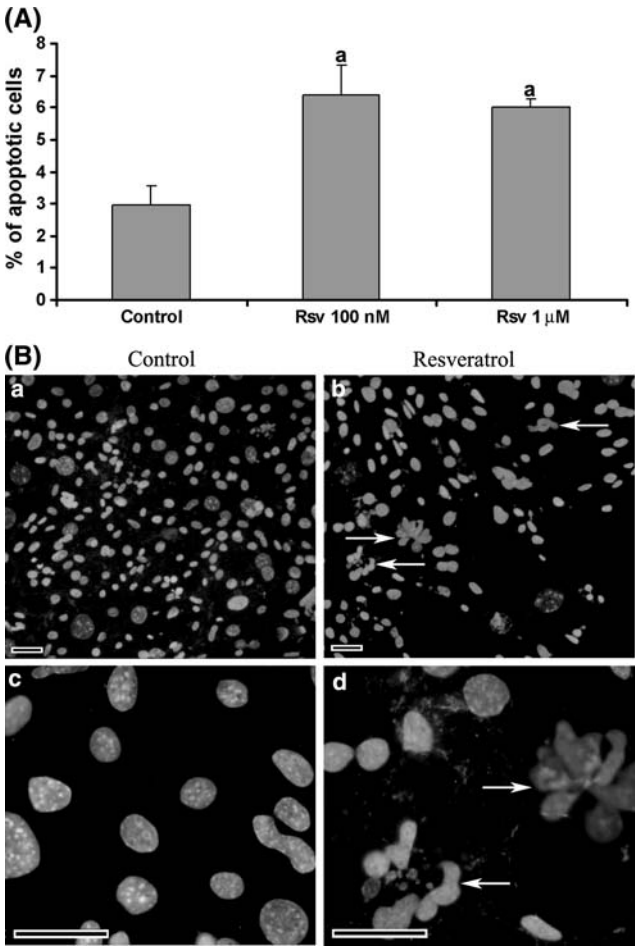

Fig. 2 Influence of RSV on apoptosis, as analyzed by subdiploid cell population and nuclear morphology of GRX cells. GRX cells were treated with RSV at indicated concentrations for 5 days. (**A**) The number of cells having a subdiploid DNA content was taken as a measurement of apoptotic cells, as described in the "Material and methods" section. Values are expressed as means ± S.E.M. of three independent experiments performed in triplicate. (**a**) significantly different from control,$P < 0.02$. (**B**) Representative images of four experimental conditions. DAPI staining was used to visualize nuclear morphology under the fluorescence microscope after exposure to RSV at 1 μM (**b**, **d**) for 5 days, compared to the control cells (**a, c**). The apoptotic cells demonstrated nuclear fragmentation or partially condensed nuclei without fragmentation (arrows). Scale bar: 50 μm

Resveratrol stimulates apoptosis in GRX cells

Cells with DNA content below G1 phase, defined as a hypodiploid sub-G1 peak, were regarded as apoptotic cells. The results shown in Fig. 2A demonstrate that RSV treatment (100 nM or 1 μM) induced a two-fold increase in the number of sub-G1 GRX cells, as compared with controls. The ability of RSV to modulate apoptosis was also studied by examination of nuclear morphology. After treatment, cells were stained with DAPI and nuclear morphology visualized under a fluorescence microscope (Fig. 2B). RSV (1 μM, 5 days), induced an increase in the number of GRX nuclei with a condensed and fragmented morphology, indicating stimulation of apoptosis (Fig. 2B, c and d).

Discussion

Resveratrol (3,5,4′-trihydroxy-trans-stilbene, RSV) is a naturally occurring polyphenol synthesized by a variety of plant species in response to injury, UV irradiation, and fungal attack [25]. Accumulating evidences indicate that an inverse relationship exists between RSV and its striking inhibition of diverse cellular events associated with cell growth [17, 26, 27]. RSV has now been recognized as a promising candidate for chemopreventive effects [17].

The major fibrogenic cell type HSC contributes to collagen accumulation during chronic liver disease. Liver regeneration, driven by hepatocyte proliferation is necessary for tissue repair and survival after acute liver injury and chronic hepatic disease, such as fibrosis and cirrhosis [28]. Induction of hepatocyte proliferation depends on cross-talk between hepatocytes and non parenchymal-liver cells, such as HSCs [28]. At sites of injury, HSC differentiate to myofibroblasts and secrete ECM and growth factors that support hepatocyte proliferation [29]. Apoptosis of activated HSC is a key factor in regression of liver fibrosis. Activated HSC are more susceptible to apoptosis and can undergo spontaneous cell death or receptor-mediated death, caused by serum deprivation or cytokine signaling [30].

Putative anti-fibrogenic drugs mainly include agents able to reduce inflammation, agents able to reduce HSC activation, agents with a pro-apoptotic potential for HSC, agents with antioxidant effects, and agents able to increase fibrillar ECM degradation. Numerous studies have attributed a beneficial role in a number of pathologies to RSV-

rich foods and beverages. In order to further understand the biochemical mechanisms of this molecule, a number of studies have been carried out with very high concentrations of RSV: In the present study, we used concentrations compatible with those found in biological fluids after consumption of RSV-rich foods and beverages, in an attempt to understand in vitro (with all the limitations of cell culture systems) the beneficial actions of the agent found in vivo by using physiologically relevant RSV concentrations [26].

With the increasing interest in developing antifibrotic therapies, there is a need for cell lines that preserve the in vivo phenotype of HSCs to elucidate pathways of hepatic fibrosis. In this context, the GRX cell line represents an interesting and efficient study tool [6, 9, 10, 12, 31–35]. In standard culture conditions, GRX cells have a myofibroblast-like phenotype that resembles activated HSCs.

The treatment of GRX cells for 5 days with variable concentrations of RSV resulted in a decrease in cell growth of about 35%. These findings are in agreement with previous studies performed in HL-60 [26], HepG2 cells [26], and C6 glioma [17, 36]. Kawada et al. [19] demonstrated that RSV dose-dependently suppressed the incorporation of [^{3}H]thymidine in rat HSC, but the value of median inhibitory concentration for RSV was 37 μM. Our results show that RSV inhibits GRX cell growth at concentrations that are lower that than those found in the majority of studies dealing with RSV effects in cells, but compatible with those reported previously in HepG2 [17]. As discussed above, we chose to use low doses of the agents that are compatible with reported concentrations of RSV after ingestion of polyphenol-rich foods [37].

In vitro studies have shown that RSV reduces cell proliferation and modulates the cell cycle in different cell lines. G1-arrest has been observed in human epidermoid carcinoma A431 cells [38], human leukemia U937 cells [39], Hep G2 cells [40], and esophageal adenocarcinoma Bic 1 [41]. Resveratrol blocked the S-G2 transition in HL-60 [42] and [17] induced a more than twofold increase in number of C6 cells in S phase. In agreement with these findings, S-phase arrest was also seen in human neuroblastoma SH-SY5Y and in Neuro-2a neuroblastoma cells [43, 44]. In rat HSCs, 100 μM of RSV reduced the level of cyclin D1, a cell cycle-related protein [19]. Since cyclin D1 regulates the activity of cdk4 and cdk6, resulting in the phosphorylation of retinoblastoma gene product Rb p110 and the successive G1-to S-phase transition, its reduction might cause G1 arrest of the cells. In general, the effect of RSV on cell cycle distribution is concentration-dependent, where lower RSV concentrations cause accumulation of the cells in S-phase and higher concentrations cause accumulation of the cells in the G0/G1 phase or G2 + M phases [27]. This may indicate that RSV has more than one target inhibiting cell division, which may be modulated at different concentrations [45].

The S-phase arrest produced by RSV is often accompanied by inhibition in [^{3}H] thymidine incorporation. RSV, at 20 μM, induced S-phase accumulation in HL-60 with a 30% inhibition of cell growth. In contrast, in HepG2, 40 μM RSV induced a maximal S-phase accumulation without a significant decrease in [^{3}H] thymidine incorporation. Here, we demonstrate that treatment of GRX cells with small doses of RSV (100 nM and 1 μM) induced a 35% inhibition in [^{3}H] thymidine incorporation and a 50% increase in S-phase cells. These S-phase arrests indicate that RSV at 100 nM and 1 μM did not induce a decrease in cyclin D1 expression like described by Kawada et al. [19] for rat HSC. Our results could be explained by the hypothesis proposed by Delmas et al. [16], in which S-phase accumulation is suggested to be caused by a decrease in the progression through the cell cycle or an inhibition of S or G2 phase transition. Furthermore, our results are in agreement with observations that RSV inhibits the production of polyamines by interfering with ornithine decarboxylase [46], which in turn catalyses the rate-limiting step in polyamine production.

In addition to its cell cycle arresting properties, another essential effect of RSV is its apoptosis-inducing ability. RSV has been previously shown to trigger apoptosis in leukemia, mammary, myeloma, epidermoid, embryonal rhabdomyosarcoma, and glioblastoma cell lines [38, 47]. The doses required for RSV apoptosis induction (100–200 μM range) are often higher than those used to induce growth inhibition and cell cycle arrest [41]. Interestingly, in GRX cells, small doses of RSV (100 nM and 1 μM) resulted in twice the number of sub-G1 cells. An obvious increase in the number of GRX nuclei with a condensed and fragmented morphology is visualized by DAPI-staining, indicating the stimulation of apoptosis by RSV.

Taken together, our observations indicate that GRX cells, when treated with RSV, are induced to enter into the S phase, but subsequent progression through the S phase is limited by the inhibitory effect of RSV on DNA synthesis. Therefore, as suggested by Kuwajerwala [45], this unique ability of RSV to exert opposing effects on two important processes in cell cycle progression, induction of S phase, and inhibition of DNA synthesis, indicate that RSV could modulate apoptotic and anti-proliferative effects in GRX. In this context, more studies are necessary to demonstrate these effects in liver fibrosis.

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References

1. Pinzani M, Rombouts K (2004) Liver fibrosis: from the bench to clinical targets. Dig Liver Dis 36:231–242
2. Friedman SL, Roll FJ, Boyles J, Bissell DM (1985) Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc Natl Acad Sci USA 82:8681–8685
3. Gutierrez-Ruiz MC, Gomez-Quiroz LE (2007) Liver fibrosis: searching for cell model answers. Liver Int 27:434–439
4. Pinzani M, Gentilini P (1999) Biology of hepatic stellate cells and their possible relevance in the pathogenesis of portal hypertension in cirrhosis. Semin Liver Dis 19:397–410
5. Borojevic R, Monteiro AN, Vinhas SA, Domont GB, Mourao PA, Emonard H, Jr.Grimaldi G, Grimaud JA (1985) Establishment of a continuous cell line from fibrotic schistosomal granulomas in mice livers. In vitro Cell Dev Biol 21:382–390
6. Guma FCR, Mello TG, Mermelstein CS, Fortuna VA, Wofchuk ST, Gottfried C, Guaragna RM, Costa ML, Borojevic R (2001) Intermediate filaments modulation in an in vitro model of the hepatic stellate cell activation or conversion into the lipocyte phenotype. Biochem Cell Biol 79:409–417
7. Margis R, Borojevic R (1989) Retinoid-mediated induction of the fat-storing phenotype in a liver connective tissue cell line (GRX). Biochim Biophys Acta 1011:1–5
8. Borojevic R, Guaragna RM, Margis R, Dutra HS (1990) In vitro induction of the fat-storing phenotype in a liver connective tissue cell line-GRX. In vitro Cell Dev Biol 26:361–368
9. Martucci RB, Ziulkoski AL, Fortuna VA, Guaragna RM, Guma FC, Trugo LC, Borojevic R (2004) Beta-carotene storage, conversion to retinoic acid, and induction of the lipocyte phenotype in hepatic stellate cells. J Cell Biochem 92: 414–423
10. da Silva FM, Guimaraes EL, Grivicich I, Trindade VM, Guaragna RM, Borojevic R, Guma FC (2003) Hepatic stellate cell activation in vitro: cell cycle arrest at G2/M and modification of cell motility. J Cell Biochem 90: 387–396
11. Chiarini LB, Borojevic R, Monteiro AN (1994) In vitro collagen synthesis by liver connective tissue cells isolated from schistosomal granulomas. Braz J Med Biol Res 27: 1193–1197
12. Guimaraes EL, Franceschi MF, Grivicich I, Dal-Pizzol F, Moreira JC, Guaragna RM, Borojevic R, Margis R, Guma FC (2006) Relationship between oxidative stress levels and activation state on a hepatic stellate cell line. Liver Int 26:477–485
13. Donnelly LE, Newton R, Kennedy GE, Fenwick PS, Leung RH, Ito K, Russell RE, Barnes PJ (2004) Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. Am J Physiol Lung Cell Mol Physiol 287:L774–783
14. Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493–506
15. Kundu JK, Surh YJ (2004) Molecular basis of chemoprevention by resveratrol: NF-kappaB and AP-1 as potential targets. Mutat Res 555:65–80
16. Delmas D, Lancon A, Colin D, Jannin B, Latruffe N (2006) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. Curr Drug Targets 7:423–442
17. Zhang W, Fei Z, Zhen HN, Zhang JN, Zhang X (2007) Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. J Neurooncol 81:231–240
18. Muir D, Varon S, Manthorpe M (1990) An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. Anal Biochem 185:377–382
19. Kawada N, Seki S, Inoue M, Kuroki T (1998) Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. Hepatology 27:1265–1274
20. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
21. Hayon T, Dvilansky A, Shpilberg O, Nathan I (2003) Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leuk Lymphoma 44:1957–1962
22. Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119: 203–210
23. Chen A, Zhang L, Xu J, Tang J (2002) The antioxidant (−)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression. Biochem J 368:695–704
24. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271–279
25. Bhat KPL, Kosmeder JW II, Pezzuto JM (2001) Biological effects of resveratrol. Antioxid Redox Signal 3:1041–1064
26. Notas G, Nifli AP, Kampa M, Vercauteren J, Kouroumalis E, Castanas E (2006) Resveratrol exerts its antiproliferative effect on HepG2 hepatocellular carcinoma cells, by inducing cell cycle arrest, and NOS activation. Biochim Biophys Acta 1760: 1657–1666
27. Stervbo U, Vang O, Bonnesen C (2006) Time- and concentration-dependent effects of resveratrol in HL-60 and HepG2 cells. Cell Prolif 39:479–493
28. Taub R (2004) Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5:836–847
29. Balabaud C, Bioulac-Sage P, Desmouliere A (2004) The role of hepatic stellate cells in liver regeneration. J Hepatol 40: 1023–1026
30. Kisseleva T, Brenner DA (2006) Hepatic stellate cells and the reversal of fibrosis. J Gastroenterol Hepatol 21Suppl 3:S84–87
31. de Aguirres AB, Mello PA, Andrade CM, Breier AC, Margis R, Guaragna RM, Borojevic R, Guma FC, Trindade VM (2007) Variations of ganglioside biosynthetic pathways in the phenotype conversion from myofibroblasts to lipocytes in murine hepatic stellate cell line. Mol Cell Biochem 303:121–130
32. Andrade CM, Trindade VM, Cardoso CC, Ziulkoski AL, Trugo LC, Guaragna RM, Borojevic R, Guma FC (2003) Changes of sphingolipid species in the phenotype conversion from myofibroblasts to lipocytes in hepatic stellate cells. J Cell Biochem 88:533–544
33. Mermelstein CS, Guma FC, Mello TG, Fortuna VA, Guaragna RM, Costa ML, Borojevic R (2001) Induction of the lipocyte phenotype in murine hepatic stellate cells: reorganisation of the actin cytoskeleton. Cell Tissue Res 306:75–83
34. Vicente CP, Fortuna VA, Margis R, Trugo L, Borojevic R (1998) Retinol uptake and metabolism, and cellular retinol binding protein expression in an in vitro model of hepatic stellate cells. Mol Cell Biochem 187:11–21
35. Vicente CP, Guaragna RM, Borojevic R (1997) Lipid metabolism during in vitro induction of the lipocyte phenotype in hepatic stellate cells. Mol Cell Biochem 168:31–39
36. dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Goncalves CA, Gottfried C (2006) Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. Arch Biochem Biophys 453: 161–167
37. Bertelli A, Bertelli AA, Gozzini A, Giovannini L (1998) Plasma and tissue resveratrol concentrations and pharmacological activity. Drugs Exp Clin Res 24:133–138

38. Ahmad N, Adhami VM, Afaq F, Feyes DK, Mukhtar H (2001) Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin Cancer Res 7:1466–1473
39. Park JW, Choi YJ, Jang MA, Lee YS, Jun DY, Suh SI, Baek WK, Suh MH, Jin IN, Kwon TK (2001) Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. Cancer Lett 163:43–49
40. Kuo PL, Chiang LC, Lin CC (2002) Resveratrol- induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sci 72:23–34
41. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB (2002) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 8:893–903
42. Ragione FD, Cucciolla V, Borriello A, Pietra VD, Racioppi L, Soldati G, Manna C, Galletti P, Zappia V (1998) Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem Biophys Res Commun 250:53–58
43. Rigolio R, Miloso M, Nicolini G, Villa D, Scuteri A, Simone M, Tredici G (2005) Resveratrol interference with the cell cycle protects human neuroblastoma SH-SY5Y cell from paclitaxel-induced apoptosis. Neurochem Int 46:205–211
44. Chen C, Zhang J, Li J, Huang J, Yang C, Huang G, Shi J (2004) Hydrodynamic-based in vivo transfection of retinoic X receptor-alpha gene can enhance vitamin A-induced attenuation of liver fibrosis in mice. Liver Int 24:679–686
45. Kuwajerwala N, Cifuentes E, Gautam S, Menon M, Barrack ER, Reddy GP (2002) Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis. Cancer Res 62: 2488–2492
46. Schneider Y, Vincent F, Duranton B, Badolo L, Gosse F, Bergmann C, Seiler N, Raul F (2000) Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Lett 158:85–91
47. Clement MV, Hirpara JL, Chawdhury SH, Pervaiz S (1998) Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood 92:996–1002