Resveratrol Enhances UVA-Induced DNA Damage in HaCaT Human Keratinocytes

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Abstract: Resveratrol, a polyphenolic phytoalexin, is a very effective antioxidant that also exhibits strong antiproliferative and anti-inflammatory properties. Recent studies have provided support for the use of resveratrol in human cancer chemoprevention, in combination with either chemotherapeutic drugs or cytotoxic factors for a most efficient treatment of drug refractory tumor cells. Resveratrol is also widely used in topical preparations, as a chemoprotective compound against development of several cutaneous disorders, including skin cancer. Nevertheless, the combined effect of resveratrol and UVA irradiation on cellular toxicity and DNA damage has never been assessed. The aim of this work was to investigate the effect of resveratrol on cell fate in immortalized human keratinocytes HaCaT cells. The results indicated that resveratrol potentiates the production of significant amounts of 8-oxo-7,8-dihydro-2'-deoxyguanosine in UVA-irradiated genomic DNA. Moreover, the combination of resveratrol with UVA significantly enhances the induction of DNA strand breaks and cell death in HaCaT keratinocytes. The conclusion is a potential hazardous effect of topical application of resveratrol, particularly on regions exposed to sunlight.

INTRODUCTION

Resveratrol (trans-3,4',-5-trihydroxystilebene, CAS number: 501-36-0) is a polyphenolic phytoalexin mainly found in grapes and red wine. The root of *Polygonum cuspidatum,* which contains an active constituent of resveratrol, also called Ko-jo-kon in Japanese, was used in traditional Japanese and Chinese medicine to treat dermatitis, favus, hyperlipemia, and gonorrhea [1]. Resveratrol is a very effective antioxidant, even stronger than α-tocopherol (Vitamin E) in some assay systems [2] that has also been shown to possess strong antiproliferative and anti-inflammatory properties [3]. Hence, resveratrol is a potentially important compound that displays interesting biological features, and some studies provide support for the use of resveratrol either alone in human cancer chemoprevention or in combination with chemotherapeutic drugs or cytotoxic factors in the treatment of drug refractory tumor cells (for review see [4]). For skin cancers in particular, resveratrol has been shown to have a chemopreventive action by inhibiting cellular events at initiation, promotion or the progression stage of carcinogenesis [5]. Recent studies indicate that resveratrol may represent a feasible and productive approach to support dermal wound healing [6]. But some toxic effects of resveratrol are also beneficial in cancer therapy. For example, resveratrol can act as a potent sensitizer for anticancer drug-induced apoptosis by inducing cell cycle arrest in S phase [7], suggesting that a combined sensitizer (resveratrol)/ inducer (cytotoxic drugs) approach may be a novel strategy to enhance the efficacy of anticancer therapy. Renal toxicity has also been observed in rats fed with resveratrol, but most of the adverse effects occurred in rats administered with the highest dose of resveratrol, i.e. 3000 mg per kilogram body weight per day [8].

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Taking into account its polyphenolic structure and the possible photo-induced effects of resveratrol, little is known about the effect of these potent antioxidant molecule on the effects of UV radiation. A few studies in mice indicate that topical application of resveratrol can prevent short-term UVB radiation-mediated damages to skin [9], and that this effect is likely to be due to the resveratrol-induced suppression of NF-kB activation in keratinocytes [10]. A recent report indicates modulations of critical cell cycle regulatory events after topical application of resveratrol on SKH-1 hairless mouse skin [11]. In these mice, resveratrol has been shown to prevent UVB-induced damage by inhibiting survivin, a member of the IAP proteins, and the associated events [12]. Nevertheless, to our knowledge, no study was previously aimed at evaluating putative relationship between resveratrol and UVA. This may be explained by the fact that the relevance of UVA for carcinogenesis and skin aging has not been fully resolved. However, UVA constitutes 95% of the total radiation received on earth, and the genotoxicity of UVA has been firmly established *in vitro* [13].

The aim of this work was to investigate the effects of resveratrol on keratinocytes HaCaT cells viability and proliferation. The results clearly indicated that resveratrol enhances UVA-induced DNA damage and cell death of keratinocytes. These events are associated with increased 8-oxodGuo formation in irradiated cells pre-treated with resveratrol. Taken together, these results suggest potential hazardous effect of topical application of resveratrol, particularly on skin regions exposed to sunlight.

RESULTS

Effect of Resveratrol on HaCaT Cells Viability and Proliferation

Resveratrol toxicity on HaCaT human keratinocyte cells was determined by an MTT assay with different concentrations of the antioxidant varying from 5 to 500 μM (Fig. **1**).

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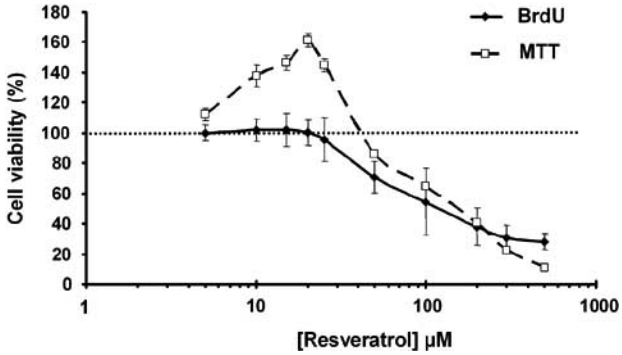

Fig. (1). Cytotoxicity and proliferation assays.

Cytotoxicity and anti-proliferative effects of resveratrol (0-500 µM) on HaCaT human keratinocytes were determined by either the MTT technique or 5-bromo-2'-deoxyuridine (BrdUrd) assay as described in the experimental procedures section. Results are expressed as percentage of cell viability, with 100% viability for non-treated cells. Results are the mean ± SD of three experiments.

The absorbance was decreased for concentrations between 40 and 500 µM, indicating a toxic effect of resveratrol, with an observed LD_{50}= 140 µM. However, an important increase in the signal was observed for low concentrations, which could be interpreted as cell proliferation. However, since a previous study reported that MTT could not be suitable for evaluation of resveratrol toxicity [14], cell viability was determined with another test using BrdUrd incorporation assay, which is based on the extent of BrdUrd incorporation during DNA synthesis. The results with the BrdUrd assay did not show any proliferation for resveratrol concentrations between 10 and 25 µM, but a plateau. For concentrations higher than 25 µM, the incorporation of BrdUrd decreased with an LD_{50}=120 µM. So, this result confirms that resveratrol has an antiproliferative property, but interferes with the MTT assay on HaCaT cells at concentration below 40 µM. Thus, the following experiments were performed with 20 µM (non toxic) and 50 µM (toxic) resveratrol concentrations, and the BrdUrd test was used rather than the MTT assay for determination of cell viability.

Resveratrol Potentiates UVA-Induced Cell Death

Resveratrol is a lipophilic polyphenolic phytoalexin, and its UV-visible spectra displays two peaks at 305 and 320 nm, i.e. in the UVB and UVA range, respectively. The BrdUrd test on HaCaT keratinocytes was performed with 0, 20 or 50 µM of resveratrol on control or UVA-irradiated cells (Fig. **2**). The same results as described above were found for non-irradiated cells, with only 50 µM resveratrol being a toxic dose. In UVA irradiated cells, cell viability slightly but significantly decreases, demonstrating a moderate effect of UVA on cell viability. However, when irradiated cells were pre-incubated with resveratrol, cell viability dropped drastically: 20 µM of resveratrol produced a decrease of 50% in cell viability, whereas almost all irradiated cells treated with 50 µM were dead. A synergistic effect of resveratrol and UVA was observed in producing cell death. Therefore, resveratrol treatment of HaCaT keratinocytes potentiates the toxicity of UVA in HaCaT keratinocytes.

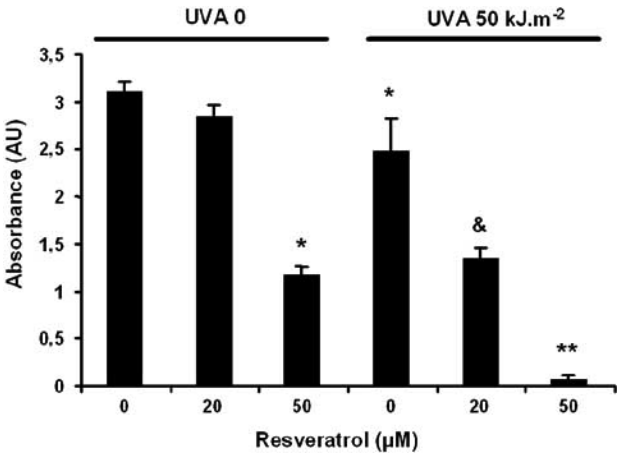

Fig. (2). Resveratrol potentiates UVA-induced cell death.

HaCaT keratinocytes were treated with 0, 20 or 50 µM of resveratrol for 24h; then cells were UVA-irradiated (50 $kJ.m^{-2}$) or not before the BrdUrd test, which measures cell proliferation. The results represent the average and S.D. of three independent experiments (* $p<0.05$ *vs* control, & $p<0.01$ *vs* control-UVA; § $p<0.001$ *vs* control-UVA).

Resveratrol Enhances DNA Strand Breaks After UVA Irradiation

The comet assay under alkaline conditions [15] was used to measure chromosomal DNA strand breaks in cells exposed to UVA radiation after resveratrol treatment. Chromosomal DNA strand breaks including alkali-labile sites were measured using the percentage DNA in the comet tail. In non-irradiated cells, strand breaks remained unchanged in presence of 20 µm resveratrol and slightly but significantly increased in HaCaT cells upon treatment with 50 µM resveratrol (Fig. **3**). UVA irradiation alone increased DNA strand breaks compared to non-irradiated control cells as expected [16], but an important increase of the percentage DNA in the comet tail was observed in cells irradiated after 24h treatment by 20 or 50 µM resveratrol. It is to note that, as it was the case above, this increase in DNA strand cleavage is much more pronounced for 50 µM resveratrol, evoking a synergistic effect between UVA and resveratrol. These results suggest that resveratrol treatment enhances UVA-induced DNA strand breaks in HaCaT keratinocytes.

8-Oxo-7,8-dihydro-2'-deoxyguanosine Formation After UVA Irradiation of Resveratrol-Treated Cells

Since resveratrol synergizes with UVA to cause DNA strand cleavage and/or alkali labile sites, the formation of a more defined oxidative damage, namely 8-oxo-7,8-dihydro-2'-deoxyguanosine, was measured in cellular DNA using HPLC-MS/MS assay. Resveratrol by itself, at both 20 and 50 µM concentrations, did not significantly change the level of lesions in non-irradiated cells compared to untreated cells, although the level of 8-oxodGuo was slightly elevated for 50 µM resveratrol (Fig. **4**). As expected, UVA irradiation of HaCaT cells significantly increased the number of lesions formed per 10^6 DNA bases. However, a much larger increase in the formation of 8-oxodGuo lesions was observed when

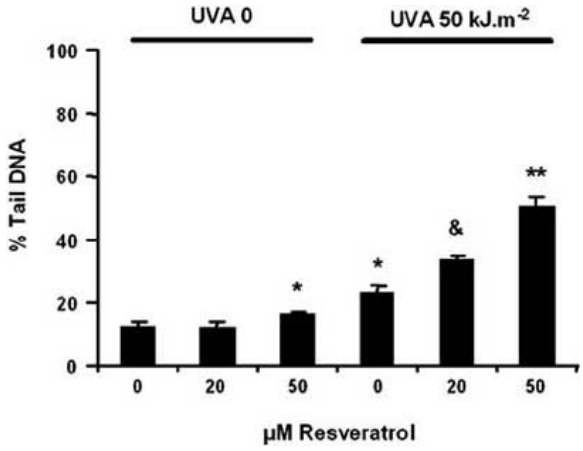

Fig. (3). Resveratrol induces strand breaks in chromosomal DNA as revealed by the comet assay.

HaCaT keratinocytes were treated with resveratrol (0, 20 or 50 µM) for 24h before irradiation by UVA (50 kJ.m^{-2}). Non-irradiated (C) or irradiated cells were processed immediately for analysis by comet assay under alkaline conditions. The extent of the DNA breakage induced by resveratrol treatment was quantified by determining the percentage of DNA in the tail of the comet. For each condition, 100 cells were scored for % DNA from random sampling. The results represent the average and S.D. of three independent experiments (* $p<0.05$ *vs* control, & $p<0.01$ *vs* control-UVA; § $p<0.001$ *vs* control-UVA).

resveratrol-treated cells were exposed to UVA radiation. Moreover, this synergistic effect is resveratrol UVA-dose dependent and consistent with the results obtained above. Therefore, the combination of resveratrol and UVA irradiation dramatically increased the amount of 8-oxodGuo lesion formed in genomic DNA of human keratinocytes.

DISCUSSION

The MTT assay may not be suitable in all cell systems to determine the effect of resveratrol on cell toxicity. This pitfall has been already reported for CEM-C7H2 lymphocytic leukemia cells [14]. In this study, this artefact was shown on HaCaT keratinocytes cells by comparing the results of the MTT and BrdUrd assays. The toxicity of resveratrol also appears to depend on the cell type. For example, resveratrol did not display any cytotoxicity in human lymphocytes at concentrations between 10 and 100 µM [17], but was a potent inducer of apoptosis in melanoma cells [18]. Resveratrol was also shown to inhibit the growth of colonic tumor cells [19], leukemic cells [20, 21], breast and prostate cancer cells [22-27]. As demonstrated in this work, resveratrol displayed an anti-proliferative and cytotoxic effect on the clonogenic but non-tumorigenic HaCaT keratinocyte cell line, in agreement with previous findings on normal human keratinocytes [28].

Resveratrol absorbs light in the UVA range, and thus may react photochemically upon UVA irradiation. Moreover, resveratrol and UVA synergize to induce death of human HaCaT keratinocytes. In addition, the toxicity of resveratrol, and its potentiation of UVA effects, has been correlated to an

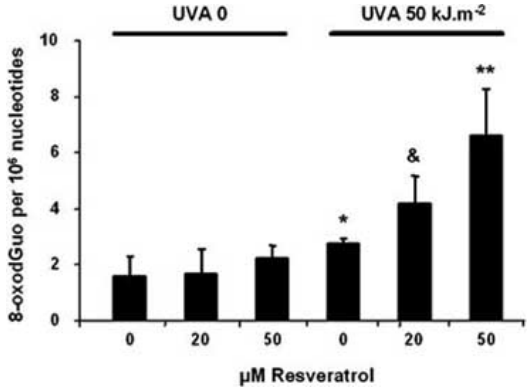

Fig. (4). Levels of 8-oxodGuo measured in cellular DNA upon treatment of cells with either 20 or 50 µM resveratrol.

$2.5.10^6$ HaCaT keratinocytes were treated with 0, 20 or 50 µM resveratrol 24h before irradiation with 50 kJ.m^{-2} UVA and trypsinized for DNA extraction. 8-Oxo-7,8-dihydro-2'-deoxy-guanosine was measured by HPLC-MS/MS from enzymatically digested DNA. Results represent the average and S.D. of six independent determinations (* $p<0.05$ *vs* control, & $p<0.01$ *vs* control-UVA; § $p<0.001$ *vs* control-UVA).

increase in DNA strand breaks, as shown by the comet assay. The same relationship was observed in parallel between resveratrol and UVA in the formation of 8-oxodGuo lesions, an oxidative damage which can lead to DNA strand breaks. All these results are in good correlation, and strongly suggest a synergistic effect between resveratrol and UVA for the generation of oxidative DNA damage, e.g. 8-oxodGuo, the occurrence of DNA strand breaks and finally cell death of HaCaT keratinocytes.

UVA irradiation is known to induce DNA damage indirectly through reactive oxygen species [29]. It was pointed out that the most important step in UVA-induced generation of strand breaks and alkali-labile sites in DNA is the production of the hydroxyl radical (˙OH) indirectly generated by UVA, and that several antioxidant molecules, e.g. glutathione, alpha-tocopherol, potently prevent UVA-induced DNA damage [30]. In another report, Mg-ascorbyl phosphate or alpha-tocopherol have been found to protect from UVA-, but not UVB-, induced DNA damage [31]. The main polyphenol found in green tea, epigallocatechin-3-gallate, has also been shown to protect HaCaT cells against the cytotoxic effects of UVA radiation [32]. In this study, a synergistic effect between UVA and resveratrol was observed, suggesting that this antioxidant molecule may act through a radical mechanism to potentiate oxidative DNA damage when irradiated by UVA. Resveratrol has been shown to penetrate skin and to protect from UVB radiation [9]. It was suggested for the prevention of UVB-mediated cutaneous damages.

Moreover, resveratrol belongs to the phytoestrogen chemical family. It is an agonist for the estrogen receptor and possesses a strong estrogenic activity [33]. There are strong pieces of evidence for the implication of estrogenic compounds in free-radical mediated cellular damage [34]; in that respect, phytoestrogens, like genistein or daidzein have

already been described as potent inducers of DNA lesions [35]. In some conditions, resveratrol can so act as an oxidant and may enhance UVA-induced oxidative stress and DNA damage.

In conclusion, these results clearly indicate that pretreatment of HaCaT keratinocytes with resveratrol increases the level of UVA-induced DNA strand breaks, including the formation of higher amounts of 8-oxodGuo in genomic DNA. Therefore, resveratrol combined with UVA irradiation, and hence with solar radiation, has potential hazardous effects on genomic DNA. Putative genotoxic effects of resveratrol would need further investigations. So, care should be taken with topical applications of resveratrol, particularly on skin regions exposed to sunlight.

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were reagent grade from either Sigma (St Quentin-Fallavier, France) or Merck (Grenoble, France). Resveratrol (Sigma) was prepared as a stock solution (200 mM) in ethanol.

Cell Culture Methods

HaCaT human keratinocyte cells [36] were grown in DMEM medium (Dulbecco's Modified Eagle's Medium, Invitrogen, Inc., Cergy-Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in a 5% humidified CO_2-enriched atmosphere and routinely split at a 1:5 ratio. Cells in Petri dishes were irradiated at 50 $kJ.m^{-2}$ under a Waldmann UVA 700L irradiator (lamp MSR 700, wavelength 330-450 nm). During irradiation, Petri dishes were maintained on ice to prevent the influence of heat. When needed, cells were treated by resveratrol or vehicle alone 24h before irradiation and then immediately trypsinized for DNA extraction.

Cell Viability Assay

Cellular viability in the presence or absence of experimental agents was determined using the Mosmans's MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide; Sigma) assay. This assay is based on the ability of living cells to convert dissolved MTT to insoluble formazan [37]. Briefly, following experimental treatment (resveratrol), 10 µL of a 5 mg/mL MTT solution was added in each well, and the plate was incubated in the dark for 3h at 37°C. The medium was then removed, and the reaction product was solubilized in 100 µL DMSO. Absorbance was measured at 570 nm using a microtiter plate reader (Labsystems Multiskan RC, Cergy-Pontoise, France). The percentage viability was calculated as follows: percentage specific viability= $[(A–B)/(C–B)] \times 100$, where A = OD_{570} of the treated sample, B = OD_{570} of the medium, and C = OD_{570} of the control (phosphate buffer saline (PBS)-treated cells). The values were expressed as percentage viability relative to vehicle-treated control cultures.

5-Bromo-2'-deoxyuridine Uptake

A cell proliferation kit (Roche Diagnostics Meylan, France) was used to determine the amount of 5-bromo-2'-deoxyuridine (BrdUrd) incorporated in the DNA. The assay was performed according to the manufacturer's instructions. The values were expressed as percentage viability relative to vehicle-treated control cultures.

Single Cell Gel Electrophoresis (Comet Assay)

After treatment in 35 mm Petri dishes, HaCaT cells were trypsinized and treated for single cell gel electroploresis as previously described [38]. The extent of DNA damage was determined after analysis with a fluorescence microscope (Axiovert 200, Zeiss, Le Pecq, France) connected to a charge-coupled device (CCD) camera and a personal computer-based analysis system (comet Analysis Software, version 4.0, Kinetic Imaging Ltd., Liverpool, UK). Results were expressed as percentage of DNA in the tail (% Tail DNA).

DNA Extraction from HaCaT Keratinocytes

Extraction of cellular DNA from keratinocytes was performed according to a previously reported procedure [39, 40] with one major modification. Typically, 10 mM sodium azide, a well-known singlet oxygen quencher, was added to the buffered solutions used to isolate DNA (buffer A, 320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM deferoxamine, 1% Triton X-100, 10 mM NaN_3, pH 7.5; and buffer B, 10 mM Tris-HCl, 5 mM EDTA-Na_2 0.15 mM deferoxamine, 10 mM NaN_3 and pH 8.0).

8-OxodGuo Measurement

The level of 8-oxodGuo in cellular DNA was assessed using a recently developed HPLC-MS/MS assay [41, 42]. This required a quantitative enzymatic digestion of DNA into nucleosides following a reported optimized procedure [41]. Accurate quantification of the level of 8-oxodGuo was obtained using an isotopically labeled M + 5 internal standard. Typically, 1.5 pmol of [M + 5] 8-oxodGuo was added to the DNA sample prior to its enzymatic digestion. The resulting hydrolysate was directly injected onto the HPLC column coupled to the mass spectrometric detector (API 3000). The output of the column was also connected to a UV detector set up at 260 nm for quantification of DNA through the measurement of dGuo. Both 284 to 168 and 289 to 173 transitions were used for the detection of 8-oxodGuo and the corresponding [M + 5]-labeled internal standard, respectively. Results, expressed as the number of oxidized dGuo lesions formed per 10^6 DNA bases, represent the average and S.D. of, at least, three independent determinations.

Statistics

When needed, results were presented as mean ± S.D. of at least 3 experiments. Significance was assessed by the *Student's t-test*.

ABBREVIATIONS

Resveratrol	=	Trans-3,4',-5-trihydroxystilebene
BrdUrd	=	5-bromo-2'-deoxyuridine
8-oxodGuo	=	8-oxo-7,8-dihydro-2'-deoxyguanosine
HPLC-MS/MS	=	High performance liquid chromatography coupled to tandem mass spectrometry

MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide
UVB radiation	=	Ultraviolet B (280 - 320 nm) radiation
UVA radiation	=	Ultraviolet A (320 – 400 nm) radiation

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