Food and Chemical Toxicology 63 (2014) 153–160

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Resveratrol contributes to chemosensitivity of malignant mesothelioma cells with activation of p53

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ARTICLE INFO

Article history:
Received 4 September 2013
Accepted 5 November 2013
Available online 14 November 2013

Keywords:
Resveratrol
Clofarabine
Mesothelioma
Apoptosis
p53
Chemopotentiator

ABSTRACT

Resveratrol is a naturally occurring polyphenolic phytoalexin with chemopreventive properties. We previously reported a synergistic anti-proliferative effect of resveratrol and clofarabine against malignant mesothelioma (MM) cells. Here, we further investigated molecular mechanisms involved in the synergistic interaction of these compounds in MM MSTO-211H cells. Resveratrol, in combination with clofarabine, time-dependently induced a strong cytotoxic effect with the nuclear accumulation of phospho-p53 (p-p53) in MSTO-211H cells, but not in normal mesothelial MeT-5A cells. Combination treatment up-regulated the levels of p-p53, cleaved caspase-3, and cleaved PARP proteins. Gene silencing with p53-targeting siRNA attenuated the sensitivity of cells to the combined treatment of two compounds. Analyses of p53 DNA binding assay, p53 reporter gene assay, and RTP-CR toward p53-regulated genes, including Bax, PUMA, Noxa and p21, demonstrated that induced p-p53 is transcriptionally active. These results were further confirmed by the siRNA-mediated knockdown of p53 gene. Combination treatment significantly caused the accumulation of cells at G_1 phase with the increases in the sub-G_0/G_1 peak, DNA ladder, nuclear fragmentation, and caspase-3/7 activity. Taken together, these results demonstrate that resveratrol and clofarabine synergistically elicit apoptotic signal via a p53-dependent pathway, and provide a scientific rationale for clinical evaluation of resveratrol as a promising chemopotentiator in MM.

1. Introduction

Apoptosis, or programmed cell death, is an active, genetically controlled cell suicide procedure which allows multicellular organisms to eliminate genome-damaged, infected, and unwanted cells through a safe, orderly process. Activation of apoptosis is a critical fail-safe mechanism designed for the cells to eliminate gene-mutated cells and to protect against uncontrolled proliferation. Therefore, it is noteworthy that acquired resistance to apoptosis or reduced apoptosis plays a vital role in carcinogenesis. Cancer cells acquire apoptosis resistance through disrupted balance of pro-apoptotic and anti-apoptotic proteins (Pepper et al., 1997), impaired death receptor signaling pathway (Fulda, 2010), p53 mutations (Slatter et al., 2011), increased cellular detoxification and antioxidant system (Lee et al., 2012b), and reduced expression of caspase family (Devarajan et al., 2002). Increased resistance of tumor cells to apoptosis contributes to therapy failure and tumor recurrence because induction of apoptosis in cancer cells is one of very important phenomenon in cytotoxicity induced by some chemotherapeutic agents or radiation (Giménez-Bonafé et al., 2009).

Malignant mesothelioma (MM) is a rare but aggressive tumor that is highly resistant to apoptosis (Fennell and Rudd, 2004). The majority of patients with MM are diagnosed at late stages when the tumor remains unresectable, the treatment option thus depending on chemotherapy. The highly resistant phenotype of this tumor to apoptosis results in a poor prognosis, and the median survival time is 10–12 months after diagnosis (Montanaro et al., 2009). Despite significant pharmaceutical advances in recent years, MM remains an incurable disease. Therefore, it is crucial for the improvement of chemotherapeutic efficacy to develop new drug

Abbreviations: MM, malignant mesothelioma; p-p53, phospho-p53; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolum bromide; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; DAPI, 4′,6-diamidino-2-phenylindole; RT-PCR, Reverse transcription-polymerase chain reaction.

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http://dx.doi.org/10.1016/j.fct.2013.11.004

or treatment strategies to enhance the apoptotic signaling pathways or restore it to normality.

The tumor suppressor p53 is a multifunctional transcription factor that drives the expression of genes for growth arrest, DNA repair, senescence, and apoptosis (Oren, 2003). Intracellular p53 is present in low concentrations, with a half-life of less than 1 h in unstressed cells. The reason for its fast turnover is its rapid ubiquitination by the E3 ubiquitin ligase Mdm2, which sends p53 to the proteasome. The p53 mainly mediates apoptosis in a transcription-dependent manner (Oren, 2003). Two distinct apoptotic pathways have been developed, namely the intrinsic and extrinsic pathways. Main signal in the intrinsic pathway is generated by DNA damage, thereby causing translocation of p53 from cytosol to the nucleus, where it activates the transcription of pro-apoptotic genes such as *Bax*, *PUMA*, *Noxa*, and $p21^{WAF1}$. The apoptosome complex consisting of cytochrome c, apoptotic protease-activating factor 1, and pre-cleaved caspase-9 subsequently activates the initiator caspase-9, which activates effector caspases-3 and -7 to execute DNA fragmentation as a hallmark of apoptosis (Henry et al., 2002). In the extrinsic pathway, p53 activates the transcription of TNF-receptor family including Fas, APO-1, and TRAIL's death receptor DR5. The binding of a ligand to its cognate receptor initiates this pathway through the formation of the death-inducing signaling complex, consisting of the receptor, adaptor protein, and Fas-associated death domain, that activates the initiator caspase-8, which proceed to activate the effector caspase-3 and -7 to induce apoptosis (Guicciard and Gores, 2009).

The importance of p53 in development of mesothelioma may be inferred by the studies of Vaslet et al., in which mice with loss of p53 function and presumably defective apoptosis develop tumors significantly faster than wild-type mice (Vaslet et al., 2002). Unlike many other types of cancer cells which evade apoptosis as a result of a mutant p53 gene, p53 mutations and/or deletions are not frequent in MM (Mor et al., 1977). Despite the wild-type genotype of *p53* in MM, its decreased function is often associated with a homologous deletion at the *INK4A/ARF* locus containing the $p14^{ARF}$ and the $p16^{INK4A}$ genes (Hirao et al., 2002). In this point, MM may be an ideal candidate for p53-targeted therapies. If strategies can be identified to enhance p53 activity, this could have significant therapeutic potential in MM.

Resveratrol, trans-3,4′,5-trihydroxystilbene, is a phytoalexin produced naturally by grapes and other plants as a defense against infection under attack by bacteria or fungi. Resveratrol possesses a wide range of health-promoting activities, including anticancer, antiviral, neuroprotective, anti-aging, and anti-inflammatory effects (Delmas et al., 2006). The potential of resveratrol as an anticancer agent, particularly its ability to sensitize cells to the cytotoxic effects of chemotherapy or radiotherapy through synergistic actions and reduced adverse effects, has been reported in several *in vivo* and *in vitro* studies (Aires et al., 2013; Hu et al., 2012; Baatout et al., 2005). We previously showed that synergistic anti-proliferative effect occurred in MM cells when resveratrol was combined with chemotherapeutic drug clofarabine; such a synergism was associated with inhibition of transcription factor Sp1 and Nrf2 activities (Lee et al., 2013, 2012b).

Based on these findings, the present study was aimed at a further investigation of the pro-apoptotic roles of resveratrol and clofarabine in MM cells. The results presented below highlight the importance of enhanced p53 activity as one of therapeutic strategies for this deadly disease.

2. Materials and methods

2.1. Cell culture and treatment

Resveratrol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolum bromide (MTT), sodium dodecyl sulfate (SDS), dimethylsuldoxide (DMSO), propidium iodide, and antibody to β-actin were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Antibodies to Fas, p53 and phospho-p53 (p-p53), horseradish peroxidase (HRP)-conjugated secondary antibodies, and enhanced chemiluminescence (ECL) kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human caspase-3, cleaved caspase-3, and PARP antibodies were from Cell Signaling Technologies (Beverly, MA, USA). Cell culture media and reagents were purchased from Hyclone Laboratories Inc. (South Logan, Utah, USA). The human mesothelioma cell line MSTO-211H and human mesothelial cell line MeT-5A were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MSTO-211H cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. MeT-5A cells were maintained in M-199 (Welgene, Daegu, Korea) medium supplemented with 3.3 nM epidermal growth factor, 10% fetal bovine serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were grown to 50% confluence in a monolayer culture in this medium for 24 h before treatment. They were then harvested with trypsin for DNA flow cytometry, DNA fragmentation, Western blotting, and RT-PCR analyses.

2.2. MTT assay

The extent of cytotoxicity induced by resveratrol and clofarabine, alone or in combination, was performed by MTT assay as described previously (Lee et al., 2011). Cells (8×10^3 cells/well) were seeded in 96-well microtiter plates and then treated with these compounds for 1, 2, 3, 4, and 5 days, after which they were exposed to MTT (final 0.1 mg/ml) for an additional 4 h. The absorbance of each well was measured by a GloMax-Multi Microplate Multimode Reader (Promega, Madison, WI, USA). The result was expressed as a percentage of the control.

2.3. Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were prepared according to the instructions provided in the NE-PER® nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA) as described previously (Lee et al., 2012c).

2.4. Western blot analysis

Western blot analyses were performed using cell lysate as described previously (Lee et al., 2012c). Cell lysate containing 40 μg of protein was separated on NuPAGE 4–12% bis-tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and then electrophoretically transferred to Immuno-Blot PVDF membranes. The signal was visualized by an ECL detection kit using X-ray films. The blots were then stripped using a stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) and reprobed with anti-β-actin antibody as a loading control.

2.5. siRNA-mediated gene silencing

RNA interference of p53 was performed using a p53-targeting small interfering RNA (siRNA) duplex from Invitrogen (Oligo ID: HSS186390). Briefly, cells were seeded in 96-well and 6-well plates and transfected at 40% confluency with siRNA duplex using lipofectamine RNAiMAX (Invitrogen as described previously (Lee et al., 2012c).

2.6. DNA binding activity of p53

The DNA-binding capacity of p53 was determined by using a Trans^{AM} p53 Transcription Factor Assay Kit according to the manufacturer's instruction (Active Motif, Carlsbad, CA, USA). The DNA binding of activated p53 protein with specific oligonucleotide sequence (5′-GGACATGCCCGGGCATGTCC-3′) coated onto a microtitre plate was revealed by the addition of a primary polyclonal anti-p53 antibody, followed by a HRP-conjugated secondary antibody and the 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was finally read at 450 nm and the results were expressed as a percentage, based on the ratio of the absorbance of treated cells to that of controls (100%).

2.7. Reporter gene assay

Cells were transfected with luciferase plasmids (p53RE-luc) or corresponding empty vector plasmids using lipofectamine plus reagent according to manufacturer's instruction (Invitrogen). Transfected cells were treated with resveratrol and clofarabine, alone and in combination, for 24 h and then lysed in a reporter assay lysis buffer (Promega). Luciferase activity in the cell lysates was measured by a GloMax-Multi Microplate Multimode Reader.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Briefly, 1 μg of the total RNA was converted to cDNA using the oligo$(dT)_{12-18}$ primer and Superscript III reverse transcriptase (Invitrogen). Each single-stranded cDNA was then diluted and subjected to PCR amplification using Ex Taq DNA polymerase (Takara, Otsu, Japan). The PCR conditions were as follows: initial