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Grape seed proanthocyanidins reactivate silenced tumor suppressor genes in human skin cancer cells by targeting epigenetic regulators

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ABSTRACT

Grape seed proanthocyanidins (GSPs) have been shown to have anti-skin carcinogenic effects in *in vitro* and *in vivo* models. However, the precise epigenetic molecular mechanisms remain unexplored. This study was designed to investigate whether GSPs reactivate silenced tumor suppressor genes following epigenetic modifications in skin cancer cells. For this purpose, A431 and SCC13 human squamous cell carcinoma cell lines were used as *in vitro* models. The effects of GSPs on DNA methylation, histone modifications and tumor suppressor gene expressions were studied in these cell lines using enzyme activity assays, western blotting, dot-blot analysis and real-time polymerase chain reaction (RT-PCR). We found that treatment of A431 and SCC13 cells with GSPs decreased the levels of: (i) global DNA methylation, (ii) 5-methylcytosine, (iii) DNA methyltransferase (DNMT) activity and (iv) messenger RNA (mRNA) and protein levels of DNMT1, DNMT3a and DNMT3b in these cells. Similar effects were noted when these cancer cells were treated identically with 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation. GSPs decreased histone deacetylase activity, increased levels of acetylated lysines 9 and 14 on histone H3 (H3-Lys 9 and 14) and acetylated lysines 5, 12 and 16 on histone H4, and reduced the levels of methylated H3-Lys 9. Further, GSP treatment resulted in re-expression of the mRNA and proteins of silenced tumor suppressor genes, *RASSF1A*, $p16^{INK4a}$ and *Cip1/p21*. Together, this study provides a new insight into the epigenetic mechanisms of GSPs and may have significant implications for epigenetic therapy in the treatment/prevention of skin cancers in humans.

Introduction

Skin cancer is the manifestation of a series of genetic and epigenetic events. Epigenetic processes result in heritable phenotypical changes in gene expression that do not involve alterations in the actual DNA sequence. Instead, epigenetic changes involve variations in DNA methylation, chromatin structure or microRNA profiles that then modify gene expression (Jones and Baylin, 2002). The hallmarks of epigenetic gene regulation are DNA methylation and histone modifications. Because epigenetic changes are reversible and can be manipulated pharmacologically, this area has become a focus of particular interest for the development of therapeutic agents to treat cancer, including cutaneous carcinogenesis. Epigenetic alterations in various genes play crucial roles in the development of cancers (Jones and Baylin, 2002). Epigenetic inactivation of genes by promoter hypermethylation has been recognized as an important mechanism by which tumor suppressor genes are shut down during development of cancers. Hypermethylation of CpG islands in the promoter region leads to silencing either by direct inhibition of transcription factor binding, or by attracting methylated-DNA binding proteins, recruiting other transcriptional repressors such as histone deacetylases (HDACs) and histone methyl transferases (Hegi et al., 2009; Jones and Baylin, 2002). DNA methylation at the 5′ position of cytosine is the most well characterized epigenetic mechanism which can be inherited without changing the DNA sequence (Hegi et al., 2009; Jones and Baylin, 2002). The mammalian DNA methylation machinery is made up of two components: DNA methyltransferases (DNMTs), which establish and maintain genome-wide DNA methylation patterns, and the methyl-CpG-binding proteins, which are involved in "reading" and interpreting the methylation patterns (Antequera and Bird, 1993; Howell et al., 2009; Jones, 2002). DNA hypermethylation is one of the major epigenetic mechanisms in the silencing of expression of tumor suppressor genes (Antequera and Bird, 1993; Herman and Baylin, 2003; Jones, 2002). DNA methylation is commonly associated with increased levels of functionally aberrant DNMTs, which then initiate the methylation of cytosine at the 5′ position in CpG dinucleotides (Counts and Goodman, 1995; Jones and Baylin, 2002). In addition to DNA methylation, histone modifications, particularly methylation and

Abbreviations: GSPs, grape seed proanthocyanidins; DNMT, DNA methyltransferase; HDAC, histone deacetylase; H3-Lys 9, lysine 9 on histone H3; PCR, polymerase chain reaction; TSA, trichostatin A; 5-aza-dc, 5-aza-2′-deoxycytidine.

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acetylation, are involved in transcriptional silencing of a number of genes in cancers. As the epigenetic mechanisms are reversible in nature, some dietary phytochemicals, which possess anti-carcinogenic properties, have been assessed for their effect of epigenetic processes (Davis and Milner, 2004; Davis and Uthus, 2004; Huang, 2002).

Bioactive phytochemicals, particularly dietary, offer promising options for the development of more effective strategies for the prevention or treatment of cancers and they can be utilized as complementary and alternative medicine. While phytochemicals have been used for thousands of years in various cultures/civilizations for the treatment of many diseases, wound healing or to preserve skin beauty, their active ingredients and mechanisms of action are not well characterized. Grape seed proanthocyanidins (GSPs) are promising bioactive molecules that have demonstrated anti-carcinogenic effects in some animal tumor models and exhibit no apparent toxicity *in vivo* (Meeran et al., 2009; Mittal et al., 2003; Nandakumar et al., 2008). As previously described, this product contains primarily proanthocyanidins (89%), which constitute dimers, trimers, tetramers, and oligomers of monomeric catechins and/or (−)-epicatechins (Mittal et al., 2003). It is likely that at least some of the components present in the GSPs act synergistically and thus this product may be more effective than any single component. GSPs have been shown to inhibit ultraviolet radiation-induced skin tumors in SKH-1 hairless mice (Mittal et al., 2003) as well as in 2,4-dimethylbenz(a)anthracene-initiated and 12-O-tetradecanoylphorbol-13-acetate-promoted skin tumors in C_3H/HeN mouse model (Meeran et al., 2009). However, an epigenetic basis for their chemopreventive effects remains unexplored.

In this study, we investigated whether GSPs would reactivate silenced tumor suppressor genes and determine the molecular mechanism underlying these effects using two well known human skin cancer cell lines, A431 and SCC13, as an *in vitro* model. Our study demonstrates that treatment of skin cancer cells with GSPs results in decreased levels of DNA methylation, inhibited histone deacetylase (HDAC) activity and increased levels of acetylated histones in cancer cells. The ultimate result of these effects was re-expression of tumor suppressor genes (*RASSF1A*, *p16^{INK4a}* and *Cip1/p21*). Interestingly, the epigenetic effects of GSPs were non-significant in normal human epidermal keratinocytes (NHEK).

Material and methods

Chemicals and antibodies. GSPs were obtained commercially from Kikkoman Corporation (Noda, Japan). The quality of this product is maintained by the vendor on a lot-to-lot basis. GSPs contain about 89% proanthocyanidins with dimers (6.6%), trimers (5.0%), tetramers (2.9%) and oligomers (74.8%), as reported previously (Mittal et al., 2003). The Methylamp™ Global DNA Methylation Quantification Kit and the EpiQuik DNA Methyltransferase Activity Assay Kit were purchased from Epigentek, Inc. (New York, NY). Standardized real-time PCR primers for DNMT1, DNMT3a, DNMT3b, *RASSF1A*, *p16^{INK4a}* and *Cip1/p21* were obtained from SuperArray Biosciences (Fredrick, MD). Antibodies were procured as follows: 5-methylcytosine (5-mC) from Calbiochem (New Jersey, NJ), DNMT1, DNMT3a and DNMT3b from Imgenex Corporation (San Diego, CA), HDAC3 and HDAC4 from Cell Signaling Technology Inc. (Danvers, MA), RASSF1A, acetyl histone H4 and H3 related antibodies were from Upstate Biotechnology and Abcam (Cambridge, MA), and p16^{INK4a} and Cip1/p21 from Santa Cruz Biotechnology (Santa Cruz, CA).

Skin cancer cell lines and cell culture. A431 and SCC 13 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured as monolayers in 100 mm tissue culture dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 100 μg/ml penicillin-streptomycin (Invitrogen). They were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The NHEK were obtained from the Cell Culture Core Facility of Skin Diseases Research Center at the University of Alabama at Birmingham, AL. The NHEK were cultured in keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Gibco/Invitrogen, Carlsbad, CA) and maintained in an incubator under the conditions as described above. Cells were seeded at a density of 1×10^6 cells per Petri dish and allowed to attach for 24 h before treatment with testing agents for either 3 or 5 days. Media and treatment agents were refreshed every 3 days. The sub-confluent cells (60–70% confluent) were treated with either varying concentrations of GSPs (0, 5, 10, 15 and 20 μg/ml) or 5-aza-2′-deoxycytidine (5-aza-dc) or trichostatin A(TSA) after dissolving in DMSO. The cells treated only with vehicle (DMSO) served as a control [maximum concentration of DMSO, 0.1% (v/v) in media].

Assay for global DNA methylation. For the analysis of global DNA methylation levels, the total genomic DNA was extracted from the cells which were treated with GSPs or 5-aza-dc using the DNeasy Kit (Qiagen Sciences, MD) following the manufacturer's protocol. The Global DNA methylation levels were determined using the Methylamp™ Global DNA Methylation Quantification Kit following the manufacturer's instructions. The methylated fraction of DNA is recognized by a 5-mC antibody. With this colorimetric assay kit, the amount of methylated DNA, which is proportional to the optical density, is quantified through an enzyme-linked immunosorbent assay (ELISA)-like reaction. This analysis provides the levels of global DNA methylation and is not specific to any particular gene.

Assay for DNMT activity. Cells were treated with various concentrations of GSPs or 5-aza-dc for 3 or 5 days. Thereafter, cells were harvested and nuclear extracts were prepared using EpiQuik Nuclear Extraction Kit (Epigentek Inc., New York, NY) following the manufacturer's instructions. DNMT activity was determined in nuclear extracts using EpiQuik DNA Methyltransferase Activity Assay Kit (Epigentek Inc., New York, NY) following the manufacturer's protocol.

Analysis of 5-mC in DNA following dot-blot assay. Cells were treated with GSPs (0, 5, 10 and 20 μg/ml) or 5-aza-dc for 5 days. Genomic DNA was isolated using the DNA Isolation Kit (Qiagen Sciences, MD), and dot-blot analysis was performed as detailed previously (Katiyar et al., 2010). Briefly, genomic DNA (1 μg) was denatured and then blotted onto Hybond-ECL nitrocellulose membranes using Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc. Hercules, CA). This was fixed by baking the membrane for 30 min at 80 °C. The membrane was incubated with an antibody specific to 5-mC (1:500, v/v) followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The membrane was then treated with enhanced chemiluminescence detection reagents and exposed to Kodak autoradiograph films. Equal DNA loading was verified by staining the membranes with 0.2% methylene blue. The intensity of each dot was measured by densitometry and normalized to total DNA.

Assay for HDAC activity. The effect of GSPs on HDAC activity in skin cancer cells was measured using the HDAC Colorimetric Activity Assay Kit (Active Motif Inc., Carlsbad, CA) following the manufacturer's protocol. Briefly, the cells were treated with GSPs or TSA for 3 or 5 days. Cells were harvested and nuclear protein fractions were isolated using EpiQuik Nuclear Extraction Kit (Epigentek Inc., New York, NY). Forty micrograms of nuclear proteins from each treatment group was incubated with a colorimetric HDAC substrate for 3 h after which the reaction was developed with assay development buffer supplied with the kit. Nuclear protein extracts treated with TSA (100 nM), an inhibitor of HDAC activity, served as a positive control.

Assay for histone acetyltransferase (HAT) activity. The effect of GSPs on HAT activity in cancer cells was measured using EpiQuik™ HAT Activity Assay Kit from Epigentek Group Inc. (Brooklyn NY, www.