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Reactivation of methylation-silenced tumor suppressor gene p16INK4a by nordihydroguaiaretic acid and its implication in G1 cell cycle arrest

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Abstract

Phytoestrogens, including the two major groups isoflavones and lignans, are chemicals with weak estrogenic activity which occur naturally in many foods and herbs. Recently, several intriguing studies reported that some isoflavones can affect DNA methylation status. However, little is known about the effect of plant lignans on epigenetic modification. Using cultured T47D and RKO human cancer cells as a model, we studied the modulating effects of nordihydroguaiaretic acid (NDGA), a member of the lignan family, on the methylation status of the gene promoter region. Our results indicated that NDGA reverses p16INK4a CpG island hypermethylation, and restores its transcription and expression in both cell lines. Cytometric analysis showed that NDGA significantly affects cell cycle progression by arresting cells at the G1 phase. Consistent with the reacquisition of p16INK4a expression, we also found that NDGA induces cellular senescence in cancer cells. This is the first study demonstrating that a member of the lignan family can induce demethylation in human cancer cell lines, suggesting a novel epigenetic mechanism in the prevention or treatment of cancer.

Keywords: Phytoestrogen; Lignan; NDGA; p16INK4a; Methylation

Introduction

Endocrine-disrupting chemicals (EDCs) in the environment, especially environmental estrogens, have been linked to human health and disease. Environmental estrogens are comprised of two main groups, phytoestrogens and synthetic substances released into the environment. It is widely accepted that the anticancer properties of phytoestrogens are due to their similarity in structure to estrogens (Setchell and Adlercreutz, 1988). Phytoestrogens may inhibit binding of the more potent endogenous estrogens, and elicit a weak estrogenic response by competing for estrogen receptors which are present in both normal and malignant tissue. However, this is not the only mechanism by which phytoestrogens exert their effects, many of which may be unrelated to the estrogenic properties of these compounds.

DNA methylation, a process by which methyl groups are added to the base cytosine in DNA, affects gene expression in many biological processes. In most studies, increased DNA methylation is associated with gene silencing, and decreased methylation is related to gene activation (Egger et al., 2004). The importance of promoter hypermethylation as well as global hypomethylation in carcinogenesis has been extensively discussed (Herman and Baylin, 2003). Currently, DNA methylation is of central interest in cancer research, as a tumor suppressor gene, p16INK4a, is the universal and key regulator of the G1–S phase transition of the cell cycle (Shapiro et al., 1998). p16INK4a

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is functionally inactivated by promoter hypermethylation in a wide variety of cancers, including breast, colon, prostate, esophageal, pancreatic carcinomas (Esteller et al., 2000; Konishi et al., 2002; Rocco et al., 1998; Xing et al., 1999), head and neck cancers (Fukushima et al., 2002), and other cell lines.

In many cases DNA methylation inhibitors can demethylate the CpG islands in the promoters, and reactivate genes silenced by aberrant methylation (Laird, 2005). Results of recent studies show that a number of isoflavones such as genistein, myricetin, apigenin, and quercetin can inhibit DNA methyltransferases (DNMT) in human cancer cell lines (Fang et al., 2007). Genistein (Fang et al., 2005) and quercetin (Ma et al., 2006) were reported to partially reverse DNA hypermethylation and reactivate genes silenced by aberrant methylation. In comparison, few studies have investigated the effects of lignans on epigenetic alteration. *Larrea tridentata* (Creosote bush) is a native plant abundant in deserts in the U.S. and Mexico. The active constituent extracted from this plant is NDGA (Fig. 1), which belongs to lignan family and has estrogenic activity (Fujimoto et al., 2004).

Bian and co-workers have demonstrated that NDGA inhibits 5-cytosine DNA methyltransferase activity (Lu and Bian, 2003) and alters the global methylation level in malignant glioma cell lines (Yao and Bian, 2004). These findings led us to hypothesize that NDGA may be involved in the regulation of gene activity by regulating the methylation of CpG islands. Earlier studies have indicated that the p16INK4a gene promoter region in RKO (Hui et al., 2000) and T47D (Luo et al., 2006) cells was hypermethylated. Using cultured T47D and RKO human cancer cells, we analyzed the methylation status of the tumor suppressor gene p16INK4a by methylation-specific PCR (MSP) analysis. Results indicated that NDGA alters promoter methylation of p16INK4a, and partially restores its expression in both cell lines. Our finding suggests that NDGA may represent a potential new class of agents for the treatment of human disease where methylation plays a role in the oncogenic process.

Materials and methods

Cell lines and cell culture

The human breast cancer cell line T47D (provided by the Cancer Institute/Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences) and human colorectal cancer cell line RKO (gifts from Dr. Dajun Deng, Peking University School of Oncology, Beijing Institute for Cancer Research) were cultured in DMEM and RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U of penicillin–streptomycin/ml. The cells were maintained at 37 °C in an incubator containing 5% CO_2. The medium was changed every 2 days.

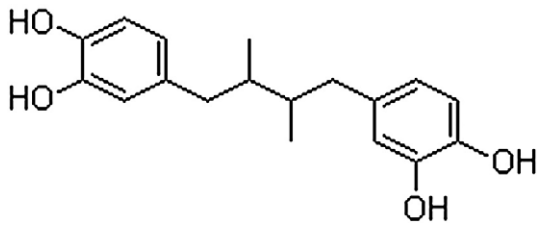

Fig. 1. Chemical structure of nordihydroguaiaretic acid (NDGA).

Chemical treatments

Cells were grown to 60–70% confluence before treatment with NDGA (Fluka N74540; Fluka Chemical Corporation, Milwaukee, WI). Cells were treated for 3 and 6 days with different concentrations of NDGA (0, 10, 20, 50, and 100 μM), which was dissolved in DMSO and diluted with the required volume of cell culture medium. A concentration of 1 μM 5-aza-dC (Sigma Chemical Co., St. Louis, MO) was chosen as a positive control as this concentration is sufficient to restore methylated gene expression (Fang et al., 2004). DNA, RNA, and protein lysates were harvested at the end of the treatment period for methylation, RT-PCR, and Western blot analyses, respectively. DNA was isolated by proteolysis and phenol–chloroform extraction, as described previously (Sambrook et al., 1989). Total RNA was extracted by using the TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

Growth inhibition assay

Cell proliferation assays were performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), as described previously (Cocker et al., 2000). In brief, 5×10^3 cells were plated in triplicate in 96-well flat bottom tissue culture plates (Costar, Corning Inc., USA), and cells were grown for 4 days. Ninety minutes before the end of the appropriate treatment periods, 10 μl of MTT (5 mg/ml in PBS) was added to each well. After incubation at 37 °C, the culture medium was removed and the resultant purple crystals were dissolved in 150 μl DMSO. The plates were gently mixed by rocking back and forth until the crystals were completely dissolved. Optical density (OD) was measured at 570 nm using an ELISA plate reader. The percent inhibition of cell proliferation was calculated as (1 − average OD value of experimental wells/average OD value of control wells) × 100%. Experiments were repeated at least three times.

Bisulfite modification and methylation-specific PCR

The MSP assay can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island. The procedure entails initial modification of DNA by sodium bisulfite, conversion of all unmethylated (but not methylated) cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA (Herman et al., 1996). Bisulfite modification of genomic DNA was carried out as reported previously (Clark et al., 1994), with slight modifications. Briefly, 2 μg of genomic DNA was denatured with NaOH, followed by incubation with 3 M sodium bisulfite for 16 h at 50 °C. After treatment, DNA was purified using a Wizard Miniprep column (Promega, Madison, WI, USA), precipitated with ethanol and resuspended in 25 μl of deionized water.