Diallyl Disulfide Inhibits N-acetyltransferase Activity and Gene Expression in Human Lung Cancer A549 Cells

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Objectives. Acetylators are believed to affect cancer risk related to environmental carcinogen exposure. Individuals can be identified as slow or rapid acetylators by measuring NAT activity. Diallyl disulfide (DADS), one of the major components of garlic (*Allium sativum*), has been reported to exhibit anti-neoplasm activity. In this study, we investigated the inhibitory effects of DADS on NAT activity and gene expresseion (NAT mRNA) in human lung A549 cancer cells.

Methods. The amounts of N-acety-2-aminofluorene (AAF) and 2-aminofluorene (AF) were measured by high performance liquid chromatography before determining NAT activity. The amounts of NAT enzymes were examined and analyzed by flow cytometry. NAT gene expression (NAT mRNA) was examined by polymerase chain reaction and cDNA microarray.

Results. DADS decreased the N-acetylation of AF in human lung A549 cancer cells in a dose-dependent manner. Flow cytometric analysis indicated that DADS decreased the levels of NAT protein in A549 cells. PCR and cDNA microarray experiments showed that DADS inhibited NAT mRNA expression in A549 cells.

Conclusions. DADS affected NAT activity by inhibiting gene expression (NAT mRNA); the result was a reduction in protein levels of NAT in A549 cells. **(Mid Taiwan J Med 2004;9:161-8)**

Key words

cDNA microarray, diallyl disulfide (DADS), human lung cancer cell line, N-acetyltransferase (NAT), 2-aminofluorene (AF)

INTRODUCTION

An initial metabolic step for 2-aminofluorene is N-acetylation, which is catalyzed by cytosolic N-acetyltransferase (NAT), using acetyl-coenzyme A as an acetyl group donor [1]. NAT1 and NAT2 genes are encoded by single open reading frames of 870 base pairs and are located on chromosone 8. Both genes exhibit genetic polymorphisms in the human population [2-5]. Multiple genetically determined variants of NAT enzyme are associated with different levels of specific activity corresponding to either rapid or slow acetylation. Therefore, individuals can be classified as rapid or slow acetylators based on NAT activity [6]. NAT1 is known to catalyze 2-aminofluorene before forming N-acetyl-2-aminofluorene. The genetic variation in NAT1 catalytic activity may have implications for genetic predisposition to cancer after exposure to arylamine carcinogens. Individuals who exhibit

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slow acetylator activity have been shown to be associated with greater susceptibility to occupational bladder cancer [7]; those with rapid acetylator activity have been shown to be associated with increased risk of colorectal cancer [8,9].

Garlic (*Allium sativum* L.) is a common food and food supplement worldwide. The pharmacological properties of garlic are well known and its anticancer properties are being extensively investigated. It has been demonstrated that garlic posses antibiotic [10], fungicidal [11], anti-helminthic and anti-thrombotic [12] functions and may be effective in the prevention and treatment of cardiovascular disease, hypertension and cancer [13-15]. Diallyl disulfide (DADS) is one of the components of garlic, and is known to act as an inhibitor of neoplastic CMT-13 cell growth [16] and in the promotion phase of DMBA induced skin tumors in mice [17]. Recently it was reported that DADS inhibited C6 glioma through the inhibition of H-ras in Sprague-Dawley rats [18] and acted as an antiproliferative agent in neuroblastoma cells by inducing apoptosis [19].

In a previous study, we showed that DADS affected NAT activity in human bladder T24 cancer cells [20], colon colo 205 cancer cells [21] and promyelocytic leukemia cells [22]. In this study, we addressed whether DADS affects NAT activity and gene expression by investigating the effects of DADS on N-acetylation and NAT gene expression of human lung cancer A549 cells.

MATERIALS AND METHODS

Chemicals

Diallyl disulfide (DADS) was purchased from Fluka Chemika Co. (Bucha, Switzerland). Acetyl-CoA (AcCoA), acetylcarnitine, leupeptin, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), Tris, dimethyl sulfoxide (DMSO), 2-aminofluorene (AF), N-acetyl-2-aminofluorene (AAF), bovine serum albumin (BSA), phenyl-methylsulfonylfluoride (PMSF), complete Freund's adjuvant, and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO). All of the chemicals were reagent grade.

Human Lung Cancer A549 Cell Line

Human lung carcinoma cell line (A549) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The A549 cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in 90% Ham's F12K medium (Sigma Chemical Company, St. Louis, MO, USA) with 2 mmol/L L-gultamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), and 2% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin).

Intact Cell NAT Activity Determination

A549 cells (in 1 mL Ham's F12K media with glutamine and 10% fetal calf serum) were individually incubated with 6.75 μmol/L 2-aminofluorene (AF) at 1 x 10^6 cells/mL in individual wells of 24-well cell culture plates with or without DADS (0.5, 5, 25, 50 and 100 μmol/L) co-treatment for 24 h at 37˚C in 95% air and 5% CO_2. Then the cells and media were isolated and centrifuged at 1500 rpm and 4˚C for 5 minutes. The supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvent evaporated under speed vacuum, and the residue redissolved in 50 μL methanol. The mixture was then assayed for N-acetyl-2-aminofluorene (AAF) by high pressure liquid chromatography as described previously [20,21].

Detection of NAT Protein in Human Lung Cancer A549 Cells by Flow Cytometry

The protein level of intracellular NAT in the human lung cancer A549 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), as described previously [23]. Cells were cotreated with various concentrations (0.5, 5, 25, 50 and 100 μmol/L) of DADS for 24 h to detect the intracellular NAT protein. The cells were harvested and washed twice, re-suspended in 100 μL of ice-cold 1% formaldehyde for 5 min, and mixed with 100 μL of ice-cold 99% methanol for 30 min. Then the cells from individual wells were washed three times with 0.1% BSA in PBS and

mixed with 100 μL of 0.1% Triton X-100 in PBS with 0.1% sodium citrate on ice for 45 min. After being washed three times with the same buffer, the cells were incubated with polyclonal antibody (anti-NAT) at 4˚C for 2.5 h, and then washed three times with 0.1% BSA in PBS. The cells were then stained with FITC-labeled secondary antibody (goat antimouse IgG; Jackson Immuno Research Laboratories, West Grove, PA, USA) at 4˚C for 35 min. Again, the cells were washed three times, re-suspended in PBS, and analyzed by flow cytometry [23].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from A549 cells which had been cotreated with or without 50 μmol/L DADS for 24 h by the Qiagen RNeasy Mini Kit as described previously [23]. The RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a microcentrifuge tube (0.5 μL) (final volume, 12.5 μL). The entire mixture was heated at 70˚C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription were the same as those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. The reactions were as follows: 50 μL of solution (1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP mix, 20 pmoles of each primer [B-MDIEA-NAT1 & VPKHGD-X-NAT1 for NAT1, FP1-NAT2 & RP1-NAT2 for NAT2, Act b1 & Act b2 for beta-actin]; cDNA template corresponded to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase). The sequences of primers were as follows: B-MDIEA-NAT1, 5'-CACCCGGATCCGGGATCATGGACATTGAAGC-3', nt 435-454, GenBank accession number X17059; VPKHGD-X-NAT1, 5'-GGTCCTCGAGTCAATCACCATGTTTGGGCAC-3', nt 1295-1278, GENBANK accession number X17059; FP1-NAT2, 5'-CTAGTTCCTGGTTGCTGGCC-3', nt 79-98, GenBank accession number NM-000015; RP1-NAT2, 5'-TAACGTGAGGGTAGAGAGGA-3', nt 1073-1054, GenBank accession number NM-000015; Act b1, 5'-GCTCGTCGTCGACAACGGCTC-3', nt 94-114, GenBank accession number NM-001101; Act2 b2, 5'-CAAACATGATCTGGGTCATCTTCTC-3', nt 446-422, GenBank accession number NM-001101 [23].

Microarray Hybridization

Human lung cancer A549 cells (1×10^7/well) in a 6-well plate co-treated with or without 50 μmol/L DADS for 24 h were harvested by centrifugation. The total RNA was extracted from isolated cells by the Qiagen Rneasy Mini Kit. The cDNA synthesis was performed from the isolated total RNA, and then the cDNA was labeled for microarray hybridization. The fluorescence-labeled cDNA hybridized to its complement on the chip, and the resulting localized concentrations of fluorescent molecules were detected and quantitated (Asia BioInnovations Corporation, Taipei, Taiwan).

Statistical Treatment of Data

Statistical analysis of the data was performed by an unpaired Student's *t* test. The results were considered to be statistically significant at $p < 0.05$.

RESULTS

Effects of Various Concentrations of DADS on NAT Activity in Human Lung Cancer Cells

The N-acetylation of AF in A549 cells with or without various concentrations of DADS (0.5, 5, 25, 50, and 100 μmol/L) for 24 h are shown in the Figure 1. In the presence of various concentrations of DADS, there was a 4% to 81% decrease in production of AAF.

Effects of Various Concentrations of DADS on the Levels of NAT in Human Lung Cancer Cells

The protein level of NAT was measured by the amounts of staining of anti-NAT antibody and then analyzed by flow cytometry. The data indicated that the percentage of NAT-antibody complex in examined cells decreased in the presence of DADS (Table 1). The profile of NAT-antibody from flow cytometric analysis is presented in Figure 2. The protein levels of NAT

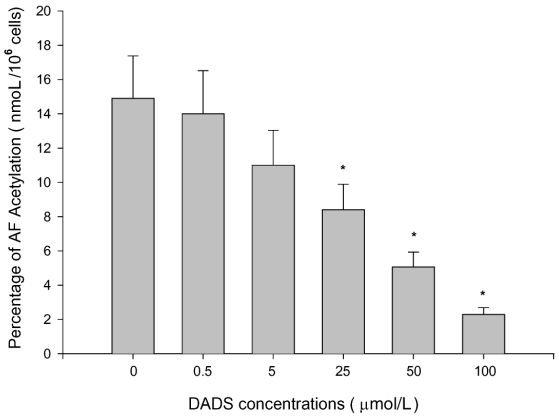

Fig 1. Effects of various concentrations of DADS on N-acetylation of AF in human lung cancer cells. A549 cells (1×10^6/mL) were incubated with 6.75 μmol/L AF and 0.5, 5, 25, 50 and 100 μmol/L DADS co-treatment for 24 h. N-acetylated AF was measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. *Mean differs between DADS treatment and control ($p < 0.05$).

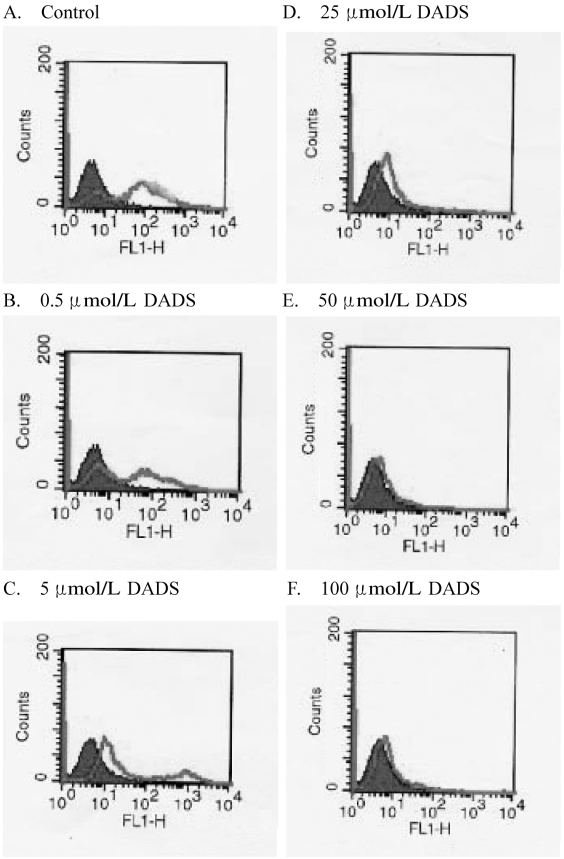

Fig 2. Effects of DADS treatment on NAT expression in human lung caner cell line. A549 cells (1 x 10^6/mL) were treated with 0.5, 5, 25, 50 and 100 μmol/L DADS for 24 h followed by evaluation of NAT expression. NAT expression was estimated by flow cytometry. Data were acquired and analyzed using flow cytometry. A detailed description is in Materials and Methods.

Table 1. Flow cytometric analysis of NAT expression in A549 cells before (control) and after 24 h treatment with DADS at the indicated concentrations

Conc. (μmol/L)	Percentage of cells stained by anti-NAT
0 (control)	64.8 ± 6.8
0.5	61.9 ± 6.9
5	50.4 ± 5.2
25	39.2 ± 4.9*
50	22.1 ± 3.3*
100	12.4 ± 1.6*

Values expressed as mean ± SD, *Compared with control, $p < 0.05$.

decreased in response to DADS concentration.

Effects of DADS on NAT1 mRNA Expression in Human Lung Cancer Cells

The ratio of NAT1 mRNA levels in response to the effect of DADS was calculated from the gel (Figure 3 A and B). Figure 3 shows that NAT1 mRNA levels decrerased after 50 μmol/L DADS was added to A549 cells.

Effects of DADS on NAT mRNA Expression in Human Lung Cancer Cells Examined by cDNA Microarray

The cDNA microarray data are presented in Figure 4. The results indicate that the NAT gene was down-regulated in the DADS-treated cells compared with the control cells. Apparently, DADS inhibited the NAT1 gene expression.

DISCUSSION

NAT has been reported to play an important role in carcinogenesis [2,24] and to affect the distribution and metabolism of AF in rats [25]. The variants of NATs are controlled by multiple genes that are involved in different levels of specific activity corresponding to rapid and slow acetylation [2]. In our laboratory we have shown that DADS affected NAT activity in human cancer cell lines (colon cancer colo 205 and bladder cancer T24) [20,25] and bacteria stains (*Helicobacter pylori* and *Klebsiella pneumoniae*) [26,27]. Therefore, the present study investigated whether DADS affected NAT activity and gene expression in human lung cancer cell line A549.

The data from HPLC analysis indicated that DADS decreased the N-acetylation of AF in a dose-dependent manner. Our previous studies

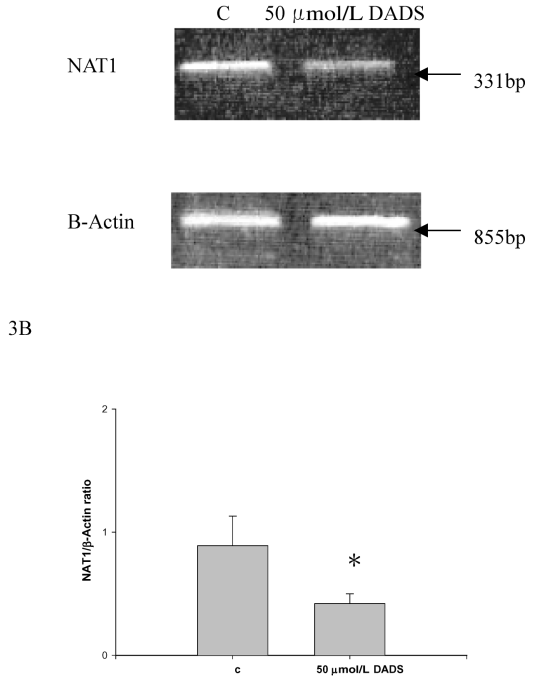

Fig 3. Effect of DADS on the expression of NAT mRNA in human lung A549 cancer cell line. The cells were incubated with 50 μmol/L DADS for 24 h. A: The cells were collected to extract RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT and β-actin, and then PCR-amplified cDNA derived from mRNA was applied to agarose gel-electrophoresis. B: The mRNA levels of NAT and β-actin on the gel were quantified by densitometric analysis of gel-photograph and expressed as NAT/β-actin ratio.

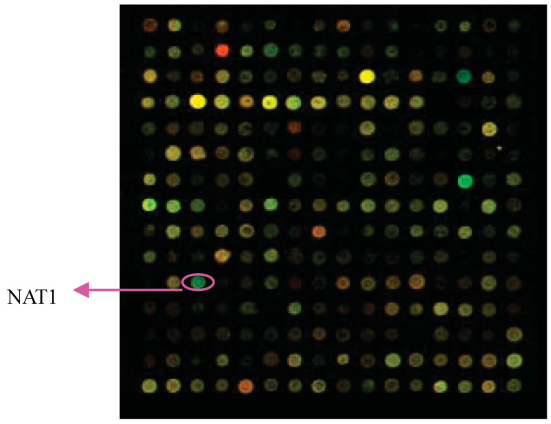

Fig 4. Down-regulation of NAT gene in human lung cancer A549 cells treated with 50 μmol/L DADS and assayed by cDNA microarray. A549 cancer cells (5 x 10^6 cells/mL) in 6-well plate treated with or without 50 μmol/L DADS for 24 h. Red color spot represents up-regulation and green color spot down-regulation. Circle marks the NAT gene down-regulation.

have shown that decreasing the cell number will lead to decreased amounts of AAF production in culture cells; however, other factors such as the amounts of NAT and the levels of mRNA of NAT may also cause the decrease in AAF production. Therefore, we also used anti-NAT antibody to stain the cells before analyzing them by flow cytometry. We also examined the levels of NAT mRNA expression in cells with and without DADS cotreatment by PCR. The results indicated that DADS inhibited NAT activity of A549 cells based on the following observations: 1) the decrease in NAT activity in A549 cancer cells; 2) the decrease in the percentage of NAT enzyme stained by anti-NAT antibody obtained from cells co-treated with DADS; 3) DADS inhibited gene expression of NAT mRNA in A549 cells. Based on those observations, DADS may affect NAT gene expression (NAT mRNA), which subsequently leads to a decrease in NAT enzymes before the decrease in the production of AAF (N-acetylation of AF) occurs.

Although it is well known that NAT plays an important role in the first step of AF metabolism [1], cytochrome P450 enzymes (CYPs) are also important factors for AF metabolism. Cytochrome P450-dependent formation of N-hydroxy-AAF is considered to play an important role in the initial rate-limiting step for the metabolism of AAF to mutagenic and potentially carcinogenic products [28]. Pathogenesis of lung diseases (lung cancer and chronic obstructive pulmonary disease) is tightly linked to exposure to environmental chemicals that require enzymatic activation to exert their deleterious effects on pulmonary cells. These activation reactions are mostly catalyzed by CYP enzymes. It is reported that after AF enters animal cells, AF is N-acetylated by NAT and then metabolized by CYP enzymes to form reactive metabolites which bind to DNA; the resultant DNA-AF metabolite adducts lead to mutation and possibly to cancer development [29].

In the current study, we were unable to positively conclude that DADS effect on NAT protein levels and gene expression leads to the decrease in lung cancer development. The reason

for this is that other enzymes, such as CYPs are involved in metabolizm of carcinogens in human lung cancer A549 cells. Some researchers have found that many CYPS are expressed in human lung tissue, such as CYP1A1 (in smokers), CYP1B1, CYP2B6, CYP2E1, CYP2J2, and CYP3A5 [30]. It is reported that qualitative reverse transcription/polymerase chain reaction (RT-PCR) showed the expression pattern of all known xenobiotic-metabolizing CYP genes in the human alveolar type II cell-derived A549 adenocarcinoma cell line. The expression of messenger RNAs (mRNAs) of CYPs 1A1, 1B1, 2B6, 2C, 2E1, 3A5, and 3A7 was detected in the A549 cells [31]. The A549 lung cell line is a valuable model for studying the mechanisms of induction of the pulmonary CYP system [31].

CYPs and NAT expression have been used as biomarkers of carcinogen-DNA adduct levels and human cancer susceptibility [32]. CYP1A1 has been shown to be particularly efficient at catalyzing the conversion of AAF to 7-OH-AAF [33,34]. Einisto et al demonstrated that increased levels of NAT activity from bacteria are associated with increased sensitivity to the mutagenic effects of many arylamines [32]; other investigators have also indicated that the attenuation of NAT activity in liver is associated with breast and bladder cancer occurrence [1,5]. However, an individual's susceptibility to the carcinogenic effects of aromatic amine carcinogens may depend on the route of excretion of metabolites among the tissues and organs, the relative rates of N-acetylation and N-hydroxylation in the liver of individuals, and possibly the rates of glucuronide hydrolysis and NAT mediated activation in the target organs [35].

We selected 50 μmol/L of DADS to examine the gene expression of NAT on A549 cells because that volume inhibited over 50% of NAT activity after A549 cells were co-treated with this dose (Fig. 1). However the exact amount of DADS in humans after exposure to DADS and the amount that finally reaches the lung in individuals may be different because individual differences (genetic variants) in enzymes exist. The PCR experiment indicated that DADS decreased NAT1 mRNA gene expression which is consistent with the results from cDNA microarray. Therefore, DADS decreased NAT gene expression which subsequently led to the decrease in the amount of NAT enzyme and finally to the decrease of the N-acetylation of AF.

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二丙烯基二硫化物抑制人類肺癌細胞A549的N-乙醯轉移酵素之活性和基因表現

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目的 N-乙醯轉移酵素(NAT)之活性決定個體乙醯轉移的快或慢，被認爲影響個體因環境暴露而產生癌症。二丙烯基二硫化物爲大蒜主要成分之一，以往被報告含有抗腫瘤的活性。本研究檢測二丙烯基二硫化物是否能夠抑制人類肺癌細胞株N-乙醯轉移酵素(NAT)酵素活性及其基因表現(NAT mRNA)。

方法 利用高壓層析儀檢測二胺螢素(AF)乙醯化和未乙醯化的量來決定N-乙醯轉移酵素(NAT)的活性。NAT 蛋白量則利用流式細胞計數儀分析。NAT mRNA 基因的表現則利用PCR 和cDNA 晶片檢測。

結果 二丙烯基二硫化物減少人類肺癌細胞對二胺螢素(AF)的乙醯化，而且有劑量依存的關係。由流式細胞計數儀分析顯示，二丙烯基二硫化物降低NAT 酵素的量。由PCR 和cDNA 晶片的實驗顯示，二丙烯基二硫化物可抑制NAT 之基因表現。

結論 本研究結果推論二丙烯基二硫化物抑制人類肺癌細胞NAT 之基因表現，導致NAT酵素量減少，因而造成AF的N-乙醯化量減少。（中台灣醫誌 **2004;9:161-8**）

關鍵詞

cDNA 晶片，二丙烯基二硫化物，人類肺癌細胞株，N-乙醯轉移酵素，二胺螢素(AF)

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