

行政院國家科學委員會專題研究計畫 期中進度報告

尼古丁暴露與台灣婦女乳癌致癌之分子機制研究(1/3)

計畫類別：個別型計畫

計畫編號：NSC94-2314-B-038-022-

執行期間：94 年 08 月 01 日至 95 年 07 月 31 日

執行單位：臺北醫學大學醫學系

計畫主持人：吳志雄

共同主持人：周燕輝

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一、中文摘要

1982年起迄今二十餘年來，國人罹患乳癌的發生率急速增加了近50-100%(Huang et al., 2001)且婦女罹患乳癌的年紀明顯較西方國家婦女要早很多(Cheng et al., 2000; Chie et al., 1995)。國人的研究發現女性乳癌發生與環境因子有密切相關如環境中少量藥物殘存(Chang et al., 2003)，荷爾蒙(避孕藥)或藥物濫用(Chie et al., 1998)，婦女抽菸或接觸二手煙等(Wu et al., 2002)。然而國人乳癌的發生與上述因子是否有關，並未獲得直接證實。本實驗室先期結果證實1. 乳癌病人癌組織中尼古丁受體($\alpha 9$ -nAChR) mRNA表現高於正常組織。2. 這些病人有接觸或抽菸習性。3. 乳癌細胞內 $\alpha 9$ -nAChR基因受到女性荷爾蒙(estrogen, E2)調控。

關鍵詞： 尼古丁， 乳癌， 抽菸。

Abstract

Nicotine [3-(1-methyl-2-pyrrolidinyl)-pyridine], a major alkaloid in tobacco, has been implicated as playing a role in carcinogenesis. Our previous study showed that cigarette smoking promoted inflammation-associated lung adeno-carcinoma formation in vivo and in vitro, and MAPK plays an important role in this process. In the present study, we aimed to investigate whether NIC and its specific nicotinic acetylcholine receptor (nAChRs)

could stimulate breast cancer cell proliferation and tumour growth and the possible mechanisms involved. We first demonstrated that the $\alpha 5$ and $\alpha 9$ nAChRs were detected in both the MCF-7 and MDA-MB-231 breast cancer cell lines. We further study the expression of the nAChR mRNA levels in breast tumour tissues collected from over-hundred cases of breast cancer patients in Taiwan and found that the $\alpha 9$ subunit of the nAChR was more significant in tumour tissue than in normal tissue. Western blotting analysis demonstrated that the Akt-regulated proteins were the major signalling pathway that involved in breast cancer cell proliferation stimulated by NIC treatment. Constructively express $\alpha 9$ -nAChR knock down breast cancer cell line caused a similar result as treatment with LY294002, a Akt/protein kinase B (PKB) inhibitor, it was not only blocked PKB down stream signal but also reduced the tumour formation and growth in vitro-soft agar assay and growth curve and in vivo-mice tumour formation assay. Another important carcinogenic factor was found to be Estrogen (E2) which directly bound $\alpha 9$ -nAChR promoter binding site at -44 bp and produced more mRNA expression in breast can cell line. Thus, this study provides evidence for a novel signalling route coupling the stimulation of $\alpha 9$ nAChR to the activation of PKB dependent manner, which implied that the $\alpha 9$ -nAChR may play some important role

involved in NIC-mediated breast tumour carcinogenesis.

Key words: NIC Breast cancer, nicotinic receptor,.

二、緣由與目的

The use of tobacco is so widespread and the health hazards deriving from it are so substantial that it has been referred to as the “global tobacco epidemic” (Bartecchi, 1995). Chronic bronchitis, emphysema, and lung cancer occur frequently in tobacco smokers. Nic, although addictive and likely responsible for the substance dependence resulting from tobacco use (Bock and Marsh, 1990), is considered to be one of the less dangerous components of tobacco smoke (Bartecchi et al., 1995; The Harvard Mental Health Letter, 1997). It has been suggested that the amount of Nic to which one is exposed as a result of tobacco smoking may not pose a serious health risk (The Harvard Mental Health Letter, 1997). Low tar cigarettes have been considered an acceptable solution to satisfy the smoker’s craving for Nic. Devices for aerosol delivery of Nic without tar-related carcinogens are actively developed and tested as safe alternatives to tobacco smoking. Nic is highly soluble in water, and its concentration in the saliva of tobacco smokers can be very high (an average of 8 mM during “smoking days”) (Lindell et al., 1993). Comparable concentrations are likely present on the bronchial and lung surface. Nic binds to and activates the nicotinic receptors for ACh. These are a family of proteins formed by five homologous or identical subunits, arranged symmetrically around a central ion channel (Conti-Fine et al., 1994; Galzi and Changeux, 1995). Different AChR isotypes exist in muscle and neurons. Muscle AChRs are composed of four different types of subunit (a, b, g or e, and d) (Conti-Fine et al., 1994; Galzi and Changeux, 1995). Neuronal AChRs may include only two types of subunits (α and β) or five copies of the same a subunit (Conti-Fine et al., 1994; Galzi and Changeux, 1995). Neurons

express at least eight different a subunits ($\alpha 1$ – $\alpha 7$, $\alpha 9$ – $\alpha 10$) and three b subunits ($\beta 2$ – $\beta 4$) (Conti-Fine et al., 1994; Galzi and Changeux, 1995). A large variety of neuronal AChRs results from the combinatorial association of different α and β subunits (Conti-Fine et al., 1994; Galzi and Changeux, 1995).

Human breast cancer cell line and breast tissue express nAChRs sensitive to Nic, similar to those expressed by ganglionic neurons, that compose the $\alpha 3$ subunit (Grando et al., 1995). We found at least three or nAChRs ($\alpha 5$, $\alpha 9$ and $\alpha 10$) exist in breast tissue for both normal or tumor in Taiwan and at least two nAChRs ($\alpha 9$ and $\alpha 10$) in breast cancer cell lines. Breast cell nAChRs $\alpha 9$ is likely to activate PI3K/AKT(PKB) pathway by NIC stimulation causing increasing cell growth curve. They seem to regulate tumour formation and malignancy because their block by nAChR-specific $\alpha 9$ siRNA and LY 294002, PI3K/AKT inhibitor, causes cancer cell decreasing anchorage independent ability and tumour growth in both culture cell and nude mice. Because of those findings and because of Luciferase reporter assay shows both NIC and estrogen can promote nAChRs $\alpha 9$ mRNA expression, we believe that nAChRs $\alpha 9$ is not only functions a calcium channel but also perform as a NIC receptor mediate signal transduction and may acts like a growth factor receptor.

三、研究報告內容

Cell culture

Human breast-carcinoma MCF-7 ,MDA MB 231 and Human normal MCF-10A cells were provided by Dr. Lin at Institute of Biochemistry/College of Medicine (NTU, Taiwan) were cultured in 75 cm² tissue culture flasks and maintained in MEM or DMEM/F12, supplemented with 10% (v/v) FBS , 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated in a 37°C and 5.0%

CO₂ atmosphere incubator. Cells were treated at a medium density. For NIC treatment, the NIC solution in sterile water was added to a final concentration of 10 μ M, and the cells were incubated for up to 72 h prior to RNA extraction. Samples used in this study were obtained from three independent culture replicates for each experimental group (i.e., control and NIC culture). Human breast tissue was obtained at Taipei Medical University Hospital. These samples were measured for nAChR subtypes mRNA expression by using rt-PCR and Quantitative real-time PCR.

Protein isolation

For cell cultures, the MCF-7 and MDAMB231 cells were seeded onto 10mm² dishes and grown in media with various pharmacologic treatments. Cell extracts were prepared as previously described (Lee et al., 1998). Cells were washed once with cold 1X PBS and resuspended in golden lysis buffer. and rocked at 4 °C for 30 min. After centrifugation (14,000 rpm) at 4 °C for 30minutes, the supernatant was collected. Protein (50 mg) from each sample was resolved on 12% SDS-polyacrylamide gel electrophoresis, transferred and analyzed by Western blotting analysis.

Immunoblot analysis

Antibodies were purchased from the following vendors: anti-phosphorylated and total ERK1/ ERK2, anti-estrogen receptor total, anti-AKT total, anti-cyclin D1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-GAPDH, anti-phosphorylated AKT (S474, T308), anti-phosphorylated p85, anti phosphorylated p110 antibodies from BD Transduction Laboratories (Lexington, KY). Immuno detection was carried by probing with proper dilutions of specific primary antibodies at room temperature for 2 h. The secondary antibodies, alkaline phosphatase-coupled anti- mouse or anti-rabbit antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) , were incubated at room temperature for 1 h at a concentration of 1:5000 dilutions, respectively. The specific protein complexes

were identified by incubating with the colorigenic substrates (nitro blue tetrazolium, NBT; and 5-bromo-4-chloro-3-indolyl-phosphate, BCIP; KPL, Inc., Gaithersburg, Maryland, USA). In each experiment, proteins were also probed with anti-GAPDH or each total-form of antibodies for protein loading control.

RT-PCR Assay

Total RNA was extracted from human cell line or tissue for using the phenol chloroform extraction procedure (TRIzol Reagent, Invitrogen Life technologies). The quantity and structural integrity of RNA samples were confirmed by spectrometer of the 260/280 nm ratio. Only samples that exhibited a 260/280 nm ratio ~1.8 were used in the experiments. two microgram of dried, 70% alcohol washed RNA was reverse-transcribed in 20 μ L of RT-PCR mix contained 25 mM dNTPs, 0.5 μ g Oligo-dt, 10 U MMLV and 1X MMLV buffer at 37°C for an hours. The PCR was carried out in a final volume of 25 μ L included 1 μ L single-strand cDNA product, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 2.5 U ProTaq DNA polymerase (all the rt-PCR reagent were purchased from ProTech Professional Technical Services,Inc), and 10 pM each of both the sense and the antisense primers (MD Bio Inc. Taipei Taiwan). For each experiment the housekeeping gene GAPDH was amplified with 30 cycles to normalize the cDNA content of the samples. Equal cDNA amounts were subsequently used for the amplification of specific genes. The amplification was performed at 94° C (30 seconds), 60 °C(30 seconds), and 72° C (30 seconds) for 35 cycles. The specific primers for human and rodent nAChR subunits α 1- α 7, α 9- α 10 and β 2- β 4 are shown in Tables 1. The amplicons were analyzed on a 1.5% agarose gel I (Amresco, Inc, Solon, Ohio, USA) stained with ethidium bromide. Pictures of the bands were taken using INFINITY- a digital imaging system (Vilber Lourmat, France) and band intensities were determined by software of PhotoCapt

Version 11.01.

Assay of Binding of 3H-Labeled NIC.

We verified the presence of nAChRs using the binding of 3H to attached of cultured MCF-7, obtained by mild trypsinization of confluent cell cultures. We used 2×10^6 cells/well and set up the samples at least in triplicate. We determined the total binding by incubating the cells with 1 to 15 nM 3H NIC (in one experiment, 7 nM) for up to 24 hrs. We chose this range of concentrations because pilot experiments that employed increasing concentrations of 3H NIC indicated that, in our experimental conditions, 1 to 15 nM 3H NIC allowed to reach binding equilibrium during the incubation time we used. In each experiment, we determined the nonspecific binding by preincubating aliquots of cells (in triplicate or more) with $10 \mu\text{M}$ unlabeled NIC for 30 minutes at 37°C . We then washed them three times by 1X PBS and collected for liquid scintillation counting.

Results:

To investigate the cellular regulatory mechanisms of apoptosis induced by NIC, human MDAMB231 cells were selected as a research model. As shown in Fig. 1, the viability of MDAMB231 cells was dose-dependently decreased at 24 hr after exposure to various concentrations of NIC (0-30 μ M), but was not affected by DMSO (0.05%, v/v) treatment. Figs. 1A and C showed that the human MDAMB231 cell was the most susceptible to NIC-induced cytotoxic effects as compared to human untransformed keratinocytes and colon cancer (COLO 205) cells.

NIC-induced apoptosis in human MDAMB231 cells

DNA fragmentation was observed in the MDAMB231 cells treated with 1 μ M NIC for 24 h (Fig. 2A, left), whereas a concentration higher than 90 μ M NIC was required for induction of DNA laddering fragmentation in the COLO205 cells (Fig. 2A, right). In consistent to Fig. 2A, a significant sub-G1 peak determined by flow cytometric analysis was observed in the MDAMB231 cells after NIC treatment for 24 hr (Fig. 2B, left). In the same conditions, the COLO 205 cells were arrested at the G0/G1 phase (Fig. 2C, left).

NIC-induced the occurrence of apoptosis in MDAMB231 cells was not through differentiation signaling pathways

To further confirm whether NIC-induced apoptosis was through differentiation processes as described previously (Martin et al., 1990; Olins et al., 2000), three recognized markers including the CD11b, CD33, and morphological changes were assessed (Stabellini et al., 2004). As shown in Fig. 3A, characteristic segmented nuclei and morphological features characteristic of blastic leukemic cell like was observed in the RA (1 μ M, 48 hrs)-treated MDAMB231 cells (Fig. 3A, middle, arrow). However, apoptotic but not differentiated cell like morphology was observed in NIC (1 μ M, 48 h)-treated MDAMB231 cells (Fig. 3A, right, arrow). Consistently, RA treatment resulted in an increase in the percentage of cells

expressing CD11b (Fig. 3B), and a time-dependent decrease in the percentage of CD33 expression (Fig. 3C).

Flow cytometric analysis of DNA content revealed that NIC (1 μ M) treatment resulted in a well-characterized and time-dependent increase in the percentage of apoptotic cells as early as after 24 h treatment (Fig. 3D). In contrast, as shown in Fig. 3D, apoptotic cells were not detected in the MDAMB231 cells until 3 days after RA (1 μ M) treatment.

NIC-induced MDAMB231 cells apoptosis was not through protein synthesis and CD95 receptors signal pathways.

Preincubation of MDAMB231 with cycloheximide (CHX, 1 μ g/ml, 1 h), a protein synthesis inhibitor (Maiani et al., 2004; Mezzanzanica et al., 2004), had no influence on NIC-mediated cell death (data not shown), indicating that protein synthesis was not pre-requested for NIC-induced apoptosis. We then examine whether a potential signaling of NIC-induced apoptosis in the MDAMB231 cells was via the cell surface CD95/Fas death receptor. Preincubation of the MDAMB231 cells with ZB4 (1 μ g/ml), a neutralizing anti-CD95 antibody (Woo et al., 2004), showed a significant reduction in apoptosis induced by soluble CD95/FasL (100 ng/ml) when compared with the cells treated with CD95/FasL only (Fig. 4A, lanes 5 and 6). In contrast, ZB4 was unable to reduce NIC (1 μ M, 24 hr)-triggered apoptosis (Fig. 4A, lane 4).

NIC treatment caused the changes of mitochondria membrane permeability in HL 60 cells

Since the CD95 death receptor seems to be not required for NIC-induced apoptosis in MDAMB231 cells (Fig. 4A), we then examined whether cytochrome c release from mitochondria into the cytosol and dissipation of the electrochemical gradient ($\Delta\Psi_m$) was involved in the NIC-mediated apoptosis. A real-time plate reader assay showed that $\Delta\Psi_m$ stayed relatively stable in untreated MDAMB231 cells, while it was rapidly (within 6 hrs) dissipated by 1 μ M NIC treatment (Fig. 4B). The $\Delta\Psi_m$ was

rapidly (within 2 hrs) dissipated by the uncoupled CCCP or by the K^+ ionophore Val and served as a positive control (Fig. 4B). As shown in the Figs. 4B and C, NIC (1 μ M) increased outer (cytochrome c release) and inner (loss of $\Delta\Psi_m$) mitochondria membrane permeability. The release of cytochrome c (Fig. 4C) kinetically paralleled a decreased of mitochondria membrane potential (Fig. 4B). These observations suggest that NIC-induced apoptosis may be initiated with early alterations in mitochondrial membrane stability.

Bcl-2 protein plays an important role in protection of NIC-induced apoptosis in HL 60 cells

We further investigated whether the observed dysfunction of mitochondria is responsible for the NIC-triggered apoptosis in MDAMB231 cells. We found that the Bcl-2 protein level was significantly decreased in the MDAMB231 cells at 6 h after treatment with 10 μ M NIC (Fig. 5A). To examine whether cytochrome c release was biologically functioning in initiating apoptosome assembly, immunoprecipitation was performed with cytosolic preparation from NIC-treated cells by using antibody specifically against the cytochrome c. As shown in Fig. 5A, a significant increase of Apaf-1 protein level in cytochrome c co-precipitates was detected from MDAMB231 cells treated with NIC for 12 h.

We therefore treated the Bcl-2 over-expressed MDAMB231 cells with NIC (0.1-30 μ M) for 24 h (Fig. 5B). In response to lower dose (0.1-1 μ M) of NIC, the MDAMB231/Bcl-2 cells, but not control (MDAMB231/PcDNA3) cells, were prevented from the occurrence of apoptosis (Fig. 5B), suggesting that down-regulation of Bcl-2 protein might be involved in the NIC-induced apoptosis through a mitochondria-dependent pathway. However, the apoptosis was not completely prevented in the MDAMB231/Bcl-2 cells treated with higher dose NIC (> 10 μ M) (Fig. 5B). As shown in the Fig. 5C, over-expression of Bcl-2 protein completely inhibited the

NIC-induced release of cytochrome c from mitochondria into cytosol in the MDAMB231/Bcl-2 cells (Fig. 5C, lanes 2 and 3). However, the results from the time-dependent experiments revealed that higher dose (10 μ M) NIC-induced apoptosis could not be completely prevented in the MDAMB231/Bcl-2 cells (Fig. 5D). These findings suggest that the results from cytochrome c release assay do not always correlate with the results from Annexin V staining. Additional signaling proteins other than Bcl-2 might be involved in the NIC-induced the occurrence of apoptosis in MDAMB231 cells.

NIC-induced apoptosis in MDAMB231 cells is through activation of caspases-3 and -9, but not caspase-8.

The MDAMB231 cells were treated with various concentrations of NIC (0.1-30 μ M) for 24 h. NIC at a lower dose (1 μ M) caused activation of the caspase-3 and degradation of the poly-ADP-ribose polymerase, the substrate for caspase-3 (Fig. 6A). To further elucidate the apoptotic pathways involved in the activation of caspase-3, we examined the changes of the protein levels of caspases 8 and 9 in the NIC-treated MDAMB231 cells. Treatment of MDAMB231 cells with NIC (> 1 μ M) activated caspase-9, but not caspase 8, evidenced by degradation of the procaspases 9 as well as the appearance of its cleavage product (Fig. 6A). To confirm that the absence of caspase-8 activation was not due to technical problem, TNF α (20 μ M)-treated MDAMB231 cells showing the cleavage of procaspase-8 as well as cleavage of its substrate, Bid protein, was served as a positive control (Fig. 6B). Caspase activity assays showed that treatment of HL 60 cells with high dose NIC (30 μ M) significantly increased caspases-3 (7.8 fold) and -9 (5.6 fold) activity as early as 12 hr after drug treatment as compared with DMSO-treated group, while the caspase 8 activity was not changed significantly even at a long-term (24 h) NIC treatment (Fig. 6C). To further confirm these findings, the MDAMB231 cells were pre-incubated for 4 h with or without the caspase-8-specific inhibitor

z-IETD-fmk, caspase-9-specific inhibitor z-LEHD-fmk, or the broad range inhibitor of caspases z-VAD-fmk, followed by NIC (30 μ M) treatment for 24 h. The percentages of apoptotic cells were analyzed by flow cytometric assay. As shown in Figure 7, both the caspase-9-specific inhibitor (z-LEHD-fmk) and the caspase general inhibitor (zVAD-fmk) reduced the NIC-induced apoptosis to a same extent. In the absence of caspase inhibitors, NIC caused a 59.6% apoptotic cell death. In the presence of z-LEHD-fmk (60 μ M) and zVAD-fmk (50 μ M), however, the percentage of NIC-induced apoptotic cell death was decreased to 6.5 and 8.3%, respectively. In contrast, the caspase-8 inhibitor (z-IETD-fmk) at a concentration of 60 μ M had no effect on the NIC-induced apoptosis (62.1%). The caspase-8 inhibitor at a concentration as high as 100 μ M still did not cause any decrease in NIC-induced apoptosis (data not shown). The TNF- α - and CD95/FasL-induced apoptosis was completely suppressed by caspase-8 inhibitor, suggesting that z-IETD-fmk at a concentration of 60 μ M is sufficient to inhibit caspase-8 activity. The specificity of these inhibitors was demonstrated by showing that vehicle (DMSO) treatment had no effect on NIC-stimulated apoptosis. Taken together, our results suggest that NIC-induced apoptosis is dependent on caspase-9 activation.

Discussion:

The ability of chemotherapeutic agents to initiate apoptosis plays an important determinant of their therapeutic response. However, significant toxicity at high doses has precluded the use of chemotherapeutic agents as a monotherapy for cancers. Combination therapy is one potential method to help in reducing a compound with undesirable toxic effects but still maintain or enhance its anti-tumor efficacy. Recently, we have demonstrated that griseofulvin, an oral antifungal agent, potentiates the anti-cancer activities of nocodazole (ND) (Ho et al., 2001). Moreover, we showed an enhancement of NIC on the ND-induced colon cancer cells apoptosis (Lee et al., 2003). NIC has been used as an orally active broad-spectrum antifungal drug, especially active in patients with histoplasmosis or nonmeningeal cryptococcosis (Caceres-Rios et al., 2000; Rademaker and Havill, 1998). A previous study has demonstrated that approximately 70% of NIC is absorbed after an oral dose (250 mg) (Jensen, 1989) and the maximum plasma concentrations of 0.5-1.5 $\mu\text{g/mL}$ are reached within 2 h (Humbert et al., 1995; Kovarik et al., 1992; Kovarik et al., 1995). Another report in a human study showed that the plasma level of NIC after daily oral receiving of 250 mg NIC for 4 weeks was $1.7 \pm 0.77 \mu\text{g/mL}$ (5.83 μM) (Kovarik et al., 1995). Here, we showed that administration of NIC at a concentration as low as 1 μM for 24h induced significant apoptosis in the HL 60 cells (Figs. 3D, 4A, and 4C). We further demonstrated that the NIC-induced the occurrence of apoptosis in the MDAMB231 cells was not mediated through differentiation process (Fig. 3A). Such results implied that administration of lower dose (1 μM) NIC could reach the therapeutic concentrations in plasma. Importantly, cytotoxicity analysis showed that NIC at the doses (0.01-30 M) used in our *in vitro* studies was not cytotoxic for the cultured untransformed human keratinocyte. Moreover, the dose (50 mg/Kg body weight) used in our previous *in vivo* study performed in the nude mice model was not cytotoxic

for the vital organs (Lee et al., 2003).

The caspase 8/ FADD (extrinsic) and mitochondrial (intrinsic) pathway are the two major signal pathways regulating apoptosis process. Both of the apoptosis routes were activated during erythroid cell differentiation (Testa, 2004) and cancer chemotherapy (Hajra and Liu, 2004). Recent studies have demonstrated that both CD95- and B cell receptor (BCR)-mediated apoptosis depend on Bax activation and cytochrome C release, although the timing and caspase-dependence of mitochondrial membrane depolarization differed considerably after CD95- or BCR-triggering (Mackus et al., 2002). However, some other death receptors in the TNF receptor family (such as TNFR1, DR3/Apo3, DR4/DR5, etc.) have been reported to be a mediator in response to cancer chemotherapy. For example, it has been shown that clinically applied anticancer drug, cisplatin, induced apoptosis of solid tumor cells through the CD95 and DR5-dependent pathways (Han et al., 2003; Lacour et al., 2004). Another study revealed that camptothecin or etoposide (VP-16) in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) substantially accelerated kinetics of apoptosis in human leukemia (HL 60) cells. The authors suggested that DR4 aggregation mediated by camptothecin or VP-16 could represent as an important mediator that accelerates TRAIL-induced apoptosis (Bergeron et al., 2004). In contrast, tumors resistant to cytotoxic drugs may occur through altered expression of death receptors DR4 and DR5 (Zhang and Fang, 2005), resistance to TRAIL-mediated apoptosis (Zhang and Fang, 2005), or sequestration of Fas/caspase-8 signaling pathways (Barnhart et al., 2005; Kim et al., 2001). In this study, although only one death receptor CD95/Fas has been examined to explore the eventual effect of NIC on the extrinsic (non mitochondrial) pathway of apoptosis. These findings suggested that NIC might be a useful salvage agent in the management of chemotherapy resistant cancer.

We have previously demonstrated that NIC

induced cell growth arrest at the G0/G1 phase through a p53-dependent signaling pathway (Ho et al., 2004; Lee et al., 2003). The NIC-induced apoptosis, however, was through a p53-independent signaling pathway. Our data suggest that intracellular regulatory proteins other than p53 may be involved in NIC-induced apoptosis. Accordingly, we investigated the p53-independent mechanisms in NIC-induced apoptosis. Our results showed that preincubation of the MDAMB231 (p53-null) cells with CHX (1 µg/ml, 1 h) had no influence on NIC-mediated cell death (data not shown), suggesting that *de novo* protein synthesis is not a prerequisite for NIC-induced apoptosis.

In turn, decrease of Bcl-2 protein expression may result in excess Bax homodimers, which will be translocated to the mitochondrial outer membrane (Fig. 5A), and then led to leakage of cytochrome c through its pore-forming activity (Wei et al., 2001). Our study provide evidences showed that Bcl-2 protein significantly prevented cytochrome c release, but cannot completely prevent the NIC-treated MDAMB231/Bcl2 cells undergoing apoptosis (Figs. 5B and 5D), suggesting that additional apoptotic factors other than Bcl2/Bax family proteins are involved in the NIC-induced the occurrence of apoptosis in MDAMB231.

In this study, our results first demonstrated that NIC induced promyelocytic (HL 60) cell apoptosis via a signaling pathway independent of cell growth arrest. We further examined the sequence of the molecular events involved in the activation of mitochondria-mediated signaling pathways during the process of NIC-induced apoptosis. Our results indicate that the leakage of cytochrome c was preceded by the translocation of Bax to mitochondria. Our data suggest that translocation of Bax to mitochondria, which lead to release of cytochrome c, is dependent on amplification of the specific caspase cascade and entry of the cell into the execution phase of apoptosis. This hypothesis is supported by our results showing that translocation of the Bax to mitochondria, release of cytochrome c into

cytosol, and the occurrence of cell apoptosis were clearly inhibited by the caspase-9-specific inhibitor (Z-LEHD-fmk) (Fig. 7). The caspase-9 might therefore play an important role in mitochondria signaling pathways for NIC-induced apoptosis. Similar results described by previous report showed that activation of caspase-9 usually occurs downstream of cytochrome c release from mitochondria (Saleh et al., 1999). Assembly of the apoptosome complex might represent the initiating step for the NIC-mediated caspase cascade activation (Saelens et al., 2004). However, whether this apoptosome directly causes the release of the different mitochondrial apoptogenic factors simultaneously is currently unknown (Martin et al., 2004). Further studies are required to understand how the compositions of the apoptosome active Bax translocation to the mitochondria and trigger the mitochondrial release of cytochrome c.

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Figure Legends:

Figure 1.

Cytotoxic effects of NIC in human normal and cancer cells. (A) Human normal keratinocyte (CCD 922SK), (B) MDAMB231, or (C) COLO 205 cells were treated with various concentrations of NIC (0.01 to 30 μ M). The cell viability was determined by trypan blue exclusion assay at the indicated time points after exposure to NIC. Results are the means $\pm$ S.E.M. of three independent experiments.

Figure 2.

Effects of NIC on DNA fragmentation in human MDAMB231 and COLO 205 cancer cells. (A) Human MDAMB231 (left) and COLO 205 cells (right) were treated with various doses of NIC for 24 h and then assayed for DNA fragmentation. Effect of NIC on cell cycle in human MDAMB231 (B) and COLO 205 (C) cells determined by flow cytometric analysis. The cells were treated with various concentrations of NIC for 24 h. Percentage of cell population in the different phase of the cell cycle were determined using established CellFIT DNA analysis software. Three samples were analyzed in each group, and values represent the mean $\pm$ S.E.M.

Figure 3.

Effects of RA and NIC on the differentiation and apoptosis of HL-60 cells. (A) HL-60 cells were treated with RA (1 μ M, middle) or NIC (1 μ M, right) for 48 h. Cells were cytopun, and then stained by Wright–Giemsa method. Magnification: 500x; scale bar: 20 μ m. (B-C) HL-60 cell differentiation was determined at the indicated time points by monitoring of the CD11b and CD33 expression on the cell surface after exposure to NIC or RA (1 μ M). (D) For apoptosis assay, the cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometric analysis as described in “Materials and Methods”. *, $P < 0.05$ versus day 0.

Figure 4.

Involvement of the CD95 receptor in NIC-induced apoptosis. (A) The MDAMB231 cells were pre-incubated for 1 h in the presence of PBS, antagonistic

anti-Fas ZB4 mAb (10 μ g/mL) or agonistic CD95L (1 μ g/mL), supplemented with either vehicle (control) or NIC (1 μ M), and then incubated for an additional 24 h. The apoptotic cells were determined by annexin V staining and analyzed by flow cytometry. The number of the apoptotic cells was expressed as a percentage of total cells. The values are means $\pm$ S.E.M. of three independent experiments. (B) The top insets represent the microscopic observations of the immunofluorescent staining in MDAMB231 cells treated with NIC (1 μ M). Mitochondria membrane depolarization in NIC-treated HL-60 cells was measured by JC-1 staining. In the lower panel, HL-60 cells were treated with NIC (1 μ M), RA (1 μ M), Valinomycin (Val, 200 μ M), or CCCP (200 μ M) for the indicated time points. After drug treatment, the MDAMB231 cells were stained with JC-1 (1 μ g/ml). Results were expressed as a change in the ratio between red JC-1 fluorescence (Em 590 nm), and green JC-1 fluorescence (Em 535 nm) over time. Each point represents the mean $\pm$ S.E.M. from three experiments. (C) The MDAMB231 cells were treated with NIC (1 μ M) for the indicated time points. The protein levels of the cytochrome c released from mitochondria into cytosol and the percentage of apoptotic cells were then determined. Annexin V-stained cells were counted as apoptotic cells and expressed as a percentage of total cells. The values are means $\pm$ S.E.M. of three independent experiments. Top inset: the representative bands of cytochrome c detected by Western blot analysis.

Figure 5.

Effects of NIC on the expression levels of mitochondria Bcl-2, Bax, and the caspase-9-associated Apaf-1 proteins. (A) The MDAMB231 cells were treated with 10 μ M NIC at the indicated time points for 24 hr. Protein extracts from the cytosolic and mitochondrial fractions were isolated from the NIC-treated MDAMB231 cells followed by immunoblotting analysis. Expression of the Bcl-2 and the caspase-9-associated Apaf-1 protein were determined by Western blot analysis, and the β -actin expression was

served as a protein loading control. (B-D) Protection effect of Bcl-2 overexpression on the NIC-induced apoptosis in the MDAMB231 cells. (B) The inset shows a representative Western blot result of Bcl-2 protein in MDAMB231 cells transfected with Bcl-2 (pBcl-2) or control (pcDNA3) plasmid. The MDAMB231/Bcl-2 and MDAMB231/pcDNA3 were then treated with NIC in a dose-dependent (0.1-30 μ M) manner for 24 hr. Annexin V-stained cells were counted as apoptotic cells and expressed as a percentage of total cells. The values are means $\pm$ S.E.M. of three independent experiments. Significant results were compared to the control group by statistic analysis described in the “Materials and Methods” as indicated as * $P < 0.05$. (C) The Bcl-2 overexpressed (MDAMB231/Bcl-2) and the control (MDAMB231/pcDNA3) cells were treated with 10 μ M NIC for 24 h, and the protein levels of the cytochrome c in cytosolic and mitochondria fractions were determined by Western blot analysis. The β -actin protein level was determined and represented as a protein loading control. (D), *In vitro* study of the effect of Bcl-2 overexpression on NIC-induced apoptosis. Apoptotic cells were quantified by Annexin V-FITC/PI stain and analyzed by flow cytometry as described in the “Materials and Methods.” section. *Data points*, the mean $\pm$ S.E.M. of three independent experiments.

Figure 6.

Dose-dependent activation of caspases in NIC-induced apoptosis in MDAMB231 cells. (A) MDAMB231 cells were treated with NIC (0.1-30 μ M) for 24 h and the expression of caspase-associated proteins were detected by Western blot analysis. The expression of β -actin protein was examined and served as a loading control. (B) MDAMB231 cells were treated with 20 μ M TNF α for 48 h. The protein levels of the caspase and Bid were detected by Western blot analysis. (C) NIC-induced caspase activities in COLO 205 cells. Cells were treated with 30 μ M of NIC for various time periods. Caspase activities were measured as described in Materials and Methods. Data

represent means $\pm$ S.E.M. for three determinations.

Figure 7.

Effects of caspase activation on mitochondria-mediated apoptosis in the NIC-treated MDAMB231 cells. For the caspase inhibitors studies, the MDAMB231 cells were incubated with 50 μ M of inhibitors specific to caspase-8 (zIETD-fmk), caspase-9 (zLEHD-fmk), or the general inhibitor of caspases (zVAD-fmk) for 4 h as indicated at the top of each profile. The HL 60 cells treated with TNF α (20 μ M) and FasL (100 ng/ml) in the presence of zIETD-fmk were performed as a positive control. The cells were then exposed to NIC at a concentration of 30 μ M for an additional 24 h. The control cells were incubated with DMSO without NIC or caspase-specific inhibitors. The DNA contents were monitored by flow cytometry as described in the Materials and Methods section.