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The study of the oncogenic Ras-mediated cancer cell survival

조선대학교 대학원

의학과

차 만 진

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Ras 암 유전자에 의한 세포 생존연구

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조선대학교 대학원

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지도교수 유 호 진

이 논문을 의학석사학위신청 논문으로 제출함.

2010 년 8 월 일

조선대학교 대학원

의학과

차 만 진

차 만 진의 박사학위 논문을 인준함

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The study of the oncogenic Ras-mediated cancer cell survival

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ABSTRACT

Point mutations in the mammalian *ras* gene are oncogenic, and occur in approximately 30 % of human tumors. Although several studies concerning the role of oncogenic H-ras in the expression of multidrug resistance (MDR) in various cell lines, the role of ras in the regulation of MDR need to be elucidated. Therefore, we investigate the role of oncogenic H-ras in rat mdr1b expression of NIH3T3 cells. The constitutive expression of oncogenic H-ras (V12-ras) was found to down-regulate mdr1b promoter activity and mdr1b mRNA expression. The mdr1b promoter activity of V12-ras-NIH3T3 cells was 75 % less than pcDNA3-NIH3T3 cells and the level of mdr1b mRNA expression in pcDNA3-NIH3T3 cells were approximately 3-fold increased than that of V12-ras-NIH3T3 cells. In addition,

treatment of pcDNA3-NIH3T3 cells with 50, 100 and 250 nM of doxorubicin led to increase mdr1b-Luc activity by 150-, 200- and 400-fold compared with untreated cells, respectively. However, in V12-ras-NIH3T3 cells, the corresponding enhancements of mdr1b-Luc activities were only 100-, 120-, and 150-fold versus untreated cells. Moreover, there is a strong correlation between the mdr1b expression and a sensitive to doxorubicin toxicity. To examine the detailed mechanism of V12-ras-mediating down-regulation of mdr1b expression, antioxidant NAC and the NADPH oxidase inhibitor DPI were used. Pretreatment of cells with either NAC or DPI was found to significantly enhance oncogenic H-ras-mediated down-regulation of mdr1b expression and to markedly prevent doxorubicin-induced cell death. As extracellular signal-regulated kinase (ERK) is a known downstream effector of Ras and to be stimulated by ROS, we next investigated whether alterations in ERK signaling affect mdr1b expression. NAC and DPI treatment led to decrease the ERK activity, and the ERK inhibitors PD98059 or U0126, caused enhancement of the mdr1b-Luc activity of V12ras-NIH3T3 and reduced doxorubicin-induce apoptosis. Taken together, these results suggest that oncogenic H-ras expression could down-regulate the mdr1b expression through intracellular ROS production, and the activation of ERK induced by ROS, at least in part, involved in the down-regulation of mdr1b expression in NIH3T3 cells.

I. INTRODUCTION

The development of acquired resistance to anticancer drugs is considered a major obstacle to the curative use of these drugs. Multiple mechanisms have been implicated in the development of multidrug resistance (mdr), and P-glycoprotein (Pgp) encoded by multidrug resistance type 1 (*MDR1*) genes has been identified as a major mechanism in the development of resistance to anticancer drugs (1). MDR1 is involved in multidrug resistance in humans, and mdr1a and mdr1b confer multidrug resistance in rodents. It is generally believed that overexpressed P-glycoprotein decreases cellular drug accumulation because of the enhanced drug efflux of various structurally and functionally unrelated anticancer drugs, these include anthracycline (e.g. doxorubicin), vinca alkaloid and antibiotics (1). Therefore, the increased expression of the human *MDR1* gene is believed to be involved in treatment failure in some human tumors, and furthermore, it has been postulated that the *mdr1* gene is a marker of tumor progression and aggressiveness.

Ras plays a key role in cellular proliferation (2) and differentiation (3), and point mutation of the *ras* gene occur at high frequencies in mammalian cells, resulting in transformation and malignant progression to cancer (4). The relationship between oncogenic Ras expression and drug resistance in some human tumors has been investigated. Although several studies concerning the expression of MDR have been performed in oncogenic Ras transformed cells, the role of Ras in the expression of MDR is far from clear. Several studies have provided evidence indicating that active Ras causes the

expression of MDR1 and leads to anticancer drug resistance (5-7). However, others have found that Ras activation was not able to up-regulate the *MDR1* gene (8), and that the overexpression of oncogenic Ras led to the down-regulation of *MDR1* gene expression and a subsequent reduction in surface-localized Pgp (9).

ROS (reactive oxygen species) is a down-stream effector of Ras, and can influence a number of intracellular pathways. As intracellular second messengers, ROS also control a variety of Ras-mediated cellular effects (10). Recently, it has been suggested that Pgp expression may be regulated by a redox-sensitive signal pathway, because rat *mdr1b* gene promoter contains redox-sensitive transcriptional factors, such as, activated protein-1 (AP-1) or nuclear factor κB (NF-κB) (11). Moreover, intracellular ROS production is able to modulate rat *mdr1b* gene expression (12, 13). Therefore, we directly addressed the question as to whether oncogenic Ras effects mdr1b expression, and whether ROS, as a down stream effector of Ras, is involved in mdr1b expression. We report here that the stable expression of oncogenic H-ras significantly decreases mdr1b expression in NIH3T3 cells, and that the treatment of V12-ras expressing cells with either antioxidant NAC or the NADPH oxidase inhibitor DPI lead to an increase in mdr1b expression and the prevention of doxorubicin-induced apoptosis. Using ERK inhibitors PD98059 and U0126, we provided evidence that activation of ERK activity is, at least in part, involved in the ROS-mediating down-regulation of mdr1b expression in V12-ras-NIH3T3 cells.

II. MATERIALS AND METHODS

1. Cell Culture and Reagents

The NIH3T3 mouse embryo fibroblast lines were obtained from ATCC (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml (Life Technologies, Inc.). Cells were cultured at 37°C in a humidified chamber containing 5% CO_2. Doxorubicin, *N*-acetylcysteine (NAC), diphenylene iodonium (DPI) and propidium iodide were purchased from Sigma (St. Louis, Mo), anti-Ras polyclonal antibody from BD Transduction Laboratories (San Diego, CA), and PD98059, U0126 and SB203580 were obtained from Calbiochem (La Jolla, CA).

2. Plasmid Constructs and Oligonucleotides

Wild type *H-ras* cDNA was cloned by RT-PCR from human Jurkat cells. The dominant positive *H-ras* (V12ras) were subjected to site-directed mutation using the wild type *H-ras* cDNA template, according to the manufacturer's instruction (Stratagene, CA). After DNA sequence confirmation, the dominant positive form of *H-ras* cDNA was cloned into a pcDNA3 mammalian expression vector driven by a CMV promoter (Invitrogen, CA). pGL3-Luc plasmid was purchased from Promega (San Diego, CA), Gal4-ElK1 and pFR-Luc plasmids were purchased from Stratagene (La Jolla, CA), and Mdr1b-Luc plasmid was generously provided by M. Tien Kuo (14). The JNK antisense oligonucleotides used in this

study were synthesized at ISIS Pharmaceuticals, Inc. (Carlsbad, Calif.). The sequences of the oligonucleotides used were as follows: Control (ISIS 17552), TCAGTAATAGCCCCACATGG; JNK1 (ISIS 15347), CTCTGTAGGCCCGCTTGG; and JNK2 (ISIS 15354), GTCCGGGCCAG-GCCAAAGTC. All oligonucleotides were 2'-O-methoxyethyl chimers containing five 2'-O-methoxyethyl-phosphodiester residues flanking a 2'-deoxynucleotide-phosphorothioate region (15).

3. Transfection and Luciferase Activity Assay

NIH3T3 cells were transfected with either mdr1b-Luc, pGL3-Luc , Gal4-Elk1 or pFR-Luc and pRL-CMV using LipofectAMINE and LipofectAMINE PLUS solution, according to the manufacturer's instruction (Life Technologies, Inc). To select Ras expressing NIH3T3 cells, the cells were transfected either with pcDNA3-V12ras or empty pcDNA3 and incubated with complete medium containing 1.2 mg/ml of G418 for 5 weeks. Cell clones resistant to G418 were isolated and analyzed. Dual luciferase activity in the cell extracts was determined according to manufacture's instructions (Promega). Briefly, each assay mixture contained 20 μl cell lysate and 100 μl firefly luciferase measuring buffer (LAR II^{R}, Promega). Firefly luciferase activity was then measured using a luminometer. The reaction mixture was then added to 100ul of renilla luciferase measuring buffer (Stop & Glo^{R}, Promega). Renilla luciferase activity was used to normalize transfection efficiency and the relative luciferase activity was calculated as a percentage of the experimental luciferase value relative to the control levels, using untreated pGL3-Luc reporter plasmids. All transfections were performed in duplicate, and repeated at least three times.

4. Western blotting

The cell were washed with phosphate-buffered saline (PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added protease Inhibitor Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13,000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 20ug of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBS-T and then incubated with appropriate primary antibodies in TBS-T at 4℃ overnight. All ; We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 2 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea)

5. Apoptosis assay

Cells were collected 24 h after being treated, fixed in 70% ethanol, and stained with

propidium iodide (PI, 50 μg/ml) after RNA digestion. 10,000 PI-stained cells were analyzed for DNA content using a FACScan flow cytometer (Becton Dickinson, San Jose, CA)

6. Measurement of ROS

The ROS were measured using previously described method (Suzukawa *et al.*, 2000) with some modification. Briefly, Cells were plated at 1×10^5/plate in 60-mm dishes and treated for the indicated times. Cells were harvested with trypsin/EDTA, washed once in PBS, and resuspended in 5 μg of 2', 7'-dichlorodihydrofluorescein diacetate (DCFHDA) /ml in Hanks' balanced salt solution. Samples were incubated for 10 min at 37 °C and The DCF fluorescence intensity was measured by a fluorescence plate reader (Bio-Tek, FL600) (excitation wavelength, 485 nm; emission wavelength, 530 nm). The mouse embryo fibroblast NIH3T3 cells were purchased from American Type Culture Collection (ATCC number CRL-1658) and grown in DMEM medium supplemented with 10% fetal bovine serum, 100 units penicillin/mL, and 100 μg streptomycin/mL (Invitrogen, Carlsbad, CA). The cells were maintained in 5 % CO_2 -95% air at 37 ^oC in a humidified incubator.

7. Statistical Analysis

All experiments were repeated at least three times to ensure reproducibility. Data in all experiments are represented as mean ± S.E. Statistical comparisons were carried out using two-tailed Student's *t* test. *p* values <0.05 were considered to be statistically significant.

III. RESULTS

1. Expression of Oncogenic H-ras Inhibits Rat mdr1b Expression in NIH3T3 cells

To understand the relationship between oncogenic H-ras and modulation of mdr1b expression in NIH3T3 cells, cells were stably transfected either with dominant positive form of H-ras through the V12-ras-pcDNA3 plasmid construct under control of CMV promoter or with empty expression vector (pcDNA3). Following selection by G418 at the concentration of 400 μg/ml for 5 weeks, we isolated several clones and chose one V12-ras-NIH3T3, which expressed oncogenic H-ras, and chose one pcDNA3-NIH3T3, which was used as a control, for further study in this investigation. Western blot analysis demonstrated that V12-ras-NIH3T3 9 cells exerted significantly overexpression of oncogenic H-ras versus that of pcDNA3-NIH3T3 cells (figure 1). To investigate whether oncogenic H-ras might modulate mdr1b expression in NIH3T3 cells, V12-ras-NIH3T3 and pcDNA3-NIH3T3 cells were transiently transfected with either mdr1b-Luc reporter plasmid or pFR-Luc plasmid and pRL-Luc plasmid containing the renilla luciferase gene, and 24 h after transfection, luciferase activities were determined by luminometer. The renilla luciferase plasmid (pRL-Luc) was used to normalize transfection efficiency. As shown in figure 1b, oncogenic H-ras markedly inhibited mdr1b-Luc activities in NIH3T3 cells. Luciferase activity analysis revealed that V12-ras-NIH3T3 cells had 75% less mdr1b-Luc activity than pcDNA3-NIH3T3 cells, but V12-ras expression had no effect on pFR-Luc. This result indicate that V12-ras could down-regulate mdr1b expression in NIH3T3 cells. To confirm the inhibitory effect of V12-ras on the mdr1b-luc activity, V12-

ras-NIH3T3 cells were transiently transfected with dominant negative ras containing plasmid (N17-ras-pcDNA3), and 12 h after transfection, cells were then transfected with mdr1b-Luc reporter plasmid and pRL-CMV plasmid, after 24 h, luciferase activities were measured. As shown in figure 1b, The decrease of mdr1b-Luc activity by V12-ras can be increased by transient transfection with dominant negative N17-ras, suggesting that the decrease of mdr1b-Luc activity results specificity from the expression of V12-ras. To further verify the effect of oncogenic H-ras on the down-regulation of mdr1b expression in NIH3T3 cells, expression of mdr1b mRNA in pcDNA3-NIH3T3 and V12-ras-NIH3T3 cells was determined by RT-PCR analysis of total RNA. The intensities of the bands derived from the GAPDH internal control gave some idea of the relative amounts of starting materials for each sample used for PCR. Based on the intensity of the band, the expression level of mdr1b mRNA seemed to be very low in V12-ras-NIH3T3 cells compare to that seen in pcDNA3-NIH3T3 cells (data not shown). Densitometric analysis revealed that the revel of mdr1b mRNA expression in pcDNA3-NIN3T3 cells was approximately 3-fold increase that that of V12-ras-NIH3T3 cells.

We next investigated whether V12-ras expression would effect on the doxorubicin-induced mdr1b-Luc activity. For this experiment, pcDNA3-NIH3T3 and V12-ras-NIH3T3 cells were transiently transfected with mdr1b-Luc reporter plasmid and pRL-Luc plasmid, and then treated with different dose of doxorubicin for 24 h after the transfection and luciferase activity was determined. As shown in figure 2, in pcDNA3-NIH3T3 cells, treatment with 50, 100 and 250 nM of doxorubicin increased mdr1b-Luc activity by 150-, 200- and 400-fold compared with untreated cells, respectively. However, in V12-ras-NIH3T3 cells, the

corresponding enhancements of mdr1b-Luc activities were only 100-, 120-, and 150-fold versus untreated cells. Taken together, these results indicates that expression of V12-ras lead to down-regulation of basal and doxorubicin-induced mdr-1b expression in NIH3T3 cells

2. Intracellular ROS Production by the Expression of Oncogenic Ras Inhibits mdr1b expression

Previously studies have shown that constitutive expression of H-ras increased ROS production in several cell lines. For this reason, we measured intracellular ROS production using DCFHDA in our system. Figure 3 showed expression of V12-ras significantly increased intracellular ROS production, which could be blocked by transient transfection with dominant negative N17-ras. In addition, treatment with either a thiol reducing agent *N*-_Acetylcysteine (NAC) or NADPH oxidase inhibitor diphenylene iodonium (DPI) dramatically reduced intracellular ROS production by expression of V12-ras. These results indicate that the activation of NADPH oxidase activity by expression of V12-ras contributes to the intracellular ROS production.

To investigate the possibility that the enhancement of ROS production by oncogenic H-ras expression was involved in the down-regulation of mdr1b expression, we examined whether antioxidant increased mdr1b expression in V12-ras-NIH3T3 cells.

For this purpose, V12-ras-NIH3T3 cells were pretreated with wither NAC or DPI for 12 h, and cells were then cotransfected with either mdr1b-Luc reporter plasmid or pFR-Luc plasmid and pRL-Luc plasmid. After 24 h transfection, mdr1b promoter activity and

mdr1b mRNA expression were then measured by luminometer and RT-PCR, respectively. The data presented in figure 4 show that both NAC and DPI were able to increase the mdr1b-Luc activity in V12-ras-NIH3T3 cells compare with non-treated cells. But DPI and NAC had no effect on pFL-Luc. Furthermore, RT-PCR data analysis revealed that pretreatment of V12-ras-NIH3T3 cells with either NAC or DPI caused up-regulate mdr1b mRNA expression. We next investigated whether intracellular ROS effects doxorubicin-induced mdr1b promoter activity. Pretreatment of cells with either NAC or DPI for 12 h, and cells were then cotransfected with mdr1b-Luc and pRL-Luc plasmid. After which cells were incubated with 50, 100 or 250 nM of doxorubicin for an additional 24 h, and the luciferase activities were measured. As shown in figure 5, mdr1b-Luc activity in V12-ras-NIH3T3 cells was found to be significantly enhanced on increasing the dose of doxorubicin in the presence of DPI and NAC versus the untreated control. These results suggest that intracellular ROS production is required for the V12-ras-mediated down-regulation of mdr1b expression in NIH3T3 cells.

3. Involvement of ERK-Mediated Signaling Pathways in the Down-regulation of mdr1b Expression

Recent evidence has implicated that ROS stimulate MAPK activities including ERK, p38 and JNK, which is a key event in many cellular processes. Therefore, we investigated whether intracellular ROS production by V12-ras expression led to activation of ERK activity, which could be involved in the down-regulation of mdr1b expression. To test this possibility, pcDNA3-NIH3T3 or V12-ras-NIH3T3 cells were pretreated with either NAC

or DPI for 12 h and cells were then cotransfected with either Gal4-Elk1 or pFR-Luc and pRL-Luc. 24 h after transfection, the level of luciferase activity was measured. These results, as shown in figure 6A, demonstrated that V12-ras-NIH3T3 cells exhibited significantly increase ERK activity versus pcDNA3-NIH3T3 cells, and the inhibition of intracellular ROS generation using DPI and NAC led to a 50 % decrease in the Ras activation of Gal4-Elk1, but NAC and DPI had no effect on the pFR-Luc. As a control, PD98059 and U0126 were found to block V12-H-ras activation of ERK activity. We next investigated whether ERK activity contributes to Ras-mediated down-regulation of mdr1b expression. For this experiment, we used the commercially available MEK1/2-inhibitory compounds, PD98059 and U0126, which are highly selective in their inhibition of the ERK pathway. V12-ras-NIH3T3 cells were pretreated with either DMSO, PD98059, or U0126 for 30 min, and cells were then cotransfected with either Gal4-Elk1 or pFR-Luc and pRL-Luc. Fours hours after transfection, the medium was replaced with fresh medium in the presence or absence of 250 nM of doxorubicin. After 24 h, the level of luciferase activity was measured. Elk1 is a transcription factor that is activated in response to activation of mitogen-activated protein kinase (MAPK). The Gal4-Elk1 construct contains the Gal4 DNA binding domain fused to the carboxyl-terminal transactivation domain of Elk1. As shown in figure 6B, pretreatment of V12-ras-NIH3T3 cells with either PD98059 or U0126 increase basal level of mdr1b-Luc activity compare with that of untreated cells. Furthermore, blocking the ERK signaling pathway led to stimulation of doxorubicin-induced mdr1b-Luc activity versus untreated cells.

4. The effect of mdr1b expression on cellular response to doxorubicin.

If intracellular ROS production and activation of ERK play an importing role in ras-mediating down-regulation of mdr1b expression, then agents capable of inhibiting ROS production and blocking ERK activity, when combined with doxorubicin treatment, should inhibit cytotoxicity. To address this possibility, we compared the doxorubicin-induced apoptosis in V12-ras-NIH3T3 and pcDNA3-NIH3T3 cells with or without inhibitors. Cells were treated with different dose of doxorubicin, after which they were stained with propidium iodide and apoptosis was measured using FACScan flow cytometry. As indicated in figure 7A, doxorubicin caused apoptosis of NIH3T3 cells in a dose dependent manner, with a concentration of 60 μM doxorubicin resulting in approximately 30 % of apoptosis by 24 h of treatment. However, V12-ras expressing cells exhibited significant sensitive to doxorubicin. The apoptosis of cells was great than 80 % at the concentration of uM doxorubicin. To investigate the effect of ROS on the doxorubicin–induced apoptosis of V12-ras-NIH3T3 cells, cells were pretreated with either NAC or DPI for 12 h prior to addition of doxorubicin. Cells treated with uM doxorubicin alone exhibited approximately 80 % of apoptotic response, however, combination with either doxorubicin and NAC or DPI induced a 50 % reduction in the apoptotic response. We next investigated whether ERK activity involved in the doxorubicin-induced apoptosis, cells were pretreated with either PD98059 or U0126 for 30 min, and uM doxorubicin was then added. As shown in figure 7B, pretreatment of cells with ERK inhibitors induced a 30 % reduction in the apoptotic response versus untreated cells

IV. DISCUSSION

Ras oncogenes are mutated in a large proportion of human cancers, and Ras pathways are activated by a variety of other mechanisms in many other cancers (Bos, 1989; Clark and Der, 1995). Rational therapies that target Ras downstream signaling molecules essential for malignant cancer cell behavior, but less critical for normal cell function, would therefore have a potential impact (Downward, 2003). Because tumorigenesis contributes to most cancer deaths, and because therapies that target Ras and its downstream signaling pathways are under active development as anticancer agents, an understanding of the biological role of Ras in the tumor progression is clinically important. The mechanisms whereby Ras oncogenes maintain the transformed characteristics of human cancer cells are poorly understood and may differ from those required for tumor initiation.

Point mutation in the Ras gene at high frequency in mammalian cells resulting in transformation and malignant progression to cancer, with oncogenic Ras mutations occurring in approximately 30% of all human tumors (Bos, 1989). Because oncogenic Ras is known to participate in the development of carcinogenesis in many human cancers, understanding the molecular basis of oncogenic Ras-regulated mdr 1b expression could lead to strategies resulting in improved anticancer therapeutic benefits. In spite of several studies concerning the role of oncogenic Ras in the expression of MDR in cancer cells, there are conflicting results about the relation between oncogenic ras and mdr expression. The expression of oncogenic ras led to increase cisplatin resistance in NIH3T3 and the appearance of atypical MDR after H-ras transfection in lung epithelial cells have been

reported. In addition, transformation of rat liver epithelial cells with ras oncogene caused appearance of MDR. Moreover, the promoter of the mdr1 gene was shown to be a potential target for H-ras oncogene, which implies that the mdr1 gene could be activated during tumor progression associated with mutations in H-Ras. Thus, it is possible that oncogenic ras could up-regulate the mdr1b gene expression. However, others have suggested that oncogenic ras transformation had no effect on the mdr-1 gene expression and expression of H-ras led to down-regulation of mdr-1 gene expression in a human colon cancer cells and acute myeloid leukemia. In the present study, we report the results of a study to direct address question about the relationship between dominant active H-ras and mdr1b expression in NIH3T3 cells. To better study the potential role of oncogenic H-ras in the mdr1b expression, NIH3T3 cells were stably transfected either with dominant active H-ras expression plasmid, V12-ras-pcDNA3 or empty expression plasmid, pcDNA3. To determine expression of mdr1b, we used reporter assay for measuring mdr1b promoter activity and RT-PCR assay for measuring mdr1b mRNA expression. We demonstrated that expression of oncogenic ras led to down-regulation of mdr1b expression and transient transfection with dominant negative ras (N17-ras) prevented V12-ras-mediated decrease of mdr1b promoter activity. In addition, V12-ras-NIH3T3 cells exhibited decrease the doxorubicin-induced mdr1b-Luc activity and potentiate doxorubicin-induced apoptosis compared with that of pcDNA3-NIH3T3 cells. These results have provided evidence that expression of oncogenic H-ras involves in the down-regulation of mdr1b expression in NIH3T3 cells.

ROS are generally considered to toxic to cells (19). However, recent studies have

demonstrated that ROS play a role as second messengers to regulate mitogenic signal transduction in various cell types (18, 20). Ras-transformed NIH3T3 cells produced intracellular ROS in NIH3T3 cell (Irani *et al.*, 1997), human keratinocyte HaCaT cells (Yang *et al.*, 1999) and human lung WI-38VA-13 cells (Liu *et al.*, 2001). This ROS production is thought to be mediated by Ras-mediated activation of NADPH oxidase, which is a multi-compartment enzyme, localized to the cellular membrane in non-phagocytic cells. We confirmed that the expression of oncogenic H-ras enhanced ROS production, and this enhancement of ROS production was blocked by transient transfection of dominant negative N17-Rac1 as well as treatment with DPI, NADPH oxidase inhibitor.

The promoter of rat *mdr1b* gene contains several transcriptional factor binding sites, such as AP-1, NF-κB, NF-Y and p53 (21-23), and these factors exhibit redox-sensitivity (24, 25). Several reports have shown that mdr1b can be induced by intracellular ROS production (12, 13) and that the activation of NF-κB redox-sensitive transcriptional factors by intracellular ROS production may be involved in mdr1b induction (14). However, other studies have provided evidence that the elevation of intracellular ROS down-regulates Pgp expression (26) and that this down-regulation is mediated by the activation of the receptor tyrosine kinase signaling pathway (27). In the present study, however, we provide evidence that intracellular ROS is important for the down-regulation of mdr1b expression in V12-ras-NIH3T3 cells. Pretreatment V12-ras-NIH3T3 cells with NAC and DPI resulted in an effective increase of mdr1b promoter activity and mdr1b mRNA expression with or without doxorubicin, suggesting that the overexpression of Ras could down-regulate mdr1b expression via the enhancement of intracellular ROS. A number of stresses and

chemicals, such as, UV irradiation (28), heat stress (29), TNF-α (30) and benzopyrine (31) are able to induce the expression of Pgp. Most of these stress factors are known to stimulate the generation of large amount of intracellular ROS. However, the induction of mdr1b expression in response to H_2O_2 treatments at concentrations in excess of 1 mM (12, 27), raises concerns regarding the biologic relevance of the observed response, as such concentrations are highly toxic to cells, and cause apoptosis (12, 27). Thus, Pgp may have an important role in the prevention of apoptosis under severe oxidative stress, presumably by regulating the anti-apoptotic or apoptotic protein (32, 33). Low concentrations of H_2O_2 (< 10 μM) are effective at activating ERK (34) and regulating cell proliferation (35). Moreover, these concentrations are significantly lower than those necessary to induce apoptosis (36). Therefore, intracellular ROS may have differential effects on the regulation of the MDR genes, which is dependent on their intracellular concentrations.

Important questions remaining are how do ROS lead to down-regulation of mdr1b expression, what are the downstream effectors involved in mediating the mdr1b expression? Indeed, a large number of signaling pathways are regulated by ROS, however the signaling molecules targeted by ROS are far from clear. There is growing evidence, however, that intracellular ROS production is functionally associated with the regulation of gene expression and the activation of MAPK. Recent study suggests that JNK activation is important component of the cellular responses to several anticancer drugs and may play a role in the MDR phenotype. More recently, Osborn et al (1999) showed that phorbol ester led to induction of MDR1 via a PKC-dependent mechanism, but ERK, p38 or JNK had no effect on the MDR1 expression in K562 cells. However, in the present study we found that

the inhibition of ERK pathway, but not p38 and JNK, led to enhanced mdr1b promoter activity and inhibited doxorubicin-induced apoptosis in V12-ras-NIH3T3 cells. The activation of ERK pathway is known to have a role in the pro-survival signal against various toxic stimuli, however, recently, several studies have shown that an inhibition of ERK signaling causes increase sensitivity of ovarican cancer cell lines and induction of apoptosis in Hela cells after exposure to anticancer drugs such as cisplatin. Such differential effects may be reflex the variation of MDR induction ability of cancer cell lines in response to anticancer drugs. In our own system using NIH3T3 cells, we found that there is a strong correlation between the inhibition of ERK pathway and a resistance to doxorubicin toxicity, suggesting ERK appears to involved in the down-regulation of mdr1b expression. Raf/ERK/MEK cascade is a major down-stream signaling pathway of oncogenic Ras, thus there is the possibility that ERK activation induced by V12-ras expression acts directly to cause the down-regulation of mdr1b expression. However, the direct contribution of ERK to mdr1b inhibition by oncogenic Ras appears not to be significant, because blocking ROS generation significantly decreased mdr1b expression and prevented doxorubicin-induced apoptosis, and treatment of V12-ras-NIH3T3 cells with either NAC or DPI led to a decrease ERK activity. Interestingly, the effect of inhibition of ERK pathway on the mdr1b promoter activity and prevention of doxorubicin-induced apoptosis is not much as those of DPI and NAC treatment. Thus another down-stream effectors of ROS, which could contribute down-regulation of mdr1b expression, may exist in ras transformed cells. Cancer cells endogenously generate ROS, which are involved in signaling pathways maintaining cell proliferation. Intracellular ROS concentration of these

cancer cells may involve in the down-regulation of mdr-1 gene expression. Thus the evaluation of ROS concentration and ERK activity could be useful in predicting which cancers will respond to anticancer therapy.

In conclusion, our current findings provide strong evidence that the overexpression of oncogenic Ras in NIH3T3 cells lead to the down-regulation of mdr1b expression through intracellular ROS production, and activation of ERK induced by ROS participates, at least in part, in the down-regulation of mdr1b expression.

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Figure 1

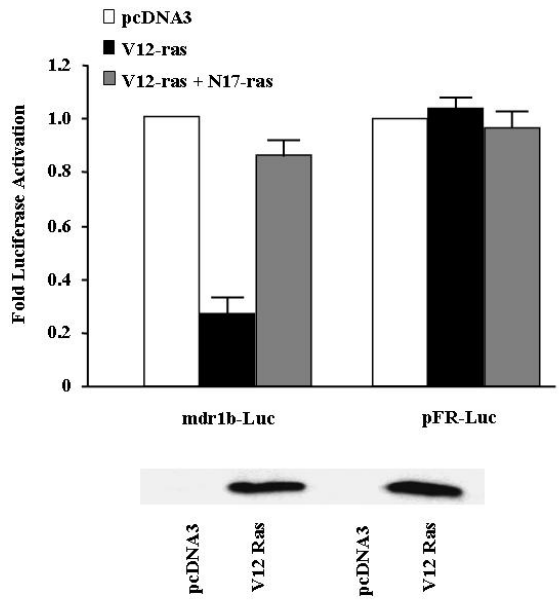

Figure 1. Effect of the overexpression of oncogenic H-ras on the expression of mdr1b. (Upper panel) pcDNA3-NIH3T3 and V12ras-NIH3T3 cells were cotransfected with either mdr1b-Luc or pFR-Luc and pRL-Luc. To blocking the Ras signaling pathway, N17-ras-pcDNA3 was transiently transfection into V12-ras-NIH3T3 cells. Luciferase activities were measured 24h after transfection, as described under "Materials and methods". Transfection with pRL-Luc plasmid was used to normalize the transfection. Fold luciferase activation is presented relative to the activity obtained from the transfection of mdr1b-Luc into pcDNA3-NIH3T3 cells, whose value was placed at 1.0. Each data bar represents the mean of five observations of three independent experiments; *error bars* indicate ± S.D. (Lower panel) Western blot analysis of pcDNA3-NIH3T3 (vector) and V12-ras-NIH3T3 (V12-ras) cells. Proteins were subjected to SDS-PAGE (12 %) and immunoblotted with anti-Ras antibody.

Figure 2

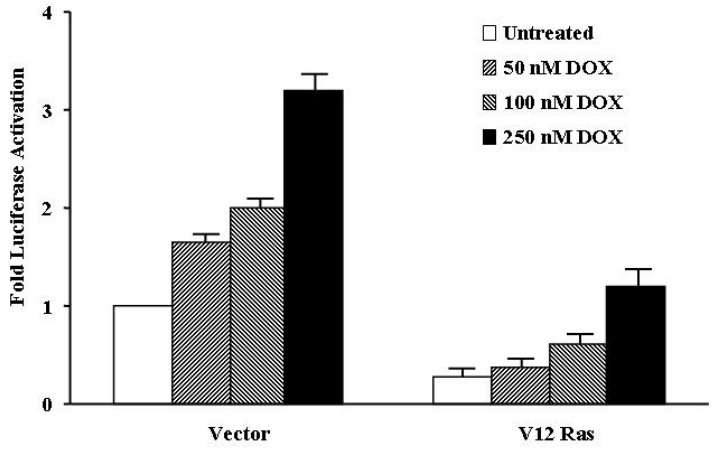

Figure 2. Oncogenic induces pRL-Luc activity. pcDNA3-NIH3T3 and V12-ras-NIH3T3 cells were cotransfected with either mdr1b-Luc reporter plasmid or pFR-Luc plasmid and pRL-Luc plasmid. 4 h after transfection cells were treated with different amounts of doxorubicin, and luciferase activity was determined 24 h later. Transfection with pRL-Luc plasmid was used to normalize the transfection. Fold luciferase activation is presented relative to the activity obtained from the transfection of mdr1b-Luc into pcDNA3-NIH3T3 cells, whose value was placed at 1.0. Each data bar represents the mean of five observations of three independent experiments; *error bars* indicate ± S.D.

Figure 3

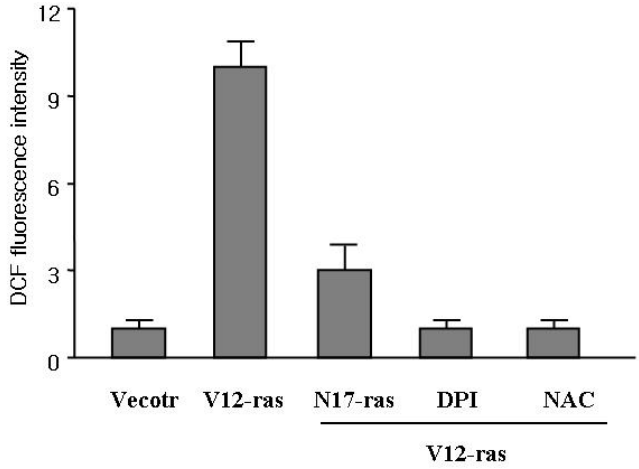

Figure3 ROS production in pcDNA3-NIH3T3 (vector) and V12-Ras-NIH3T3 (V12-ras) cells. Cells were pretreated with 20 mM NAC or 500 nM DPI, or transiently transfected with N17-ras-pcDNA3 (N17), and then incubated with DCFHDA, and ROS assay was carried out as describe in "Material and methods". Each point is the average of multiple independent experiments; *error bars* represent ± S.D. *P* values were less than 0.05 *vs* specific parental control

Figure 4

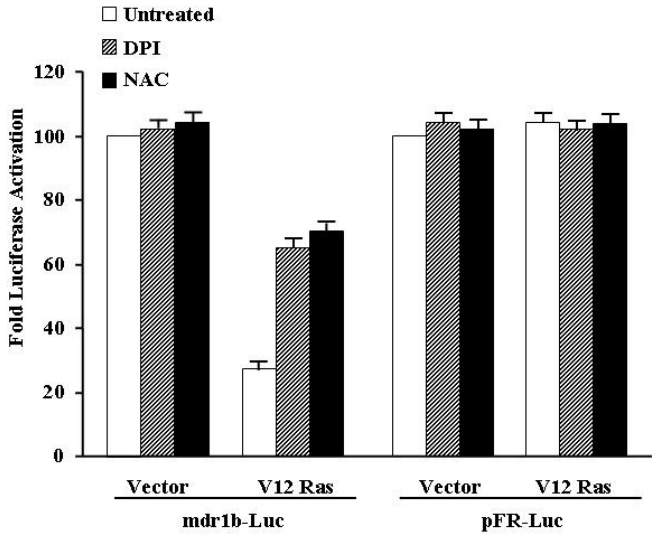

Figure 4. Effect of NAC and DPI on basal level of mdr1b expression. V12-ras-NIH3T3 cells were pretreated with either NAC or DPI for 12 h, and cells were then cotransfected with either 1μg of mdr1b-Luc or 1ug of pFR-Luc and 0.1 μg of pRL-Luc and luciferase activity was measured 24 h later. Transfection with renilla luciferase plasmid was used to normalize the transfection. Fold luciferase activation is shown relative to the luciferase activity of pcDNA3-NIH3T3 cells, which was placed at 1.0. . Each point is the average of multiple independent experiments; *error bars* represent ± S.D. *P* values were less than 0.05 *vs* specific parental control.

Figure 5

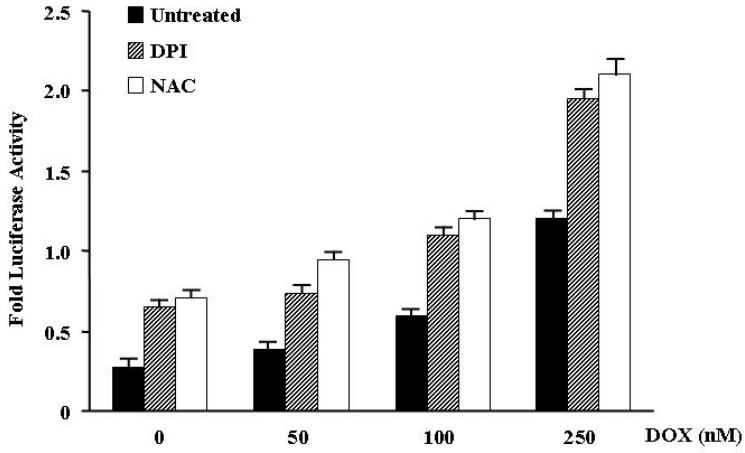

Figure 5 Effect of NAC and DPI on the induction level of mdr1b expression by doxorubicin. V12ras-NIH3T3 cells were pretreated with either NAC or DPI for 12 h and cells were then cotransfected with either 1μg of mdr1b-Luc or 1ug of pFR-Luc and 0.1 μg of pRL-Luc. Four hours after transfection, the cells were treated with 50, 100 or 250 nM of doxorubicin for 24 h, after which luciferase activity was determined. Fold luciferase activation is presented relative to the activity obtained from the transfection of mdr1b-Luc into pcDNA3-NIH3T3 cells, whose value was placed at 1.0. Each point is the average of multiple independent experiments; *error bars* represent ± S.D. *P* values were less than 0.05 *vs* specific parental control

Figure 6A

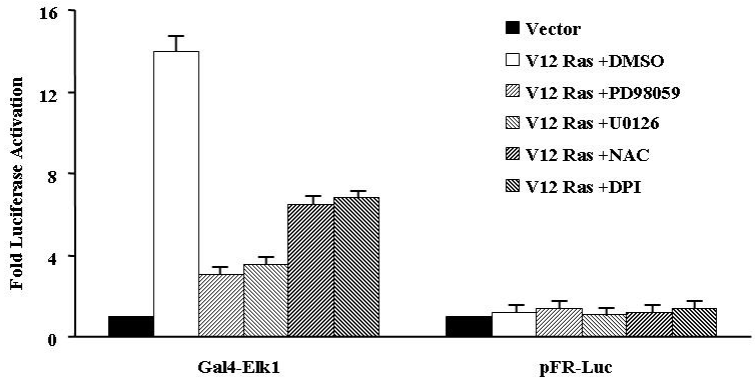

Figure 6B

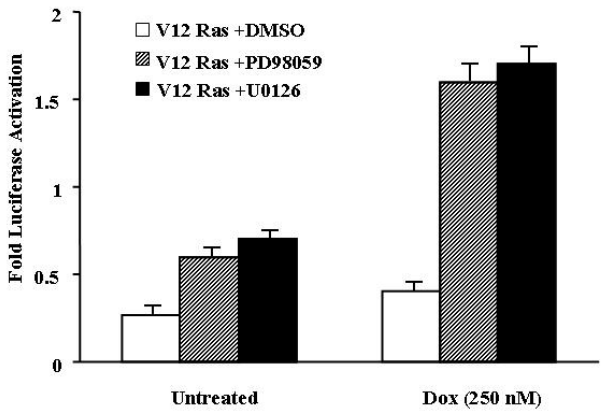

Figure 6 The effect of inhibitors on the Ras-induced pRL-Luc activity. (A) pcDNA3-NIH3T3 (vector) and V12-ras-NIH3T3 cells were pretreated with either DMSO or the indicated compounds for 12 h, at the following concentrations: 50 μM PD98059, 20 μM U0126, 10 mM NAC, and 500 nM DPI. After which cells were cotransfected with either Gal4-Elk1 or pFR-Luc plasmid and pRL-CMV. Luciferase activities were then measured 24 h after transfection. (B) V12ras-NIH3T3 cells were pretreated with either DMSO, or ERK inhibitor PD98059 or U0126, or p38 inhibitor SB2303580 for 30 min or transiently transfection with either control oligo (ISIS17552), JNK1/2 antisense oligos (ISIS 15347, ISIS 15354). Cells were then cotransfected with mdr1b-Luc and pRL-Luc. Four hours after transfection the medium was replaced with fresh medium in the presence or absence of 250 nM of doxorubicin. Cells were harvested 24 h after this addition, and luciferase activities were measured. Transfection with renilla luciferase plasmid was used to normalize the transfection. Fold luciferase activation is shown relative to the luciferase activity of pcDNA3-NIH3T3 cells, whose value was placed at 1.0. Each point is the average of multiple independent experiments; *error bars* represent ± S.D. *P* values were less than 0.05 *vs* specific parental control.

Figure 7A

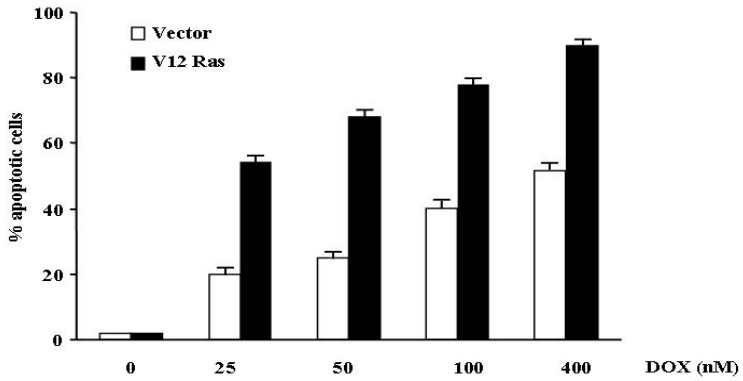

Figure 7B

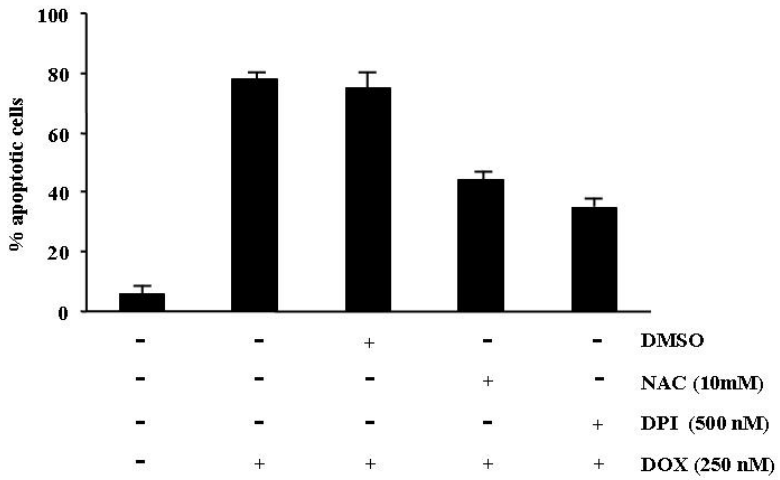

Figure 7. The effect of ROS inhibitors on the Ras-mediated cell death (A) pcDNA3-NIH3T3 (control) and V12-ras-NIH3T3 cells were treated with the indicated dose of doxorubicin for 12 h, and the cells were stained with propidium iodide, after which apoptosis was analyzed by folwcytometry. (B) Pretreatment of V12-ras-NIH3T3 cells with either DPI and NAC for 12 h or PD9850 and U0126 for 30 min, and the apoptosis was measured. *Bars* represented S.D. values determined from at least three independent experiments.

<국문초록>

Ras 암유전자에 의한 세포 생존연구

차 만 진

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의학과

Ras의 점돌연변이는 인간 암세포 전체의 약 30%에서 발견된다. 돌연변이를 통한 활성화된 Ras 종양유전자는 정상세포를 형질전환 할 수 있고 악성 사람 종양들의 많은 개체의 성장에 관련되어있다. Ras 암 유전자 가 활성화된 암세포는 암세포의 생존률을 조절하는데 아직 그 기작이 밝혀지지않았다. 본 연구 논문에서는 활성화된 Ras 암유전자에 의한 암세포 생존증가가 어떠한 기작에 의하여 발생되는지를 규명하고자 하였다. 그 결과 본 연구 논문에서 는 항암제 내성을 유발하는 mdr1의 발현이 Ras 암유전자에 의하여 발현이 감소되었다. 또한 H-RasV12-transformed NIH3T3 세포들은 대조세포에비하여 mdr1 프로모터 활성 (mdr1-luc)이 현저하게 감소되었다. Ras 암 유전자에의한 mdr1 발현 감소기작을 규명하기 위하여 활성산소 억제제를 투여한 결과 Ras 암유전자에의하여 활성이 감소된 mdr1 프로모터는 활성산소 억제제인 NCA 또는 DPI투여시 정상화

되는것을 관찰되었으며 항암제인 doxorubicin에의한 암세포 생존에 영향을 미침을 규명하였다. 또한 ERK 억제제인 PD98059와 U0126을 투여 시 Mdr1의 프로모터 활성이 정상화 되었다. 이러한 결과들로 Ras 암유전자는 Ras downstrem 신호전달경로인 ERK활성을 증가시켜 활성산소를 발생시키며 이러한 활성산소가 mdr1의 발현을 조절하여 암세포 생존에 영향을 줄것으로 판단되었다.

저작물 이용 허락서

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논문제목	한글 : Ras 임유전자에 의한 암세포 생존연구 영문 : The study of the oncogenic ras-mediated cancer cell survival				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의 여부: 동의 (0) 반대 ( )

2010 년 8 월 일

저작자: 차 만 진(서명 또는 인)

조선대학교 총장 귀하