Inhibition of NADPH oxidase 4 induces apoptosis in malignant mesothelioma: Role of reactive oxygen species

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Abstract. Malignant pleural mesothelioma (MPM) is an aggressive tumor that is characterized by dysregulated growth and resistance to apoptosis. Reactive oxygen species (ROS)-generating NADPH oxidase (Nox) family enzymes have been suggested to be involved in neoplastic proliferation. Both the antioxidant N-acetylcysteine (NAC) and the inhibitor of flavoprotein-dependent oxidase, diphenylene iodonium (DPI), inhibited the cell viability of MPM cells in a dose-dependent manner. To examine whether Nox-mediated ROS generation confers antiapoptotic activity and thus a growth advantage to MPM cells, we analyzed the mRNA expression of Nox family members using quantitative RT-PCR in 7 MPM cell lines and a normal mesothelial cell line. *Nox4* mRNA was expressed in all of the examined MPM cell lines, whereas little or no *Nox2, Nox3* and *Nox5* mRNA expression was detected. In 2 MPM cell lines, *Nox4* mRNA expression was significantly higher than that in a normal mesothelial cell line. siRNAs targeting *Nox4* suppressed ROS generation and cell viability in the MPM cell lines. In addition, DPI treatment and knockdown of Nox4 attenuated phosphorylation of AKT and ERK. Taken together, our results indicate that Nox4-mediated ROS, at least in part, transmit cell survival signals and their depletion leads to apoptosis, thus highlighting the Nox4-ROS-AKT signaling pathway as a potential therapeutic target for MPM treatment.

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Abbreviations: DCF, dichlorofluorescin; Duox, dual oxidase; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DPI, diphenylene iodonium; MPM, malignant pleural mesothelioma; NAC, N-acetylcysteine; Nox, NADPH oxidase; PBMC, peripheral blood mononuclear cell; ROS, reactive oxygen species

Key words: malignant mesothelioma, NADPH oxidase, reactive oxygen species, apoptosis

Introduction

Malignant pleural mesothelioma (MPM) are incurable thoracic malignancy, that has poor prognosis because it is frequently diagnosed at an advanced stage (1,2). The world-wide incidence of mesothelioma is expected to increase, particularly in Europe and Japan (3-5). The primary cause of MPM is often linked to asbestos exposure, and the number of patients worldwide is predicted to peak in the next 2 decades (6,7). Investigations for the molecular pathogenesis of MPM has begun (8-12). Recent whole-exome sequencing revealed frequent genetic alterations in *BAP1, NF2, CDKN2A* and *CUL1* in 22 MPMs (13). The latent period between the first exposure to asbestos and the onset of this disease is ~30 years, and the first symptom is insidious and may include chest pain and breathlessness. Many clinical trials including surgery, radiotherapy, and chemotherapy were reported, but the prognosis of patients remains poor. Although there was recent progress in clinical treatment with combination chemotherapies, a curative therapy for MPM remains unknown; the median survival ranges between 9 and 17 months after diagnosis (14-17). Combinations of cisplatin and pemetrexed appear to be the best chemotherapy regimen for MPM. Thus, effective clinical approaches such as molecular-targeted therapy are needed to treat MPM.

The recently-discovered epithelial NADPH oxidases (Noxs) mediate critical physiological and pathological processes including cell signaling, inflammation and mitogenesis by generating reactive oxygen species (ROS) (18). The Nox enzyme complex was first described in neutrophils, where it is normally quiescent but generates a large quantity of ROS upon activation during phagocytosis and plays a vital role in non-specific host defense against ingested pathogens (19,20). Many non-phagocytic cells contain NADPH oxidases (20). There are 7 identified family members in the NADPH family: 5 Noxs and 2 dual oxidases (DUOXs) (20). Noxs and the mitochondria are major sources of cellular ROS (21). Cancer cells produce ROS that act as signaling molecules to promote cell survival (22,23). Nox4-mediated ROS inhibit apoptosis and promote tumor cell growth in pancreatic cancer cells (24,25). However, our understanding of the roles of the Nox family members in the development and growth of human cancer is limited (26-30).

We hypothesized that intracellular ROS conferred anti-apoptotic activity and thus a growth advantage to MPM cells. In this study, we demonstrated that treatment with diphenylene iodonium (DPI), a flavoenzyme inhibitor (31) and knockdown of Nox4 suppressed ROS production in MPM cells, which induced apoptosis, suggesting that Nox4-generated ROS at least in part, transmits cell survival signals and provides a useful clinical approach for MPM treatment.

Materials and methods

Cell culture and materials. Seven MPM cell lines (ACC-MESO-1, ACC-MESO4, Y-MESO-8A, MSTO-211H, NCI-H28, NCI-H290 and NCI-H2052) and a normal mesothelial cell line (Met-5A) were kindly provided by Dr Y. Sekido, Division of Molecular Oncology, Aichi Cancer Center Research Institute. Cells were maintained at 37˚C under 5% CO_2 air atmosphere in DMEM culture medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin. Heparinized peripheral blood was collected from normal individuals after informed consent was obtained, and PBMCs were separated using density-gradient centrifugation.

Analysis and quantification of Nox4 mRNA levels by RT-PCR and real-time PCR. Reverse transcription (RT) was conducted as follows: 8 μl water containing 1 μg total RNA was added to 50 ng random primers (Life Technologies) and incubated at 65˚C for 5 min. cDNA was prepared with SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carsbad, CA, USA) according to the manufacturer's protocol.

Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio, Otsu, Shiga, Japan), and PCR amplifications were performed in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Briefly, a solution of SYBR Premix Ex Taq II (10 μl; Takara Bio) containing sense and antisense primers (10 μM each) was prepared and 2 μl cDNA was added to a final volume of 20 μl. Conditions for PCR included 42˚C for 5 min, 95˚C for 10 sec, and 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. Data were analyzed with Sequencer Detector version 1.6 software (ABI-PE). The threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission by nuclease activity of Taq polymerase. β-actin was used as an internal control. Relative transcripts were determined by the following formula: $1/2^{(CTtarget - CTcontrol)}$ (32). Specific primers for *Noxs* 1-5 and *β-actin* were synthesized (Star Oligo Rikaken, Nagoya, Japan). PCR primer pairs were as follows: *Nox1*, sense 5'-AGCGTC TGCTCTCTGCTTGAA-3' and antisense 5'-GGCTGCAAA ATGAGCAGGT-3'; *Nox2*, sense 5'-TGCCTTTGAGTGGTTT GCAGAT-3' and antisense 5'-ATTGGCCTGAGACTCAT CCCA-3'; *Nox3*, sense 5'-GAACCCTCGGCTTGGAAAT-3' and antisense 5'-TGGCTTACCACCTTGGTAATGA-3'; *Nox4*, sense 5'-CCCTCACAATGTGTCCAACTGA-3' and antisense 5'-GGCAGAATTTCGGAGTCTTGAC-3'; *Nox5*, sense 5'-AAGAGTCAAAGGTCGTCCAAGG-3' and antisense 5'-GCTTTCTTTTCTGGTGCCTGT-3'; *β-actin*, sense 5'-GAT GACCCAGATCATGTTTGAGACC-3' and antisense 5'-CGG TGAGGATCTTCATGAGGTAGT-3'.

Cell viability assay. The viability of the cells transfected with *Nox4* siRNAs or treated with NAC, DPI, or specific inhibitors for protein kinases was determined using the MTT assay. MPM cells ($1x10^3$) were incubated with each reagent at each concentration in triplicate in 96-well culture plates at 37˚C in humidified air with 5% CO_2. Three wells contained MPM cells in drug-free medium to determine the control cell survival and the percentage of cells after culture. Three wells contained medium only to blank the spectrophotometer. After 2 days, 10 μl (5 mg/ml) MTT salt was added for 6 h. Formazan production was quantitated using a spectrophotometer at 562 nm. The optical density (OD) is linearly related to the cell number. Cell survival (CS) was calculated at each drug concentration by the equation CS = (OD treated well/mean OD control wells) x 100%.

Flow cytometric analysis of apoptosis. To analyze apoptosis, the externalization of phosphatidylserine was measured by flow cytometric staining with FITC-conjugated Annexin V (BD Pharmingen). Cells in 6-well plates ($2x10^5$ cells per well) were treated for 48 h, washed, resuspended in 100 μl Annexin-binding buffer, and stained with 5 μl Annexin V-FITC and propidium iodide for 20 min. Flow cytometric analysis was performed using FACSCalibur (BD Biosciences) and Cell Quest Pro Version 4.0.2 (BD Biosciences) software. Cells that were positively stained with Annexin V were counted as apoptotic populations.

Measurement of intracellular ROS production. Cells ($2x10^5$ per well) were seeded in 6-well plates and treated with 10 μM DPI for 48 h or transfected *Nox4* siRNAs. Then, cells were incubated with 2.5 μM of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) for 30 min at 37˚C in the dark, washed with Hank's buffer, and fixed in 1% paraformaldehyde. The fluorescence intensity was measured using FACS, with the excitation source at 488 nm and an emission wavelength of 580 nm. An analysis was performed with the software program BD FACStationt System Data Management System (Becton-Dickinson). Background fluorescence from the blank was subtracted from each reading.

Transfection and immunoblotting. Cells were transfected with *Nox4* siRNAs or scramble siRNAs utilizing Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. siRNAs were designed from the human *Nox4* cDNA sequences as follows (Integrated DNA Technologies, Coralville, IA, USA): 5'-GCUGAAGUAUCAAACUAUUUAGAT-3' and 5'-AUCUA AAUUAGUUUGAUACUUCAGCAG-3' for Nox4RNAi-1, and 5'-GAAUUACAGUGAAGACUUUGUUGAA-3' and 5'-UUC AACAAAGUCUUCACUGUAAUUCAC-3' for Nox4RNAi-2. Universal scrambled siRNA sequences, which have no significant homology to mouse, rat, or human genome databases, were used as controls (Invitrogen).

For western blot analysis, equal amounts of reduced proteins (20 μg) were loaded on 10% Bis-Tris-buffered polyacrylamide gels. After gel electrophoresis, proteins were transferred to PVDF membranes (Invitrogen) by electroblotting. The membranes were preincubated for 1 h in 5% low-fat dried milk in TBS and 0.1% Tween-20 (TBS-T) to block non-specific binding sites. After washing with TBS-T,

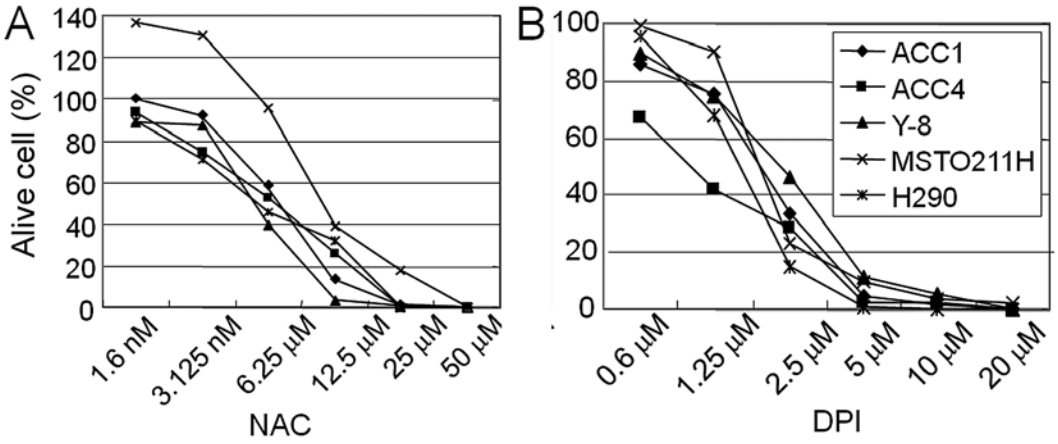

Figure 1. Effects of NAC and DPI on the viability of MPM cells. MPM cells ($1x10^3$) were treated with NAC (A) or DPI (B) at the indicated concentrations for 48 h. The viability of the cells was determined by an MTT assay. The IC_{50} values of these drugs were extrapolated from the respective dose-response curves. The data are means (n=3).

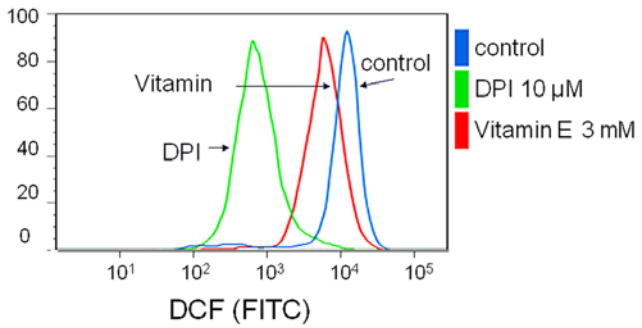

Figure 2. Inhibition of ROS production by DPI or vitamin E. ACC4 cells ($1x10^5$) were cultured in a 24-well plate for 48 h. Cells were treated with or without DPI (10 μM) or vitamin E (3 mM). The cells were labeled with DCFH-DA and alterations in the intracellular ROS level were measured by FACS analysis. A typical profile of FACS analysis is shown. The horizontal axis indicates the fluorescence intensity with the excitation source at 488 nm and an emission wavelength of 580 nm.

membranes were incubated overnight with a 1:1,000 dilution of primary antibody in TBS-T containing 5% BSA at 4˚C (Cell Signaling Technology), and probed with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) for 1 h at room temperature. The bound antibodies were visualized with the ECL reaction (GE Healthcare).

Statistical analysis. Data were analyzed by the Welch t-test, Fisher's exact-test, or ANOVA using Statview software (SAS, Cary, NC, USA), and P-values at <0.05 were considered to be statistically significant.

Results

Inhibition of cell growth, suppression of ROS generation, and induction of apoptosis by antioxidants. The flavoenzyme inhibitor, DPI inhibits membrane-bound, flavoprotein-containing Noxs. We examined whether the antioxidant NAC and the flavoenzyme inhibitor DPI, affected the cell viability of mesothelioma cell lines. Five MPM cell lines (ACC1-MESO, ACC4-MESO, Y8-MESO, MSTO-211H and H290) were treated with various concentrations of NAC or DPI for 48 h. Both NAC and DPI treatments inhibited the cell viability in a dose-dependent manner (Fig. 1). The IC_{50} values for DPI were as follows: 2.1 μM (ACC1-MESO), 0.8 μM (ACC4-MESO), 2.5 μM (Y8-MESO), 2.2 μM (MSTO-211H) and 2.2 μM (H290).

To verify that antioxidants affect ROS generation, we evaluated ROS production using flow cytometry. With vitamin E and DPI treatment, DCF fluorescence intensity, $2.1x10^4$ in untreated cells was reduced to $0.7x10^4$ and $0.1x10^4$, respectively (Fig. 2). Thus, MPM cells regularly generated ROS, and both vitamin E and DPI treatment suppressed ROS generation.

We further examined the effect of DPI on the induction of apoptosis of MPM cells using Annexin V assay. DPI treatment significantly induced apoptosis (27 and 26%) in ACC1 and MSTO-221H cells, respectively (Fig. 3). Our results strongly suggest that depletion of ROS leads to the apoptosis of MPM cells.

Expression and quantification of Nox 1-5 mRNAs in MPM cell lines. The Nox family members produce ROS that are though to be pivotal for cell proliferative signaling. To examine the role of the Nox family in proliferation of MPM cells, we analyzed the mRNA expression of Nox family members in 7 MPM cell lines and a non-malignant mesothelial cell line (Met-5A) (Fig. 4). *Nox4* mRNA was expressed in all of the examined MPM cell lines, whereas little or no *Nox2*, *Nox3* and *Nox5* mRNAs were detected (Fig. 4). In the ACC-MESO4 cell line, *Nox1* mRNA expression was readily detected.

Subsequently, the expression levels of *Noxs* 1-5 relative to β-actin were measured by quantitative real-time RT-PCR (Fig. 5). Expression was arbitrarily graded as low (Nox copy number/β-actin copy number $<500x10^{-8}$), intermediate (ratio >500 but $<2,000x10^{-8}$) or high (ratio $>2,000x10^{-8}$). Nox genes with expression ratios $>500x10^{-8}$ were routinely visible by RT-PCR analysis using ≥40 μg total RNA. High- or intermediate-level *Nox4* mRNA expression was observed in all examined MPM cell lines. Especially, 2 ACC-MESO4 and MSTO-211H cell lines expressed *Nox4* mRNA at a high level. For comparison, we examined expression of the Nox family in

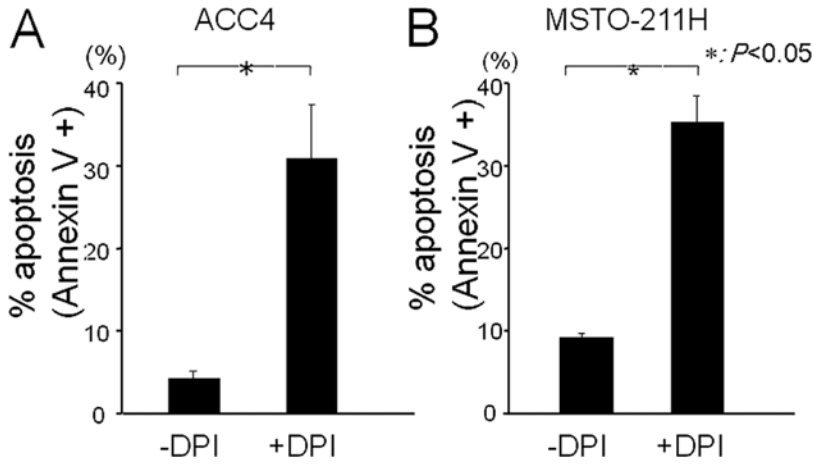

Figure 3. Induction of apoptosis in MPM cells by DPI. (A) ACC4 cells and (B) MSTO-211H cells ($1x10^5$/ml) were inoculated and, cultured in 24-well plates, and treated with 10 μM DPI for 48 h. The numbers of apoptotic cells were determined by Annexin V staining. Error bars represent the SD for 3 samples. An asterisk (*) indicates a significant difference with a P-value <0.05 (n=3).

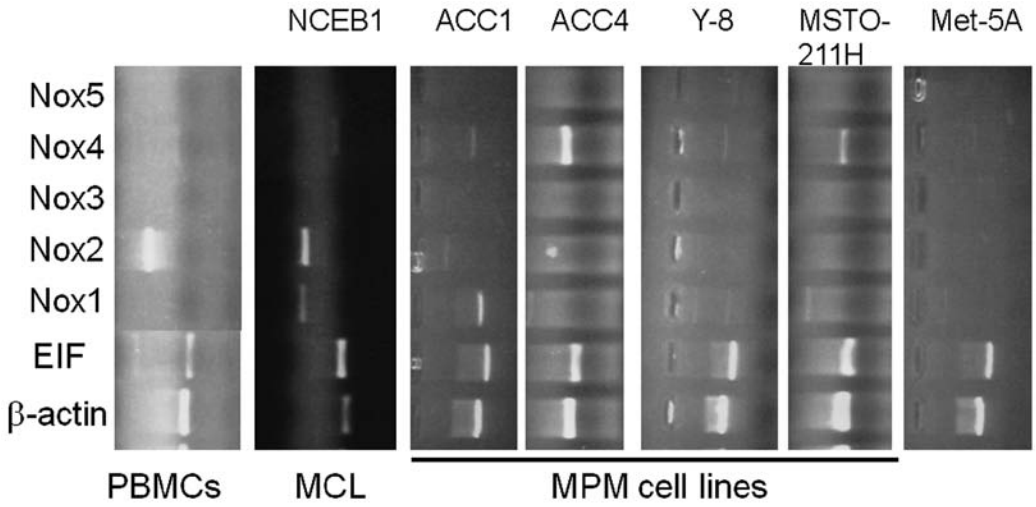

Figure 4. mRNA expression of Nox family members in human MPM cell lines. Total RNAs were extracted from MPM cells, and the mRNA expressions of Nox family members were analyzed by RT-PCR with *β-actin* and *EF1-α* expression as internal controls.

several lymphoma/leukemia cell lines; mantle lymphoma cells and Jurkat leukemia cells expressed low levels of *Nox2* mRNA (Fig. 5). High levels of *Nox2* were detected in human PBMCs (Fig. 5) and the Jurkat cell line (data not shown).

Nox4 mediates ROS production in MPM cells. We utilized an RNA interference approach to verify whether Nox4 mediates ROS production in MPM cells. The expression of endogenous *Nox4* mRNAs in ACC-MESO4 and MSTO-211H cells was significantly suppressed upon transfection of *Nox4* siRNAs, (Fig. 6A). Intracellular ROS production was evaluated by flow cytometry. The transfection of *Nox4* siRNAs significantly suppressed ROS levels compared to controls (Fig. 6B), indicating that Nox4, at least in part, is responsible for intracellular ROS generation. However, the ROS generation was not completely inhibited by transfection of *Nox4* siRNAs, suggesting the possibility that other Nox members and mitochondria sources may also contribute to ROS generation.

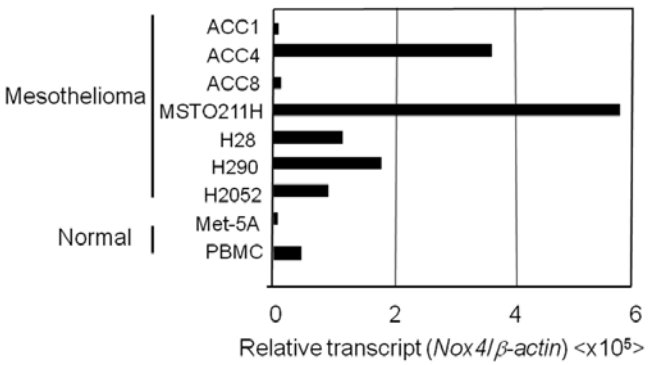

Figure 5. Quantitative analysis of *Nox4* mRNA expression in MPM cells. Real-time PCR was performed using SYBR green assay in an ABI PRISM 7700 Sequence Detection System. The threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission by nuclease activity of Taq polymerase. *β-actin* was used as an internal control for mRNA expression. Real-time PCR efficiency of target (*Nox1-5*) and reference (*β-actin*) was approximately equal over a concentration range of 0.1-200 ng cDNA. Relative transcripts were determined by the formula: $1/2^{(CTtarget - CTcontrol)}$.

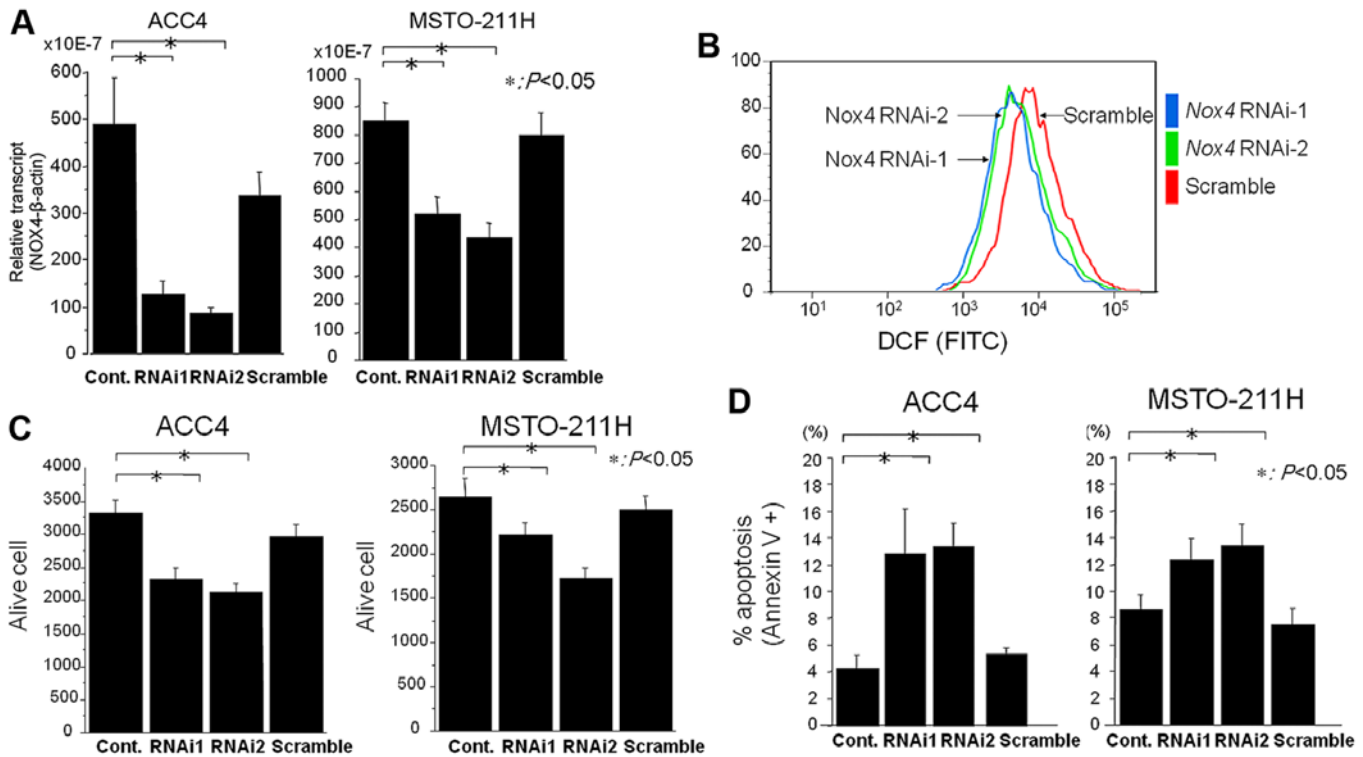

Figure 6. Inhibition of ROS production and induction of apoptosis by *Nox4* siRNAs. ACC4 and MSTO-211H cells ($5x10^6$) were transfected with *Nox4* siRNA-1, *Nox4* siRNA-2 and scrambled siRNA. (A) The expression levels of endogenous *Nox4* mRNAs, relative to *β-actin* mRNA as a control, were examined by quantitative RT-PCR at 48 h after transfection. (B) ACC4 cells were seeded in 6-well plates ($2x10^5$ cells/well) and, incubated overnight, and transfected with *Nox4* siRNA-1, *Nox4* siRNA-2 and scrambled siRNAi. After 48 h, the cells were labeled with DCFH-DA, and alterations in the intracellular ROS level were measured by FACS analysis. A typical profile of FACS analysis is shown, and DCF fluorescence was normalized to that in cells transfected only with scrambled siRNAs. The viability and induction of apoptosis in ACC4 and MSTO-211H cells ($2x10^5$) transfected with *Nox4* siRNA were assayed for MTT (C) or apoptosis (D) assay as per protocol. Error bars represent the SD for 3 samples. An asterisk (*) indicates a significant difference with a P-value <0.05 (n=3).

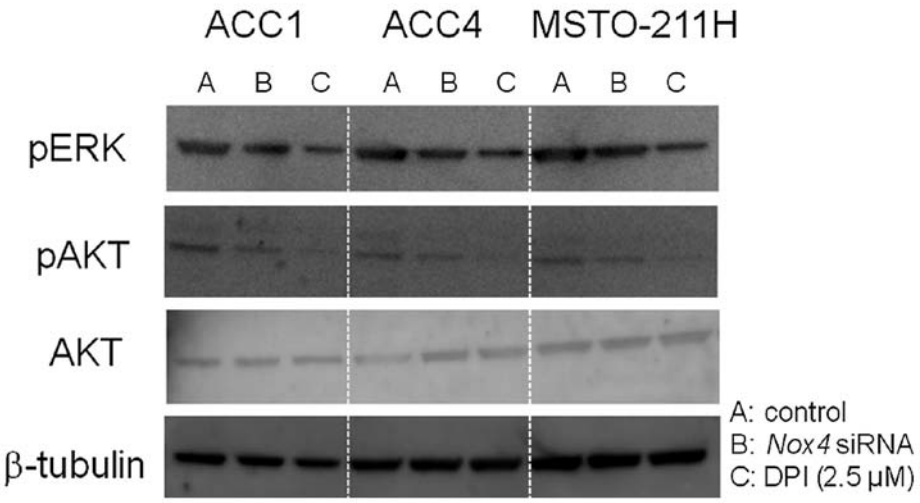

Figure 7. Attenuated phosphorylation levels of AKT and ERK by *Nox4* siRNAs or DPI. ACC1, ACC4, and MSTO-211H cells were transfected with *Nox4* siRNA-1 or treated with DPI (2.5 μM) for 24 h, and the cell lysates were prepared. Twenty micrograms of proteins was subjected to western blot analysis to detect the phosphorylation levels of AKT and ERK. β-tubulin was used as an internal control for protein expression.

Suppression of ROS generation by Nox4 siRNAs induces apoptosis. To explore whether Nox4-generated ROS regulate cell survival, we examined the effect of *Nox4* siRNAs on cell viability and apoptosis. The knockdown of Nox4 significantly reduced the cell viability of ACC-MESO4 and MSTO-211H cells by 30% (Fig. 6C). To verify whether the inhibitory effect of *Nox4* siRNAs is associated with apoptosis, an Annexin V assay was performed. The transfection of *Nox4* siRNAs induced apoptosis by 13% in MPM cells (Fig. 6D). Thus, *Nox4* siRNAs suppressed ROS production, and the depletion of ROS by *Nox4* siRNAs and DPI treatment induced apoptosis in MPM cells.

The role of protein kinases in cell survival signaling in MPM cells. Both PI3K/AKT and MEK/ERK1/2 signaling cascades have important roles in cell proliferation, but they also mediate apoptosis (33). Western blot analysis showed that ACC1, ACC4, and MSTO-211H cell lines expressed phospho-AKT and phospho-ERK (Fig. 7). *Nox4* siRNAs transfection and DPI treatment attenuated the phosphorylation of AKT and ERK. Given that transfection of *Nox4* siRNAs and DPI treatment induced apoptosis in MPM cells, the inactivation of PI3K/AKT and MEK/ERK1/2 signaling cascades likely plays an important role in the induction of apoptosis.

Discussion

It is well established that the development of MPM is associated with asbestos exposure (34,35). Chronic inflammation accelerates the development and progression of malignant mesothelioma, possibly because of cytokine release and ROS generation. The inflammation that infiltrate into tissue areas containing asbestos deposits consists largely of phagocytic macrophages that internalize asbestos and release numerous cytokines and mutagenic ROS.

In this study, we first examined the role of ROS in cell proliferation. Although ROS are thought to cause stress-induced apoptosis, ROS often provide cancer cells with a survival advantage. In fact, we showed that suppression of ROS levels by NAC and DPI treatment reduced the viability of MPM cells. Similar results were observed in other cancer cells including pancreatic cells. Second, we examined whether the Nox4-mediated generation of intracellular ROS conferred anti-apoptotic activity and thus a growth advantage to MPM cells. To this end, we analyzed the expression levels of *Nox* genes in 7 MPM cell lines, and a normal mesothelial cell line. RT-PCR analysis revealed that *Nox4* mRNA was expressed in all MPM cell lines, whereas little or no *Nox2*, *Nox3* and *Nox5* mRNAs were detected. Quantitative real-time RT-PCR also revealed that a high or intermediate level of *Nox4* mRNA expression was observed in all MPM cell lines; high-level expression was detected in ACC-MESO4 and MSTO-211H cells compared to the normal mesothelial cell line Met-5A ($P<0.01$).

The siRNAs targeting *Nox4* in 2 MPM cell lines reduced intracellular ROS generation by 50%, and cell viability by 30%. The depletion of ROS by DPI treatment or knockdown of Nox4 induced apoptosis. Collectively, our findings suggest that ROS generated by Nox4, at least in part, transmit cell survival signals and their depletion leads to apoptosis. High-level *Nox1* mRNA expression was observed in colorectal cancer cell lines. Growth inhibition profiling of DPI revealed a modest positive correlation with *Nox1* levels (31). Exposure of HT-29 colon cancer cells, which expresses *Nox1*, to DPI had inhibitory effects on the steady-state ROS levels, and decreased STAT, ERK1/2, and AKT signaling activity (31). Nox4 overexpression is also reported in primary breast, ovarian, prostate, melanoma, and glioblastoma cancer cell lines (36-38). Nox4 expression was intermediate to high in 2 of the 4 tested ovarian cancer cell lines. Notably, in A2780/DDP cells, high level acquired resistance to cisplatin was associated with a marked decrease in the expression level of Nox4. Recently, Nox4 was shown to be an oncoprotein localized to mitochondria (39). Together with these studies, our study suggests that *Nox4* may act as an oncogene, and Nox4-related signaling molecules may be good candidates for molecular-targeted therapy for MPM. Given a possible role of Nox4 in tumorigenesis, it will be of particular interest to investigate the correlation of Nox4 expression in primary mesotheliomas and prognosis in the patients.

AKT (protein kinase B) is a regulator of cell survival in response to a growth factor. AKT is activated through its phosphorylation, and it inhibits apoptosis-inducing proteins, thereby promoting cell survival. The ERK pathway is mostly activated by growth factors and mediates cell proliferation, but it also mediates apoptosis by stress stimuli (33). To examine the role of AKT and ERK in the apoptosis of MPM cells, we evaluated the phosphorylation state of AKT and ERK. Both DPI treatment and knockdown of Nox4 attenuated their phosphorylation levels, suggesting that AKT and ERK play a role in cell survival signals in MPM cells. Consistent with our results, the PI3K-AKT pathway was reported to be activated in human malignant mesothelioma (40). In addition, both selective inhibitors for PI3K/AKT and MEK/ERK1/2 were effective in downregulating the expression of prometastasis phenotypes of MPM cells (41).

In conclusion, we demonstrated that Nox4-mediated ROS generation, at least in part, transmits cell survival signals, and ROS depletion by the knockdown of Nox4 and DPI treatment leads to apoptosis in a subset of MPM cells. Our study raises the possibility of the Nox4-ROS-AKT signaling pathway as a novel therapeutic target for MPM. Antioxidant treatment targeted to this signaling pathway has the potential to enhance the therapeutic index of cisplatin-based therapy. Further studies are warranted to contribute to the knowledge required to ultimately develop targeted therapies for MPM.

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