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Adenoviral Transfer of Rho Family Proteins to Lung Cancer Cells Ameliorates Cell Proliferation and Motility and Increases Apoptotic Change

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Lung cancer is still a very severe disease which has a low survival rate due to local invasion and metastasis potentials in spite of many clinical challenges using anti-cancer drugs. Rho family small GTPases play pivotal roles in cell invasion and metastasis during carcinogenesis. In this study, we explored the inhibitory effect of adenoviral vector encoding dominant negative mutants of Rac, RhoA, and ROCK in human non-small cell lung carcinoma cell lines (A549 and SQ5) and mouse carcinoma cell line (Lewis lung carcinoma, LLC). These cells showed high expression of Rac, Rho, and ROCK, whereas only faint bands were detected in normal human lung epithelial cells, BET-1A. The efficiency of adenoviral vector transfer was stronger in A549 and SQ5 cells than LLC cells. Dominant negative forms of RhoA (Rho-DN) and Rac (Rac-DN) decreased cell proliferation in WST-8 assay and increased the number of apoptotic cells in both A549 and SQ5 cells by Hoechst 33258 and TUNEL staining. On the other hand, DN form of ROCK (ROCK-DN) did not show any apparent changes compared with the other proteins. Transwell® chamber analysis showed that migration/invasion activity was significantly suppressed by gene transfection both in A549 and SQ5 cells and that ROCK-DN gene transfer required a higher multiplicity of infection to show effects similar to Rho and Rac. Although the effect of gene therapy is cell-dependent, these data suggest that adenoviral gene transfer with Rho family small GTPases is one good approach to lung cancer therapy.

Ras-homologous (Rho) GTPases play a pivotal role in the regulation of numerous cellular functions such as cell growth, apoptosis, lipid metabolism, cytoarchitecture, membrane trafficking and transcriptional regulation, associated with malignant transformation and metastasis (2, 7). The Rho family includes RhoA, RhoB, RhoC, Cdc42, TC10, Rac1, Rac2, Rac3, RhoG, Rho6/Rnd1, Rho7/Rnd2, Rho8/Rnd3, RhoE, RhoD and RhoH. These proteins are localized at membranes and become activated upon stimulation of cell surface receptors. They cycle between GTP-bound active forms and GDP-bound inactive forms and have intrinsic GTPase activities.

The small GTP-binding proteins RhoA and Rac are downstream effectors of the oncogene product p21-ras, one of the most important oncogenes involved in human tumorigenesis. RhoA promotes stress fiber formation and cell motility. In GTP-bound state, Rho proteins bind to effector proteins such as Rho-kinase (ROCK), thereby triggering

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specific cellular responses. Rho guanine exchange factors (GEFs) are often oncogenic and the expression level of Rho GTPases frequently increases with malignancy. Rac is involved in actin polymerization, Jun kinase activation and intracellular superoxide anion production, through distinct pathways in tumor cells (14). Therefore, specific inhibitors of individual Rho functions are predicted to be of great therapeutic benefit.

So far, many researchers have explored the cell signaling cascade in the involvement of carcinogenesis and metastasis. As one therapeutic approach, we constructed the adenovirus vectors encoding dominant negative forms of RhoA, ROCK, and Rac, and examined the possibility of controlling lung cancer.

MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell line A549, human lung squamous carcinoma cell line SQ5, human lung epithelial cell line BET-1A and murine lung cancer cell line Lewis lung cancer cells (LLC) were obtained from American Type Culture Collection (Manassas, VA, USA). A549 and LLC cells were maintained in DMEM containing 10% fetal bovine serum (FBS), whereas SQ5 was in RPMI-1640 medium containing 10% FBS and BET-1A was in serum-free modified LHC-9 medium (Clonetics, San Diego, CA, USA) with supplements as described previously (9).

Recombinant adenovirus-mediated gene transfer

Point mutations were introduced in the COOH-terminal portion of Rho-kinase containing Rho-binding (RB) and pleckstrin homology (PH) domains [RB/PH (TT)], to abolish the Rho binding activity (1). C-myc-tagged Rho kinase RB/PH (TT), from Professor K. Kaibuchi (Nagoya University, Nagoya, Japan), or LacZ was placed into pAdex1CAwt under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter (12) to give pAdex Rho kinase RB/PH (TT) and pAdex LacZ, respectively. A recombinant adenovirus was constructed by in vitro homologous recombination in 293 cells using pAdex Rho kinase RB/PH (TT) or pAdex LacZ and the adenovirus DNA-terminal protein complex by a method previously described (11). The adenovirus vector encoding a c-myc-tagged dominant-negative mutant of RhoA (RhoA T19N) was described previously (8). The adenovirus vector encoding a dominant-negative mutant of Rac (RacN17) was kindly provided by W. Ogawa (Kobe University Graduate School of Medicine, Kobe, Japan).

After cells had attained confluence, they were infected with recombinant adenoviral vectors encoding dominant-negative forms of RhoA (RhoA-T19N, RhoA-DN), ROCK/Rho kinase (Rho kinase RB/PH (TT), ROCK-DN), Rac (Rac-N17, Rac-DN) and LacZ as control, at a multiplicity of infection (moi) of 30, 60 or 100 for 1 hour, and incubated for 48 hours after washing twice with serum free medium, at 37ºC in 5% CO_2 atmosphere.

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl, pH7.4, 0.15 M NaCl, 1 mM EDTA, 1 μM leupepsin, 1 μM pepstatin, 1% CHAPS, 1% TritonX-100). After centrifugation at 15,000 rpm for 20 minutes, the supernatant was collected, separated on an SDS-polyacrylamide gel (7.5-15%) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was incubated for at least 1 hour in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) supplemented with 5% dry milk to block non-specific binding sites. Mouse monoclonal antibodies against RhoA (Santa Cruz Biotechnology, Santa-Cruz, CA, USA), ROCK2 (BD Biosciences), c-myc-tag (Upstate, Charlottesville, VA, USA), and rabbit polyclonal antibodies against Rac1 (Santa Cruz Biotechnology) and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA,

USA) were used as primary antibodies. Following overnight incubation with each antibody, the membrane was washed three times with TBS-T. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour and washed again. The blots were then developed with the ECL Advance Kit (Amersham Biosciences, Buckinghamshire, England).

Proliferation assay

Virus-infected cells ($2x10^4$ per well) were cultured in 96-well plates in 100 μl of medium for 48 hours. Then, the number of viable cells was examined by a colorimetric assay using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] cell-counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Two hours after incubation with WST-8 solution at 37ºC, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm.

Apoptotic cell count

Apoptotic change in tumor cells treated with adenovirus vectors was analyzed by Hoechst 33258 stain solution (Sigma-Aldrich, St. Louis, MO, USA). The number of cells with chromatin cohesion into nuclei was counted and the percentage of apoptotic cells was compared.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining

End labeling of exposed 3′-OH ends of DNA fragments in paraffin-embedded tissue was undertaken with the *in situ* apoptosis detection TUNEL kit (Takara, Shiga, Japan) according to the manufacturer's recommended protocol. The specimens were further detected by diaminobenzidine and counterstained by methyl green.

Migration/invasion assay

The tumor cell migration activity was assayed in Transwell® cell-culture chambers with polycarbonate filters of 8.0 μm pore size (Corning, Acton, MA, USA). Forty-eight hours culture after virus infection, tumor cells were harvested and suspended to a final concentration of 1-2 × 10^5/ml in serum free medium with 0.1% BSA. Cell suspensions (100 μl) were added to the upper compartment, 5% fetal bovine serum (FBS) in medium was added to the plate well as a chemoattractant, and incubated for 48 hours at 37ºC in 5% CO_2 atmosphere.

For the analysis of the cell invasion activity, we used BD BioCoat Matrigel® Invasion chambers (BD Biosciences, Bedford, MA, USA) coated with basement membrane matrix, instead of uncoated chambers.

The filters were fixed with methanol, and stained with Diff-Quik. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded to the lower surface areas were counted under a microscope (average of 5 semi-random non-overlapping fields at × 400 magnification).

Statistical analysis

Results are presented as means ± standard deviations. Data were analyzed using one-way analysis of variance followed by an unpaired Student's t test for comparison between groups. Differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

In this study, we examined the effect of adenovirus infection expressed dominant negative form of Rho family proteins. First, we confirmed whether these proteins were expressed in lung cancer cells by western blot analysis (Fig. 1). The expressions of RhoA, ROCK and Rac were all detected strongly in cancer cell lines, such as human lung

adenocarcinoma cells, A549, human lung squamous carcinoma cells, SQ5, and mouse Lewis lung carcinoma cells (LLC). On the other hand, normal human lung epithelial cell line, BET-1A, showed faint bands.

Next, the efficiency of adenovirus infection was determined by X-gal staining after LacZ vector gene transfer. Almost all of A549 and SQ5 cells infected at 30 moi of LacZ vector showed blue staining which indicated high efficiency. The number of damaged cells infected at 100 moi was small and similar to non-infected cells.

RhoA-DN, ROCK-DN and Rac-DN were inserted in adenovirus vector and transfected to A549, SQ5 and LLC cells, and the expression of each protein was determined by western blotting analysis (Fig. 2). A549 and SQ5 cells showed strong expression of dominant negative mutants by gene transfer, however LLC cells showed only weak bands even at higher moi.

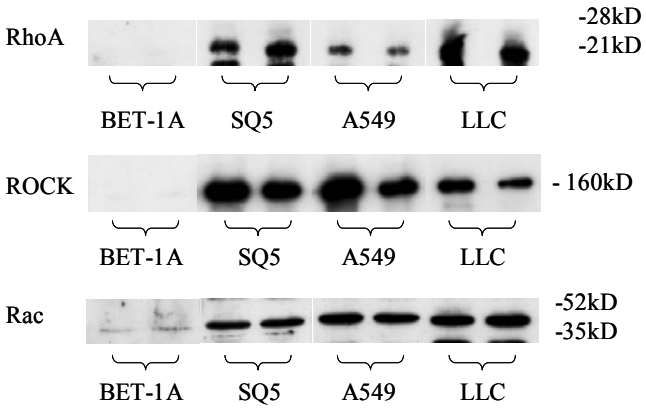

Fig. 1. The expression of endogenous RhoA, ROCK and Rac in lung cancer cells. Cell lysate from A549, SQ5 and LLC cells were blotted with anti-RhoA (upper panel), anti-ROCK2 (middle panel) or anti-Rac1 antibody (lower panel).

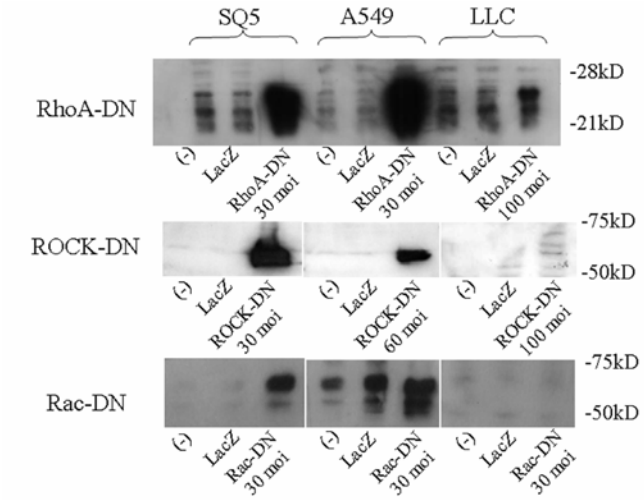

Fig. 2. The expression of dominant forms of RhoA, ROCK, and Rac after gene transfer. Forty-eight hours after transfection, cell lysate from A549, SQ5 and LLC cells were collected and blotted with anti-RhoA or anti-c-myc antibody.

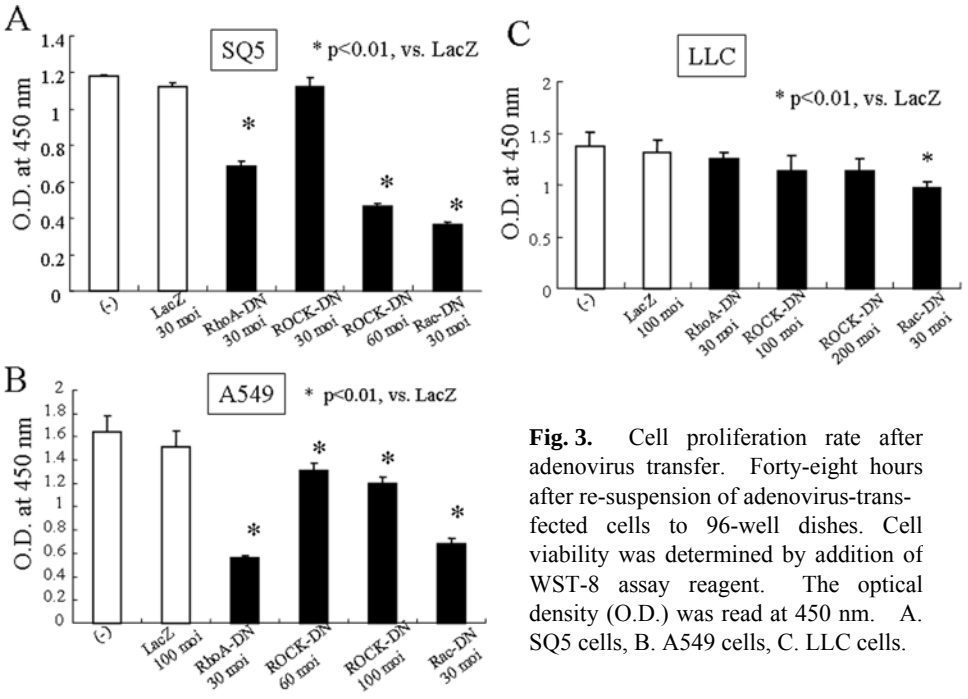

Fig. 3. Cell proliferation rate after adenovirus transfer. Forty-eight hours after re-suspension of adenovirus-transfected cells to 96-well dishes. Cell viability was determined by addition of WST-8 assay reagent. The optical density (O.D.) was read at 450 nm. A. SQ5 cells, B. A549 cells, C. LLC cells.

In Fig. 3, we explored the cell proliferation rate after adenovirus infection by using 96-well colorimetric assay. Over-expression of dominant negative form of RhoA or Rac inhibited the cell proliferation of lung cancer cell lines, A549 and SQ5. The transfection of RhoA-DN and Rac-DN significantly inhibited the proliferation of both A549 and SQ5 at 30 moi. The inhibitory effect of ROCK-DN was weak compared with the other transfections.

These data indicated that adenovirus transfer caused apoptotic changes in cancer cells. We counted the number of apoptotic cells whose nuclei showed chromatic cohesion as shown in Fig. 4. The percentage of apoptotic cells was increased in cancer cells transfected with RhoA-DN and Rac-DN, whereas ROCK-DN transfer showed lower apoptosis rate even at higher moi. In similar, TUNEL positive cells were increased in Rho protein-transfected A549 cells (Fig. 5A, b-d), as well as SQ5 cells (data not shown). The intensity of cleaved caspase-3 was slightly increased after transfection of adenovirus vectors, however this change was not statistically significant. These results suggested that dominant negative forms of RhoA, ROCK and Rac induced apoptotic changes in cancer cells.

To explore the effect of adenoviral infection on cell motility, we used Transwell® chambers coated with or without basement membrane matrices (Figs. 6 and 7). Under Matrigel®-coated condition, the transfection of RhoA-DN and Rac-DN inhibited cell invasion activity, whereas ROCK-DN also inhibited at higher moi. Without coating, migration activity was also inhibited by transfection of dominant negative forms.

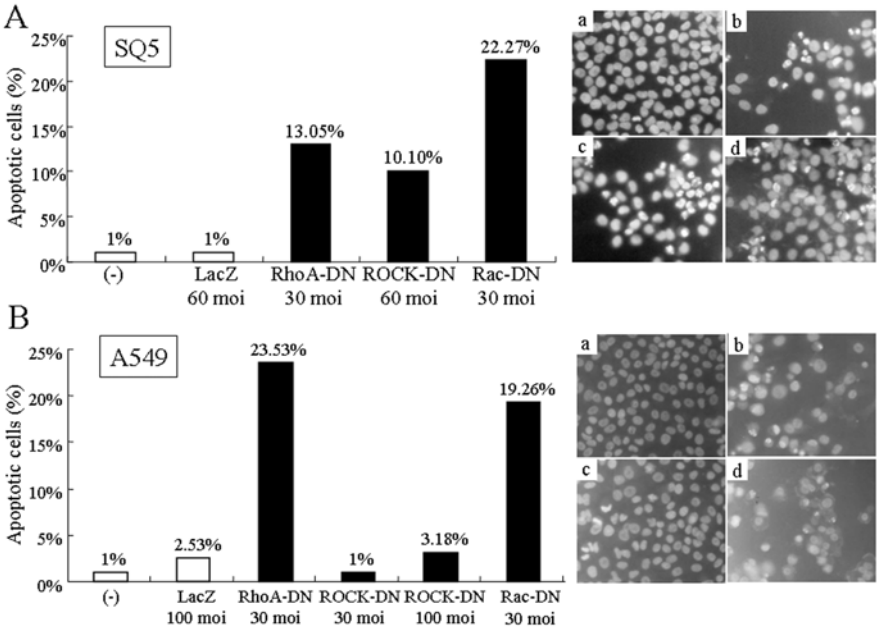

Fig. 4. Apoptotic cell counts in SQ5 and A549 cells. The cells (A: SQ5 cells, B: A549 cells) were stained with Hoechst 33258 stain solution (a: LacZ, b: Rho-DN, c: ROCK-DN, d: Rac-DN). The number of apoptotic cells after gene transfer was calculated and the average data among several fields (at least 4) are shown.

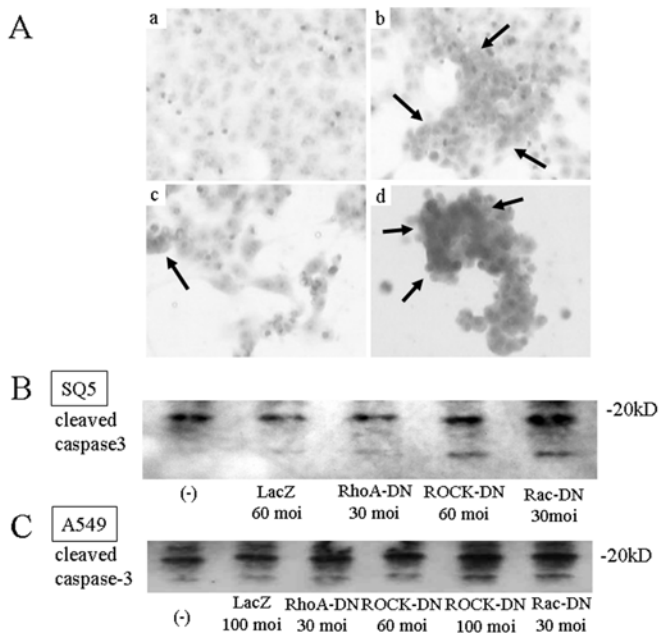

Fig. 5. TUNEL assay and western blotting data of cleaved caspase-3. A. Transfected cells were stained by an *in situ* apoptosis detection TUNEL kit (a: LacZ, b: Rho-DN, c: ROCK-DN, d: Rac-DN). TUNEL-positive cells were indicated as arrows. Cell lysate in SQ5 (B) and A549 (C) cells from each group were blotted with anti-cleaved caspase-3 antibody.

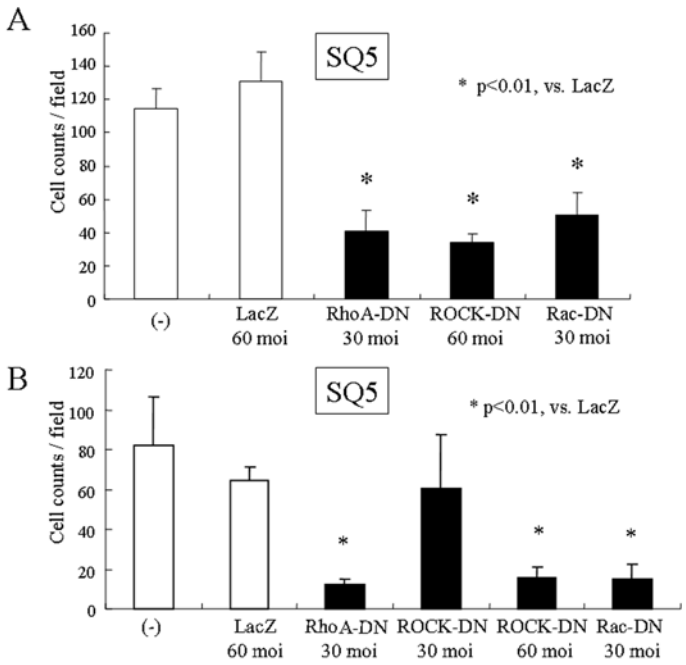

Fig. 6. Cell migration/invasion activity in SQ5 cells. Forty-eight hours after plating of adenovirus-transfected cells to Transwell® cell-culture chambers without (A. migration activity) or with (B. invasion activity) Matrigel® coating, the number of cells that had migrated to the lower surface area were counted under afs light microscope.

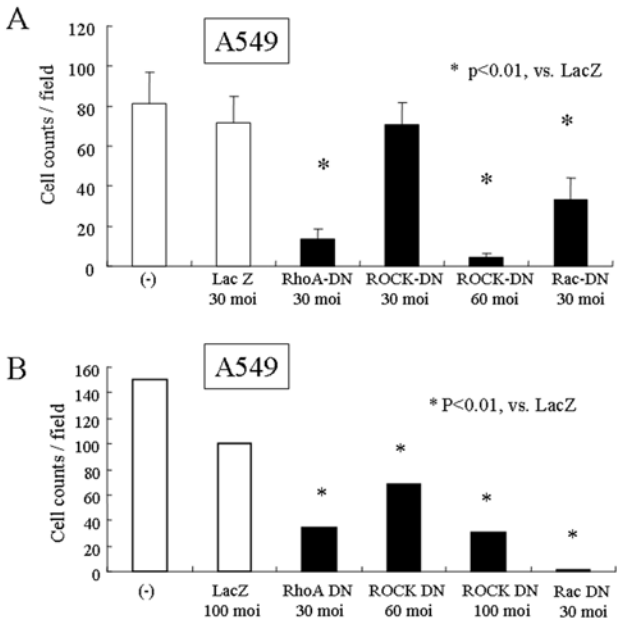

Fig. 7. Cell migration/invasion activity in A549 cells. Forty-eight hours after plating of adenovirus-transfected cells to Transwell® cell-culture chambers without (A. migration activity) or with (B. invasion activity) Matrigel® coating, the number of cells that had moved to the lower surface area were counted under a light microscope.

DISCUSSION

Cell migration is a critical step in tumor invasion and metastasis, and the understanding of this process may lead to appropriate therapies for treating cancer (5, 21). The actin cytoskeleton and its regulatory proteins are crucial for cell migration in most cells. It is well known that Rho family small GTPases play pivotal roles in reorganization of actin cytoskeleton. Among them, Cdc42, Rac and Rho are key regulators of actin assembly and control the formation of filopodia, lamellipodia and stress fibers, respectively (5). During cell movement, Rac and Cdc42 stimulate formation of protrusions at the leading edge, with localized actin polymerization, which has finger-like structures known as filopodia and sheet-like structures known as lamellipodia. On the other hand, Rho induces retraction at the trailing edge and regulates the assembly of contractile acto-myosin filaments through downstream effectors, such as ROCK/Rho kinase (13).

It is reported that cancer cells have two different types of migration morphology, Rac-dependent elongated cell motility and Rho/ROCK-dependent rounded bleb-associated mode of motility (16, 21). For example, NIH 3T3 cells transformed by the activated human Rac or RhoA genes have metastatic potential when injected into the mouse blood stream (4). In breast tumor, the expression of RhoA-DN, ROCK-DN or Rac-DN inhibited tumorigenesis in rat mammary adenocarcinoma cells (3). The invasive form of tumor cells is dependent on cell types and tissues. In our experiment, the migration and invasion activity of non-small cell lung carcinoma (NSCLC) cells, A549 and SQ5 cells, were significantly attenuated by adenoviral transfer of dominant negative mutant of Rho and Rac. On the other hand, it has recently been shown that small cell lung carcinoma (SCLC) cells expressed higher amount of RhoA than NSCLC cells (19). The inhibition of RhoA by C3 exoenzyme did not show detectable compaction of NSCLC cells, unlike SCLC cells. Although gene transfer was not tried in that study, this evidence suggested that it is useful to check the amount of Rho family small GTPases in each tumor cell to get more effective treatments.

In fact, a previous report analyzed the expression of Rho family proteins in colon, breast, and lung cancers (6). All breast tumors showed high levels of RhoA, Rac and Cdc42 proteins, whereas in the corresponding normal tissue these Rho proteins were hardly or not detectable. As shown in lung cancer in this study, all tumors showed largely enhanced levels of RhoA protein, whereas normal tissues showed only very low amounts of RhoA.

Transfection of MM1 rat hepatoma cells with cDNA encoding a constitutive active mutant of ROCK increased invasive activity, and ROCK-DN substantially attenuated the invasive phenotype. A specific ROCK inhibitor (Y-27632) blocked invasive activity of these cells (18). ROCK plays an essential role in tumor cell invasion, and these data demonstrate its potential as a therapeutic target for the prevention of cancer invasion and metastasis (10). Our western blotting analysis showed that the expression of ROCK in A549 and SQ5 cells was not increased strongly compared with Rho and Rac. In addition, relative low expressions of ROCK, ROCK-DN did not show complete inhibition of cell migration and invasion in these cell types.

Rho GTPases are also regulators of cell cycle progression (15). It was firstly demonstrated by observations that C3 exoenzyme inhibition of RhoA, or dominant negative inhibition of Rac or Cdc42 blocked serum-induced DNA synthesis in rodent fibroblasts (13, 20). Moreover, a previous study showed that transfection of a constitutively active form of Rac inhibits tumor cell response to apoptosis in M14 melanoma cells. Transfection of Rac-DN in the HaRas-expressing bladder carcinoma cell line decreased superoxide anion concentration, and resulted in a significant increase in tumor cell sensitivity to apoptosis (14).

Suppression of Rac activity via Rac-DN induced the death of glioma cells, primary glioblastoma and oligodendroglioma cells but not normal human adult astrocytes (17). In the some ways, all dominant negative forms of Rho small GTPases increased apoptotic responses in lung cancer cells as demonstrated in our study. These observations imply that gene therapy of Rho small GTPases into cancer cells can specifically control cell proliferation as well as cell migration and invasion.

In conclusion, we demonstrated that adenovirus-mediated gene transfection of dominant negative mutant form of Rho family GTPases with high efficiency caused significant inhibition of cell proliferation and motility in lung cancer cells. This was the first time to perform transfection into lung cancer cells, therefore our data supported the data that Rho family GTPases are promising targets to treat as well as other types of cancer. Since the cell responses were not identical among cell types and vectors, we suppose that the profile of small GTPases expression in each tumor cell is one of important determinants of cell destiny.

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