Reversal of the Malignant Phenotype of Gastric Cancer Cells by Inhibition of RhoA Expression and Activity

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ABSTRACT

Purpose: The small GTPase RhoA has been implicated in the regulation of cell morphology, motility, and transformation, but the role of RhoA protein in the carcinogenesis of gastric cancer remains unclear. In the present study, we have analyzed the expression status of the RhoA protein in human gastric cancer cells and tissues and investigated the possible involvement of RhoA in regulating the malignant phenotype of gastric cancer cells.

Experimental Design: RhoA expression was analyzed by immunohistochemistry and Western blot in gastric cancer tissues and cell lines. The RhoA-specific small interfering RNA (siRNA) vector was designed and constructed. We examined the role of RhoA in the malignant phenotype of gastric cancer cells by using siRNA knockdown and dominant-negative RhoA mutant suppression of endogenous RhoA activity.

Results: RhoA was found frequently overexpressed in gastric cancer tissues and cells compared with normal tissues or gastric epithelial cells. RhoA-specific siRNA could specifically and stably reduce RhoA expression up to 90% in AGS cells. Both RhoA-specific siRNA and dominant-negative RhoA expressions could significantly inhibit the proliferation and tumorigenicity of AGS cells and enhance chemosensitivity of the cancer cells to Adriamycin and 5-fluourouracil.

Conclusion: RhoA may play a critical role in the carcinogenesis of gastric cancer, and the interference of RhoA expression and/or activity could provide a novel avenue in reversing the malignant phenotype of gastric cancer cells.

INTRODUCTION

Rho family proteins are prominent members of the well-known Ras superfamily of small GTPases that can cycle between inactive GDP-bound state and active GTP-bound state and that exhibit intrinsic GTPase activities (1–3). Several Rho GTPases have been shown to regulate diverse signal transduction pathways and are involved in a variety of biological processes, including cell morphology (4–5), motility (6), proliferation (7), and apoptosis (8–9). Recently, a number of reports have shown that RhoA expression was up-regulated in a group of malignancies, including breast cancer, colon cancer, lung cancer, and ovarian cancer (10–14) and that the expression level of RhoA seemed to be positively correlated with the progress of these carcinomas, suggesting that RhoA may play an important role in tumorigenesis and tumor progression.

Gastric cancer is one of the most common malignancies throughout the world, especially in East Asian countries such as China, Japan, and Korea (15, 16). It has been known that gastric carcinogenesis is a multi-step process with morphological progression involving multiple genetic and epigenetic events, and an intestinal metaplasia-dysplasia-invasive carcinoma sequence exists (17). But the precise molecular mechanism of this progress remains largely unknown, and in particular, there have been little available data on the expression and function of Rho GTPase in human gastric cancer. In a previous study, our laboratory reported that the mRNA expression level of RhoA in gastric cancer tissue specimens were significantly higher than those in the adjacent nontumorous tissue specimens (18). In this study, we further analyze RhoA expression at the protein level in gastric cancer tissues and adjacent normal tissues by Western blotting and immunohistochemistry. We also show that the interference of RhoA expression and activity may provide an effective way to reverse the malignant phenotypes of gastric cancer cells.

MATERIALS AND METHODS

Tissue Collection. For immunostaining of RhoA, archival paraffin blocks of gastric specimens from 45 gastric cancer patients were collected from the Department of Pathology in our hospital. Tissue array containing 60 dots of gastric cancer tissues was purchased from Cybrdi Co. (Xi’an, China). For Western blotting, fresh surgical gastric cancer and adjacent normal tissues were obtained from 19 patients who underwent surgery at the Department of General Surgery in our hospital. All gastric cancer cases were clinically and pathologically proved. The protocols used in the studies were approved by the Hospital’s
Protection of Human Subjects Committee. Patients having fresh surgical tissue for the study signed informed consent.

Cell Lines, Drugs, and Animals. Gastric cancer cell lines AGS, MKN45, SGC7901, KATOIII, and SV40-transformed immortal gastric epithelial cell GES-1 were preserved in our institute. XGC-9811 was established from tumor cells isolated from a human malignant ascites sample in our institute. Adriamycin (ADR) and 5-fluorouracil (5-Fu) were freshly prepared before each experiment. BALB/c nude mice, 4 to 6 weeks old, were provided by Shanghai Cancer Institute for the in vivo tumorigenicity study.

Immunohistochemistry. The avidin-biotin complex immunoperoxidase method was used to examine RhoA expression by immunostaining (12). Briefly, 4 μm-thick tissue slides were deparaffinized in xylene and rehydrated serially with alcohol and water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes, followed by microwave antigen retrieval. The slides were then incubated with anti-RhoA monoclonal antibody (1:75 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 37°C for 1 hour. The Vectastain ABC kit, mouse IgG, Elite Series (Vector Laboratories, Burlingame, CA) was used according to the manufacturer’s instructions for detection. Preimmune serum was used instead of first antibody as negative control.

Expression of RhoA was evaluated to the ratio of positive cells per specimen and staining intensity as described previously (19). The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of 0–2%, 1 for staining of 2 to 25%, 2 for staining of 26 to 50%, 3 for staining of 51 to 75%, and 4 for staining >75% of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total score of 0 to 12 was finally calculated and graded as negative (-; score: 0–1), weak (+; 2–4), moderate (+ +; 5–8), and strong (+ + +; 9–12).

Western Blotting. Tissues or cells were lysed in a lysis buffer. Proteins were separated on 12% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were then first stained with first antibodies, followed by incubation with a biotinylated secondary antibody. Enhanced chemiluminescence (Amersham, Freiburg, Germany) was used for detection. Antibodies of RhoA (diluted 1:100) and RhoC (diluted 1:50) were purchased from Santa Cruz Biotechnology. Antibodies of Rac (diluted 1:1000) and Cdc42 (diluted 1:100) were obtained from Upstate Biotechnology (Lake Placid, NY), and antibody of β-actin was obtained from Sigma Chemical Co. (St. Louis, MO).

Structures. Two pairs of hairpin small interfering RNA (siRNA) oligos for RhoA were designed according to the principles described before (20). For construct U6/siRhoA-1 (targeted to nucleotides 120–138), sense sequence was 5’-ttgattatgcatctgctagcacaatgtggcagatcttttt3’ and antisense sequence was 5’-etgaaaaagatatgcgtagctgcagcagtacttttt3’. For U6/siRhoA-2 (targeted to nucleotides 186–204), sense sequence was 5’-ttgattatgcatctgctagcacaagatgtggcagatcttttt3’ and antisense sequence was 5’-etgaaaaagatactgcgtagctgcagcagagttatgttttt3’. Sense and antisense oligos were annealed and ligated between the BbsI and XbaI sites into mU6pro (kindly provided by Professor Dave Turner, University of Michigan, Ann Arbor, MI), respectively.

Cell Transfection. Cells were plated and grown to 70 to 90% confluency without antibiotics. Transfections were done with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. For stable expression of siRNAs, cells transfected with 1 μg of siRNA expressing vectors and 100 ng of pCEFL-GST-neo plasmid were selected with 700 μg/ml G418 for 12 days. Clones were picked and expanded for an additional 2 months. Transient transfection was done similar to what was described above for stable transfections, but without the neomycin-resistant vector. Experiments with the transiently transfected cells were done 72 hours after the transfection.

Pull Down Assay. Active RhoA in cell lysates (200 μg) was precipitated with 15 μg GST-RBD (containing amino acids –8–89 of Rhotekin), which was expressed in Escherichia coli and bound to agarose beads. The precipitates were washed three times in washing buffer [50 mmol/L Tris (pH 7.2), 150 mmol/L NaCl, 10 mmol/L MgCl2, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin], and after adding the loading buffer and boiling for 5 minutes, the bound proteins were resolved in 12% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-RhoA antibody as described above.

Flow Cytometry. Cells were fixed with 70% ethanol at –20°C overnight, washed with 1× PBS, and stained with propidium iodide (50 μg/ml) in 1× PBS supplemented with RNase (10 mg/ml) for 30 minutes. Flow cytometry was done on a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with Cellquest software, and cellular DNA content was determined for 1 × 104 cells.

Monolayer Growth Rate. Monolayer culture growth rate was determined as described previously (21) by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) to a water-insoluble formazan by viable cells. Three thousand cells in 200 μL of medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 4, and 5 days and absorbance values were determined on the enzyme-linked immunosorbent assay reader (DASIT, Milan, Italy) at 492 nm.

Soft Agar Clonogenic Assay. Anchorage-independent growth as a characteristic of in vitro tumorigenicity was assessed by soft agar clonogenic assay. Briefly, cells were detached and plated in 0.3% agarose with a 0.5% agarose underlay (1 × 104/well in 6-well plates). The number of foci >100 μm was counted after 17 days.

Tumor Growth in Mice. For analysis of the tumorigenicity of AGS cells in vivo, 1 × 107 cells were injected into peritoneal cavity of athymic nude mice. Survival time of mice was recorded from the day of tumor cells injection.

In vitro Drug Sensitivity Assay. MTT assay as described previously (22, 23) was used to evaluate the sensitivity of gastric cancer cells to anticancer drugs. In brief, cells were treated with different concentrations of ADR or 5-Fu for 48 hours. Relatively inhibitory rate of cell growth by drugs was calculated according to the following formula: R = (A2–A1)/ A2 × 100% in which R is relatively inhibitory rate of cell growth; A1 is absorbance value of cells in the presence of drugs; and A2 is absorbance value of control cells without any drug treatment.
Wound-Healing Assays. For wound-healing assays, cells were plated at $2 \times 10^5$/dish density in 60-mm diameter dishes. A plastic pipette tip was drawn across the center of the plate to produce a clean 1-mm-wide wound area after the cells had reached confluency. After a 48 hours culturing in medium with 1% FBS, a phase-contrast microscope was used to examine cell movement into the wound area (24).

Invasion Assays. A transwell plate (Costar, Corning, NY) precoated with Matrigel (Becton-Dickinson) was used to perform cell invasion assays. Briefly, cells were suspended in 0.2 ml of culture medium with 1% FBS and were added to the upper chamber. Ten percent FBS in the culture medium was plated in the lower chamber as chemoattractant. Cells in the invasion chambers were incubated in a humidified incubator for 24 hours. The cells that traversed membrane pore and spread to the lower surface of the filters were stained with 5% Giemsa solution for visualization (24).

Statistical Analysis. Statistical analysis was performed with Kruskal-Wallis rank test and the Mann-Whitney U test was used to calculate the P value and to compare the differences of groups for immunohistochemistry. Assays for characterizing phenotype of cells were analyzed by Student’s test. Statistical SPSS software package (SPSS Inc, Chicago) was used to analyze data. Differences were considered statistically different at $P < 0.05$.

RESULTS

Immunohistochemical Analysis of RhoA Expression in Human Gastric Cancer Tissues. To examine whether the RhoA expression level is altered in gastric cancer, the expression and subcellular localization of RhoA were studied in a set of gastric cancer patient specimens derived from 20 normal gastric mucosae (NG), 6 intestinal metaplasias (IM), 7 dysplasias (D), and 102 gastric cancer tissues (GC). RhoA was found expressed predominantly in the cytoplasm of the lower two-thirds of the normal epithelium (Fig. 1A). The RhoA staining in epithelial cells from the NG samples was weak, and the average staining score was $3.6 \pm 1.13$. In intestinal metaplasia and dysplasia, RhoA could be detected in most epithelial cells examined with a higher expression level than that of the NG samples with an average staining score $4.4 \pm 2.65$ (Figs. 1B). In gastric cancer cells, RhoA was found mostly in the cytoplasm with a minor population in the plasma membrane, and staining was consistently stronger than that of the NG samples as well (Figs. 1C-D) with an average score $6.3 \pm 2.90$ (Fig. 2; $P = 0.003$).

To investigate if the RhoA expression might be associated with the progression of gastric cancer, the RhoA expression levels and the clinicopathologic characteristics of 102 gastric cancer patients were compared as summarized in Table 1. It is apparent that RhoA expression did not correlate with differen-

![Fig. 1](https://cancerres.aacrjournals.org) Immunohistochemical staining of RhoA in normal gastric tissue and in gastric cancer at different stages of differentiation. The monoclonal goat anti-RhoA antibodies (SC-418) were used to stain paraffin sections. A, normal epithelium exhibiting positive RhoA immunostaining only in lower glands. B, intestinal metaplasia; C, dysplasia; D, well-differentiated; E, moderately-differentiated; and F, poorly-differentiated gastric cancer tissues showing moderate or strong RhoA immunosignals in most epithelial cells. Original magnifications, $\times 10$. 

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tiation grade, Tumor-Node-Metastasis stage, gross type, or metastasis with a statistic $P < 0.05$ in each parameter. These results provide evidence that RhoA expression level positively correlates with gastric cancer onset but is not associated with the progressiveness of the cancer.

**Analysis of RhoA Expression in Gastric Cancer Cells and Tissues.** Expression levels of RhoA, Rac1, and Cdc42 were examined by Western blotting in the gastric cancer and adjacent normal tissues taken from 19 patients (Fig. 3A). In 15 of the 19 cases or 79% samples, RhoA was found overexpressed in cancerous tissues, consistent with the result from immunohistochemistry analysis. In contrast, there were no apparent differences in Rac1 or Cdc42 expression between the gastric cancer and adjacent normal tissues (Fig. 3A), suggesting that the increased expression of RhoA might be a unique feature in gastric cancer. We further compared the relative RhoA expression level in five different gastric cancer cell lines, i.e., AGS, MKN45, XGC-9811, SGC7901, and KATOIII, with that of the normal gastric cell line GES-1. Again, RhoA was found expressed at a higher level in all five gastric cancer cell lines than in GES-1 cells (Fig. 3B). These results suggest that RhoA may have a role in the carcinogenesis of gastric cancer.

**siRNA Targeting the RhoA Expression in AGS Cells.** To further analyze the involvement of RhoA in gastric cancer malignant phenotype, we took advantage of the powerful siRNA technology. AGS cells were transiently transfected with two different siRNA constructs, and the protein knockdown effects were compared by anti-RhoA Western blot. The construct U6/siRhoA-2, but not U6/siRhoA-1, significantly suppressed RhoA expression up to 90%, whereas $\beta$-actin protein levels were unaffected. Control experiments with the empty vector alone showed a negligible effect on RhoA expression (Fig. 4A). To further confirm the stable suppression effect of siRNAs, AGS cells were cotransfected with U6/siRhoA-2 and pCEFL-GST-neo. After G418 selection for 2 months, Western blot analysis revealed that the selected stable clones of the siRhoA transfectants also showed substantial reduction in RhoA level, but the RhoA-specific siRNA did not detectably affect the expression of three closely related Rho proteins, RhoC, Rac1, and Cdc42, in AGS cells (Fig. 4B). By pull-down assay, we found that the endogenous RhoA activity was also inhibited by siRNA expression as well as the dominant-negative RhoA (N19RhoA) expression (Fig. 4C).

**Interference of RhoA Expression or Activity Inhibited AGS Cell Proliferation.** Because RhoA is known to be involved in regulating cell growth (25), the inhibition of RhoA expression or activity may result in suppression of the cancer cell growth. We found that AGS/siRhoA or AGS/N19RhoA cells grew significantly slower than the control cells (AGS/U6 and AGS/neo; Fig. 5). Cell cycle analysis revealed that the population of cells in the S phase was remarkably decreased by U6/siRhoA-2 expression (15.43% of asynchronous AGS/siRhoA cells in the S phase of the cell cycle compared with

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**Table 1** Clinicopathological associations of RhoA expression in patients with gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th>Normal gastric mucosae</th>
<th>Intestinal metaplasia and dysplasia</th>
<th>Gastric carcinoma</th>
<th>Differentiation</th>
<th>Gross type (Borrman)</th>
<th>TNM</th>
<th>Metastasis</th>
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<tr>
<td>Total number of cases</td>
<td>20</td>
<td>13</td>
<td>102</td>
<td>39</td>
<td>13</td>
<td>18</td>
<td>53</td>
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<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>–</td>
<td>3 (15.0%)</td>
<td>4 (30.8%)</td>
<td>12 (60.0%)</td>
<td>16 (84.2%)</td>
<td>8</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>+</td>
<td>12 (60.0%)</td>
<td>5 (38.5%)</td>
<td>5 (5.0%)</td>
<td>15 (78.9%)</td>
<td>13 (84.6%)</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>++</td>
<td>5 (25.0%)</td>
<td>2 (15.4%)</td>
<td>43 (42.2%)</td>
<td>16 (84.2%)</td>
<td>22 (80.0%)</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>+++</td>
<td>0 (0%)</td>
<td>1 (7.7%)</td>
<td>21 (20.5%)</td>
<td>15 (78.9%)</td>
<td>11 (37.9%)</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

NOTE. Interpretation of RhoA staining was described in Materials and Methods. RhoA staining was graded as negative (–; score: 0–1), weak (+; 2–4), moderate (++; 5–8), and strong (+++; 9–12).
27.96% of control cells) or N19RhoA mutant expression (17.57% of AGS/N19RhoA cells compared with 25.50% of controls; Table 2). These results indicate that RhoA plays an important role in driving cell cycle progression to promote gastric cancer cell proliferation.

Interference of RhoA Expression or Activity Inhibited Tumorigenicity of AGS Cells. Anchorage-independent growth could be a sensitive marker for tumor growth in vivo; therefore, we examined whether interference of RhoA expression or activity would cause an inhibition of AGS cell growth in soft agar. As shown in Fig. 6, both siRNA transfection and N19RhoA-mutant expression resulted in a substantial reduction in the colony number (0–4 and 0–3 colonies, respectively) when compared with the control cells (AGS/U6 and AGS/neo, 37–46 and 43–55, both \(P < 0.001\)). To confirm this effect in vivo, we injected the siRNA or N19RhoA-mutant expressing AGS cells into peritoneal cavity of athymic nude mice to see if they might affect tumor induction. We found that mice receiving an injection with control cells (\(n = 5\) each group) lose weight quickly, and the survival time was 36.4±5.77 days for the AGS/U6 mice and 37.0±8.51 days for the AGS/neo mice, both significantly shorter than that of mice receiving injection with AGS/siRhoA or AGS/N19RhoA cells (63.2±6.98 and 58.8±8.91 days, \(P < 0.001\) and \(P = 0.004\), respectively). These results show that interference with RhoA expression or down-regulation of RhoA activity could lead to inhibition of gastric tumor cell growth in vivo.

Interference of RhoA Expression or Activity Sensitized AGS Cells to Anticancer Chemotherapy. To explore the role of RhoA in the regulation of chemosensitivity of human-gastric cancer cells, we compared the drug sensitivity of AGS/siRhoA or AGS/N19RhoA cells with that of control cells, using the MTT assay. As shown in Fig. 7, either U6/siRhoA-2 or N19RhoA expression significantly enhanced the sensitivity of AGS cells to ADR or 5-Fu treatment at three differently doses, suggesting that down-regulation of RhoA may have a beneficial effect in chemosensitivity.

Interference of RhoA Expression Did Not Affect Migration and Invasion of AGS Cells. To see if the elevated RhoA level/activity might have an effect on cell movement, we compared the migration rate of the tumor cells in a wound-healing assay. Figure 8A shows that the N19RhoA mutant, but not the siRNA construct, significantly decreased cell migration from the...
edge of the wound. Similarly, when the cell invasion potential was measured in a Matrigel-coated transwell assay (Fig. 8B), we found that RhoA expression knockdown by siRNA expression did not affect the invasion ability of AGS cells ($P = 0.326$), but N19RhoA was able to significantly inhibit the tumor cell invasion ($P = 0.000$). Therefore, it seems that the use of RhoA knockdown by siRNA has a differential effect from that of dominant-negative RhoA mutant in tumor cell migration/invasion, which may reflect the relative nonspecific nature of the N19RhoA application. Combined with the above cell proliferation results, these data further suggest that RhoA may be specifically involved in the cancer cell cycle regulation but not invasion.

**DISCUSSION**

RhoA, a prominent member of Rho family GTPases, is responsible for the function of reorganizing the actin to form stress fiber and focal complex formation. It is also involved in the process of tumorigenesis and tumor progression. Although no activate RhoA mutation similar to that of oncogenic Ras has been found in human tumors, some researchers reported recently that RhoA was overexpressed in various types of tumors (10–14). The current study was designed to evaluate the expression and biological significance of RhoA in human gastric cancer, because little information on this subject is available. We show that the expression level of RhoA protein is significantly higher in gastric cancer tissues and cells than that in normal gastric mucosae or immortal gastric epithelial cells by immunohistochemical examination and Western blotting analysis. In a number of previous reports, it was shown that RhoA was involved in progression of testicular germ cell, upper urinary tract, bladder, and ovarian cancer (11–13, 26), suggesting that RhoA might be a useful prognostic factor in these diseases. We found that RhoA expression was not associated with the differentiation grade, Tumor-Node-Metastasis stage, gross type or metastasis, suggesting that although up-regulation of RhoA may be associated with gastric cancer properties, the expression level of RhoA does not correlate with the progressiveness of the disease.

To further show the contribution of RhoA to the malignant phenotype of gastric cancer cells, we attempted to interfere with...
RhoA function by using the dominant-negative RhoA mutant and siRNA method. Dominant-negative mutants are conventional molecular tools to study the function of Rho GTPases. However, the limit of the specificity of dominant-negative mutant such as N19RhoA, which acts to sequester the upstream guanine nucleotide exchange factors, makes distinguishing the closely related Rho GTPases such as RhoA, RhoB, and RhoC difficult. A useful method, named RNA interference, has been developed recently to allow introduction of double-stranded RNA to the host cells leading to the silencing of specific cellular gene (27–28). Additional studies showed that 21 to 23 nucleotides resembling the processing products from long double-stranded RNA, called siRNAs, could induce RNA interference directly in mammalian cells (29). Compared with other gene expression interference strategies, the siRNA method could inhibit target gene expression with exquisite specificity, efficiency, and endurance, which could be advantageous in elucidating subtype-specific functions of closely related family members (30–31).

By applying dominant-negative RhoA-mutant and RhoA-specific siRNA constructs, we discovered a role of RhoA in the proliferation and tumorigenesis of gastric cancer cells. Previously, it has been shown that RhoA activation is necessary for normal cell growth as well as for maintaining the transformed phenotype of fibroblasts (32–34). RhoA was also shown to be important in regulation of cell cycle progression through inactivation of the cyclin-dependent kinase inhibitors p27kip1 and p21cip1 and increasing cyclin D1 promoter activity (35, 36). We observed that AGS cells transfected with siRNA or dominant-negative RhoA-mutant constructs grew significantly slower than control cells and showed a much shortened S phase. This is distinct from the observed cell growth arrest in the G2 phase described by Ghosh (25) and may indicate that the RhoA signals responsible for cell cycle change might differ among different cell types. We further show that RhoA activity was necessary for the anchorage-independent growth of AGS cells, and inhibition of RhoA suppressed the tumorigenicity of the cells in nude mice. All these data suggest that RhoA contributes to the malignant phenotype of gastric cancer, and it could be a useful therapeutic target for gastric cancer.

Chemotherapy is one important strategy in the treatment of gastric cancer, but it often fails because of the resistance of gastric cancer cells to anticancer agents. It would be very helpful to develop new chemo-sensitizing agents. RhoA plays a critical role in the regulation of the assembly and organization of the actin cytoskeleton and gene transcription that may contribute to the survival signals (1). It was reported that overexpression of a constitutively active form of RhoA protein enhanced resistance

**Fig. 7** Role of RhoA in regulating chemosensitivities of AGS cells. Inhibitory effects of different concentration of ADR or 5-Fu on cells were evaluated by MTT assay. Relative inhibitory rate was calculated as described in Materials and Methods. Results showed that siRNA and N19RhoA both enhanced the cytotoxicity of AGS cells to ADR and 5-Fu. Values represent mean (SEM) from at least three separate experiments. *, P < 0.05; **, P < 0.01. □, AGS/U6; ■, AGS/siRhoA; ◊, AGS/neo; ●, AGS/N19RhoA.

**Fig. 8** Role of RhoA in regulating the migration and invasion ability of AGS cells. A, cell migration was measured by the wound-healing assay described in Materials and Methods. The cells were incubated for 48 hours after cell wounding, and then the photos were taken. B, the invasive activity of cells was assayed in a Matrigel-coated transwell. The cells that succeeded in invading the Matrigel were quantified 24 hours after plating. Data were expressed relative to the invasion ability of AGS cells. Values represent the mean (SEM) from at least three separate experiments. *, P = 0.000.
of HeLa cells to the cytotoxic effects of Clostridium difficile toxins. Blockade of RhoA function by exoenzyme C3 led to a reduction of human endothelial cell survival (37). Here we show for the first time that inhibition of RhoA expression or activity could lead to the substantial enhancement of sensitivity of AGS cells to two different types of anticancer drugs, ADR or 5-Fu, and this provides new leads to potential chemo-sensitizing strategies.

Although some researchers showed that RhoA was involved in tumor invasion and metastasis, and overexpression of RhoA could promote tumor cell invasion (1, 38), inhibition of RhoA expression by siRNA construct did not affect the migration and invasion activity of gastric cancer cells, in consistent with results from RhoA expression analysis in gastric cancer tissues. However, dominant-negative RhoA expression did cause the suppression of AGS cell migration and invasion, in contrast to the RhoA-specific siRNA effect. This discrepancy may come from the relative nonspecific nature of N19RhoA mutant. For example, N19RhoA mutant is an antagonist of the guanine-nucleotide exchange factors for multiple Rho proteins including RhoC, which was revealed as a key regulator of migration and metastasis in human tumors (39). These results also highlight the extraordinary specificity of the siRNA technology that allows the distinction between RhoA and other related molecules. Consistent with this interpretation, our previous work using the RhoGAP fused with the carboxyl terminal intracellular localization sequences of Rho proteins also helps establish that distinct subtypes of Rho GTPases contribute to different phenotypes of cancer cells (24).

In conclusion, overexpression of RhoA protein was found to be a frequent event in human gastric cancer. With siRNA technology, we show that down-regulation of RhoA expression could suppress gastric cancer cell growth and increase the chemosensitivity of the cancer cells to chemotherapeutic drugs. This effect was similar, but greater than that with the use of a dominant-negative mutant of RhoA. Therefore, RhoA is likely to play an important role in the tumorgenesis of human gastric cancer, and therapeutic strategies targeting RhoA and RhoA-mediated pathways such as the use of RhoA-specific siRNA may be a novel avenue in gastric cancer intervention.

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