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Anti-Invasion Effect of Rosmarinic Acid via the Extracellular Signal-Regulated Kinase and Oxidation–Reduction Pathway in Ls174-T Cells

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ABSTRACT

Rosmarinic acid is a major phenylpropanoid isolated from *Prunella vulgaris* L., which is a composition of herbal tea for centuries in China. However, the anti-invasion activity on Ls174-T human colon carcinoma cells has not been studied. In this study, we investigated the anti-metastasis functions according to wound healing assay, adhesion assay, and Transwell assay and found that rosmarinic acid could inhibit migration, adhesion, and invasion dose-dependently. Rosmarinic acid also could decrease the level of reactive oxygen species by enhancing the level of reduced glutathione hormone. In addition, rosmarinic acid repressed the activity and expression of matrix metalloproteinase-2,9. According to Western blot and quantitative real-time PCR assay, rosmarinic acid may inhibit metastasis from colorectal carcinoma mainly via the pathway of extracellular signal-regulated kinase. In animal experiment, intraperitoneal administration of 2 mg of rosmarinic acid reduced weight of tumors and the number of lung nodules significantly compared with those of control group. Therefore, these results demonstrated that rosmarinic acid can effectively inhibit tumor metastasis in vitro and in vivo. J. Cell. Biochem. 111: 370–379, 2010.

KEY WORDS: ROSMARINIC ACID; ANTI-METASTASIS; MATRIX METALLOPROTEINASES; EXTRACELLULAR SIGNAL-REGULATED KINASE; OXIDATION–REDUCTION

Colorectal carcinoma was reported to have high incidence and mortality rates worldwide, with approximately 940,000 new cases and nearly 500,000 deaths annually. It has also become one of the most common malignant tumors in China, ranking fourth currently in cancer incidence. Nearly 40% of patients with colorectal cancer would either present with metastases or develop recurrences and eventually die from the disease [Kohne et al., 1998]. However, the treatment to metastasis is still far from satisfaction, mainly derived from a lack of effective drugs. So, it is critical to find new effective drugs to fight against metastasis for colorectal carcinoma.

Traditional Chinese herbal medicines have a long history and some of the Chinese herb medicine have been used in tumor treatment and showed promising results. *Prunella vulgaris* L., a composition of heat tea, is considered as an effective remedy for the treatment of some "heat-like" symptoms. It possesses a wide spectrum of biological effects including anti-microbial, anti-inflammatory, and immunomodulatory activity [Han et al., 2009]. In particular, *P. vulgaris* L. has a certain effect for many malignant tumors, for example, human gingival fibroblasts [Zdarilová et al., 2009] and human thyroid cancer cell line SW579 [Du et al., 2009]. It has been found that rosmarinic acid (RA), a phenylpropanoid

Abbreviations: RA, rosmarinic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BCIP/NBT, 5-bromo-4-chloro-3-indolyl -phosphate/nitroblue tetrazolium; ECM, extracellular matrix; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; ROS, reactive oxygen species; GSH, reduced glutathione hormone; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; EGFR, endothelial growth factor receptor; VEGFR, vascular endothelial growth factor receptor; RI, rate of inhibition; LLC, Lewis lung carcinoma.

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isolated from *P. vulgaris* L., shows the biological activities involved in anti-cancer [Ma et al., 2006], anti-oxidative [Qiao et al., 2005], anti-inflammatory [Youn et al., 2003], anti-mutagen, anti-bacterial, and anti-viral activities [Parnham and Kesselring, 1985]. However, the anti-metastatic ability of RA has not been studied. Therefore, in the present study, we examined the effect of RA on metastasis of cancer cells in vitro and in vivo, and further investigated the anti-metastatic mechanisms of it with special reference to the process of cell invasion.

MATERIALS AND METHODS

MATERIALS

RA was isolated and provided by Pharmacy Department, Shuguang Hospital Affiliated with Shanghai University of Traditional Chinese Medicine. The structure of the compound is shown in Figure 1. Structure was assessed by NMR and ESI-MS and purity was assessed by HPLC as >98%.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) were purchased from Sigma Chemical Co.(St. Louis, MO); human reactive monoclonal antibodies, anti-MMP-2, anti-MMP-9, anti-NF-κB, and anti-ERK, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and human reactive polyclonal antibodies anti-Akt, anti-phosphor-Akt, and anti-phosphor-ERK were provided by Cell Signaling Technology (Beverly, MA).

CELLS AND ANIMALS

Human Colon Cancer Cell Line Ls174-T cells were obtained from Cell Bank of Chinese Academic of Science and were cultured in PRIM medium 1640 (Gibco Industries, Inc.) with 10% (v/v) dialyzed heat-inactivated bovine serum (BS) (Gibco Industries, Inc.) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. KM mice (6 weeks old) and male C57BL/6 mice (6 weeks old), License number: 2007-0002 SCXK, were obtained from the Animal Center of Fudan University, and maintained on standard chow and water.

CELL VIABILITY ASSAY

Cell viability was determined by the MTT assay [Mareel and Leroy, 2003]. Half inhibition concentration (IC_{50}) means the concentration of the drug when survival rate of cells reaches 50%.

WOUND HEALING ASSAY

For wound healing assay, Ls174-Tcells were plated in 24-well microtiter cell culture plates. A plastic pipette tip was drawn across the center of the plate to produce a clean 1-mm wide wound area after the cells have reached confluence. Cell movement into the wound area was examined. After a 24-h culturing with and without RA, the migration distances between the leading edge of the migrating cells and the edge of the wound were compared. Migration rate = (migration distances of drug treated cells/migration distances of untreated cells) × 100% [Zhang et al., 2005].

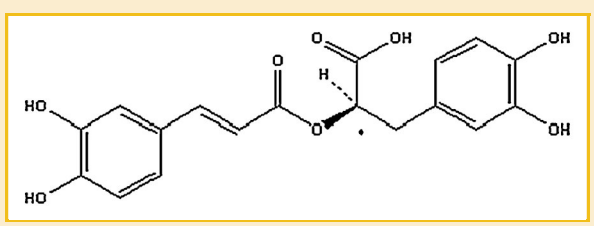

Fig. 1. Structure of RA.

CELL ADHESION ASSAY

Ls174-T cells were pre-treated with or without different concentrations of RA for 24 h. Then cells were suspended in serum-free RPMI medium 1640 to form a single-cell suspension, 10 mM of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 ml of DMSO and the absorbance at 570 nm was measured on a microplate reader [Zhang et al., 2005].

INVASION ASSAY

Ls174-T cells were incubated with or without different concentrations of RA for 24 h. Invasiveness into the reconstituted basement membrane Matrigel (Becton Dickinson Labware) [Liu et al., 2000] was assayed.

GEL ZYMOGRAM ASSAY

Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in the 9% polyacrylamide gel containing 0.1% (m/v) gelatin [Wu et al., 2008]. Clear bands of gelatinolytic activity were visualized after staining the gel with Coomassie blue. At last, the gel was photographed.

INTRACELLULAR ROS AND GSH LEVELS ANALYSIS

Reactive oxygen species (ROS) determination was performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described [Yang et al., 2005]. Cellular GSH levels were analyzed following the instruction of the Glutathione Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

WESTERN BLOT ANALYSIS

Cells (1×10^6/dish) were seeded in 10-cm dishes. After incubation for 12 h, cells were treated with or without different concentrations of RA for an additional 24 h. The Western blotting assay was performed as described previously [Zhang et al., 2005].

EGFR AND VEGFR LEVELS ANALYSIS

The activities of EGFR and VEGFR kinase were analyzed using HTScan® EGF Receptor Kinase Assay Kit and HTScan® VEGF Receptor 2 Kinase Assay Kit (Cell Signaling Technology).

DELFIA® Streptavidin Microtitration Plates were prepared, and the other steps were followed by instructions. The concentrations of RA were used as 0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}. After the addition of the primary antibody for 1 h, DELFIA® Eu-N1 rabbit anti-mouse-IgG was added. At last, DELFIA® Enhancement Solution was mixed in, and then detected by a fluorescence multiwell plate reader.

QUANTITATIVE REAL-TIME PCR ASSAY

Total RNA of cells treated without or with different concentrations of RA was extracted using TRIZOL TM reagent (Promega Corporation) according to the supplier's instructions. And then

RNA was reverse-transcribed with the Reverse Transcription System (Takara Shuzo, Shiga, Japan). Real-time PCR was performed using SYBR Green Supermix with an iCycler® thermal cycler (Bio-Rad). Primers were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China) and their sequences were: 5′-GGTCGGAGTCAACGGATTTG-3′ (sense) and 5′-ATGAGCCCCAGCCTTCTCCAT-3′ (anti-sense) for GAPDH; 5′-TTGCTGCTCTACCTCCAC-3′ (sense) and 5′-AATGCTTTCTCCGCTCTG-3′ (anti-sense) for VEGF; 5′-CCCTGATGTCCAGCG-3′ (sense) and 5′-AGCAGCCTAGCCAGTC-3′ (anti-sense) for MMP-2; 5′-CACTGTCCACCCCTCAGAGC-3′ (sense) and 5′-GCCACTTGTCGGCGATAAGG-3′ (anti-sense) for MMP-9.The cycling conditions included an initial 3 min polymerase activation at 94°C followed by 40 cycles at 94°C for 30 s, reannealing temperature (55, 48, and 55°C for VEGF, MMP-2, and MMP-9, respectively) for 40 s and 72°C for 30 s. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted CT).

ACUTE TOXICITY ASSAY

The index generally used to evaluate acute toxicity is the LD_{50}. In this study, the LD_{50} was determined by improved Karber's method [El-Sayed et al., 2009]. Acute toxicity is usually tested in two steps: (1) in an initial investigation, the range of doses producing the toxic effects is established; (2) based on the results, further specific doses are administered to calculate the LD_{50}. Briefly, in the initial investigation, KM mice at 6 weeks of age were randomized into six groups each comprising two mice, one male, and the other female. These groups were treated with RA by tail intravenous injection at doses of 31.25, 62.5, 125, 250, 500, and 1,000 mg/kg, respectively. The mortality of the mice was recorded for up to 7 days in each group after the treatment. Based on the results of the initial investigation, new dosages, 62.5, 94.7, 143.6, 217.6, 329.9, and 500 mg/kg, were separately administered to the groups (10 animals for each group, 5 male, and 5 female mice) in the second test. The mortality was recorded. The LD_{50} was calculated according to these formulas:

$$LD_{50}\ \log^{-1}[X_m - i(\sum P - 0.5)]$$

where X_m is the logarithm of the maximal dose; i the logarithm of ratio between the neighboring two dose group (high dose and low dose); P the mortality for each group; $\sum P$ the summation of the mortalities; and n the number of animals for each group. $S_{X50} = i\sqrt{\frac{(\sum P - \sum P^2)}{n-1}}$ where S_{X50} is the standard error for log LD_{50}; X_{50} the log LD_{50}; 95% confidence interval of $LD_{50} = \log^{-1}(X_{50} \pm 1.96 S_{X50})$; average confidence interval of LD_{50} = (higher limit of 95% confidence interval of LD_{50} − lower limit of 95% confidence interval of LD_{50})/2.

ANTI-METASTATIC ACTIVITY IN VIVO

The mouse implanted with the Lewis lung carcinoma (LLC) cells were killed by cervical dislocation. Under sterile environment, tumor tissues were dissected. Harvested tumor cells were suspended in PBS. Then the suspension was implanted in the flanks of experimental mice (10^5 cells per mouse in 200 μl of PBS) [Tang et al., 2006]. The tumor-bearing mice were randomly divided into five groups, and each group consisted of eight mice. The tumor-bearing mice were treated with RA via celiac injection at the dosage of 1, 2, and 4 mg/kg for 20 days. And the control positive group was treated with vehicle mixture and vinblastine 2 mg/kg, respectively. At the end of the experiment, mice were euthanized by cervical dislocation, and the solid tumors were picked up and weighed. Data were statistically analyzed by solid tumor weight. The rate of inhibition (RI) was calculated according to the formula: RI = [(mean tumor weight of the experimental group − mean tumor weight of the control group)/mean tumor weight of the control group] × 100%. Meanwhile, their lungs were excised, rinsed, and fixed in Bouin's solution. The total number of visible nodules on the lung surface per mice was counted under light microscope.

STATISTICAL ANALYSIS

Each experimental value was expressed as means ± standard deviation (SD). Significant differences between the groups were determined using the unpaired Student's *t*-test. Statistical analysis was performed using the Origin 7.5 software to evaluate the significance of differences between groups considered as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. All data points represented the mean of triplicates.

RESULTS

CYTOTOXICITY EFFECT OF RA ON Ls174-T CELLS

To rule out the possibility whether the restrictive metastasis effect of RA on Ls174 cells was caused by the cytotoxicity, the cells' viability of RA on Ls174-T cells was evaluated. The cytotoxicity of RA against Ls174-T cells was shown in Figure 2, when the concentration of RA was varied from 0 to 80 μg/ml, the viability of cells was found to maintain above 70% all along, which indicated that the inhibition effect of RA on metastasis was not accompanied with cytotoxicity.

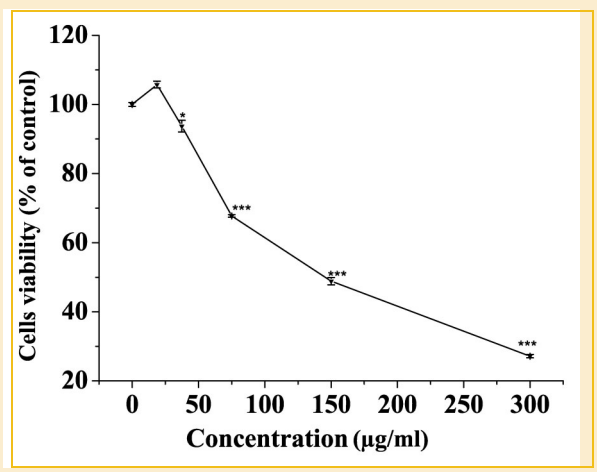

Fig. 2. Cell toxicity of RA on Ls174-T cells. All data were presented as the mean ± SD of three parallel samples in each team. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

EFFECT OF RA ON THE MIGRATION, ADHESION, AND INVASION OF Ls174-T CELLS

To evaluate the anti-metastatic effect of RA on Ls174-T cells, we first assessed the inhibitory effect of RA on the migration of Ls174-T cells by wound healing assay. As shown in Figure 3A,B, the cellular motility was obviously inhibited in a dose-dependent manner by RA. RA at the concentration of 80 μg/ml decreased the migration of Ls174-T cells by 83.33% ($P < 0.001$). Previous studies have demonstrated that the adhesion of cancer cell to ECM components plays important roles in cancer metastasis [Rosalyn et al., 2005]. We examined the effects of RA on the adhesion of Ls174-T cells to ECM proteins by similar cells treatment as provided in cell migration test.

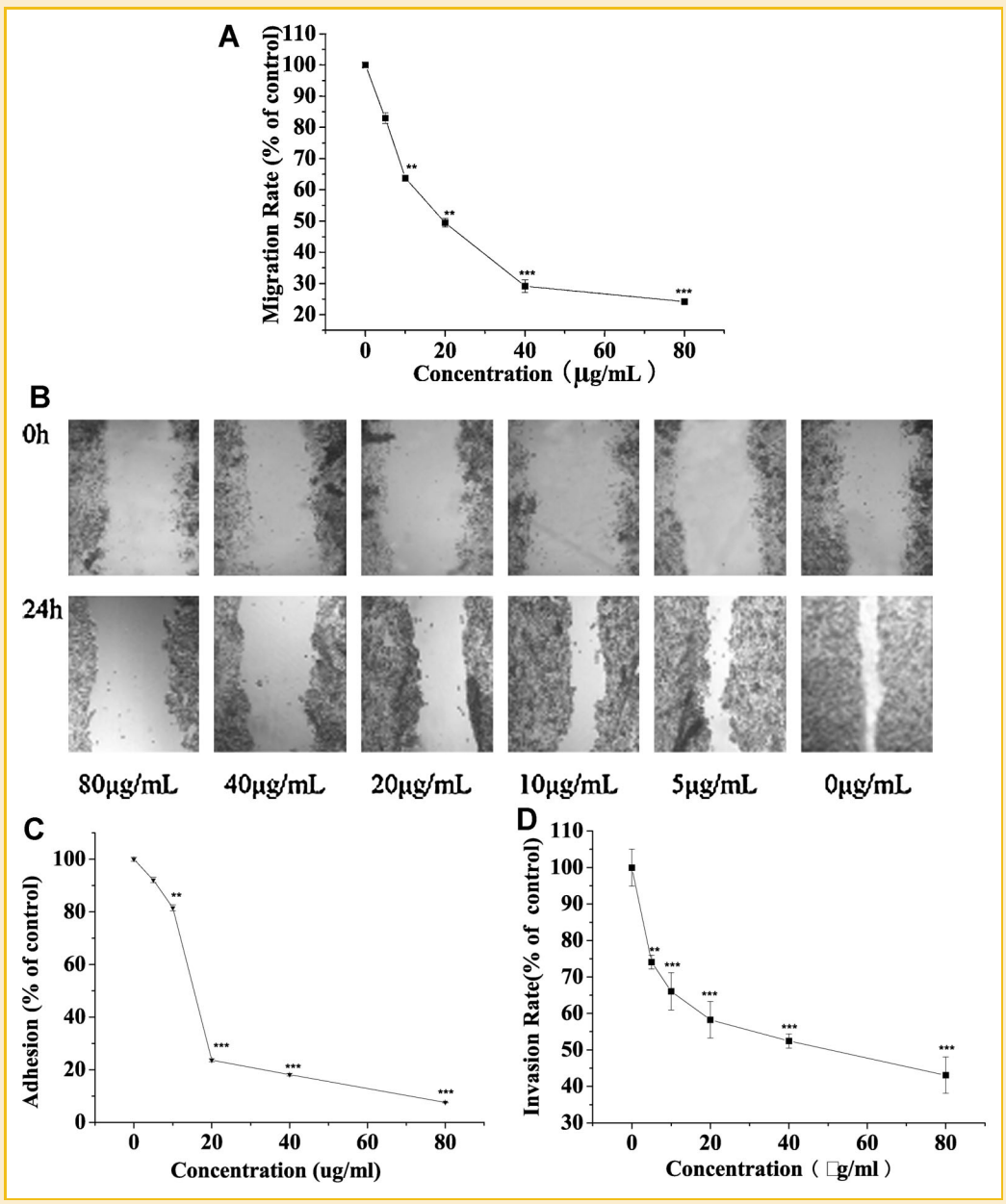

Fig. 3. Effect of RA on migration, adhesion, and invasion of Ls174-T cells. A,B: Effect of RA on migration of Ls174-T cells. It was tested by wound healing assays. A wound was introduced by scraping with a pipette tip when the cells have reached confluence. After 24 h incubation with or without different concentrations of RA, the migration distances were measured. Cells were photographed under phase-contrast microscopy (40×). Migration rate = (migration distances of drug treated cells/migration distances of untreated cells) × 100%. C: Effect of RA on cell adhesion of Ls174-T cells. Ls174-T cells were treated with or without different concentrations of RA for 24 h and placed onto the wells pre-coated with Matrigel®. After a 45-min incubation at 37°C, the amount of adhering cells was determined by MTT method. Adhesion rate = (absorbance of drug treated team/absorbance of untreated team) × 100%. D: Effect of RA on invasion of Ls174-T cells. Ls174-T cells were pre-treated with or without different concentrations of RA for 24 h. The cells then were seeded into the upper compartment of a transwell cell culture chamber. After 8 h of incubation, the invading cells on the lower surface were dyed and counted. Invasion rate = (the number of invading cells in drug treated team/the number of invading cells in drug untreated team) × 100%. Experiments were repeated independently three times with similar results. The data shown are results of a representative experiment. All data were presented as the mean ± SD of three parallel samples in each team.

RA with concentration at 80 μg/ml decreased the adhesion of Ls174-T cells by 92.83% ($P < 0.001$; Fig. 3C). To investigate whether RA can restrict the invasiveness of cancer cell, the Transwell assay was conducted. As illustrated in Figure 3D, 80 μg/ml of RA suppressed the invasiveness of Ls174-T cells to 56.92% of the corresponding treatment without RA. From these results obtained, it was revealed that RA was capable of inhibiting the migration, adhesion, and invasion of Ls174-T cells dose-dependently.

EVALUATION OF MMP-2 AND MMP-9 ACTIVITIES ON Ls174-T CELLS

We tested the effects of RA on ECM degradation catalyzed by MMPs with gel zymogram. Figure 4A,B showed that gelatin zymogram of concentrated serum-free conditioned medium revealed the bands of lysis at 72 kDa (MMP-2) and 92 kDa (MMP-9). And a dose-dependent decrease in gelatinase activity was observed in secretions from RA-treated cells.

EFFECT OF RA ON INTRACELLULAR ROS AND GSH LEVELS

ROS levels in RA incubated with different concentrations were shown in Figure 5A. Treatment with RA decreased the ROS content in a concentration-dependent manner. The ROS content in the test groups (IC_{50} or 1/2 IC_{50}) was evidently decreased compared with the control group. However, no significant effect ($P < 0.05$) on ROS content was seen with 1/4 IC_{50} of RA. A positive modulation of GSH levels was observed with RA in a concentration-dependent manner. Significant effect ($P < 0.01$) on GSH level was seen with RA (IC_{50}) as compared to the control group (Fig. 5B).

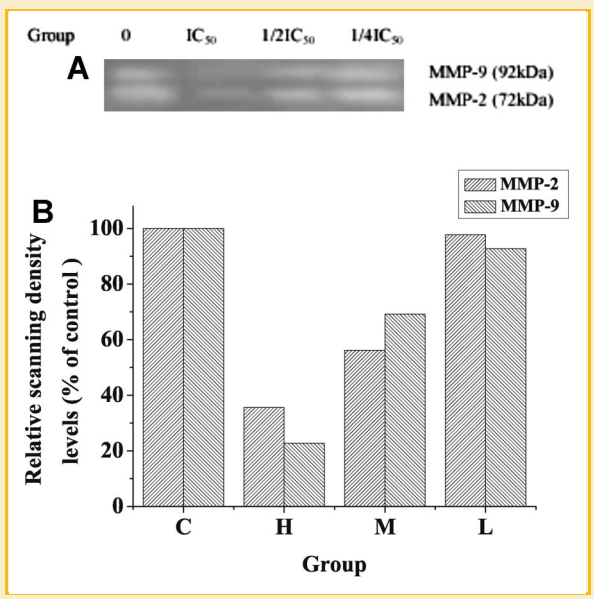

Fig. 4. A,B: Effect of RA on activity of MMP-2 and MMP-9. After 24 h incubation without or with different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}), MMP-2, and MMP-9 on the degradation of gelatin was imaged. Experiments were repeated independently three times with similar results. The data shown are results of a representative experiment. All data were presented as the mean ± SD of three parallel samples in each team. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

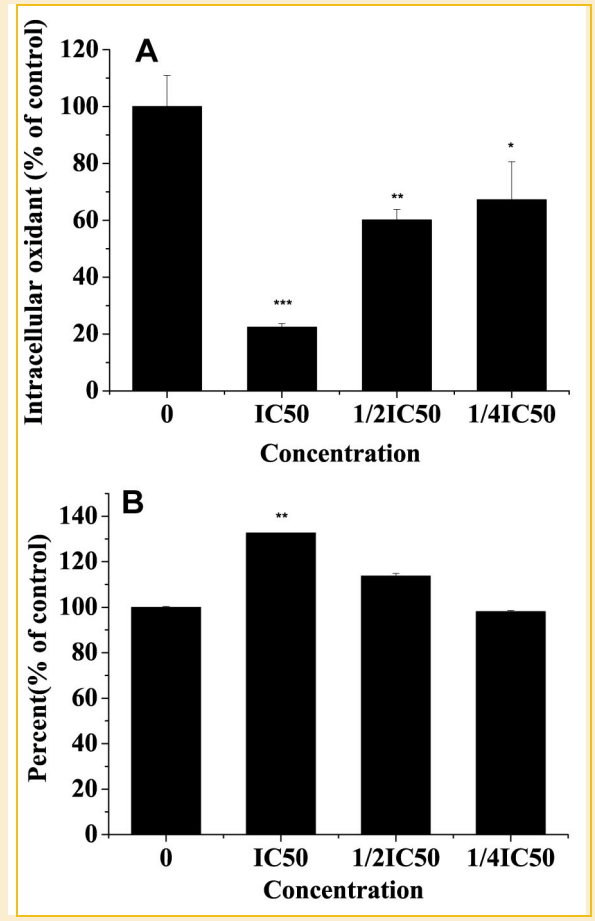

Fig. 5. A: Effect of RA on intracellular ROS contents in Ls174-T cells. Ls174-T cells were treated without or with different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}) for 24 h, then seeded into a 96-well microplate, and added with the redox indicator dye CDCFH, followed by 50-min redox reaction and fluorometry with excitation at 485 nm and emission at 525 nm. Experiments were repeated independently three times with similar results. The data shown are results of a representative experiment. All data were presented as the mean ± SD of three parallel samples in each team. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. B: Effect of RA on GSH contents in Ls174-T cells. Ls174-T cells were treated without or with different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}) for 24 h, followed by seeding into a six-well microplate, and experiments were repeated independently three times with similar results. The data shown are results of a representative experiment. All data were presented as the mean ± SD of three parallel samples in each team. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

EFFECT OF RA ON EXPRESSION OF MMP-2 AND MMP-9

To further investigate the molecular changes involved in RA, the inhibition of metastasis in Ls174-T cells was mediated and the expression of MMP-2 and MMP-9 analyzed by Western blot. The analysis results of MMP-2 and MMP-9 proteins treated with IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50} of RA (Fig. 6A) showed that RA could induce obvious changes in MMP-2 and MMP-9.

EFFECT OF RA ON NUCLEAR TRANSLOCATION OF NF-κB AND THE EXPRESSION OF ERK, p-ERK, Akt, AND p-Akt

To understand the effect of the molecular events involved in the effect of RA activity on cell metastasis, investigation was performed

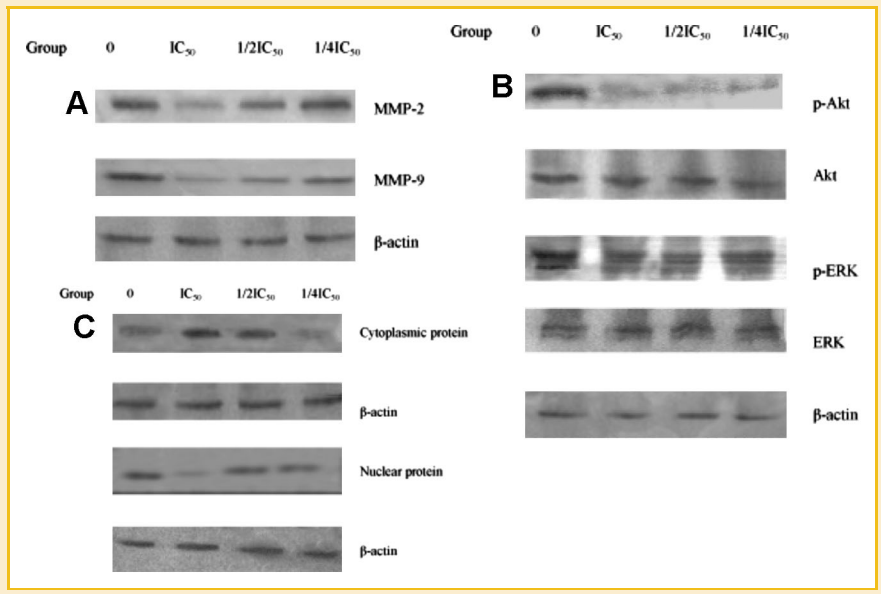

Fig. 6. Western blot analysis. A: Effect of RA on the expression of MMP-2 and MMP-9 protein in Ls174-T cells. After 24 h incubation with or without different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}), the protein from the supernatant and the extract of Ls174-T cells were prepared. Effect of RA on the expression of MMP-2 and MMP-9 protein was assessed by Western blot. B: Effect of RA on the expression of Akt, p-Akt, ERK, and p-ERK protein in Ls174-T cells. After 24 h incubation with or without different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}), then the total protein of Ls174-T cells was prepared. Effect of RA on the expression of Akt, p-Akt, ERK, and p-ERK protein was assessed by Western blot. C: Effect of RA on the expression of NF-κB protein in Ls174-T cells. After 24 h incubation with or without different concentrations of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}), cytoplasmic protein and nuclear extracts were prepared, assessed by Western blot. β-Actin was used as a loading control. Western blots were representative of three independent experiments. Experiment was repeated three times with similar results.

for the effect of RA on the expression and the nuclear translocation of NF-κB, and the expression of proteins including p-Akt and p-ERK that were pivotal for metastasis. The results explained that nuclear translocation of NF-κB was reduced, but no distinct changes were found on its expression (Fig. 6B). Meanwhile, the down-regulations of p-Akt and p-ERK were observed, but there was no difference in the expression of proteins of Akt and ERK (Fig. 6C).

EFFECT OF RA ON EGFR AND VEGFR

The activities of tyrosine kinases (TKs) were tested by DELFIA. As shown in Figure 7A, RA bound to EGFR and VEGFR in a dose-independent manner, and the activities of binding to EGFR and VEGFR under the IC_{50} of RA were significantly higher ($P < 0.001$), indicating a good inhibition of RA against EGFR and VEGFR.

EFFECT OF RA ON mRNA LEVELS OF VEGF, MMP-2, AND MMP-9

Accumulating evidence has indicated that VEGF, MMP-2, and MMP-9 were target genes regulated by ERK pathway. When we found that RA was involved in the anti-metastatic effects, expression of VEGF, MMP-2, and MMP-9 was measured by quantitative real-time RT-PCR (Fig. 7B) to further guarantee if inhibition has accounted for the anti-metastatic effects of RA. Hence, the results suggested that the expression of VEGF, MMP-2, and MMP-9 could be inhibited significantly by RA.

ACUTE TOXICITY OF RA IN KM MICE

In an initial investigation, six groups, each of which contained one male and one female mouse, were treated with RA by tail intravenous injection at doses of 31.25, 62.5, 125, 250, 500, and 1,000 mg/kg, respectively. Based on the results of the initial investigation, new dosages (62.5, 94.7, 143.6, 217.6, 329.9, 500 mg/kg) were administered to the animals (10 animal per group, 5 male and 5 female mice) in the second test, and their mortality rates were 10%, 20%, 50%, 60%, 80%, and 90%, respectively. The LD_{50} value of RA calculated using the above formulas was about 169.6 mg/kg, and standard error for log LD_{50} was 0.042, while 95% confidence interval of LD_{50} ranged from 140.3 to 205.0 mg/kg and average confidence interval of LD_{50} was 169.6 ± 32.4 mg/kg, indicating that RA was slightly toxic.

ANTI-METASTATIC EFFECT OF RA IN VIVO

When the solid tumors in C57BL/6 mice were treated with the RA, the suppression of tumor growth was observed. Table I listed the inhibition ratios of the tumor growth for the RA treatments in C57BL/6 mice. The results showed that when the tumor-bearing mice were treated with 1, 2, and 4 mg/kg of RA for 20 days, the inhibition ratios were 55.40%, 56.83%, and 29.98%, respectively. The inhibitory rates of the formation of metastasis nodules reached 33.11%, 59.53%, and 25.75% in the case of administration with 1, 2, and 4 mg/kg of RA relatively. In addition, the treatments with 1 or 2 mg/kg of RA did not result in a body weight loss in the mice during the whole treating period, while a delivery of 4 mg/kg led to small amount of weight loss, suggesting a little cytotoxicity to mice. The results demonstrated that RA could suppress tumor growth in vivo, and a dose of 2 mg/kg would be recommended.

DISCUSSION

Metastasis is one of the major causes of mortality in cancer patients. The initial steps of metastasis include detachment of malignant cells

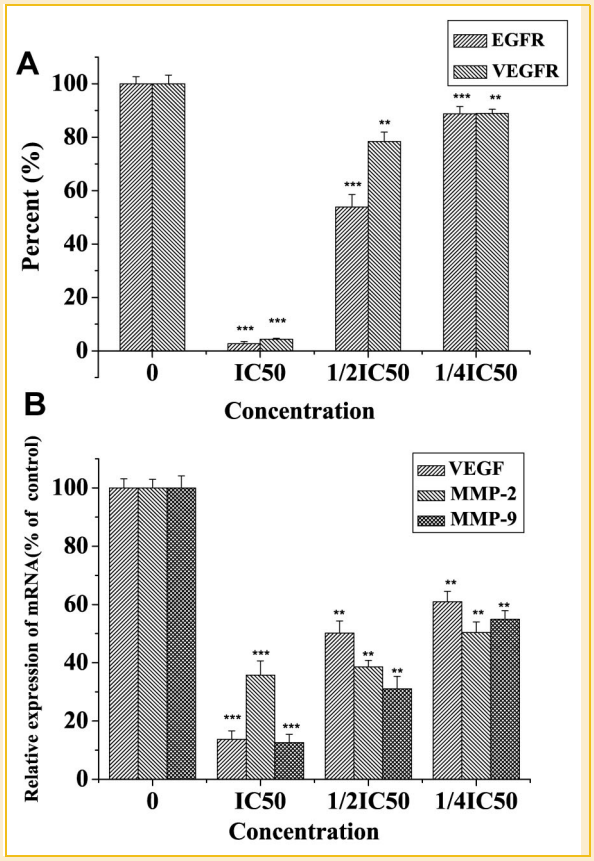

Fig. 7. A: Effect of RA on activities of protein tyrosine kinase. The inhibition effects of RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}) against EGFR and VEGFR were examined according to DELFIA. B: Effect of RA on mRNA expression of VEGF, MMP-2, and MMP-9 in Ls174-T cells. Cells were treated with RA (0, IC_{50}, 1/2 IC_{50}, and 1/4 IC_{50}) for 24 h and then harvested. The total RNA was extracted, and then cDNA was synthesized from total RNA (2 μg), which was amplified by using the sets of primers for examining the expression of VEGF, MMP-2, and MMP-9, according to quantitative real-time RT-PCR. Data were presented as means ± SD and representative of an average of three independent experiments per concentration. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

from the primary tumor and invasion into surrounding tissues [Glinsky et al., 2000]. In normal tissues, cells are so tightly associated with each other so that they are generally not allowed to migrate freely. However, the malignant cells are more loosely associated and can freely detach from the primary tumor and migrate out [Matsuyoshi et al., 1992]. In this study, using wound healing assay and adhesion assay, we found that RA could effectively inhibit migration of tumor cells in vitro with EC_{50} <20 μg/ml (Fig. 2B). After that, transwell assay also showed that RA can significantly inhibit the invasion ability of Ls174-T cells. The next important step in the invasive progress is the degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs) [Liotta, 1986]. The invasive process of cancer cells requires the tumor cells to adhere to ECM components first, and then secrete MMPs to degrade ECM, and then, finally migrate through the ECM [Zagzag and Zhong, 2000]. We focused on this process and demonstrated that RA could interfere with multiple steps of invasive progress. It might represent a potential strategy for cancer therapy.

Physiological and pathological tissue remodeling processes such as wound healing, embryo implantation, tumor invasion, metastasis, and angiogenesis are associated with MMPs which constitute a family of secreted, zinc-dependent endopeptidases that are required for ECM degradation [Ray and Stetler-Stevenson, 1994; Stetler-Stevenson, 1999]. A central role in this step is played by MMP-2 and MMP-9, which cleaves primarily type IV collagen in the basement membrane. Both proteins are correlated with an aggressive, invasive, or metastatic tumor phenotype [Cockett et al., 1998; Papathoma et al., 2001]. Therefore, inhibiting the activity of MMP-2 and MMP-9 is regarded as a rational approach to metastatic disease therapy. In this study, RA suppressed the activity of MMP-2 and MMP-9 and eventually inhibited cancer cell metastasis.

Angiogenesis is essential for the efficient dissemination of primary tumor cells during metastasis. The formation of new blood vessels provides nutrients. The early steps of angiogenesis and tumor metastasis are nearly identical, as both processes involve degradation of the ECM and directed migration of either vascular or neoplastic cells. Vascular endothelial growth factor (VEGF) is a crucial growth and permeability factor for tumor angiogenesis and is also frequently up-regulated in human solid tumors. The data presented here suggested that RA may inhibit colon cancer's progression by interfering with tumor angiogenesis mediated by MMPs and VEGF, thereby providing a novel mechanism for the anti-cancer action of RA.

In the study, RA decreased protein level of NF-κB in Ls174-T cells. NF-κB, as an important signal molecule, might play a key role in the signaling pathway triggered by RA. With the results of cell adhesion assay, it was possible that RA suppressed cell adhesion to ECM through down-regulating NF-κB expression, and then eventually inhibited cell metastasis. After adhesion, another key step in the invasive progress is the degradation of a variety of ECM proteins by MMPs. The invasive process of cancer cells requires that tumor cells first adhere to ECM components, and then secrete MMPs to degrade ECM, and finally migrate through the ECM. We focused on this process and demonstrated that RA could interfere with multiple steps of invasive progress. The anti-metastatic effects of RA were associated with the inhibition of cell invasion, which was evidenced by inhibition of cell adhesion and motility as well as suppression of MMP-2 and MMP-9 activation. The down-regulation of NF-κB expression may be involved in the possible molecular mechanisms that underlie the anti-invasive effect of RA. In this study, it was

TABLE I. Anti-Metastatic Effect of RA In Vivo

Group	Treatment	Tumor weight (g) (mean ± SD)	Number of lung nodules
C	0.2 ml NS/one/day	5.21 ± 1.21	37.37 ± 9.33
P	Vinblastine 2 mg/kg/day	1.57 ± 1.30***	12.25 ± 3.85***
H	RA 4 mg/kg/day	3.65 ± 1.71	27.75 ± 6.69*
M	RA 2 mg/kg/day	2.25 ± 1.17***	15.12 ± 3.14***
L	RA 1 mg/kg/day	2.33 ± 1.55**	25.00 ± 6.30**

Eight C57BL6 mice per group were inoculated with LLC cells (10^5 cells per mouse) and was injected i.p. with drugs for 20 days and lastly, they were sacrificed. The data shown are results of a representative experiment. Values are mean ± SD for each group of mice. Significant differences from control were indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

demonstrated that RA suppressed the activities of MMP-2 and MMP-9 and ultimately inhibited the cancer cell metastasis.

It is generally believed that ROS is the total saying of super-oxide anion, hydroxyl radical, peroxy nitrite, hydrogen peroxide, and other peroxides. Tumor cells can produce a large number of ROS, which is an important messenger molecule for downstream of many signaling pathway, which eventually led to cancer cell invasion and the occurrence of metastasis. It has been confirmed that the activation of NF-κB is associated with ROS; NF-κB activity can be induced in most cell types upon exposure to certain stimuli of which ROS is one of the very important factors [Yang et al., 2001]. On the other hand, ROS is reported to be involved in the invasion of cancer: invasive and metastasis properties of malignant cells were inhibited by oxygen radical scavengers, such as r-Hsod [Noaka et al., 1993], DMSO, and allopurinol [Salim, 1992], and by redox-regulating agents such as glutathione [Anasagasti et al., 1998] and *N*-acetylcysteine [Albini et al., 1995]. Several reports have indicated that oxidative stress is a prime cause of cancer. Cellular reduced glutathione (GSH) has been shown to be crucial for regulation of cell proliferation, cell-cycle progression, and apoptosis. GSH functions intracellularly reduce numerous oxidizing compounds, including ROS [Yu et al., 2009]. In the present study, we found that RA dose-dependently decreased the intracellular ROS of Ls174-T cells and enhanced the level of GSH. It can be concluded that the inhibition of the translocation of NF-κB by RA in Ls174-T cells is correlated with its scavenging ROS, and the anti-invasion and anti-metastasis activities of RA in Ls174-T cells are also possibly related to its scavenging ROS, while GSH is the main factor to reduce the level of intercellular ROS.

Extracellular signal-regulated kinase (ERK) was reported to be important to the activation of NF-κB, which was critically involved in the regulation of tumor cell proliferation, apoptosis, and oncogenesis [Nakano et al., 1998]. Many previous studies have shown that anti-tumor activities of RA may be mediated in part through the suppression of NF-κB activation [Enk et al., 2007; Hu et al., 2008; Pandey et al., 2008; Yi et al., 2008]. Similarly, some observations found that tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) activated MMPs via the ERK signaling pathway [Yu and Wen, 2009]. Studies by Irie et al. [2005] demonstrated that Akt inhibited the motility of MCF10A cells by attenuating extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) activities. Also, there was a report about ERK signaling pathway that mediated the downstream of MMPs [Giannelli et al., 2008]. Akt had been shown to play a key role in multiple processes of VEGF-mediated angiogenesis, both in physiological and pathological contexts. In endothelial cells, Akt phosphorylated the substrate protein girdin thereby promoting VEGF-dependent cell migration, which was essential for sprouting, formation, and branching of vessels during angiogenesis [Kitamura et al., 2008]. In addition, VEGF enhanced the survival of human endothelial cells via the Akt signaling pathway [Gerber et al., 1998]. Akt activation in turn promoted the expression of VEGF in endothelial cells as well as tumor cells [Jiang et al., 2000; Zundel et al., 2000]. Otherwise, EGFR-PI3K/Akt signaling path joined in adjusting the process of invasion and metastasis on melanoma [Zhang et al., 2006]. These results clearly suggested that ERK signaling cascade is a critical target for RA. As members of TKs family, epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VEGFR) possess intrinsic protein TK activities, which are essential in the regulation of signal transduction in the cells. In the ERK pathway, it is firstly activated by some growth factors or their receptors, for example, EGF/EGFR and VEGF/VEGFR. Due to the abnormal expression of EGFR and VEGFR, a certain type of cancer may be developed and progressed [Zhang et al., 2008]. Meanwhile, inhibitors of the EGFR and/or VEGFR would efficiently reduce cell migration and invasion, with a similar pattern of TK receptors relevant to ERK pathway [Giannelli et al., 2008]. Otherwise, ROS plays a role in activation of ERK pathway [Samavati et al., 2002], and it could stimulate all the pathways of MAPK [Kim and Choi, 2010]. Mitochondria-derived ROS always acts as an upstream factor of other signaling pathways [Pelicano et al., 2004].

In addition, the host environment also influences the process of drug getting to its target. So taking the influence of host into account, we further tested anti-metastatic activity of RA in the animal model. In line with the results of cultured cell model, RA showed the capacity to inhibit cancer metastasis in vivo (Table I). Intraperitoneal administration of 2 mg/kg RA decreased the number of metastatic nodules by 59.53% and the weight of tumors by 56.83% with control group. The statements above supported the conclusion that RA could be a promising candidate for cancer chemotherapeutic agent. Based on the result of LD_{50}, a dosage of 2 mg/kg would be safely used with little toxicity.

In conclusion, our present study has provided evidences that RA could inhibit cell adhesion, migration, invasion, secretion of MMPs, gene expression of MMP-2, MMP-9, and VEGF, and protein expression of MMP-2 and MMP-9. Furthermore, our research focused on the molecule mechanism of RA, and we found that ERK signaling pathway played an important role in the mediating metastasis of colon carcinoma. RA inhibited the activities of EGFR and VEGFR, and then suppressed the nuclear translocation of NF-κB via p-Akt and p-ERK de-phosphorylation (Fig. 8). In addition, a suitable concentration of RA had extremely good anti-tumor and anti-metastatic effects, which were similar to those acted by vinblastine. Meanwhile, the result of LD_{50} indicated that the toxicity of RA was slim.

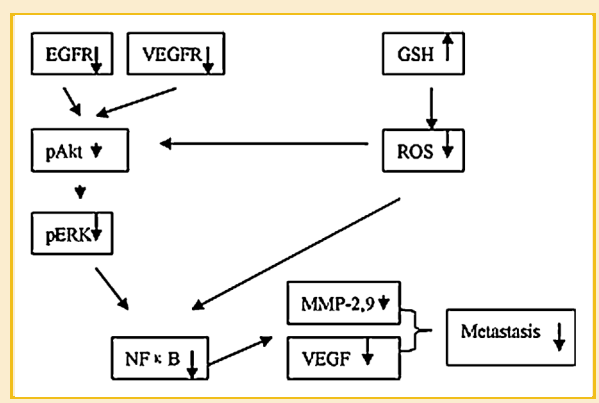

Fig. 8. The anti-invasion mechanism of RA in Ls174-T cells.

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