

Generation and Characterization of Monoclonal Antibody to Ginsenoside Rg3

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Received September 12, 2008; accepted January 14, 2009; published online January 20, 2009

While studying the mechanism of ginsenoside Rg3 (G-Rg3) on tumor inhibition, we produced monoclonal antibody to G-Rg3 for more specific investigation. We immunized Balb/c mice to G-Rg3 conjugated bovine serum albumin (BSA) by intraperitoneal injection and hybridized splenocytes from those immunized mice and myeloma cells. From those fusion cell lines, we selected productive monoclonal clones and obtained culture media containing monoclonal antibody to G-Rg3. After purification, we performed enzyme-linked immunosorbent assay (ELISA) to verify the sensitivity and specificity of the antibody. When compared with G-Rh2 having a very similar structure as a metabolite of G-Rg3, the antibody worked only with G-Rg3 in a concentration-dependent manner. We confirmed that the monoclonal antibody to G-Rg3 can be applied to immunocytochemistry for detection of the treated G-Rg3 inside A549 human lung adenocarcinomas. Thus, the monoclonal antibody to G-Rg3 would be a useful tool for measuring the bioactivity of G-Rg3 in various fields.

Key words ginsenoside Rg3; monoclonal antibody; enzyme-linked immunosorbent assay

Ginseng and its extract have been used as one of the most popular traditional medicines for the promotion of general health in Eastern Asian countries for thousands of years. The pharmacological and therapeutic effects of ginseng have been reported to have anti-aging, anti-diabetics, anti-stress, maintenance of homeostasis, and to effect on central nervous system and immune function.¹⁾ As its extensive effectiveness and usefulness have been well-noticed, numerous researchers have investigated about its constituents, chemical and physical characteristics as well as its pharmacological activities and their mechanisms. The constituents of ginseng are identified as saponins called ginsenosides, phytosterols, peptides, polysaccharides, fatty acids, polyacetylenes, vitamins, and minerals. Among them, the main biological components are more than 60 kinds of ginsenosides that have been isolated from *Panax* genus.²⁾

There have been reports on the productions and applications of monoclonal antibodies to ginsenoside Rb1 (G-Rb1),³⁾ G-Rg1,⁴⁾ and G-Rf⁵⁾ as well as polyclonal antibodies to G-Rg1⁶⁾ and G-Rf/G-Rg2.⁷⁾ They were developed to quantify or trace specific ginsenosides in the body fluids or in mixed ginseng extracts by immunoassay without cross reactivity.

In this study, for the first time, we report the monoclonal antibody to G-Rg3. G-Rg3 is a type of protopanaxadiol having glucose (2→1) glucose attached at the C-3 hydroxyl group (Fig. 1). Both G-Rg3 and its metabolite G-Rh2 have already been sold as anti-cancer drugs in the Chinese and Taiwanese markets.⁸⁾ Although many researchers have revealed that G-Rg3 shows the effects such as anti-angiogenic activity,⁹⁾ anti-proliferative activity,¹⁰⁾ apoptosis¹¹⁾ and protective actions to chemotherapy¹²⁾ both *in vitro* and *in vivo*, its mechanism has yet to be clearly established. Therefore, the proteins to 'give and take' the influences with G-Rg3 related to tumor inhibition draws attention to understand the mechanism of G-Rg3.

Herein, we produce, purify and characterize monoclonal antibody to G-Rg3. In our investigation, we proved that the

monoclonal antibody works specifically to G-Rg3, not G-Rh2. G-Rh2, having a glucose at the C-3 hydroxyl group, possesses very similar structure to G-Rg3 and also shows anti-cancer activity. Therefore, the monoclonal antibody to G-Rg3 is a very useful tool not only for the study *in vitro* and *in vivo* at the cellular level but also it would be a good immunological approach to quality control or quantitative determination of the mixed samples containing G-Rg3.

MATERIALS AND METHODS

Preparation of Ginsenosides G-Rg3 (Fig. 1A) was preparatively purified from the steamed ginseng extract by high speed counter current chromatography.¹³⁾ G-Rh2 (Fig. 1B) and G-Rg2 (Fig. 1C) were purchased from Chengdu Cogon Bio-tech Co., Ltd. (Chengdu, China).

Synthesis of Antigen Conjugates Each ginsenoside-bovine serum albumin (BSA) conjugate was synthesized by the previous method⁷⁾ with slight modification. After dissolving 4 mg NaIO₄ (Sigma, St. Louis, MO, U.S.A.) in 0.5 ml H₂O, the mixture was added to 80% methanol solution (0.7 ml) of each ginsenoside (10 mg) at room temperature for 1 h. For the conjugation, 1 ml of 100 mM carbonate buffer (pH 9.0) containing 10 mg BSA (Santa Cruz, CA, U.S.A.) was then reacted with the mixture at room temperature for 5 h. The reaction mixture was dialyzed to phosphate buffered saline (PBS) 5 times and lyophilized to obtain the ginsenoside-BSA conjugate.

Immunization and Hybridization Balb/c mice (6-week-old, male) were used for the immunization of G-Rg3-BSA conjugate. The first immunization was carried out by intraperitoneal injection using the emulsifying mixture of G-Rg3-BSA conjugate (100 μg) and complete Freund's adjuvant (Sigma, St. Louis, MO, U.S.A.). The second and third immunizations were performed with the emulsifying mixture of G-Rg3-BSA conjugate (100 μg) and incomplete Freund's adjuvant (Sigma, St. Louis, MO, U.S.A.) every two weeks.

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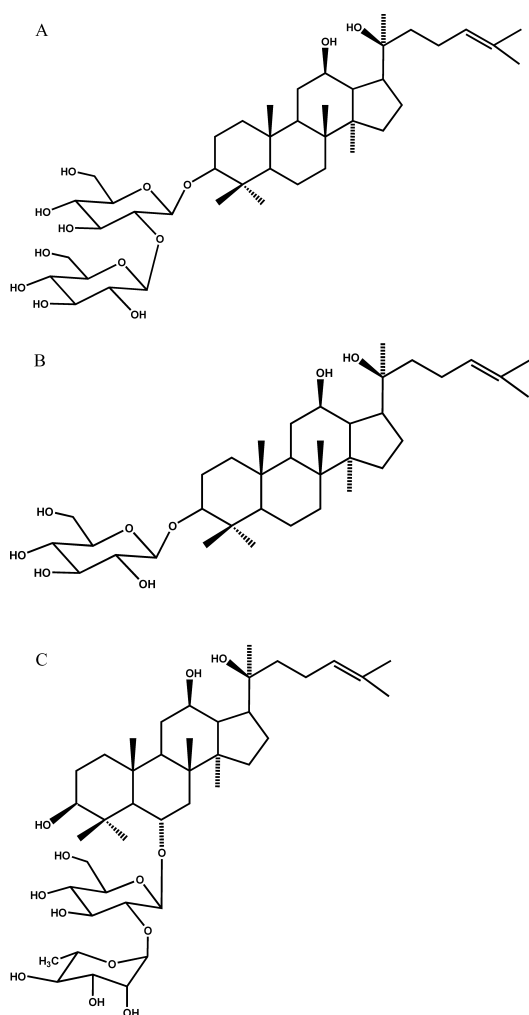


Fig. 1. Chemical Structures of Ginsenoside Rg3 (G-Rg3) (A), G-Rh2 (B) and G-Rg2 (C)

On the third day after the final immunization, the splenocytes were isolated and fused with a hypoxanthin-aminopterin-thymidine (HAT)-sensitive mouse myeloma cell line, P3-X63-Ag8-U1, by the polyethylene glycol method.¹⁴⁾ Hybridomas producing monoclonal antibody to G-Rg3 were cloned by the limited dilution method.¹⁵⁾ The cloned hybridomas were then cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) containing 10% FBS (Hyclone, Logan, UT, U.S.A.).

Purification of Monoclonal Antibody to Ginsenoside Rg3 The produced monoclonal antibody was purified by immobilized protein G (Pierce, Rockford, IL, U.S.A.) column, following the manufacturer's instruction. Briefly, the cultured supernatant containing the IgG was adjusted to 1 M Tris buffer (pH 7.5) and subjected to the equilibrated protein G resin. The immunoglobulin G (IgG) bound to the resin was eluted with 0.1 M glycine buffer (pH 2.5) and the eluted IgG was neutralized with 1 M Tris buffer. The purified monoclonal antibody was dialyzed to PBS (pH 7.4) three times and stored at 4 °C.

Calculation of Hapten Number on Ginsenoside Rg3-BSA Conjugate by Matrix Assisted Laser Desorption/Ionization (MALDI-TOF) MS To calculate the conjugation number of each ginsenoside per BSA, 10 pmol of each con-

jugate was dissolved in a 1000-fold molar excess of sinapinic acid solution containing 0.1% trifluoroacetic acid. After the mixtures were dropped on a gold-coated plate and air dried, the samples were analyzed using MALDI-TOF mass spectrophotometer (Voyager-DE™ STR Biospectrometry Workstation, Applied Biosystems Inc.) by linear mode for BSA and each conjugate and by reflector mode for each ginsenoside. The ions formed by each pulse were accelerated by a 25 kV potential into a 2.0-evacuated tube and detected using compatible computer.

Direct Enzyme-Linked Immunosorbent Assay (ELISA)

The reactivity of monoclonal antibody to G-Rg3 was confirmed by direct ELISA method. Fifty microliters of G-Rg3-BSA conjugate diluted in 0.2 M sodium carbonate buffer (coating buffer) were adsorbed to the 96 well plate overnight at 4 °C. The plate was treated with 2% skim milk in tris-buffered saline containing 0.05% of Tween 20 (TBST) to reduce nonspecific adsorption and washed three times with TBST. The plate was reacted with the monoclonal antibody diluted in TBST at 37 °C for 2 h. After washing the plate with TBST 3 times, the monoclonal antibody to G-Rg3 was then reacted with anti-mouse IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL, U.S.A.) at 37 °C for 1 h. After washing the plate with TBST 5 times, the substrate of *ortho*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, U.S.A.) was added to each well and incubated for 30 min at room temperature. After the incubation period, the absorbance of each well was read at 450 nm on the spectrophotometer.

Competitive ELISA The 96 well plate was coated with 50 μ l of G-Rg3-BSA conjugate diluted in 0.2 M sodium carbonate buffer overnight at 4 °C. After washing 3 times, the plate was blocked with 2% skim milk in TBST to reduce nonspecific adsorption. Fifty microliters of various concentrations of G-Rg3, G-Rh2 and G-Rg2 were incubated with 50 μ l of monoclonal antibody to G-Rg3 for 2 h. The mixture was incubated to the G-Rg3-BSA conjugate precoated plate for 2 h. After washing 3 times with TBST, secondary anti-mouse IgG conjugated with horseradish peroxidase was added to each well and incubated for 1 h. After washing 5 times with TBST, the substrate solution was added to the well and its absorbance was read at 450 nm. All of the processes were carried out at room temperature except for the preparation of the precoated plates.

Immunocytochemistry A549 human lung adenocarcinomas (ATCC, Manassas, VA, U.S.A.) were cultured in RPMI (Sigma, St. Louis, MO, U.S.A.) media containing 10% FBS. After the stabilization of cells in a 24 well plate, 500 μ l of 50 μ M G-Rg3 was treated to each well and incubated for 10 h at 37 °C in 5% CO₂ incubator. The cells were washed with ice-cold Dulbecco's phosphate buffered saline (DPBS) (Sigma, St. Louis, MO, U.S.A.) and fixed with 2% paraformaldehyde solution in DPBS for 20 min. After washing 3 times with ice-cold DPBS, each well was blocked by 3% BSA in DPBS for 2 h. Without washing, the cells were incubated with monoclonal antibody diluted in 1 : 10 at 4 °C for overnight. After washing 3 times with ice-cold DPBS, the cells were incubated with secondary anti-mouse IgG conjugated with horseradish peroxidase. After washing 5 times by ice-cold DPBS, the working solution of 3,3'-diaminobenzidine (Vector Laboratories, CA, U.S.A.) was treated to each

well and incubated at room temperature for 15 min. The stained cells were observed using an inverted microscopy (Olympus CKX41, Japan). All of the processes were carried out at room temperature except for the incubation of monoclonal antibody to G-Rg3.

RESULTS

Calculation of Hapten Number by MALDI-TOF MS

The bands loaded with ginsenoside-BSA conjugates appeared broad, whereas the lane with only BSA was just one band of approximately 67000 Da (data not shown). For the calculation of hapten number, the conjugates of G-Rg3-BSA, G-Rh2-BSA and G-Rg2-BSA were analyzed each 5 times by MALDI-TOF MS under the same conditions. The peak ranges appeared from m/z 65000 to 80000 centering at about m/z 69028 (Fig. 2A), m/z 70189 (Fig. 2B) and m/z 75582 (Fig. 2C), respectively, as marked by arrows. The molecular weights of G-Rg3, G-Rh2, G-Rg2 and BSA were also determined to be 785, 623, 785 and 66436 Da, respectively. Based on these results, the number of G-Rg3 conjugated with BSA was determined to be approximately four and that of G-Rh2 was six. Also, the number of G-Rg2 was twelve.

Direct ELISA The 96 well plates were coated with G-Rg3, G-Rh2, G-Rg2, each ginsenoside-BSA conjugate and BSA diluted in coating buffer and reacted with various concentrations of monoclonal antibody to G-Rg3 by direct enzyme-linked immunosorbent assay (ELISA). Expectedly, the wells coated with only each ginsenoside and BSA were not detected by the antibody. The monoclonal antibody to G-Rg3 was reacted within the concentrations of G-Rg3-BSA conjugate ranged from 25 $\mu\text{g/ml}$ to 0.25 ng/ml and the minimum concentration of the diluted antibody was 1 : 1000 (Fig. 3A). For sensitivity and specificity, direct ELISA to G-Rh2-BSA and G-Rg2-BSA was carried out under the same conditions. They were not detected even under the maximum concentration (1 : 10) of monoclonal antibody to G-Rg3 (Fig. 3B).

Competitive ELISA As another assay to assess the specificity of monoclonal antibody to G-Rg3, the plates were prepared for competitive ELISA. The wells were coated with 50 μl of G-Rg3-BSA conjugate (2.5 $\mu\text{g/ml}$) and various concentrations of tested ginsenosides were preincubated with 50 μl of monoclonal antibody to G-Rg3 (1 : 10). The concentrations of G-Rg3 and its antibody were selected from the results of direct ELISA. Because, after a 2-h incubation period, G-Rh2 and G-Rg2 were not reacted to monoclonal antibody to G-Rg3, it allowed free antibody directly to bind with G-Rg3-BSA conjugate coated in the plate (Fig. 4). This result indicates that the cross-reactivity was not found between monoclonal antibody to G-Rg3 and other ginsenosides, whose structures are similar to G-Rg3.

Immunocytochemistry In order to trace G-Rg3 treated to cells, A549 human lung adenocarcinomas were seeded in a 24 well plate. Five hundred microliters of 50 μM G-Rg3 showed no cytotoxicity within 12 h. After incubation with 50 μM G-Rg3 and A549 cells for 10 h, the cells were fixed, washed and reacted with the antibody. The localization of G-Rg3 inside cells appeared in the form of the brown color of 3,3'-diaminobenzidine. It suggested that G-Rg3 passed across the cell membrane and was detected clearly around the nuclear membrane (Fig. 5B). G-Rg3 was not observed in

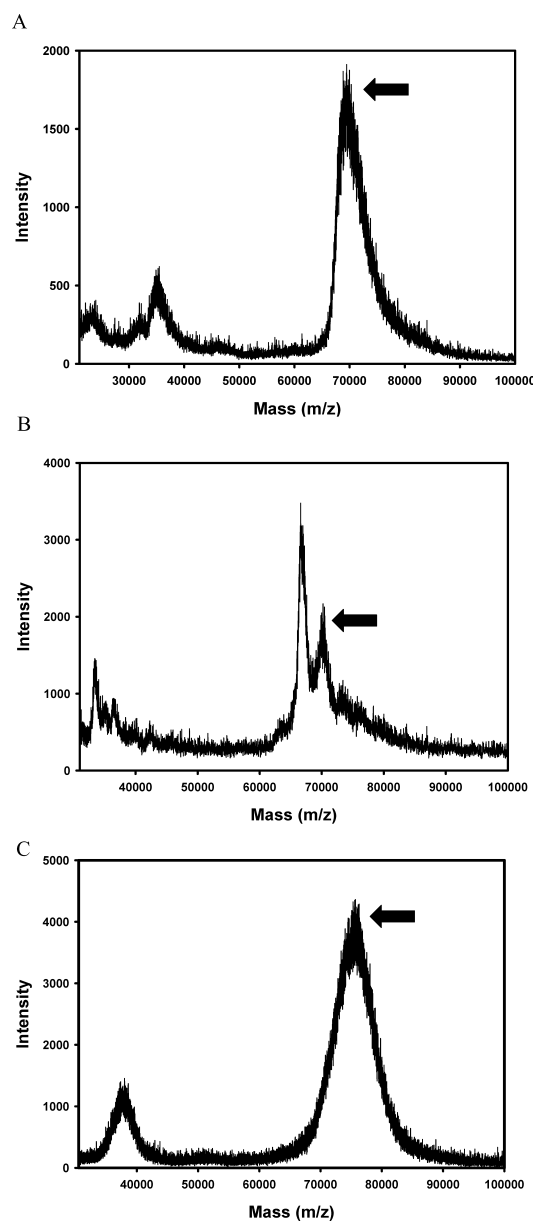


Fig. 2. Determination of Ginsenoside-BSA Conjugates Using MALDI-TOF MS

The conjugates of G-Rg3-BSA (A), G-Rh2-BSA (B) and G-Rg2-BSA were analyzed and determined by MALDI-TOF MS. The hapten numbers of G-Rg3, G-Rh2 and G-Rg2 were calculated, according to their spectra and molecular weights, as four, six and twelve, respectively.

the cytoplasm or on the cell surface membrane under this condition. It proves that monoclonal antibody to G-Rg3 can be applied to the molecular study such as immunocytochemistry.

DISCUSSION

In the present study, we describe the production and characterization of the monoclonal antibody to G-Rg3. Since the monoclonal antibody to G-Rb1 was developed in 1999,³⁾ a few monoclonal antibodies to ginsenosides have been prepared for immunoassay or further pharmacokinetic study in rat model.

First, we determined by MALDI-TOF MS that the calculated numbers of G-Rg3 and G-Rh2 are four and six mole-

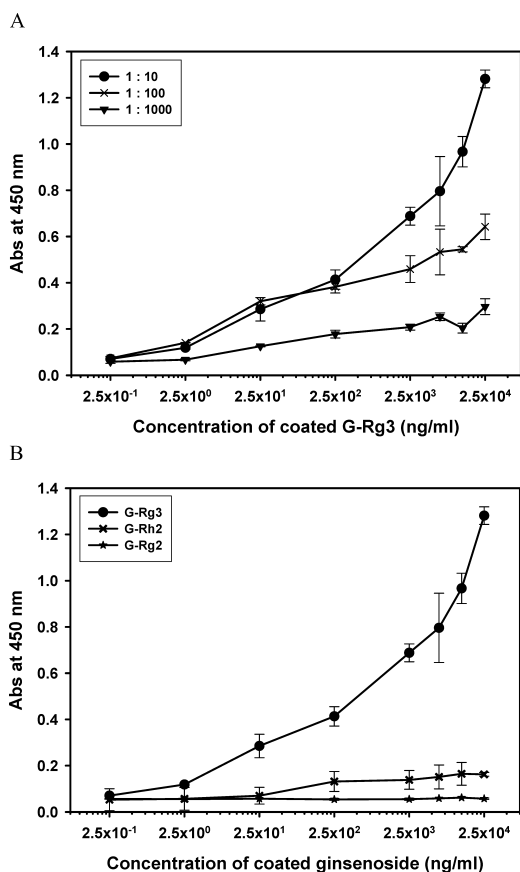


Fig. 3. The Reactivities of G-Rg3 by Direct ELISA
The reactivities of monoclonal antibody to G-Rg3 were tested by direct ELISA against various concentrations of G-Rg3 (A) and against other ginsenosides (B).

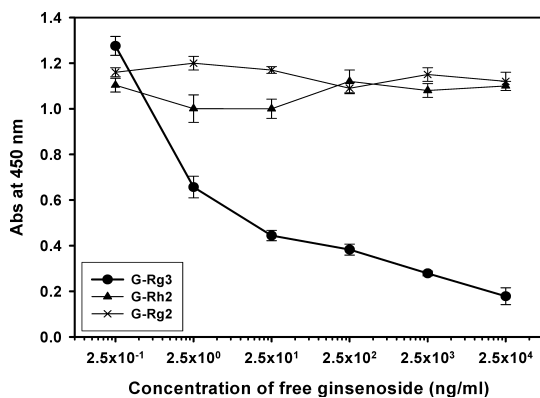


Fig. 4. Competitive ELISA
Because G-Rh2 and G-Rg2 did not react with monoclonal antibody to G-Rg3, the free monoclonal antibody bound to the coated G-Rg3 in the same concentration.

cules, respectively. These values are lower than those of the previously reported ginsenosides. It seems that the hapten number is related not to the type but rather the number of terminal sugars. Second, we also tested the sensitivity and specificity of monoclonal antibody to G-Rg3 by both direct and competitive ELISA. Expectedly, as the concentration of antibody was progressively diluted, the reactivity to G-Rg3 was lowered. Also, the antibody was rarely reacted to G-Rh2 and G-Rg2 having rhamnose at the C-6 terminus. As shown above (Fig. 1), G-Rg3 is a kind of protopanaxadiol having glucose (2→1) glucose attached at C-3 hydroxyl group and

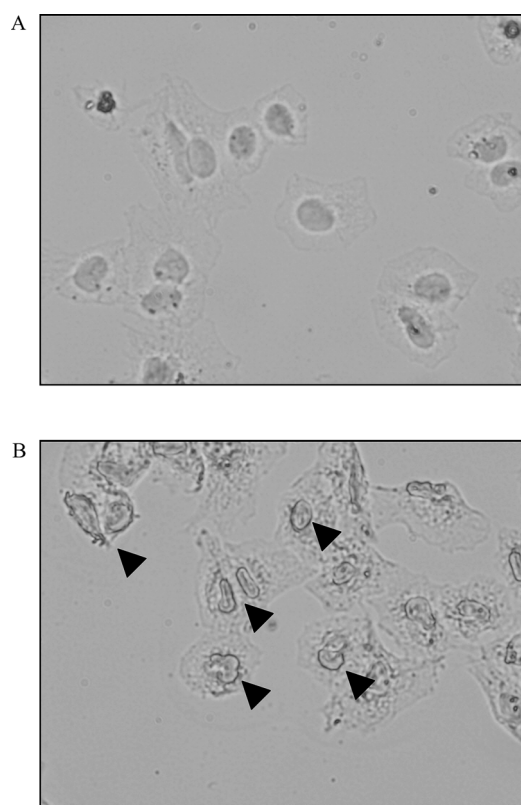


Fig. 5. The Cellular Localization of G-Rg3 Using Immunocytochemistry
After G-Rg3 was treated to A549 lung adenocarcinomas for 10h, the cells were washed, fixed and incubated with monoclonal antibody to G-Rg3. Compared with non-G-Rg3 treated cells (A), the brown color (B, arrows) indicated the localization of G-Rg3 around the nuclear membrane in the treated cells.

G-Rh2 possesses a structure by which one glucose is eliminated from the structure of G-Rg3. 20(S)-G-Rg3 has been reported to be metabolized to G-Rh2 by human intestinal bacteria such as *Bacteroides* sp., *Eubacterium* sp., *Bifidobacterium* sp. and *Fusobacterium* sp.¹⁶⁾ In addition, G-Rg3 and G-Rh2 have been produced in singular and mixed capsules as anti-cancer drugs. Therefore, it is very meaningful to differentiate G-Rg3 from its metabolite, G-Rh2 by means of monoclonal antibody to G-Rg3.

As ginseng is one of the most well-known medicinal plants in both Eastern and Western countries, ginseng and its extract have showed pharmacologically multiple effects. In the past, they had been used in drugs for the promotion of general health, such as adaptogen. However, as their chemical and physical characteristics have been revealed, studies on individually active compounds have been attempted in various fields. Among those characteristics, anti-cancer activity of G-Rg3 has been reported to exhibit by the effects such as anti-proliferative effect to prostate cancer cells,¹⁰⁾ cell cycle arrest, apoptosis, anti-angiogenic effect,¹⁷⁾ the effects of combination therapy with cyclophosphamide.^{18,19)} In 2000, G-Rg3 was introduced into the marketplace as an anticancer drug named 'Shenyi Capsule' and its pharmacological effects have been also proved both in laboratories^{20,21)} and clinics.²²⁾ According to a recent report using microarray hybridization, the most effective pathway of G-Rg3 related to tumor inhibition is the Eph/ephrin pathway.²³⁾ Herein, we investigate the trafficking of G-Rg3 in A549 human lung adenocarcinomas through immunocytochemistry. Taken together, we first re-

port the generation and characterization of monoclonal antibody to G-Rg3. The antibody might be a very effective tool to achieve their goals in various fields such as immunoassay, mechanism study, quality control and pharmacokinetics.

Acknowledgements This work was supported by the grant of the Ministry of Commerce, Industry and Energy in 2007.

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