Ginsenoside Rg3 attenuates cell migration via inhibition of aquaporin 1 expression in PC-3M prostate cancer cells

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1. Introduction

Prostate carcinoma is among the most common malignant cancers worldwide (Jemal et al., 2010). Whereas localized prostate carcinoma can be treated by radical prostatectomy or radiation therapy, patients are at increased risk of cancer metastasis (Cooperberg et al., 2009). Hormone manipulation has been developed as an effective resolution for advanced metastatic prostate carcinoma, however, the occurrence of loss or mutation of the androgen receptor in cancer cells results in failure of androgen deprivation therapy (Devlin and Mundy, 2009). Many other therapeutic agents and protocols have been developed and utilized clinically, however, few of them have been shown to be dramatically effective. Moreover, drug resistance and cytotoxic side effects also impede the use of alternative therapies (Mahon et al., 2011).

Today, natural products have become a valuable resource for the development of new drugs (Craig et al., 2009). Ginseng, which refers to the root of Panax ginseng and its related species, has been used for thousands of years in Asian countries and been reported to exhibit a wide range of medicinal effects. Ginsenoside Rg3, which has been posited to be one of the active ingredients in ginseng, has been shown to have various biological effects including anti-cancer activities (Jia and Zhao, 2009; Jia et al., 2009). Many studies have demonstrated that Rg3 has an anti-proliferation effect in various cancer cell lines including prostate cancer (Chen et al., 2008; Kim et al., 2004). An anti-metastasis activity for Rg3 has also been reported in several models (Ishi et al., 1997; Mochizuki et al., 1995). In this report, we sought to elucidate the effect of Rg3 treatment on a highly metastatic androgen receptor-negative prostate cancer cell line, PC-3M.

Aquaporin (AQP) is a water channel protein family eliciting fundamental functions in water transportation and osmotic homeostasis. The first member to be discovered, Aquaporin 1 (AQP1) is ubiquitously expressed in the human body (Borgnia et al., 1999). In addition to its basic function, AQP1 facilitates cell migration in a variety of cell types (Papadopoulos et al., 2008). Overexpression of AQP1 is common to malignancies from various organs and tissues, and several cancer cell lines expressing high levels of AQP1 exhibit enhanced migration in vitro and greater metastatic potential in vivo (Verkman et al., 2008). In addition, AQP1 promotes endothelial cell migration and angiogenesis, which is another important factor in tumor progression (Clapp and Martinez de la Escalera, 2006).

ABSTRACT

Ginsenoside Rg3 (Rg3), one of the bioactive extracts found in ginseng root, was reported to have anti-cancer activity in various cancer models. The anti-proliferation effect of Rg3 on prostate cancer cells has been well reported. To test whether Rg3 has an anti-metastatic effect on prostate cancer, we treated a highly metastatic PC-3M prostate cancer cell line with Rg3. We found that Rg3 (10 μM) led to remarkable inhibition of PC-3M cell migration. Simultaneously, exposure to Rg3 suppressed expression of the aquaporin 1 (AQP1) water channel protein, which has previously been reported to be involved in cell migration. Overexpression of AQP1 attenuated Rg3-induced inhibition of cell migration, and introduction of a shRNA targeting AQP1 abrogated the inhibitory effect of Rg3, although the basal level of cell migration was decreased by RNA interference. In mechanism study, estrogen receptor- and glucocorticoid receptor-dependent pathways are proved involved in the AQP1 regulation by Rg3. However, Rg3 treatment triggered the activation of p38 MAPK, and SB202190, a specific inhibitor of p38 MAPK, antagonized the Rg3-induced regulation of AQP1 and cell migration, suggesting a crucial role for p38 in the regulation process. Deletion analysis of the promoter region of AQP1 was also conducted using dual-luciferase assay, which indicated that the −1000 bp to −200 bp promoter region was involved in the AQP1 regulation by Rg3. In all, we conclude that Rg3 effectively suppresses migration of PC-3M cells by down-regulating AQP1 expression through p38 MAPK pathway and some transcription factors acting on the AQP1 promoter.
Cellular AQP1 expression is controlled through a number of mechanisms. Osmotic change is a principal factor in AQP1 regulation and can be elicited through transcription (Jenq et al., 1998, 1999) or proteasome-mediated degradation (Leitch et al., 2001). Additionally, toxicity-independent mechanisms, such as glucocorticoid triggered AQP1 expression, have been described (Moon et al., 1997). Because previous studies have identified Rg3 as having glucocorticoid-like actions in cancer cells (Hien et al., 2010) and glucocorticoid is thought to modulate AQP1 expression, which is important in cell migration, we hypothesized that Rg3 may affect cell migration through AQP1 in PC-3M cells.

We found that Rg3 significantly suppresses cell migration and AQP1 expression in PC-3M cells. Furthermore, we showed that Rg3 treatment activated p38 MAPK, whereby PC-3M cell migration was mediated. We also offer possible mechanisms on the regulatory pattern of AQP1 through deletion analysis of the promoter of AQP1 using luciferase assay.

2. Materials and methods

2.1. Drugs and reagents

Ginsenoside Rg3 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and the purity was at least 95% as determined by HPLC. Rg3 was dissolved in dimethyl sulfoxide (DMSO) in a 100 mM stock solution and stored at −20 °C. Aliquots of stock solution were added directly to the culture media.

The proteasome inhibitor MG132 (z-Leu-Leu-Leu-al, no. D7156) and the proteasome inhibitor MG132 (z-Leu-Leu-Leu-al, no. D7156) and dexamethasone (no. D1756) were purchased from Sigma-Aldrich. SB202190 (4-(4-hydroxy-17-(1-propynyl)­estra-4,9-dien-3-one) was kindly provided by Prof. Bin He (National Research Institute for Control of Pharmaceutical and Biological Products, Beijing, China), 100 U/ml penicillin, and 100 μg/ml streptomycin (Inalco Pharm) were added to the lower chamber. The plate was incubated at 37 °C for 12 h and the cells were fixed in 4% formaldehyde. The upper chamber was gently wiped with a cotton swab to remove non-migrated cells, and the migrated cells on the lower side of polycarbonate filters were stained with crystal violet and counted under an Olympus microscope (×200 magnification).

2.2. Cell culture

The PC-3M prostate cancer cells were obtained from the American-type culture collection (ATCC) and cultured in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Inalco Pharmaceuticals) in a humidified incubator with 5% CO2 at 37 °C. When treated with Rg3 or other reagents, cells were cultured in low-serum medium (containing 1% FBS) with the indicated concentration of additives.

2.3. PC-3M cells were plated on 96-well plates at a density of 5 × 104 cells/well and were cultured with DMEM. Twenty-four hours later, cells were treated with a range of concentrations of Rg3 for 24 h. After treatment, cell viability was determined via the MTT (3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) cell proliferation assay. Briefly, 20 μl of sterile MTT dye (5 mg/ml, Sigma, no. M2128) was added per well and cells were incubated at 37 °C for an additional 4 h. Next, culture media were removed and the crystalized formazan was dissolved in 100 μl of DMSO. The optical density (OD) value of the solute was measured at a wavelength of 570 nm.

2.4. In vitro wound healing assay

Wound healing assays were performed as previously described (Liang et al., 2007) to assess the capacity for cell migration. Briefly, PC-3M cells were plated on a 6-well plate. When cells reached 90–95% confluence, scratches were made with a 200-μl sterile pipette tip. Cells were washed with phosphate buffered saline (PBS) three times and the initial wounds were recorded using an Olympus microscope. Cells were then incubated with medium containing Rg3 for 24 h and the wounds were photographed again. The rate of cell migration was evaluated by the rate of wound closure.

2.5. Transwell migration assay

Cell migration was further investigated using the transwell migration assay. PC-3M cells were suspended in serum-free DMEM (1 × 105 cells) and placed in the upper chamber of a 24-well transwell insert (8 μm pore size) (Corning-Costar). DMEM containing 10% FBS was added to the lower chamber. The plate was incubated at 37 °C for 12 h and the cells were fixed in 4% formaldehyde. The upper chamber was gently wiped with a cotton swab to remove non-migrated cells, and the migrated cells on the lower side of polycarbonate filters were stained with crystal violet and counted under an Olympus microscope (×200 magnification).

2.6. Western blot analysis

After treatment, cells were washed twice with PBS and lysed in RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). Protein concentrations were determined using a BCA protein assay kit (Pierce). Equal amounts of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Anti-AQP1 antibody (no. sc-208810) was purchased from Santa Cruz Biotechnology. Anti-ERK (no. 4695), anti-phospho-ERK (no. 4370), anti-p38 (no. 9212), anti-phospho-p38 (no. 4511), anti-JNK (no. 9258) and anti-phospho-JNK (no. 9255) antibodies were purchased from Cell Signaling Technology. Anti-β-actin antibody (no. A8481) was purchased from Sigma. After incubation with primary antibodies, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies. Finally, protein bands were detected and visualized by 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Amresco)/nitro blue tetrazolium (NBT, Amresco), and they were then scanned using an Epson electronic scanner.

2.7. Preparation of total RNA and real-time PCR analysis

Total RNA was isolated from PC-3M cells using TRIzol reagent (Invitrogen, no. 15596-026) according to the manufacturer’s directions. Total RNA was reverse transcribed to cDNA using Superscript III First-strand Synthesis Kit (Invitrogen, no. 18080-051) and oligo(dT) primers. For the real-time PCR analysis, a THUNDERBIRD SYBR qPCR Mix (Toyobo, no. QPS-201) was used according to the manufacturer’s instructions, and PCR was performed using an Mx3005P QPCR system (Agilent). The selective primers for human AQP1 and GAPDH were as follows: 5′-CATCCCTCTACGGCATCAC-3′ (forward) and 5′-GGTACTACGAGGTTTGC-3′ (reverse) for human AQP1 (GenBank ID: 293703114); 5′-CAAAGCTGAAACGCCGAA-3′ (forward) and 5′-GGCCAGAGATGACCCCTT-3′ (reverse) for human GAPDH (GenBank ID: 83641880). The relative amount of AQP1 mRNA was normalized on the basis of the amount of GAPDH.

2.8. Overexpression of AQP1 in PC-3M cells

A pcDNA3-AQP1 plasmid for overexpression of AQP1 was kindly provided by Prof. Bao-Xue Yang (Peking University Health Science Center, Beijing, China). PC-3M cells were transfected using Lipofectamine LTX and PLUS reagent (Invitrogen, no. 15338-100) and prepared for experiments after 24 h of transfection.
2.9. Knockdown of AQP1 by RNA interference

A pSUPER.GFP.neo vector (Oligoengine, no. VEC-PBS-0006) was kindly contributed from Prof. Yuxin Yin (Peking University Health Science Center, Beijing, China). The AQP1 specific and scrambled siRNA sequences were designed by the pSUPER RNAi system (Oligoengine) and cloned into the vector using BglII and XhoI restriction enzyme sites. The siRNA sequence targeting AQP1 was as follows: 5′-CATCAGCATTGCTCTGCGC-3′. PC-3M cells were transfected with plasmid expressing AQP1 specific or scrambled shRNA and cultured for an additional 24 h to silence AQP1 expression. Lipofectamine LTX and PLUS reagent (Invitrogen) were used for transfection.

2.10. Dual-luciferase reporter assay

To test the binding activity of Rg3 on estrogen receptor and glucocorticoid receptor, we inserted the consensus sequences of estrogen responsive element (ERE) and glucocorticoid responsive element (GRE) into a pGL3-Promoter vector (Promega, E1761) upstream of the luciferase cassette. The consensus sequences were as follows: 5′-AGCTCACTGTGACCCGTCATGTGACCGCAGCCGACCTG-3′ for ERE; 5′-GTGATCACTTGCCTTCTGAGACAAATGTTCCGCTTACCGATCTC-3′ for GRE. As for the analysis of the promoter activities of AQP1, several segments (−2026/+78, −1519/+78, −968/+78, −526/+78 and −200/+78) from the 2 kb sequences 5′ upstream of the first exon of human AQP1 (GenBank ID: 224589819) were cloned and inserted into pGL3-Basic vector (Promega, E1751). These constructs (designated as pGL3−2026, −1519, −968, −526 and −200, respectively) were employed to test the promotor sequence responsible for AQP1 regulation by Rg3. A Dual-Luciferase Reporter (DLR) Assay System (Promega, no. E1910) was used to assess luciferase activity. Briefly, PC-3M cells were co-transfected with different pGL3 constructs and pRL-TK control plasmid (Promega, no. E2241). After transfection, cells were treated with Rg3 for 24 h. Cells were then lysed and processed according to the manufacturer’s instructions. The luciferase activity was measured by a FlexStation 3 microplate reader (Molecular Devices). For each sample, the pGL3 firefly luciferase activity was normalized by the Renilla luciferase activity from the pRL-TK control.

2.11. Statistical analysis

Results were presented as means ± S.E.M. For experiments with more than two groups, the one-way analysis of variance (ANOVA) test was performed for multiple comparisons. Single treatment and control groups were compared using a two-tailed t-test. All the experiments were repeated at least three times. P-values less than 0.05 were considered to be significant.

3. Results

3.1. Ginsenoside Rg3 inhibits cell migration but does not influence the proliferation of PC-3M cells

To evaluate the anti-metastatic effect of Rg3, we treated the metastatic prostate PC-3M cell line with various concentrations of Rg3 (up to 10 μM). Results from the MTT proliferation assay showed similar cell viability between the control group and the groups treated with various concentrations of Rg3 (Fig. 1B). However, in wound healing assays, the groups treated with 1 μM and 10 μM of Rg3 migrated much slower compared with the control group (Fig. 1C, D), suggesting anti-migration effects of Rg3. To confirm this result, we performed further cell migration assays using the transwell chamber model. As shown in Fig. 1E and F, the cells treated with either 1 μM or 10 μM Rg3 had dramatically fewer migrating PC-3M cells compared with the control group. These results indicate that treatment with Rg3 at concentrations between 1 μM and 10 μM results in a substantial anti-migration effect without affecting proliferation of PC-3M cells.

3.2. Ginsenoside Rg3 inhibits expression of AQP1 in PC-3M cells

To investigate whether Rg3 regulates AQP1 protein expression in PC-3M cells, we treated PC-3M cells with various concentrations and durations of Rg3 and assessed AQP1 expression by immunoblotting. We found that the protein level of AQP1 in PC-3M cells was significantly inhibited following a 24 h treatment with 1 μM and 10 μM of Rg3 (Fig. 2A, B); and in the time curve, when cells were treated with 10 μM of Rg3, the inhibitory effect began to appear after 24 h treatment (Fig. 2C, D).

Because AQP1 expression can be modulated transcriptionally or post-translationally through proteasome-mediated degradation (Leitch et al., 2001), we tested both of these pathways following Rg3 treatment. Pretreatment of cells with the proteasome inhibitor MG132 did not affect the inhibitory effect of Rg3 on AQP1 expression in PC-3M cells (Fig. 2E), however, quantitative RT-PCR analysis showed that Rg3 treatment led to a down-regulation of AQP1 mRNA after treatment 18 h (Fig. 2F). These results suggest that Rg3 suppresses AQP1 protein expression in PC-3M cells in a transcriptional manner.

3.3. AQP1 mediates the anti-migration effect of Rg3

As Rg3 inhibits PC-3M cell migration and AQP1 expression simultaneously, we sought to test whether AQP1 mediates the Rg3-induced inhibition of PC-3M cell migration. To counteract the suppression of AQP1 by Rg3, we transfected PC-3M cells with an AQP1 expression vector. Transfection of AQP1 successfully increased AQP1 expression level in PC-3M cells (as determined by Western blot analysis) and led to a marked attenuation of inhibition of cell migration induced by Rg3 (Fig. 3B), suggesting that migration suppression was due to AQP1 inhibition induced by Rg3 treatment. For loss of function studies, we constructed a plasmid encoding an AQP1-targeted shRNA that effectively silenced AQP1 expression (Fig. 3C). The resulting cell migration of AQP1 knockdown cells was diminished and was no longer responsive to Rg3 treatment (Fig. 3D), suggesting that AQP1 is required for Rg3-induced inhibition of cell migration. Together, the loss and gain of function studies demonstrate that AQP1 is a critical mediator in the anti-migration effect of Rg3.

3.4. Estrogen receptor- and glucocorticoid receptor-dependent pathways are not involved in the inhibitory effect of Rg3 on AQP1 expression

Recent studies suggest that Rg3 and other ginsenosides can exert estrogen- and glucocorticoid-like activity (Hien et al., 2010; Leung et al., 2009). Moreover, the correlation between steroid hormones and AQP1 expression has also been well elucidated (Jablonski et al., 2003; Moon et al., 1997; Richard et al., 2003). Therefore, we determined Rg3’s estrogen receptor- and glucocorticoid receptor-dependent transcriptional activities in PC-3M cells. By the luciferase assay, we found that Rg3 had no significant effect on ERE activity (Fig. 4A). As for glucocorticoid receptor-dependent pathway, Rg3 activated it in a dose-dependent manner, but the activation level was much lower than the positive control (Fig. 4B). To test whether glucocorticoid receptor-related pathway was involved in the regulation process, we utilized RU486, a glucocorticoid receptor antagonist, to block the pathway. As shown in Fig. 4C, although slightly agonizing GRE activity, RU486 effectively blocked the effect of Rg3 on GRE. However, the blocking of glucocorticoid receptor-dependent pathway did not antagonize the inhibitory effect of Rg3 on AQP1 expression (Fig. 4D). These results indicate that neither estrogen receptor- nor glucocorticoid receptor-triggered pathway mediates Rg3-induced suppression of AQP1 expression.
3.5. p38 MAPK is crucial for Rg3-induced inhibition of AQP1 expression and cell migration

As previous studies have linked MAPK signaling pathways to AQP1 modulation (Umenishi and Schrier, 2003), we sought to elucidate the relationship between MAPK pathways (ERK, p38 and JNK) and Rg3-induced inhibition of AQP1. Following treatment with Rg3, the p38 MAPK was robustly activated, while JNK remained unaltered and ERK was only transiently activated at 30 min (Fig. 5A). This result suggests that p38 MAPK may mediate the inhibition of AQP1 by Rg3.

To further address the role of p38 MAPK in Rg3-induced inhibition of AQP1 and cell migration, we used a specific inhibitor of p38 MAPK, SB202190. Pretreating PC-3M cells with 10 μM of SB202190 significantly inhibited the effect of Rg3 on AQP1 expression in PC-3M cells, as confirmed by Western blot (Fig. 5B). Moreover, the inhibition of cell migration by Rg3 was also attenuated (Fig. 5C). These results demonstrate that p38 MAPK is involved in the inhibitory effect of Rg3 on AQP1 expression and cell migration.

3.6. Deletion analysis of the AQP1 promoter

Because AQP1 is transcriptionally downregulated following Rg3 exposure, some transcription factors and their binding elements are likely involved in the regulatory process. Thus, we employed a deletion analysis approach to determine the required segments of the AQP1 promoter. By isolating different lengths of the 5′ genomic flanking region of AQP1 and constructing them into the pGL3 luciferase reporter plasmid, the AQP1 expression rates under normal and Rg3 treated conditions were determined (Fig. 6). Under basal conditions, the luciferase protein (luminescence) was stably expressed in the presence of the 2000 bp AQP1 promoter sequence. As the promoter was truncated to 1500 bp, the luminescence markedly increased. This increase in luciferase activity was maintained until the promoter region inserted was truncated to 200 bp, which led to a dramatic loss of luminescence. However, when the promoter was longer than 1000 bp, the Rg3 treated groups showed suppressed luciferase activity compared with the respective control groups. When the promoter was truncated to 500 bp, the suppression by Rg3 was partly attenuated. Further, this Rg3-induced loss of...
Fig. 2. The effect of Rg3 on the expression of AQP1 in PC-3M cells. (A) Cells were cultured with the indicated concentration of Rg3 for 24 h, and cell extracts were subjected Western blot analysis for AQP1 expression. (B) Statistical results for (A). (C) Cells were cultured with 10 μM of Rg3 for different times and Western blot analysis for AQP1 was performed. (D) Statistical results for (C). (E) Proteasome-mediated degradation pathway is not involved in AQP1 down-regulation by Rg3. Cells were left untreated or treated with 20 μM of MG132 for 1 h. Next, medium was replaced and cells were treated by DMSO or Rg3 (1 μM and 10 μM) for 24 h, after which AQP1 level was measured by Western blot. (F) AQP1 is transcriptionally regulated by Rg3. Cells were treated with DMSO or Rg3 (1 μM and 10 μM) for 18 h and total RNA was extracted by TRizol reagent and analyzed by quantitative RT-PCR. Results represent the means ± S.E.M. of triplicates. * P < 0.05 and ** P < 0.01, significantly different from control.

Fig. 3. Effect of overexpression and silencing of AQP1 on PC-3M cell migration. (A and B) Overexpression of AQP1 attenuates cell migration suppression induced by Rg3. (A) Cells transfected with pcDNA3-AQP1 plasmid or empty vector (EV) control were treated by Rg3 (1 μM and 10 μM) or DMSO for 24 h, and AQP1 expression level was evaluated by Western blot. (B) After transfection and Rg3 (10 μM) treatment, cells were suspended and seeded into transwell chambers for 12 h. Next, the migrated cells were counted under microscopy. (C and D) Knockdown of AQP1 inhibits cell migration and abrogates the effect of Rg3. (C) Cells were transfected with pSUPER plasmid expressing shRNA against AQP1 (si AQP1) or scrambled shRNA control (si control), and both groups were treated with Rg3 (1 μM and 10 μM) or DMSO for 24 h and then AQP1 content was tested. (D) AQP1 silenced and control cells were treated with 10 μM of Rg3 or DMSO, and then subjected to a transwell assay to evaluate the cell migration rate. Statistical results represent the means ± S.E.M. of triplicates. * P < 0.05 and ** P < 0.01, significantly different from control.
luminescence disappeared after the promoter being cut down to 200 bp. As a negative control, pGL3 empty plasmid was also transfected into PC-3M cells. The luciferase activities of those groups were negligibly regardless of the Rg3 treatment.

4. Discussion

Ginsenoside Rg3, a principal bioactive component of ginseng extract, has been reported to exhibit anti-proliferative effects in cancer cell lines derived from various tissues (Wang et al., 2007). Previous reports have shown that Ginsenoside Rg3 and Rh2, its metabolite found in the human intestine, suppress proliferation and cell attachment of prostate cancer cells (Kim et al., 2004; Liu et al., 2000). Similar to other cancer subtypes, patients suffering from prostatic carcinoma are at risk for metastasis. Thus, in addition to hormone deprivation and chemotherapy, anti-metastatic therapy should also be considered. Cell migration is critical to cancer metastasis. In this study, we demonstrate that Rg3 markedly suppresses cell migration.
in PC-3M cells, a well-described in vitro metastatic prostate cancer model. Moreover, the concentration under which Rg3 inhibits cell migration (1 μM and 10 μM) was lower than the concentration required for its anti-proliferation activity (above 100 μM) (Kim et al., 2004; Wang et al., 2007). This result indicates that Rg3 may be a potential anti-cancer agent with both anti-proliferation and anti-metastasis activity.

Despite previous in vivo and in vitro experiments demonstrating an anti-metastasis activity of Rg3 (Chen et al., 2011; Ishii et al., 1997; Mochizuki et al., 1995; Shinkai et al., 1996), few results have been reported specifying the mechanism. In the present study we first linked the anti-migration function of Rg3 to AQP1 expression. AQP1 was initially discovered as a channel protein for water transportation (Walz et al., 1997), but was later found to be a regulator of cell migration (Papadopoulos et al., 2008). Reports have shown that AQP1 is overexpressed in various types of cancer including prostate cancer (Mobasher et al., 2005), and in PC-3M cells we also detected AQP1 expression. We found that Rg3 markedly suppressed AQP1 protein expression in PC-3M cells, indicating a link between AQP1 and Rg3-induced migration inhibition.

Furthermore, we confirmed that Rg3 regulated cell migration of PC-3M cells through the inhibition of AQP1 expression. As expected, overexpression of AQP1 promoted PC-3M cell migration. Increased AQP1 expression also compensated for Rg3-induced inhibition of cell migration, whereas knockdown of AQP1 led to an attenuation of cell migration similar to Rg3 treatment alone. However, following knockdown of AQP1, Rg3 treatment no longer affected cell migration. These results correlated AQP1 and Rg3-induced migration inhibition, suggesting that AQP1 is the mediator for the anti-migration effect of Rg3.

While defining the regulatory mechanism of Rg3 on AQP1 expression, we first tested the estrogen receptor- and glucocorticoid receptor-dependent pathways because Rg3 has been reported to have activity on estrogen receptor and glucocorticoid receptor (Hien et al., 2010) and steroid hormones are proved to be able to regulate AQP1 expression (Jablonski et al., 2003; Moon et al., 1997). We found that glucocorticoid receptor, but not estrogen receptor, dependent transcriptional activity was weakly agonized by Rg3 in PC-3M cells. However, the specific antagonist of glucocorticoid receptor cannot block the effect of Rg3 on AQP1 expression. These results indicate that the estrogen receptor- and glucocorticoid receptor-dependent pathways are not involved in the inhibitory process of AQP1.

After that we investigated the role of the MAPK signaling pathway as it is a common regulatory system and was reported to play some role in AQP1 regulation (Umenishi and Schrier, 2003). We found that Rg3 remarkably up-regulated p38 MAPK in PC-3M cells, while JNK remained unaltered and ERK was only transiently activated. Furthermore, a specific inhibitor of p38 MAPK significantly suppressed the effects of Rg3 on AQP1 and cell migration, providing evidence for the role of p38 MAPK in the suppression of AQP1 and cell migration by Rg3. Previously, activation of p38 MAPK has been shown to be followed by up-regulation of AQP1 (Umenishi and Schrier, 2003), which is in disagreement with our results. Similarly opposing results of JNK-mediated AQP1 regulation have also been reported. Under hypertonicity stimulation, phosphorylated JNK promoted AQP1 expression (Umenishi and Schrier, 2003); however, activation of JNK was also shown to be involved in LPS-induced down-regulation of AQP1 in the salivary gland (Yao et al., 2010). The MAPK pathway is a common route leading to a large array of downstream activities. It is possible that different cell types and upstream stimulation may explain these contradictory results. Thus, further studies are required to clarify the mechanism downstream of the MAPK pathway that mediates the suppressive effect of Rg3 on AQP1 and cell migration.

During our experiments on the ability of Rg3 to inhibit AQP1, we demonstrated that Rg3 inhibited AQP1 through transcriptional adjustment and not through the proteasome-related pathway. Since Rg3 suppresses AQP1 transcriptionally, some transcription factor that directly affects the promoter region of AQP1 gene is indispensable for the regulation of AQP1. Previous studies have focused on the promoter of AQP1 and several transcriptional factors known to bind to this region. Among them, most research results involved osmotic changes and toxicity related transcriptional elements, such as toxicity-responsive enhancer (TonE) (Lanaspa et al., 2010) and the hypertonicity response element (HRE) (Umenishi and Schrier, 2002). In this study, these elements were less likely to be involved in the AQP1 regulation by Rg3, as the osmotic pressure of the culture medium was not affected by Rg3. Deletion analysis of the AQP1 promoter offered some hints as to the regulatory pattern of AQP1, specifically that some element located between 1000 bp to 200 bp upstream of the first exon of AQP1 gene might be responsible for the AQP1 regulation by Rg3. Also, the sequence within the first 200 bp of the promoter may not be implicated in the regulatory process. Several sites for transcription factor binding are found in the region from −1000 bp to −200 bp, including GATA, CCAAT, E-box, SP1 and AP2 (Umenishi and Verkman, 1998). To clarify the mechanism...
underlying the suppression of AQP1 by Rg3, further studies should be performed to identify specific transcription factors involved.

In summary, we have shown that Ginsenoside Rg3 effectively suppresses the expression of AQP1 in PC-3M cells. Through the inhibition of AQP1, Rg3 also exerts anti-migration effect in PC-3M cells. In mechanism study we exclude the involvement of estrogen receptor- and glucocorticoid receptor-dependent pathways in Rg3’s effect on AQP1, although Rg3 shows weak agonistic activity on glucocorticoid receptor. However, we find that p38 MAPK is activated by Rg3 and it is essential for Rg3 exerting its effect on AQP1 expression and cell migration. Further, the deletion analysis of AQP1 promoter suggests that the 1000 bp to 200 bp segment of AQP1 promoter is crucial for the regulation of AQP1 by Rg3. Further study on this promoter region may identify more specific mechanism underlying this regulation process.

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References


