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Transient Receptor Potential Melastatin 7 Channels are Involved in Ginsenoside Rg3-Induced Apoptosis in Gastric Cancer Cells

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Abstract: Ginsenosides play a role in a number of physiological and pharmacological functions in the gastrointestinal tract. The aim of this study was to clarify the potential role for transient receptor potential melastatin 7 (TRPM7) channels in ginsenoside Rg3-inhibited growth and survival of AGS cells, the most common human gastric adenocarcinoma cell line. The AGS cells were treated with varying concentrations of Rg3. Sub-G1 analysis, caspase-3 activity and poly(ADP-ribose) polymerase (PARP) cleavage analysis were conducted to determine whether AGS cell death occurs by apoptosis. TRPM7 channel blockers (La^{3+} or 2-APB) and small interfering RNA (siRNA) were used in this study to confirm the role of TRPM7 channels. Furthermore, TRPM7 channels were over-expressed in human embryonic kidney (HEK) 293 cells to identify the role of TRPM7 channels in AGS cell growth and survival. The addition of Rg3 to the culture medium inhibited AGS growth and survival. Experimental results showed sub-G1 was markedly increased, caspase-3 activity was elevated, and degree of PARP cleavage was increased. TRPM7 channel blockade, either by La^{3+} or 2-APB or by suppressing TRPM7 expression with siRNA, blocked the Rg3-induced inhibition of cell growth and survival. Furthermore, TRPM7 channel over-expression in HEK 293 cells exacerbated Rg3-induced cell death. These findings indicate that ginsenoside Rg3 inhibits the growth and survival of gastric cancer cell which is because of the blockade of TRPM7 channel activity. Therefore, TRPM7 channels may play an important role in the survival of gastric cancer.

Ginseng, the root of *Panax ginseng* C. A. Meyer *(Araliaceae)*, is a well-known folk medicine that has been used as a tonic agent. The molecular components primarily responsible for the mechanism of action of ginseng are ginsenosides, also known as ginseng saponins. Ginsenosides are derivatives of triterpenoid dammarane and consist of 30 carbon atoms [1]. Ginsenosides have a four-ring, steroid-like structure and attached sugar moieties. About 30 different ginsenosides have been isolated and identified from the *P. ginseng* root [2]. In a study that used purified compounds of ginsenosides, ginsenosides Rb1 and Rg3 were observed to exert a number of biological activities that affected central and peripheral nervous systems as well as cardiovascular and immune systems [3–7]. A common belief in Korea is that ginseng may augment conventional cancer treatments; it is frequently used as a complementary cancer drug after operation [8]. *Panax ginseng* extracts exhibit anticarcinogenic activity, like inhibiting tumour angiogenesis and metastasis [9–11] and inducing tumour cell apoptosis [12]. Another study has shown that a *P. ginseng* extract activates multiple immunostimulation effector pathways for anti-tumour activity [13].

Gastric cancer is a leading cause of cancer-related mortality in Korea. In previous studies, we suggested that human gastric adenocarcinoma cells express the transient receptor potential melastatin 7 (TRPM7) channel, which is essential for cell survival and is a potential target for pharmacological gastric cancer treatment [14]. TRPM7 is a member of the large TRP channel superfamily that is expressed in nearly every tissue and cell type [15–17]. Many reports suggest that activated TRPM7 channels contribute to a number of physiological and pathophysiological processes [18–20]. However, the role of the TRPM7 channel in the survival of gastric cancer cells after incubation with Rg3 is unknown.

In this study, we examined the potential role of TRPM7 channels in Rg3-inhibited growth and survival of AGS cells, the most common human gastric adenocarcinoma cell line. Our data suggest that TRPM7 channels play an important role in the survival of these tumour cells.

Materials and Methods

Materials. The ginsenoside Rg3 was provided by the AMBO Institute (Seoul, Korea) with a purity of about 95%. The remaining agents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells. The AGS cell line, the most common human gastric adenocarcinoma cell line, was used. AGS cell line was established at the Cancer Research Center, College of Medicine, Seoul National

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University, Korea. The cell line was propagated in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum and 20 μg/mL penicillin and streptomycin in an atmosphere of 5% CO_2 at 37°C.

Patch-clamp experiments. Whole-cell configuration of the patch-clamp technique experiments was performed at room temperature (22–25°C). The AGS cells were transferred to a small chamber on an inverted microscope stage (IX70; Olympus, Tokyo, Japan) and were constantly perfused with a solution containing (mM) KCl 2.8, NaCl 145, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2, and N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (mM) Cs-glutamate 145, NaCl 8, Cs-2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid 10, and HEPES-CsOH 10, adjusted to pH 7.2 with CsOH. Axopatch I-D (Axon Instruments, Foster City, CA, USA) was used to amplify membrane currents and potentials. For data acquisition and the application of command pulses, PCLAMP software v.9.2 and DIGIDATA 1322A (Axon Instruments) were used. Results were analysed using PCLAMP and ORIGIN software (Microcal Origin version 6.0).

TRPM7 expression in human embryonic kidney (HEK) 293 cells. Human embryonic kidney-293 cells were transfected with the Flag-murine LTRPC7/pCDNA4-TO construct and grown on glass coverslips in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, blasticidin (5 μg/mL) and zeocin (0.4 mg/mL). TRPM7 (LTRPC7) expression was induced by adding 1 μg/mL tetracycline to the culture medium. Whole-cell patch-clamp experiments were performed at 21–25°C with cells that were grown on glass coverslips.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting. Western blot was performed using the AGS cell lysates. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis by using 8% polyacrylamide gels, transferred to a polyvinylidene difluoride membrane and quantified by incubation with anti-poly(ADP-ribose) polymerase (PARP) (1:500) antibodies. All procedures used standard methods.

RNA interference. All the synthetic siRNA sequences were designed by Qiagen with the BIOPREDsi algorithm licensed from Novartis. All siRNA target sequences that were used to silence the *TRPM7* gene (GenBank accession number NM_017672) and in other experimental procedures were identical to those used in a previous study [14].

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cell viability was assessed by MTT assay. The AGS cells were seeded into each well of 12-well culture plates and then cultured in RPMI-1640 supplemented with other reagents for 72 hr. After incubation, 100 μL of MTT solution [5 mg/mL in phosphate-buffered saline, (PBS)] was added to each well, and the plates were incubated at 37°C for 4 hr. After removing the supernatant and shaking with 200 μL of dimethyl sulfoxide (Jersey Lab Supply, Livingston, NJ, USA) for 30 min., absorbance was measured at 570 nm. All experiments were repeated at least three times.

Flow cytometric analysis. To investigate whether the cell cycle of AGS cells was redistributed, flow cytometric analysis was used with propidium iodine (PI) stain [21,22]. A total of 1×10^6 cells were placed in an e-tube. A volume of 700 μL of ice-cold fixation buffer (ethyl alcohol) was slowly added with vortexing. Tubes were sealed with parafilm and incubated at 4°C overnight. Samples were spun for 3 min. at $106 \times g$ at 4°C, and the supernatant was aspirated and discarded. The cell pellet was resuspended by 200 μL of PI staining solution [PI (5 mg/mL) 2 μL and RNase 2 μL in PBS 196 μL] at $20{,}817 \times g$ for 5 sec. After 30 min. in the dark at room temperature, samples were analysed in a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Moutain View, CA, USA) at λ = 488 nm using CELL-QUEST software (Becton-Dickinson). The DNA content distribution of normal growing cells is characterized by two peaks: the G1/G0 and G2/M phases. The G1/G0 phase comprises the normal functioning and resting state of the cell cycle with the most diploid DNA content, while the DNA content in the G2/M phase is more than diploid. Cells in the sub-G1 phase have the least DNA content in cell-cycle distribution; this is termed hypodiploid. The hypoploid DNA contents represent the DNA fragmentation [23].

Caspase-3 assay. Caspase-3 assay kits (Cellular Activity Assay Kit Plus) were purchased from BioMol (Plymouth, PA, USA). After experimental treatment, cells were centrifuged ($10{,}000 \times g$, 4°C, 10 min.) and washed with PBS. Cells were resuspended in ice-cold cell lysis buffer and incubated on ice for 10 min. Samples were centrifuged at $10{,}000 \times g$ (4°C, 10 min.), and the supernatant was removed. Supernatant samples (10 μL) were incubated with 50 μL of substrate (400-μM Ac-DEVD-pNA) in 40 μL of assay buffer at 37°C. Absorbance at 405 nm was read at several time-points. pNA concentration in samples was extrapolated from a standard created with absorbances of sequential pNA concentrations.

Statistical analysis. Data are expressed as mean ± S.E.M. Differences between the data were evaluated by Student's *t*-test. A *p*-value of 0.05 was taken to indicate a statistically significant difference.

Results

Inhibition of cell survival by Rg3.

We investigated Rg3 influence on the survival of AGS cells. After incubation for 24 hr with 100, 200, 300, 400 or 500 μM Rg3 in the culture medium, AGS cell survival was inhibited by 0.1 ± 0.1%, 1.1 ± 0.2%, 9.2 ± 1.3%, 16.3 ± 3.1% or 23.2 ± 3.3%, respectively, in the MTT assay (n = 5; fig. 1A). After incubation for 48 hr with 100, 200, 300, 400, or 500 μM Rg3 in the culture medium, AGS cell survival was inhibited by 0.1 ± 0.1%, 5.1 ± 1.2%, 17.3 ± 3.3%, 19.4 ± 4.2% or 47.5 ± 5.3%, respectively, in the MTT assay (n = 5; fig. 1B). After a 72-hr incubation with 100, 200, 300, 400 or 500 μM Rg3 in the culture medium, AGS cell survival was inhibited by 0.3 ± 0.1%, 6.1 ± 1.2%, 35.2 ± 1.5%, 61.3 ± 2.1% or 73.2 ± 1.3% in MTT assay (n = 5; fig. 1C). These results suggested that Rg3 plays an important role in the survival of AGS cells.

Inhibition of cell survival by Rg3 leads to increased apoptosis.

To determine whether AGS cell death occurs by apoptosis, we conducted sub-G1 analysis and used a method involving specific proteolytic cleavage of the DNA repair enzyme, PARP [24,25]. In this protocol, cells were incubated with Rg3 and stained with a fluorescent DNA stain (PI). The action of endogenous endonucleases in apoptotic cells cleaves DNA into endonucleosomal fragments of typical size, which are extracted from the cells. The loss of DNA is detected by FACS analysis, as the reduced nuclear staining in apoptotic cells, which results in a novel (sub-G1) fluorescence peak to the left of the regular fluorescence peak. Rg3 induced an increase in sub-G1 levels to 20.8 ± 1.5% with 300 μM Rg3, 32.8 ± 3.5% with 400 μM Rg3, and 42.2 ± 1.5% with 500 μM Rg3 (n = 5; fig. 2A). Caspase-3 activation is one of the hallmarks of apoptotic cell death. We also measured the enzyme activity in AGS cells after Rg3 incubation. Using a

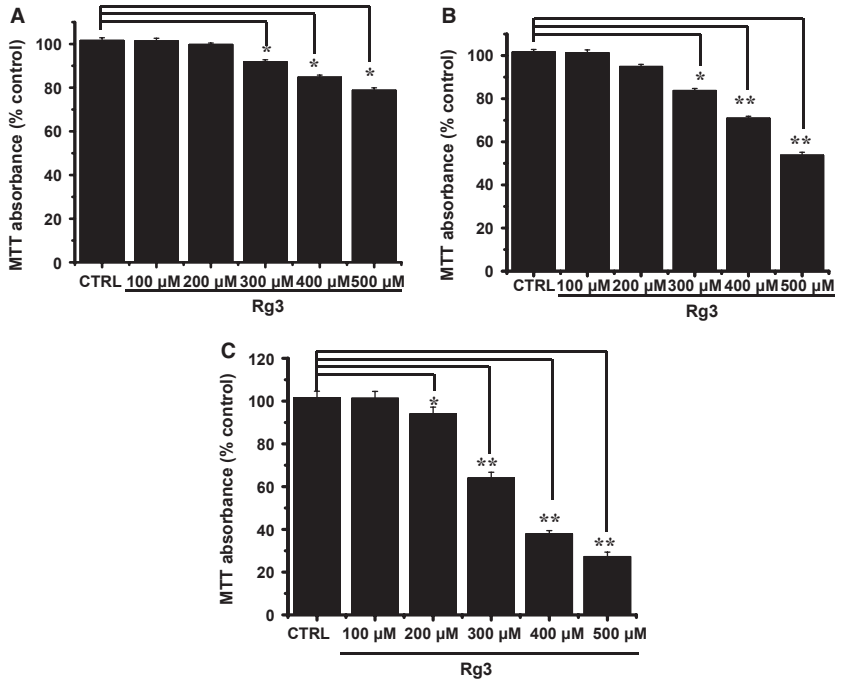

Fig. 1. Effects of Rg3 on AGS cell viability. (A) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based viability assay. AGS cells were treated with increasing concentrations of Rg3 for 24 hr. (B) MTT-based viability assay. AGS cells were treated with increasing concentrations of Rg3 for 48 hr. (C) MTT-based viability assay. AGS cells were treated with increasing concentrations of Rg3 for 72 hr. Changes in cell viability were observed by performing the MTT assay. A significant reduction in cell viability was observed after application of 300, 400 or 500 μM Rg3. CTRL: AGS cells. *$p < 0.05$. **$p < 0.01$.

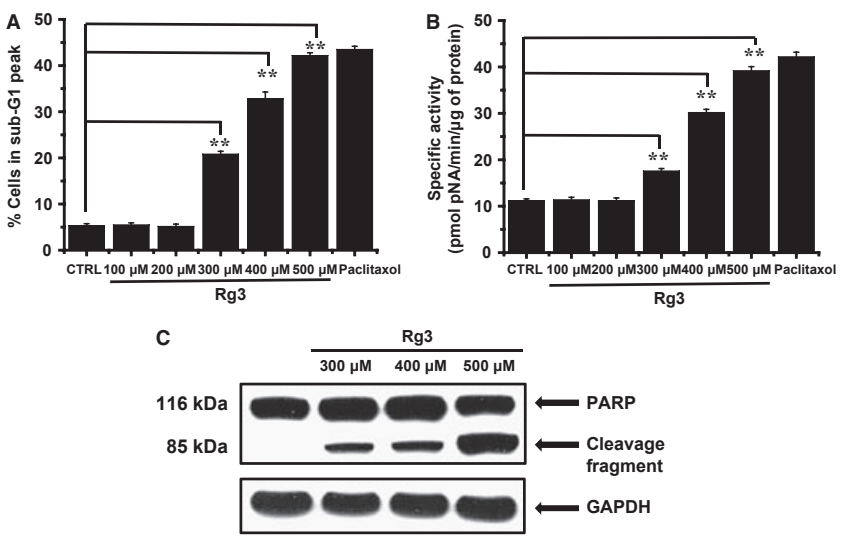

Fig. 2. Inhibition of cell survival by Rg3 leads to increased apoptosis. (A) Sub-G1 peak measured by using FACScan. Quantitative data of three independent experiments. (B) Caspase-3 activity measured by performing enzyme assays. The specific activity was obtained from three samples per group. (C) A representative western blot of incubated cells with anti-poly (ADP-ribose) polymerase (PARP) antibody. Cells incubated with Rg3 represent increased PARP cleavage. Glyceraldhyde-3-phosphate dehydrogenase was used as an internal control. Paclitaxel was used as positive control. CTRL: AGS cells. **$p < 0.01$.

synthetic substrate, we detected the caspase-3 activity in AGS cells to be 11.2 ± 0.8 nmol pNA per min./μg protein. After incubation for 72 hr with Rg3, AGS cells exhibited elevated caspase-3 activity to 17.6 ± 1.4 nmol pNA per min./μg protein with 300 μM Rg3, 30.2 ± 1.5 nmol pNA per min./μg protein with 400 μM Rg3 and 39.2 ± 1.9 nmol pNA per min./μg protein with 500 μM Rg3 ($p < 0.01$, n = 5; fig. 2B). To confirm apoptosis, we used a positive control, paclitaxol, which is an anticancer drug. Paclitaxol induced increases in the sub-G1 level and caspase-3 activity.

We further characterized the changes in caspase-3 activity by western blot analysis of its natural substrate, PARP, which has been shown to function as a cellular target of caspase-3 and other caspases. During apoptosis, PARP is proteolytically cleaved from a 116-kDa-intact form into 85 and 25 kDa fragments. After incubation with Rg3 for 72 hr, AGS cells showed increased PARP cleavage (fig. 2C).

Inhibition of cell death by TRPM7 blockade.

It has been proposed that TRPM7 is required for cell survival, on the basis of experiments in genetically engineered DT-40 B-cells [16]. Furthermore, Wykes *et al.* [26] suggested that TRPM7 is critical to human mast cell survival. We recently suggested, as in previous reports, that AGS cells express the TRPM7 channel and suppression of the TRPM7 channel induces cell death [14]. Therefore, we investigated the influence of TRPM7 channels on the survival of AGS cells after they were incubated with Rg3 for 72 hr. Firstly, we tested the effect of 2-APB, a non-specific TRPM7 channel inhibitor, on AGS cell survivals. The addition of 100 μM 2-APB in the culture medium inhibited the death of AGS cells by 45.3 ± 1.9% in the MTT assay (n = 5; fig. 3A). Similar to 2-APB, La^{3+} also inhibited the death of AGS cells (n = 5; fig. 3A). We performed whole-cell voltage-clamp recordings to investigate the effect of Rg3 in TRPM7-like current in AGS cell. A voltage ramp with voltage ranging from +100 mV to −100 mV evoked small inward currents at negative potentials, whereas larger outward currents were evoked at positive potentials, showing outward-rectifying cation currents (n = 10; fig. 3B). In the presence of 500 μM Rg3, the amplitude of the currents was inhibited outwardly by 36.1 ± 2.5% and inwardly by 4.2 ± 1.9% (n = 7; fig. 3B).

Effects of RNAi on AGS cells.

We used RNA interference (RNAi) to determine whether the TRPM7 channel was actually important to cell viability after incubation with Rg3. To prevent non-specific effects of the

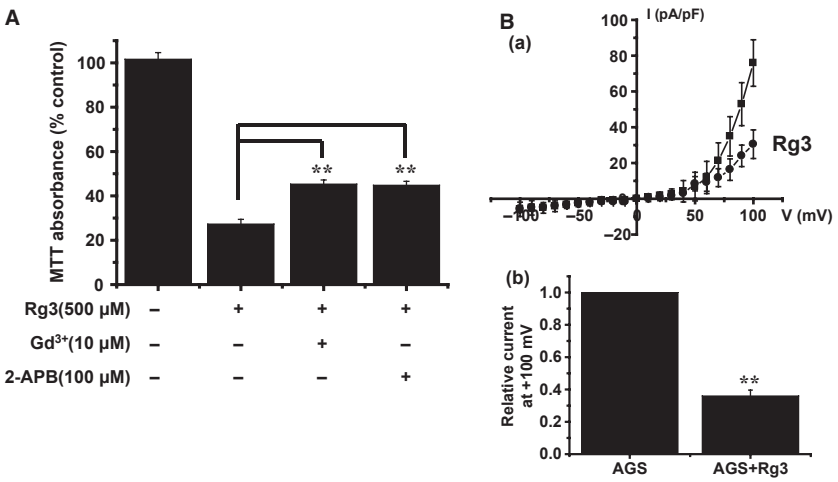

Fig. 3. Inhibition of cell death by transient receptor potential melastatin 7 (TRPM7) blockade. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay induced by different treatment as indicated. Inhibition of TRPM7 channels by Gd^{3+} or 2-APB reduced Rg3 induced apoptosis. (B) Effect of 2-APB on TRPM7-like current. I–V curves and summary bar graph in the absence (■) or presence (●) of 500 μM Rg3. $^{**}p < 0.01$.

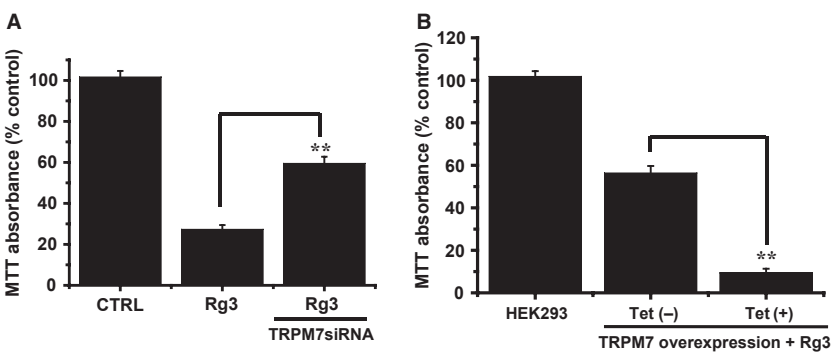

Fig. 4. Effects of RNA interference (RNAi) in AGS cells and the effect of Rg3 on transient receptor potential melastatin 7 (TRPM7) channel over-expression in human embryonic kidney cells. (A) Cell viability was increased 72 hr after transfection with TRPM7siRNA and incubation with Rg3. (B) TRPM7 cells were treated or not treated with tetracycline for 1 days. Cells were incubated with Rg3, followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. $^{**}p < 0.01$.

siRNA sequence used here, we generated three types of 21-nucleotide siRNA that targeted human TRPM7 specifically, TRPM7siRNA1, TRPM7siRNA2 and TRPM7siRNA3 [14]. In our previous report, we studied the effects of these TRPM7siRNA sequences in AGS cells. Only the TRPM7siRNA3 sequence silenced TRPM7 protein expression by 70–80% without reducing glyceraldhyde-3-phosphate dehydrogenase expression [14]. We used the TRPM7siRNA3 sequence in this experiment. After cell incubation with Rg3 and transfection with TRPM7siRNA3, viability increased from $27.3 \pm 2.1\%$ to $59.5 \pm 2.3\%$ in the MTT assay ($p < 0.01$, n = 5; fig. 4A). To provide additional evidence that supports the contribution of the TRPM7 channel to Rg3 toxicity, we investigated changing expression levels of TRPM7 channel and its influences on Rg3-mediated cell death. We used HEK293 cells with inducible TRPM7 channel expression [16,19]. In the absence of induced TRPM7 channel expression [TRPM7(−) cells, Tet(−)], HEK293 cells incubation with Rg3 induced cell death in the MTT assay (n = 5; fig. 4B). However, when TRPM7 channel over-expression was induced by adding tetracycline [TRPM7(+) cells, Tet(+)], HEK293 cells incubation with Rg3 induced cell death at an increased rate in the MTT assay, which suggests that increased expression of TRPM7 channels leads to increased rate of Rg3-induced cell death. Taken together, our data suggest that TRPM7 channels play important roles in the survival of AGS cells.

Discussion

Ion channels play an important role in a wide variety of biological processes. In addition to supporting these life-supporting activities, ion channels are associated with several diseases [21,22]. Ion channels are crucial to tumour development and cancer growth. Epithelial cells change from normal to cancerous while a series of genetic alterations occur, which may also affect ion channel expression or cause changes in ion channel activity [27]. Voltage-gated potassium ion channels are over-expressed in colon cancer [28], and voltage-gated sodium ion channels are involved in the growth of prostate cancer [29]. Volume-regulated Cl^- channels were observed in a human prostate cancer cell line and in lung cancer cells [30,31]. TRP proteins have diverse functional properties and have profound effects on a number of physiological and pathological conditions. TRPV6 is involved in prostate adenocarcinoma and is a promising target for new therapeutic strategies to advanced prostate cancer [32]. TRPM1 appears to be a prognostic marker for melanoma metastasis in human cutaneous melanoma [33]. In addition, the TRPM8 channel protein has been used as a prostate-specific marker; the loss of TRPM8 is considered a sign of poor prognosis [34,35]. TRPM7 is endogenously expressed in a wide variety of tissues, including brain, hematopoietic tissues [36], kidney and heart [17,37,38]. The TRPM7 cation channel supports multiple cellular and physiological functions, including cellular Mg^{2+} homoeostasis [20,39], cell viability and growth [16,39,40], anoxic neuronal cell death [41], synaptic transmission [42], cell adhesion [43] and intestinal pacemaking [44]. Wykes *et al.* [26] suggested that TRPM7 channels are critical to human mast cell survival. Jiang *et al.* [19] suggested that activation of TRPM7 channels is involved in the growth and proliferation of human head and neck carcinoma cells. Abed *et al.* [45] proposed the importance of TRPM7 in human osteoblast-like cell proliferation. Guilbert *et al.* [46] suggested that TRPM7 is required for breast cancer cell proliferation. As in previous studies, we suggested that TRPM7 channels play an important role in the growth and survival of gastric cancer cells [14]. In line with these studies, our studies show that ginsenoside Rg3 induces apoptosis in human gastric adenocarcinoma cells and may be because of the blockade of TRPM7 channel activity.

Ginsenosides, the active ingredients in *P.* ginseng, have been used widely as invigorating agents, and many reports describe a variety of physiological or pharmacological effects in various regions [47]. However, only a few reports have described the effects of ginsenosides on gastric cancer. In a previous report, evidence indicated that TRPM7 channel activation influences the growth and survival of human gastric adenocarcinoma cells [14].

An anti-cancer compound isolated from *P.* ginseng has an effect on human pancreatic tumours [48]. Ginsenoside Rh2 induces apoptosis and paraptosis-like cell death in colorectal cancer cells by activation of p53 [49]. Ginsenoside Rg3 inhibits colorectal tumour growth by down-regulation of Wnt/ß-catenin signalling [50]. These anti-cancer ginsenosides provide reason for further development of this compound as a chemotherapeutic agent.

In summary, we have shown that ginsenoside Rg3 induces apoptosis in human gastric adenocarcinoma cells, which is, at least partially, attributable to the blockade of TRPM7 channel activity.

Conclusion

Ginsenoside Rg3 inhibited the growth and survival of AGS cells. Sub-G1 analysis was markedly increased, caspase-3 activity was elevated, and degree of PARP cleavage was increased. Blockade of TRPM7 channels by La^{3+} or 2-APB or suppression of TRPM7 expression by siRNA blocks Rg3 induced inhibition of cell growth and survival. Furthermore, over-expression of TRPM7 channels in HEK 293 cells increases the rate of Rg3-induced cell death. These findings indicate that ginsenoside Rg3 inhibits the growth and survival of gastric cancer, and that the Rg3-induced apoptosis is because of the blockade of TRPM7 channel activity. Therefore, TRPM7 channels may play an important role in survival in cases of gastric cancer.

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