Facilitation of H_2O_2-induced A172 Human Glioblastoma Cell Death by Insertion of Oxidative Stress-sensitive TRPM2 Channels

MASAKAZU ISHII[1], AKINORI OYAMA[1], TAMIO HAGIWARA[2], AKIRA MIYAZAKI[2], YASUO MORI[3], YUJI KIUCHI[1] and SHUNICHI SHIMIZU[1]

[1]*Department of Pathophysiology, School of Pharmaceutical Sciences and*
[2]*Department of Biochemistry, School of Medical Sciences, Showa University, Shinagawa-ku, Tokyo 142-8555;*
[3]*Laboratory of Molecular Biology, Department of Synthetic and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan*

Abstract. *The melastatin-like transient receptor potential M2 (TRPM2) channel is a Ca^{2+} permeable channel that is activated by reactive oxygen species (ROS), and its activation induces necrotic cell death. The effect of insertion of TRPM2 into A172 human glioblastoma cells (A172 cells) was investigated. The insertion of TRPM2 channels enhanced cell death induced by H_2O_2 in the A172 cells. An H_2O_2-induced Ca^{2+} increase was observed in TRPM2-expressing cells, but not in wild-type cells. Proliferation, migration and invasion activities were not affected by the expression of TRPM2. TRPM2 seems to be a candidate for gene therapy in glioblastoma cells, since the insertion of TRPM2 into A172 cells can facilitate cell death through Ca^{2+} increase after H_2O_2 treatment without increasing malignancy.*

Glioblastoma is a highly malignant glioma that is resistant to many treatment modalities including chemotherapy, radiation and other adjuvant therapies (1, 2). Standard therapy consists of surgical resection to an extent that is safely feasible, followed by chemotherapy, radiation and other adjuvant therapies (3, 4). However, despite advances in diagnosis and treatment, the prognosis of patients with malignant glioma remains very poor (5). Therefore, new therapeutic agents or alternative therapeutic approaches should be explored.

Melastatin-like transient receptor potential M2 (TRPM2) is the second member of the TRPM subfamily and a Ca^{2+}-permeable non-selective cation channel (6). Two extracellular signals are known to activate TRPM2 channels, oxidative stress and tumor necrosis factor α (TNFα) (6, 7). We have previously reported that Ca^{2+} influx through TRPM2 induced by H_2O_2 mediates necrotic cell death in TRPM2-expressing the human embryonic kidney cell line HEK293, the insulinoma cell line RIN-5F, and the monocytic cell line U937 (6), and similar effects have been demonstrated by others (7, 8). Reactive oxygen species (ROS) such as H_2O_2 are generated by γ-radiation (9, 10) and anti-cancer drugs (11). The physiological character of TRPM2 channels may allow the development of a gene therapy that in combination with γ-radiation and/or chemotherapy may facilitate the death of cancer cells, particularly those that are difficult to treat such as glioblastoma.

In this study, we investigated whether insertion of TRPM2 into the malignant glioma cell line A172 could enhance cell death induced by H_2O_2.

Material and Methods

Materials. H_2O_2 (30% solution) was purchased from Wako Pure Chemicals (Osaka, Japan). Fura-2 acetoxymethyl ester (Fura-2/AM) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of the highest grade commercially available.

Plasmid construct. The human TRPM2 cDNA fragment derived from pCI-neo (Promega, Tokyo, Japan)/human TRPM2 (6) was subcloned into the expression vector pCMV-TAG4 (Stratagene, La Jolla, CA, USA) to generate a cDNA allowing cytomegalovirus promoter-driven expression of a TRPM2-Flag protein (pCMV-TAG4/human TRPM2).

Cell culture and stable expression of TRPM2 in A172 cells. The human glioblastoma cell line A172 (American Type Culture Collection, Manassas, VA, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C.

Correspondence to: Shunichi Shimizu, Department of Pathophysiology, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan. Tel: +81 3 3784 8041, Fax: +81 3 3786 0481, e-mail: shun@pharm.showa-u.ac.jp

Key Words: H_2O_2, glioblastoma, TRPM2, calcium, cell death.

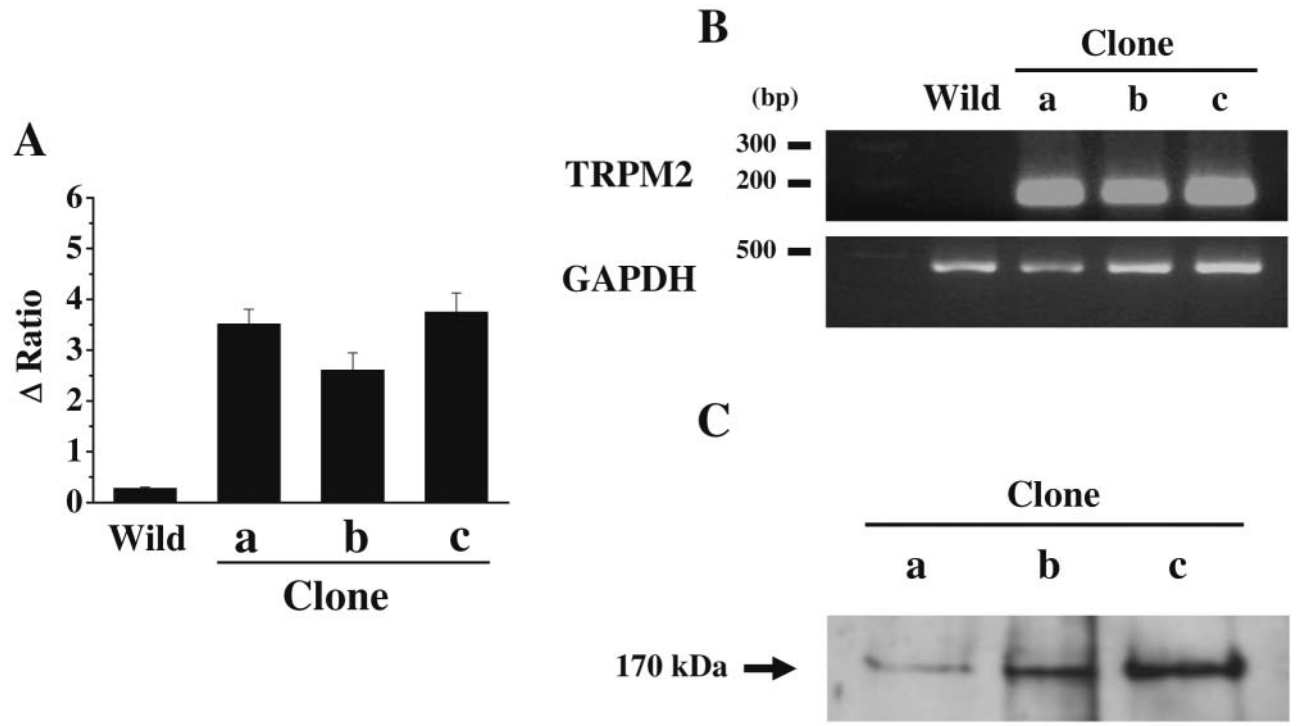

Figure 1. *Establishment of TRPM2-expressing A172 cells. A, cytosolic Ca^{2+} in wild-type A172 cells and three TRPM2-transfected neomycin resistant clones (a-c) that all contained responsive cells to 1 mM H_2O_2. Δ ratio, change in Ca^{2+} (peak ratio – resting ratio). B, TRPM2 mRNA (185 bp) and GAPDH mRNA (loading control) following amplification by RT-PCR. C, Western blot of TRPM2 protein from clones a, b and c.*

Transfection of TRPM2 into A172 cells was carried out using the Amaxa electroporation system with Cell Line Nucleofector kit V (Amaxa Inc., Gaithersburg, MD, USA). Cells were collected by trypsinization, washed with culture medium, and then suspended at a concentration of $5x10^5$ cells in 100 μl of Nucleofector solution V. The cell suspension was immediately mixed with 3 μg of the recombinant plasmid pCMV-TAG4/TRPM2 and electroporated using program U-29 of the Nucleofector device. After electroporation, cells were incubated in culture medium for 3 days. The culture medium was replaced with fresh culture medium containing 500 μg/ml geneticin difulfate (G418) and thereafter the medium was changed every 3 days for 10 days. The surviving cells were cloned and cultured.

Measurement of changes in $[Ca^{2+}]_i$. Cells on coverslips were loaded with fura-2 by incubation in culture medium containing 5 μM fura-2/AM and 10% FBS at 37˚C for 40 min. The fura-2 loaded cells were washed with the HEPES-buffered saline (HBS) containing (in mM) 107 NaCl, 6 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 11.5 glucose, 20 HEPES, all adjusted to pH 7.4 with NaOH. The coverslips were then placed in a perfusion chamber mounted on the stage of a microscope. Fluorescence images of the cells were recorded and analyzed with a video image analysis system (Meta Fulora, Nippon Ropper, Tokyo, Japan), as previously described (6). Results were depicted as a 340/380 ratio or Δ ratio (Δ ratio (340/380 nm) = peak ratio - resting ratio).

Measurement of TRPM2 mRNA levels by RT-PCR. Total RNA from the cells was extracted by a modified guanidium isothiocyanate method with Trizol Reagent (Invitrogen Co., Ltd., Carlsbad, CA, USA). Reverse transcription (RT) and PCR amplification from 0.5 μg of total RNA were performed using rTth DNA polymerase (RT-PCR high Plus, Toyobo Co. Tokyo, Japan). The primer pair used for the amplification of TRPM2 (NM_001011559.1) was 5'-AGCTCATCACCATCGGAGTCGC-3' and 5'-TCCACGAGAGG ATGAAGTGA-GTG-3'. The thermocycler was programmed to give an initial cycle consisting of reverse transcription at 60˚C for 30 min and denaturation at 94˚C for 2 min, followed by 30 cycles of denaturation at 94˚C for 1 min and annealing/extension at 58˚C for 1.5 min. As control of the amounts of total RNA, parallel RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as a reference, using the primers 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATG CCATCAC-3'. PCR products were electrophoresed on a 3% Agarose S (Wako Pure Chemicals) gel containing ethidium bromide and visualized by UV-induced fluorescence.

Western blot analysis. Cells were lysed in lysis buffer (50 mM Tris-HCl, [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1% NP-40 (Sigma Chemical Co., St. Louis, MO, USA) and protease inhibitor mix [Roche, Tokyo, Japan]) for 30 min on ice. The obtained supernatant was incubated with 2.5 μg of monoclonal anti-Flag antibody (Sigma Chemical Co.) for 30 min at 4˚C and then added to protein A-agarose beads (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). The immuno-precipitates were separated on an 8% SDS-polyacrylamide gel under reducing conditions. The proteins were transferred to a

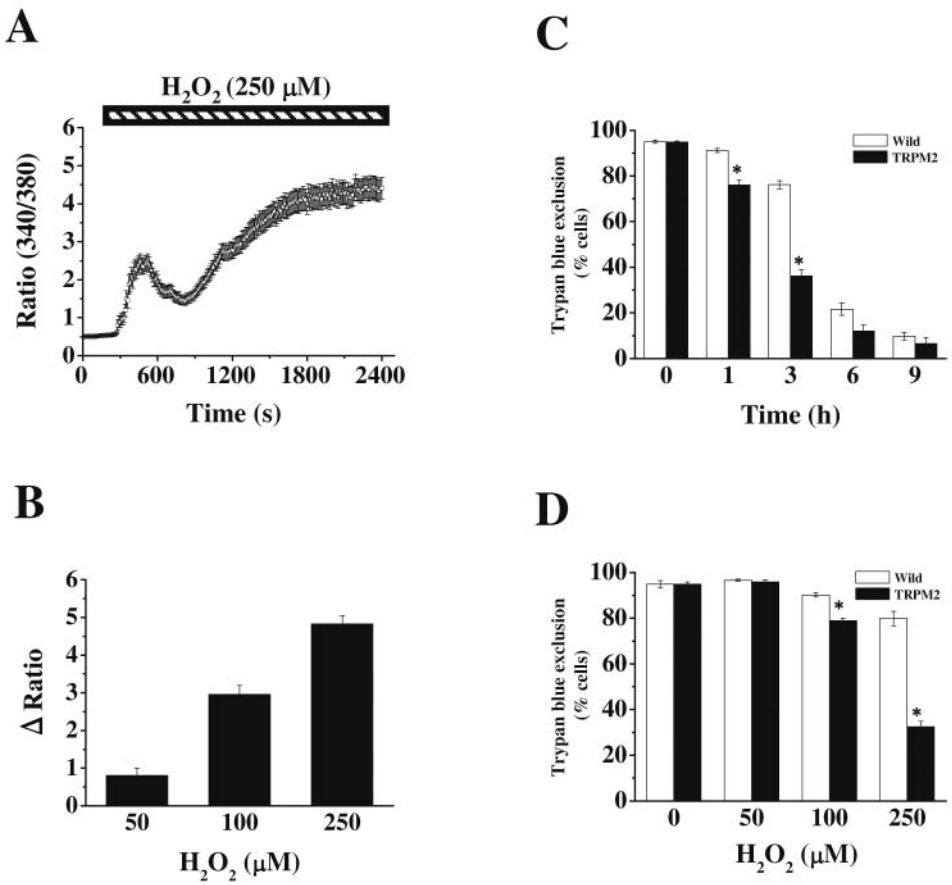

Figure 2. *Facilitation of H_2O_2-induced cell death by insertion of TRPM2. Cytosolyic Ca^{2+} in TRPM2-expressing A172 cells treated with 250 μM H_2O_2 (A) or 50-250 μM H_2O_2 (B). Mean±SEM of 25-93 cells. Δ ratio, change in Ca^{2+} (peak ratio-resting ratio). Cell death of wild-type and TRPM2-expressing cells assessed by Trypan blue exclusion assay at various times after H_2O_2 (250 μM) addition (C) or with various concentrations of H_2O_2 (50-250 μM) incubated for 3 h (D). Mean±SEM of 3-4 experiments. *p<0.05, vs. wild-type group.*

nitrocellulose membrane (Hybond-ECL, GE Healthcare, Tokyo, Japan). Non-specific binding was blocked with 20 mM Tris-HCl [pH 7.5], 0.15 M NaCl and 0.1% Tween 20 (TBS-T) containing 5% skim milk for 1 h. The membrane was incubated with a 1:1,000 dilution of anti-flag antibody. As the second antibody, a 1:10,000 dilution of horseradish-conjugated anti-mouse IgG was used. The membrane was then developed with an enhanced chemiluminescence Western blotting detection system (ECL, GE Healthcare).

Assay of cell death. Cell death was estimated by Trypan blue exclusion assay (6).

Proliferation assay. Proliferation was determined by counting the cell number. The cells ($3x10^4$ cells) were seeded on a 6-well culture plate and incubated with culture medium supplemented with 10% FBS at 37˚C for 1-7 days. The number of viable cells in Trypan blue solution was counted using an optical microscope (100-fold magnification).

Invasion assay. Invasive potential of the cells was assessed using Transwell® (Corning Life Sciences, NY, USA), in which each chamber includes a cell insert and its well. The bottom of the cell insert consists of a filter with multiple 5 μm pores that is coated with a type I collagen. The cells were collected and suspended to a final concentration of $2x10^5$ cells/ml. The cell suspension (100 μl) was seeded in the cell insert, and 600 μl of culture medium containing 10% FBS was added to the lower chamber. After incubation for 12 h, the non-invading cells on the upper surface of the filter were wiped out with a cotton swab. The invasive cells that migrated to the lower surface of the filter were fixed and stained with Diff Quik (Sysmex International Reagents Co., Ltd., Kobe, Japan), and the number of the cells was counted.

Migration assay. The spreading and migration capabilities of the A172 cells were examined using a scratch wound assay. Confluent cells in 60-mm tissue culture dishes were incubated with DMEM containing 0.1% FBS for 2 h at 37˚C. Then, the cells were carefully wounded by a surgical blade to cut the sheet of cells and mark the plate. The cells from one side of the wound were removed with cell scrapers. The cells were incubated for 22 h at 37˚C and then photographed. Cellular migration was evaluated by counting the number of cells that moved to the field from which cells had been removed, and also by measurement of the distance to the furthest cell from the wound line.

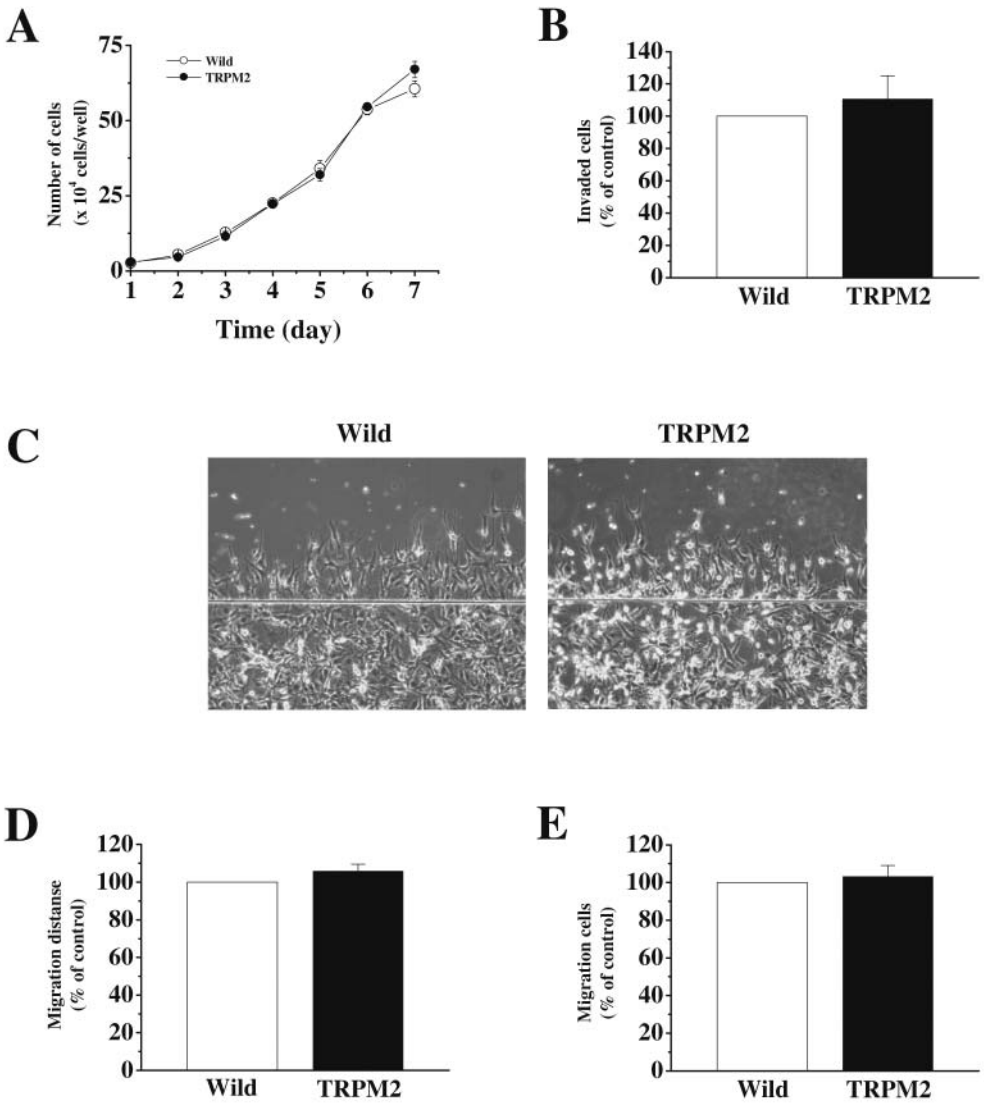

Figure 3. *Influence of the insertion of TRPM2 on the proliferation, invasion, and migration of A172 cells. A, total number of wild-type and TRPM2-expressing A172 cells cultured in DMEM containing 10% FBS for 1-7 days. B, percentage of cells that invaded through the type I collagen-coated membrane after 12 h incubation in comparison with control (2x10^5 cells/ml). C, representative photographs of migrated cells 22 h after the beginning of the wound assay. Cell migration calculated in terms of the distance of migration (D) and number of migrated cells (E). Control, wild-type. Mean±SEM of 4-8 experiments.*

Data analysis. Data are presented as the means±SEM of n observations. The statistical significance of observed differences was determined by analysis of variance followed by Bonferroni's method. Differences between means were considered significant when p was less than 0.05.

Results

Establishment of TRPM2-expressing A172 cells. The screening for TRPM2 expression in the TRPM2-transfected A172 cells was performed by measuring the Ca^{2+} response to H_2O_2 (1 mM). In the wild-type A172 cells, $[Ca^{2+}]_i$ elevation was not induced by 1 mM H_2O_2 (Figure 1A), and the expression of TRPM2 mRNA was not observed (Figure 1B). Three out of a total of 87 neomycin-resistant clones contained all responsive cells with 1 mM H_2O_2 (Figure 1A). Moreover, in these clones (a, b and c), TRPM2 mRNA and protein were detected (Figure 1B and 1C) and the expression of TRPM2 protein in clone c was the highest (Figure 1C). Thus, three clones of TRPM2-expressing A172 cells were obtained, and clone c was used throughout subsequent experiments.

Facilitation of H_2O_2-induced A172 cell death by insertion of TRPM2 channels. As shown in Figure 2A, addition of 250 μM H_2O_2 elicited biphasic $[Ca^{2+}]_i$ elevation, and the $[Ca^{2+}]_i$

elevation was concentration-dependent (Figure 2B). By the trypan blue exclusion assay, H_2O_2 (250 μM) caused the death of wild-type A172 cells from 3 h after its addition, and the H_2O_2-induced cell death was markedly enhanced in the TRPM2-expressing cells at 1 and 3 h after addition (Figure 2C). Moreover, H_2O_2-induced cell death was enhanced by TRPM2 insertion when the H_2O_2 concentration was 100 and 250 μM (Figure 2D).

Influence of TRPM2 expression on proliferation, invasion, and migration. As shown in Figure 3A, the proliferation activity of TRPM2-expressing A172 cells was similar to wild-type A172 cells. Invasion activity was not affected by the insertion of TRPM2 (Figure 3B). Moreover, the insertion of TRPM2 had no effect on the migration activities of A172 cells as determined by two different criteria, migrated distance and cell number (Figure 3C-E).

Discussion

The present study clearly showed that the insertion of TRPM2 could facilitate Ca^{2+} influx and H_2O_2-induced cell death in the human glioblastoma cell line A172.

It is widely accepted that sustained $[Ca^{2+}]_i$ elevation induces cell death in various types of cells including vascular endothelial cells, pancreatic β-cells and astroglioma cells (6, 12, 13). ROS including H_2O_2 are known to cause Ca^{2+} overload and cell death (6, 12, 13). We have previously reported that H_2O_2 evokes Ca^{2+} overload through TRPM2 activation in transiently TRPM2-expressing HEK293 cells, but not in wild-type HEK293 cells (6). In the present study, the insertion of TRPM2 into A172 cells induced the sustained increase of $[Ca^{2+}]_i$ by the treatment with H_2O_2. It is therefore likely that the Ca^{2+} overload through TRPM2 activation mediates H_2O_2-induced human glioblastoma A172 cell death.

TRPM2 activation is generally thought to mediate necrotic cell death (6). Togashi *et al.* (14) reported that glucose-induced insulin secretion from pancreatic islets is regulated by Ca^{2+} influx not only through L-type voltage-gated Ca^{2+} channels, but also through TRPM2 channels. Therefore, TRPM2 activation is likely to be able to mediate physiological cell functions other than cell death. It is known that cell proliferation is regulated by the increase of $[Ca^{2+}]_i$ in vascular smooth muscle cells and glioblastoma cells (15, 16). Migration and invasion of glioblastoma cells are also regulated by $[Ca^{2+}]_i$ elevation (15, 17). However, the capacities of TRPM2-expressing A172 cells for cell proliferation, migration, and invasion were not enhanced compared to wild-type A172 cells. These results suggest that the insertion of the TRPM2 gene into the A172 cells facilitated H_2O_2-induced cell death without the stimulation of malignancy of the glioblastoma cells.

In conclusion, the insertion of TRPM2 can facilitate Ca^{2+} influx and H_2O_2-induced cell death in human glioblastoma A172 cells, and may therefore enhance glioblastoma cell death by H_2O_2 generating γ-radiation and/or anti-cancer drugs. Thus, the TRPM2 gene may be a candidate for gene therapy to facilitate glioblastoma cell death.

References

1 Berger F, Gay E, Pelletier L, Tropel P and Wion D: Development of gliomas: potential role of asymmetrical cell division of neural stem cells. Lancet Oncol *5*: 511-514, 2004.

2 Carpentier AF: Neuro-oncology: the growing role of chemotherapy in glioma. Lancet Neurol *4*: 4-5, 2005.

3 Walker MD, Alexander E, Hunt WE, MacCarty CS, Mahaley MS, Mealey J, Norrell HA, Owens G, Ransorhoff J, Wilson CB, Gehan EA and Strike TA: Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas: a cooperative clinical trial. J Neurosurg *49*: 333-343, 1978.

4 Walker MD, Green SB, Byar DP, Alexander E, Batzdorf U, Brooks WH, Hunt WE, MacCarty CS, Mahaley MS, Mealey J, Owens G, Ransohoff J, Robertson JT, Shapiro WR, Smith KR, Wilson CB and Strike TA: Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med *303*: 1323-1329, 1980.

5 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups and National Cancer Institute of Canada Clinical Trials Group: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*: 987-996, 2005.

6 Hara Y, Wakamori M, Ishii M, Maeno E, Nishida M, Yoshida T, Yamada H, Shimizu S, Mori E, Kudoh J, Shimizu N, Kurose H, Okada Y, Imoto K and Mori Y: LTRPC2 Ca^{2+}-permeable channel activated by changes in redox status confers susceptibility to cell death. Mol Cell *9*: 163-173, 2002.

7 Zhang W, Hirschler-Laszkiewicz I, Tong Q, Conrad K, Sun SC, Penn L, Barber DL, Stahl R, Carey DJ, Cheung JY and Miller BA: TRPM2 is an ion channel which modulates hematopoietic cell death through activation of caspases and PARP cleavage. Am J Physiol Cell Physiol *290*: C1146-C1159, 2005.

8 Zhang W, Chu X, Tong Q, Cheung JY, Conrad K, Masker K and Miller BA: A novel TRPM2 isoform inhibits calcium influx and susceptibility to cell death. J Biol Chem *278*: 16222-16229, 2003.

9 Leach JK, van Tuyle G, Lin PS, Schmidt-Ullrich R and Mikkelsen RB: Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. Cancer Res *61*: 3894-3901, 2001.

10 Lyng FM, Seymour CB and Mothersill C: Oxidative stress in cells exposed to low levels of ionizing radiation. Biochem Soc Trans *29*: 350-353, 2001.

11 Doroshenko N and Doroshenko P: The glutathione reductase inhibitor carmustine induces an influx of Ca^{2+} in PC12 cells. Eur J Pharmacol *497*: 17-24, 2004.

12 Shimizu S, Nomoto M, Naito S, Yamamoto T and Momose K: Stimulation of nitric oxide synthase during oxidative endothelial cell injury. Biochem Pharmacol *55*: 77-83, 1998.

13 Smith MA, Herson PS, Lee K, Pinnock RD and Ashford MLJ: Hydrogen-peroxide-induced toxicity of rat striatal neurons involves activation of a non-selective cation channel. J Physiol *574*: 417-425, 2003.

14 Togashi K, Hara Y, Tominaga T, Higashi T, Konishi Y, Mori Y and Tominaga M: TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. EMBO J *25*: 1804-1815, 2006.

15 Ishiuchi S, Tsuzuki K, Yoshida Y, Yamada N, Hagimura N, Okado H, Miwa A, Kurihara H, Nakazato Y, Tamura M, Sasaki T and Ozawa S: Blockage of Ca^{2+}-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. Nat Med *8*: 971-978, 2002.

16 Naito S, Shimizu S, Maeda S, Wang J, Paul R and Fagin JA: Ets-1 is an early response gene activated by ET-1 and PDGF-BB in vascular smooth muscle cells. Am J Physiol *274*: C472-C480, 1998.

17 Wakabayashi K, Kambe F, Cao X, Murakami R, Mitsuyama H, Nagaya T, Saito K, Yoshida J and Seo H: Inhibitory effects of cyclosporine A on calcium mobilization-dependent interleukin-8 expression and invasive potential of human glioblastoma U251MG cells. Oncogene *23*: 6924-6932, 2004.

Received August 1, 2007
Revised October 9, 2007
Accepted October 24, 2007