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Involvement of TRPV1 and TRPV4 Channels in Enhancement of Metastatic Ability Induced by γ -Irradiation in Human Lung Cancer A549 Cells

Kurumi Washiya,^a Rie Sasaki,^{a,b} Yuto Nakanishi,^a Kazuki Kitabatake,^a Keisuke Nishino,^{a,b} Sei-ichi Tanuma,^b Shuji Kojima,^a and Mitsutoshi Tsukimoto^{*,a}

^aDepartment of Radiation Biosciences; ^bDepartment of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi Chiba 278-8510, Japan

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Radiation therapy is an important local treatment for malignant tumors, but ionizing radiation may also facilitate tumor invasion and metastasis. The transient receptor potential (TRP) superfamily, which is a diverse group of ion channels activated by various stimuli, has a variety of pathophysiological functions, including a role in malignancy. However, it is not clear whether TRP channels influence radiation-induced biological effects. Here, we show that TRPV1 and TRPV4 channels contribute to the γ -irradiation-induced enhancement of migration of human lung cancer A549 and mouse melanoma B16 cells. We found that γ -irradiation induced both cell migration and actin stress fiber formation of A549 cells, but both effects were suppressed by the TRPV1 inhibitors capsazepine AMG9810, SB366791, and BCTC, and by the TRPV4 inhibitors RN-1734 and GSK2193874. γ -Irradiation induced migration was also suppressed by knockdown of TRPV1 and TRPV4 channels. Furthermore, γ -irradiation of B16 mouse melanoma cells increased the number of lung metastases in C57BL/6 mice, compared to non-irradiated B16 cells, and TRPV1 and TRPV4 channel inhibitors suppressed this increase. These results suggest that TRPV1 and TRPV4 channels are potential targets for intervention to block the acquisition of motility by cancer cells during radiotherapy in order to reduce the risk of metastasis.

Key words γ -irradiation, lung cancer, migration, transient receptor potential vanilloid channel, radiation therapy

INTRODUCTION

Although high-energy ionizing radiation, such as γ -rays, X-rays, and electron beams, is utilized to treat various malignancies by damaging DNA, thereby causing cells to die or stop dividing, it can also have long-term side effects, causing injury to normal tissues, inflammation, fibrosis,¹⁾ and even tumorigenesis.²⁾ There is also an increased risk of metastasis after local tumor irradiation under experimental or clinical conditions,³⁾ probably because of the migratory ability and invasiveness of surviving cancer cells are enhanced after irradiation.⁴⁾

Irradiation activates multiple signaling pathways that regulate cellular functions,⁵⁾ and there appear to be complex relationships among the tumor microenvironment, production of proteases, and induction of epithelial–mesenchymal transition (EMT). EMT is a morphological program that enables epithelial cells to acquire a highly motile mesenchymal phenotype, including dissolution of intracellular junctions, reorganization of the actin cytoskeleton, and acquisition of increased cell motility.⁶⁾ EMT is an important mechanism of radiation-induced cell migration/invasion.^{7,8)} Thus, induction of EMT may be one of the reasons why ionizing radiation enhances the metastatic potential of breast cancer cells,⁹⁾ hepatocellular carcinoma,¹⁰⁾ glioma cells,¹¹⁾ and colorectal cancer cells.¹²⁾ Transforming growth factor β 1 (TGF- β 1) also plays a significant role in the induction of EMT.¹³⁾ TGF- β 1 is a multifunctional cytokine that is involved in regulating many biological pro-

cesses, including cell growth, differentiation, apoptosis, homeostasis,¹⁴⁾ angiogenesis,¹⁵⁾ and tissue fibrosis.¹⁶⁾ We have shown that TGF- β 1 also induces EMT in human lung cancer A549 cells,^{17,18)} and it was recently reported that ionizing radiation promotes migration/invasion through TGF- β 1-mediated induction of EMT in human lung cancer cells.^{19,20)} Moreover, plasma TGF- β 1 levels and lung metastasis of mice are increased by irradiation.²¹⁾ Therefore, it seems likely that activation of TGF- β 1 signaling is involved in the enhancement of tumor metastasis following irradiation.

In humans, the transient receptor potential (TRP) channel superfamily is subdivided into six subfamilies, consisting of the canonical TRP (TRPC), vanilloid TRP (TRPV), melastatin TRP (TRPM), ankyrin TRP (TRPA), polycystic TRP (TRPP), and mucolipin TRP (TRPML) groups.²²⁾ TRP channels have an extraordinary diversity of functional properties and individual channels have roles in various physiological and pathological conditions.²³⁾ TRP channels are activated by chemical or physical stimuli, including oxidative stress, intracellular calcium influx, temperature, pH, mechanical stimulation, osmotic pressure, pain, etc.^{24,25)} Increased expression of members of the TRPV, TRPM, and TRPC families is associated with proliferation, malignancy, and metastasis of certain epithelial cancers.^{26–28)} Activation of TRPV family members contributes to multiple physiological and pathological cellular responses, including promotion of cell migration/invasion in various types of cancer cells via Ca^{2+} signaling.^{29–31)}

*To whom correspondence should be addressed. e-mail: tsukim@rs.noda.tus.ac.jp

We have reported the involvement of the transient receptor potential vanilloid 1 (TRPV1) channel in cellular responses to DNA damage caused by irradiation in human lung cancer A549 cells, showing that γ -irradiation activates the TRPV1 channel.³²⁾ However, the role of TRPV channels in cellular responses to irradiation remains to be fully established. In particular, although it is clear that TRPV family members are closely related to tumor progression, it has not been established whether TRPV1 and TRPV4 channels are directly involved in irradiation-induced cell migration/invasion and tumor metastasis. Therefore, in this study, we investigated the involvement of TRPV1 and TRPV4 channels in γ -irradiation-induced migration of human lung cancer cells.

MATERIALS AND METHODS

Reagents and Antibodies Dulbecco's modified Eagle's medium (DMEM), human recombinant SB431542, AMG9810, SB366791, BCTC, GSK1016790A and RN-1734 were purchased from Wako Pure Chemical (Osaka, Japan). Capsazepine (CPZ) was purchased from Cayman Chemical Co. (Michigan, USA). GSK2193874 was purchased from Sigma Aldrich (USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France) and Life Technologies (Gibco BRL, Grand Island, NY, USA). Rhodamine-phalloidin was purchased from Cytoskeleton, Inc. (Denver, CO). The primary antibodies used were anti- β -actin monoclonal antibody (FUJIFILM Wako Pure Chemical, Osaka, Japan), TRPV1 antibody (Novus), and TRPV4 antibody (Sigma Aldrich, USA).

Animals Male C57BL/6 mice were purchased from Sankyo Labo Service (Japan) and used at 6 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of $23 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$ under a 12 h light-dark cycle. They were allowed free access to tap water and normal diet, CE-2 (CLEA Co. Ltd.). The mice were treated and handled according to Tokyo University of Science's institutional ethical guidelines for animal experiments and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee (permission numbers S19006, S18008, S17008).

Cell Culture and Irradiation A549 human adenocarcinoma cells and B16 mouse melanoma cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL) in a humidified atmosphere of 5% CO_2 in air at 37°C . The cells were irradiated with 1.0-10.0 Gy of γ -rays from a ^{137}Cs source (0.72 Gy/min) for 100-900 sec at room temperature in a Gammacell 40 γ -irradiation system (Nordin International, Inc.).

Cell Migration Assay γ -Irradiation-induced cell migration was analyzed by using 24-well Transwell plates (6.5 mm diameter; 8 μm pore size polycarbonate membrane, Corning, Lowell, MA). The upper compartment was seeded with A549 cells (2×10^4 cells) in basal culture medium. After 24 h, the medium was replaced with fresh medium. The medium in the upper chamber contained 5% FBS instead of 10% FBS. After incubation for a further 24 h, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and incubated with 1 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI) and 50 $\mu\text{g/mL}$ propidium iodide (PI) for 30 min at room temperature. Non-migrated cells on the upper surface of the membrane were removed and cells that had migrated through the mem-

brane to the lower surface were counted using a fluorescence microscope (BZ-9000; Keyence).

Fluorescence Imaging For F-actin staining and immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 for 5 min. For staining of F-actin, fixed cells were incubated with 100 nM Rhodamine-phalloidin for 30 min at room temperature. Counterstaining with Hoechst 33342 (10 $\mu\text{g/mL}$) was used to verify the location and integrity of nuclei. Stained cells were analyzed using a confocal laser-scanning microscope (TCS SP2; Leica, Mannheim, Germany) equipped with a HCX PL Apo 63 $\times$ 1.32 NA oil objective lens. Leica software (TCS SP2, version 2.6.1) was used for image acquisition and processing. Some images were obtained by using a confocal laser-scanning microscope (FV1000-D, Olympus, Tokyo, Japan). We estimated actin remodeling in terms of the formation of actin stress fibers, not increase of fluorescence intensity.

Small Interfering RNA (siRNA) Transfection siRNAs targeting human TRPV1 and TRPV4 channels and negative control siRNA (TriFECTa Kit[REMOVED EQ FIELD] DsiRNA Duplex; Duplex Name for TRPV1: NM_080704 duplex 1-3, Duplex Name for TRPV4: hs.Ri.TRPV4.13.1-3) were purchased from Integrated DNA Technologies. Cells (5×10^4 cells per 40 mm dish) were incubated in culture medium for 48 h. The siRNA duplex oligonucleotides (10 nM) for knockdown of human TRPV1 channel and TRPV4 channel were transfected into A549 cells by using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Forty-eight hours after transfection, the reduction of TRPV1 was confirmed by western blotting. The expression of TRPV1 and TRPV4 channels in each A549 cells transfected with 3 different siRNA targeting TRPV1 were decreased to 51, 50, or 41% and targeting TRPV4 were decreased to 81, 66 or 60% compared with cells transfected with scramble siRNA.

B16 Melanoma Pulmonary Metastasis Model Cells were pretreated with 10 μM BCTC and 1 μM RN-1734 for 30 min, and then irradiated with 1 Gy of γ -rays. After incubation for 48 h, the cells were suspended at 5.0×10^5 cells/mL in PBS. Then 200 μL of this cell suspension was injected into C57BL/6 mice via the tail vein. The mice were sacrificed two weeks later and the lungs were harvested. The number of black colonies in the lungs was counted.

Statistics Values are given as the mean $\pm$ SE. Comparison between two values was performed by means of the unpaired Student's t-test. The statistical significance of differences between control and other groups was calculated by using Dunnett's test with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA, USA). The criterion of significance was set at $P < 0.05$.

RESULTS

γ -Irradiation Induces Increased Motility of A549 Cells It is well established that irradiation enhances the migration of cancer cells.^{4,9-12)} We examined the effect of γ -irradiation in the range of 1.0-10.0 Gy on the motility of A549 cells by using Transwell assay, which can measure cell migration regardless of cell proliferation, because the migration is dependent on the concentration gradient of FBS. The number of migrated cells was maximum at 2.0 Gy, while migration was decreased at 8.0 Gy (Fig. 1A). These results are consistent with the

Fig. 1. Effect of TRPV1 and TRPV4 Inhibitors on γ -Irradiation-Induced Migration of A549 Cells

(A) A549 cells were irradiated with 1-10 Gy of γ -irradiation, and cell migration was examined by Transwell assay as described in Materials and Methods. The lower membrane surfaces were photographed through a microscope, and migrated cells in each field were counted. Error bars indicate $\pm$ SE. Significant difference from control: ** ($P < 0.01$). (B) A549 cells were pretreated with CPZ (10 μ M), BCTC (10 μ M), SB366791 (1 μ M) or AMG9810 (10 μ M) at 30 min before γ -irradiation, and cell migration was examined as above. Typical data of 3 independent experiments are shown. Error bars indicate $\pm$ SE. Significant difference from non-irradiated cells: *** ($P < 0.001$). Significant difference from irradiated cells: ### ($P < 0.001$). (C) A549 cells were pretreated with RN-1734 (10, 20 μ M) or GSK2193874 (1, 5 μ M) at 30 min before γ -irradiation, and cell migration was examined as described above. Error bars indicate $\pm$ SE ($n=3$). Significant difference from non-irradiated cells: *** ($P < 0.001$). Significant difference from irradiated cells: ### ($P < 0.001$).

Fig. 2. Effect of TRPV1 and TRPV4 Inhibitors on γ -Irradiation-Induced Actin Remodeling in A549 Cells

A549 cells were pretreated with CPZ (10 μ M), BCTC (10 μ M), SB366791 (1 μ M) or AMG9810 (10 μ M) (A) or with RN-1734 (10, 20 μ M) or GSK2193874 (1, 5 μ M) (B) at 30 min before γ -irradiation. The cells were irradiated with 2 Gy γ -rays and incubated for 48 h. F-actin was stained with Rhodamine-phalloidin, and images were acquired with a confocal laser-scanning microscope at 63 $\times$ magnification. Typical data from several independent experiments are shown.

γ -irradiation-induced decrease of cell viability due to irreversible cell damage.³⁶ Based on the results in Fig. 1A, we chose a dose of 2.0 Gy for subsequent experiments using A549 cells.

Involvement of TRPV1 and TRPV4 Channels in the γ -Irradiation-Induced Motility Increase of A549 Cells To investigate the role of TRPV1 and TRPV4 channels in cancer cell migration, we employed Transwell assay to examine the effects of TRPV1 and TRPV4 channel inhibitors. In addition, it is well known that Rho/ROCK signaling plays a critical role in the regulation of cell motility, invasion, and metastasis of cancer via regulation of actin remodeling.^{33–35} Actin remodeling is a method in which spherical proteins polymerize to form filamentous actin stress fibers, and cells move due to the tension. This is used as an indicator of the metastatic potential of cancer cells. Four highly specific TRPV1 inhibitors, capsaizipine, BCTC, SB366791 and AMG9810, all suppressed the γ -irradiation-induced increase of cell motility and actin remodeling. The TRPV4 inhibitors RN-1734 and GSK2193874 had similar effects (Fig.1B, 1C, 2A, 2B). In addition, the proliferation ability of A549 cells did not decrease significantly by TRPV1 and TRPV4 channels inhibitors³⁶ (Supplementary Fig. 1).

To confirm the involvement of TRPV1 and TRPV4 channels in γ -ray-induced migration, A549 cells were transfected with siRNA targeting each channel. The number of migrated cells was not significantly different in irradiated knockdown cells from that in the corresponding non-irradiated knockdown cells (Fig. 3A, 3B). These results support the idea that knockdown of expression of TRPV1 and TRPV4 blocks the γ -irradiation-induced enhancement of cell motility.

Fig. 3. Knockdown of TRPV1 and TRPV4 Channels Suppresses γ -Irradiation-Induced Migration of A549 Cells

siRNA duplex oligonucleotides for knockdown of human TRPV1 (A) or TRPV4 (B) were transfected into A549 cells. Then, at 48 h after transfection, the cells were irradiated with 2.0 Gy. After incubation for 48 h, cell migration was examined using Transwell systems. (A) The data are presented as percentages of the negative control (non-irradiated cells transfected with scramble siRNA). Values are means $\pm$ S.E. (n=10-30) from 2-6 independent experiments (scramble siRNA; 6 independent experiments, siRNA1; 2 independent experiments, siRNA2; 5 independent experiments, siRNA3; 6 independent experiments). Significant difference from non-irradiated cells: *** (P < 0.001). Significant difference from irradiated cells: # (P < 0.05) or ## (P < 0.01). (B) Values are means $\pm$ S.E. (n=5). Typical data from three independent experiments. Significant difference from non-irradiated cells: *** (P < 0.001). Significant difference from irradiated cells: ### (P < 0.001).

Involvement of TRPV1 and TRPV4 Channels in Enhancement of Metastasis Induced by γ -Irradiation *in Vivo* Finally, we examined whether TRPV1 and TRPV4 channel inhibitors can suppress the γ -irradiation-induced enhancement of metastatic ability of B16 cells *in vivo*. B16 cells were subjected to 1 Gy of γ -irradiation and incubated for 48 h prior to injection into the tail vein of C57BL/6 mice. The number of lung colonies was increased in the mice injected with

Fig. 4. Effect of TRPV1 and TRPV4 Channel Inhibitors on γ -Irradiation-Induced B16 Melanoma Cell Metastasis to the Lungs

B16 cells were pretreated with BCTC (10 μ M) (A) or RN-1734 (1 μ M) (B) at 30 min before irradiation with 1.0 Gy γ -rays and incubated for 48 h. Then, B16 cells in 5.0×10^5 cells/mL of PBS (200 μ L) were injected into the tail vein of C57BL/6 mice (n=5). The mice were sacrificed 14 d after injection and the lungs were harvested. The number of lung metastatic colonies was counted. Error bars indicate $\pm$ SE. Significant difference from non-irradiated cells: * (P < 0.05) or *** (P < 0.01). Significant difference from irradiated cells: ### (P < 0.001).

γ -irradiated cells compared with that in the mice injected with non-irradiated cells, indicating that metastatic ability of B16 cells is enhanced by irradiation.

When the B16 cells were treated with TRPV1 and TRPV4 channel inhibitors before γ -irradiation, the increase of the number of lung colonies was suppressed (Fig. 4A, B), strongly supporting the view that TRPV1 and TRPV4 channels are involved in the irradiation-induced enhancement of metastatic ability.

DISCUSSION

Activation of TRPV channels, which are nonselective cation channels, can mediate increased Ca^{2+} influx under pathological conditions, and this can lead to increased cell motility, resulting in metastasis of epithelial cancer.²⁹⁻³¹ Therefore,

in this study, we investigated the involvement of TRPV1 and TRPV4 channels in irradiation-induced lung cancer cell migration.

TRPV1 inhibitors suppressed the γ -irradiation-induced cell migration and actin remodeling, as did TRPV4 channel inhibitors. Since the enhancement of cell migration induced by γ -irradiation was also suppressed by TRPV1 and TRPV4 channel knockdown, we conclude that activation of TRPV1 and TRPV4 channels plays a role in γ -irradiation-induced cell migration.

Since TRP channels exhibit high calcium ion permeability,^{37–39} activation of TRPV1 and TRPV4 channels by γ -irradiation may cause Ca^{2+} influx; this in turn activates the MAPK pathway,^{40–45} which is involved in cancer growth and migration. In our results, both TRPV1 channel and TRPV4 channel are involved in γ -irradiation-induced cell migration. The results suggest that the regulation of intracellular calcium ions by both TRPV1 and TRPV4 channels are needed to enhance the cell motility, because the inhibition of TRPV1 channel or TRPV4 channel almost completely suppressed the increase of cell migration.

However, the mechanism underlying TRPV1 and TRPV4 channel activation by γ -irradiation is unclear. One possibility is that reactive oxygen species (ROS) produced by ionizing irradiation⁴⁶ activate the TRP channel and cause Ca^{2+} influx.^{47–51} In addition, we have recently reported that a TRPV1 channel inhibitor suppressed DNA repair after irradiation,³² suggesting that TRPV1 inhibitors might increase the radiosensitivity of cancer cells.³⁶ Therefore, administration of TRPV channel inhibitors concomitantly with radiotherapy might reduce the risk of metastasis, as well as amplifying the cytotoxic effect of radiation.

Notably, we found that the number of metastatic lung colonies after injection of γ -irradiated B16 cells into the tail vein of mice was larger than that after injection of non-irradiated B16 cells, and the increase was suppressed by pretreating the cells with TRPV1 and TRPV4 channel inhibitors. Overall, our results represent the first evidence that activation of TRPV1 and TRPV4 channels plays an important role in ionizing radiation-induced enhancement of cell motility and metastatic ability. Further work will be needed to assess whether activation of other TRP channels, such as TRPC, TRPM or TRPA, might also influence migration or invasion of cancer cells.^{52–56} Nevertheless, our results suggest that TRPV1 and TRPV4 channels are potential targets for intervention to block the acquisition of motility by cancer cells during radiotherapy in order to reduce the risk of metastasis.

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Conflict of interest The authors declare no conflict of interest.

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