SUPPRESSION OF TWO-STAGE CELL TRANSFORMATION BY ELECTROLYZED REDUCED WATER CONTAINING PLATINUM NANOPARTICLES

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Abstract: According to the two-stage cell transformation theory, cancer cells first receive initiation, which is mainly caused by DNA damage and then promotion, which enhance transformation. Since murine Balb/c 3T3 cells lose contact inhibition by cell transformation, the cells have been widely used for transformation experiments. Electrolyzed reduced water (ERW) is a health beneficial alkaline drinking water containing high concentration of dissolved hydrogen, and can scavenge intracellular reactive oxygen species (ROS). ERW contains a small amount of platinum (Pt) nano-particles as atomic hydrogen donor and ROS-scavenger. Therefore, ERW supplemented with Pt nanoparticles (ERW/Pt) can be considered as a model of strong ERW. Here, we report that ERW/Pt can prevent transformation of Balb/c 3T3 cells. ERW was prepared by electrolysis of 0.002 M NaOH. Balb/c 3T3 cells were treated with 3-methyl cholantrene (MCA) as an initiation compound, followed by the treatment with phorbol-12-myristate-13-acetate (PMA) as a promotion compound. The cell transformation induced by MCA/PMA was strongly suppressed by ERW/Pt treatment, especially at the stage of promotion. Analysis of intracellular ROS level showed that ERW/Pt could decrease excess intracellular ROS induced by PMA. These results suggested that ERW/Pt suppressed cell transformation at promotion stage by its ROS scavenging effect.

Key words: active hydrogen; Balb/c 3T3 cells; electrolyzed reduced water; platinum nanoparticles; reactive oxygen species; two-stage cell transformation

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1. INTRODUCTION

Intracellular ROS cause irreversible damage to biological macromolecules, resulting in many diseases and cell transformation[1]. Electrolyzed reduced water (ERW) is a health beneficial alkaline drinking water which contains high concentration of dissolved hydrogen, and can scavenge intracellular ROS[2-5]. ERW was known to improve various diseases such as diabetes[5,6], angiogenesis and cancer, and reduce hemodialysis-induced oxidative stress in end-stage renal disease patients[7]. We have revealed that ERW contains a small amount of platinum (Pt) nanoparticles as atomic hydrogen (active hydrogen) donors and ROS-scavengers. Therefore, ERW containing synthesized Pt nanoparticles (ERW/Pt) can be considered as a model of strong ERW. *In vitro* cell transformation assay with Balb/3T3 or C3H10T1/2 cells, has been recognized as being directly relevant to carcinogenesis[8-10] and regarded as a useful method for the screening of potential carcinogens[11-13]. According to the two-stage cell transformation theory, normal cells first receive initiation, which is mainly caused by DNA damage, and then promotion, which enhances cell transformation[14]. Here, we report that ERW/Pt can prevent cell transformation of Balb/c 3T3 cells.

2. MATERIAL AND METHODS

2.1 Cell culture

A murine cell line, Balb/c 3T3 A31-1-1 cells was obtained by Japanese Collection of Research Bioresources, and were cultured in a MEM medium (Nissui Pharmaceutical Co. Ltd., Tokyo Japan) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5 % CO_2.

2.2 ERW, platinum nanoparticles and preparation of medium

ERW preparation was previously described[5]. Briefly, ERW was prepared by electrolysis of 0.002 M NaOH for 1 hr. using a batch-type electrolysis device (Type TI-200S, Nihon Trim Co., Osaka, Japan). Platinum nanoparticles of average size 2 nm were obtained from SENEKA Co., Tokyo, Japan. In order to investigate the two-stage cell transformation assay, culture medium was prepared using ERW instead

of Milli-Q water, and then filtrated by 0.22-μm membrane for sterilization.

2.3 Two-stage cell transformation assay

Balb/c 3T3 cells (1.0×10^4 cells ml^{-1}) were inoculated into 60-mm culture dish. Next, the cells were cultured in the presence of 1.0 μg ml^{-1} 3-methyl cholantrene (MCA) from day 1 to day 3 as the initiation stage. After initiation, the cells were cultured with 300 ng ml^{-1} phorbol-12-myristate-13-acetate (PMA) from day 6 to day 21 as the promotion stage. Culture medium was changed every 3 or 4 days intervals. When the culture period reached to 25 ~ 35 days, cells were fixed using methanol and Giemsa stain was performed. Transformed foci were observed by naked eyes, and focus numbers and their sizes were measured. ERW supplemented with various concentrations of Pt nanoparticles were added in the culture medium from day 1 to day 21 and the effect against two-stage transformation was compared.

2.4 Measurement of intracellular ROS

Amount of intracellular ROS, especially the intracellular H_2O_2 produced by PMA was determined by using a fluorescent dye, 2',7'-dichlorofluorescin-diacetate (DCFH-DA)[5]. Balb/c 3T3 cells were incubated for 15 min in the MEM medium with 300 ng ml^{-1} PMA with or without ERW/Pt. After removal of the supernatant, the cells were incubated in 5 μM DCFH-DA in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution for 10 min. The cells were then harvested by trypsinization, washed with PBS, resuspended in PBS and analyzed immediately using a flow cytometer with excitation and emission wavelength of 495 and 525 nm, respectively. Gating was performed to remove cellular debris and apoptotic cells before data were collected.

2.5 Statistical analysis

Statistical analysis was done using the Student's *t*-test. Probabilities of 0.05 or less were considered statistically significant. Statistically significant was represented as *; $P<0.05$ and ***; $P<0.005$, compared with treatment of MCA/PMA only.

3. RESULT AND DISCUSSION

3.1 Co-treatment of ERW/Pt suppresses two-stage cell transformation foci

Few foci appeared in the control without MCA/PMA treatment. In the presence of MCA/PMA treatment, many large foci significantly appeared in the culture dishes. Appearance of transformation foci was strongly suppressed by treatment of ERW/Pt (Table 1). Transformation foci were suppressed by addition of Pt nanoparticles to ERW in concentration-dependent manner. ERW supplemented with 1 ppm, 3 ppm and 10 ppm of Pt nanoparticles showed 57.1%*, 65.4%* and 100%*** of transformation foci suppression compared with MCA/PMA only, respectively. However, Pt nanoparticles without ERW could not effectively suppress cell transformation induced by MCA/PMA. It suggested that both ERW and Pt nanoparticles were needed to suppress two-stage cell transformation.

Table 1. Co-treatment of ERW/Pt suppresses two-stage cell transformation foci

MCA/PMA	Treatment	Foci/Dish (Mean ± SD)	% of Transformation
−	−	1.3 ± 0.57	2.5*
+	−	52 ± 10.8	100
+	Pt 1 ppm	57 ± 7.1	109.6
+	Pt 3 ppm	42 ± 2.0	80.8
+	Pt 10 ppm	51.7 ± 5.1	99.4
+	ERW/Pt 1 ppm	22.3 ± 3.5	42.9*
+	ERW/Pt 3 ppm	18 ± 1.6	34.6*
+	ERW/Pt 10 ppm	0 ± 0	0***

*:$P < 0.05$, ***:$P < 0.005$

3.2 ERW/Pt suppress transformation at promotion stage

Treatment of ERW containing 10 ppm of Pt nanoparticles suppressed the cell transformation at the stage of promotion but not at the stage of initiation. ERW containining no syntheisized Pt nanoparticles could not suppress the cell transformation. On the treatment of both ERW and Pt nanoparticles, the amounts of transformation foci were suppressed to 1.4%*** at promotion stage, compared to the MCA/PMA treatment only. During all stage treatment of ERW/Pt, transformation was suppressed to 4.3%***. However, the transformation foci were not sufficiently suppressed to 91.3% by the ERW/Pt treatment at initiation stage only. It was almost similar value (91.3%) compared to the MCA/PMA treatment only (Figure 1). Since PMA was well known as a strong inducer of intracellular ROS, it was suggested that both ERW and Pt nanoparticles suppressed the augmentation of intracellular ROS induced by PMA.

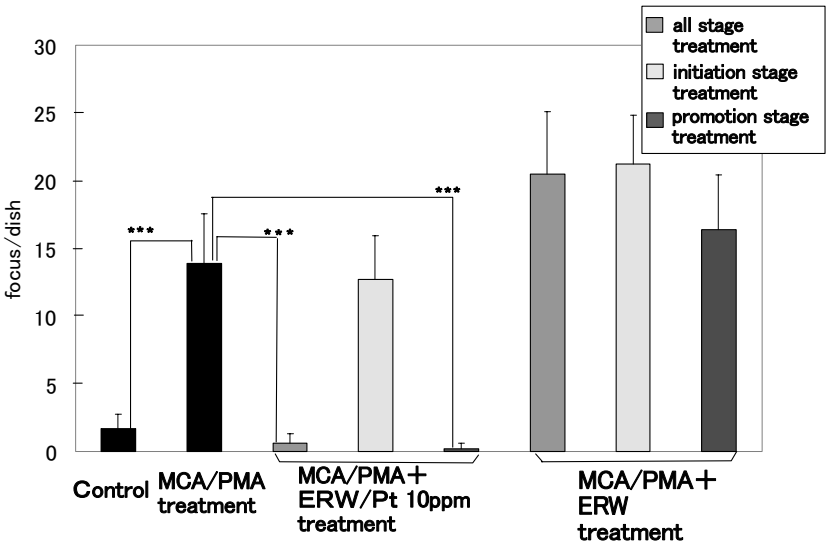

Figure 1. ERW/Pt suppressed cell transformation at promotion stage
*** $P<0.005$ compared with MCA and PMA only.

3.3 ERW/Pt did not suppress growth of Balb/c 3T3 cells

To clarify the effect of ERW/Pt on cell proliferation of Balb/c 3T3 cells, the cell growth was examined in the presence of ERW, Pt nanoparticles and both. The growth curve of Balb/c 3T3 cells under the all conditions showed no difference (data not shown). Therefore, ERW, Pt nanoparticles and both did not affect proliferation of Balb/c 3T3 cells, the treatment of ERW/Pt suppressed cell transformation without growth arrest or cell death. The cells treated with only ERW slightly grew faster than control (data not shown).

3.4 ERW/Pt suppress the augmentation of intracellular ROS induced by PMA as well as ascorbic acid-2-phosphate (ASA-2P)

ASA-2P suppress the transformation foci under the condition of 0.2 μg ml^{-1} of MCA initiation from day 1 to day 3 and 100 ng ml^{-1} of PMA promotion from day 6 to day 21 (data not shown). ASA-2P is a stable antioxidant, and suppressed Balb/c 3T3 two-stage cell transformation in promotion stage[15]. The treatment of ERW with 1 ppm of Pt nanoparticles

suppressed cell transformation stronger than 100 μM ASA-2P (data not shown). To evaluate the intracellular ROS scavenging effect of ERW/Pt, intracellular ROS was induced by the PMA treatment for 15 min and determined by using DCFH-DA and flow cytometer (Figure 2). ASA-2P, ERW, Pt nanoparticles and ERW/Pt significantly reduced excess intracellular ROS induced by PMA treatment. We have revealed that ERW contains a small amount of Pt nanoparticles as active hydrogen donors and ROS-scavengers[2,5]. These results suggested that ERW/Pt acted as antioxidant in the cells and suppressed tumorigenesis.

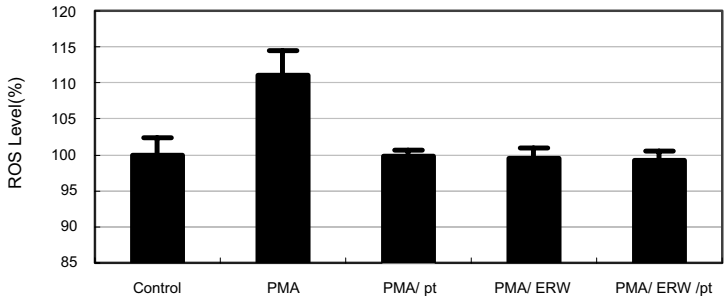

Figure 2. ERW/Pt suppressed intracellular ROS 15 min after PMA treatment. Values were statistically significant at $P<0.005$ compared with samples additionally treated with PMA.

It was suggested that active hydrogen produced from hydrogen molecules by catalysis on the surface of Pt nanoparticles would scavenge intracellular ROS. ERW/Pt also suppressed ERK^{MAPK} phosphorylation and excess intracellular ROS induced by PMA in murine epidermal JB6 cells (data not shown). In the recent study, intracellular ROS was known to activate MAP kinase, such as ERK^{MAPK} phosphorylation, and then AP-1, NFκB, iNOS and NOS were activated by MAP kinase. This cascade was related to the cell transformation in promotion stage[1]. It was suggested that suppression mechanism for cell transformation of ERW/Pt was similar to effective antioxidant for tumorigenesis such as ASA-2P via reducing excess intracellular ROS.

In conclusion, this is the first report about the suppression of two-stage cell transformation by ERW/Pt nanoparticles. We confirmed that ERW/Pt could scavenge excess intracellular ROS and strongly suppressed cell transformation at promotion stage. The detailed action mechanism of the reducing agents in ERW responsible for the ROS scavenging activity will be reported elsewhere.

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