Cytotechnology (2005) 47:97–105
DOI 10.1007/s10616-005-3759-2

Electrolyzed reduced water supplemented with platinum nanoparticles suppresses promotion of two-stage cell transformation

Ryuhei Nishikawa[1], Kiichiro Teruya[1], Yoshinori Katakura[1], Kazuhiro Osada[1], Takeki Hamasaki[1], Taichi Kashiwagi[1], Takaaki Komatsu[1], Yuping Li[1], Jun Ye[1], Akira Ichikawa[4], Kazumichi Otsubo[2], Shinkatsu Morisawa[2], Qianghua Xu[3] and Sanetaka Shirahata[1,*]

*[1]Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan; [2]Nihon Trim Co. Ltd., 1-8-34 Oyodonaka, Kita-ku, Osaka 531-0076, Japan; [3]College of Life Sciences, Zhejiang University, No. 268 Kaixuan Road, Hangzhou 310029, Zhejiang, P.R. China; [4]Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan; *Author for correspondence (e-mail: sirahata@grt.kyushu-u.ac.jp; phone: +81-92-642-3045; fax: +81-92-642-3052)*

Received 1 February 2005; accepted in revised form 7 March 2005

Key words: Active hydrogen, Antioxidant, Electrolyzed reduced water, 3-Methyl cholanthrene, Phorbol-12-myristate-13-acetate, Pt nanoparticles, Reactive oxygen species, Two-stage cell transformation assay

Abstract

In the two-stage cell transformation theory, cancer cells first receive initiation, which is mainly caused by DNA damage, and then promotion, which enhances transformation. Murine Balb/c 3T3 cells are widely used for transformation experiments because they lose contact inhibition ability when transformed. Electrolyzed reduced water (ERW), which is produced near a cathode during electrolysis of water, is an alkaline drinking water that is beneficial to health. ERW contains a high concentration of dissolved hydrogen and scavenge reactive oxygen species (ROS), along with a small amount of platinum (Pt) nanoparticles (Pt nps) derived from Pt-coated titanium electrodes. Pt nps stably disperse in aqueous solution for a long time, and convert hydrogen molecules to active hydrogen (atomic hydrogen) that can scavenge ROS. Therefore, ERW supplemented with synthesized Pt nps is a model strong reduced water. This is the first report that ERW supplemented with synthesized Pt nps strongly prevents transformation of Balb/c 3T3 cells. ERW was prepared by electrolysis of 0.002 M NaOH solution using a batch-type electrolysis device. Balb/c 3T3 cells were treated with 3-methyl cholanthrene (MCA) as an initiation substance, followed by treatment with phorbol-12-myristate-13-acetate (PMA) as a promotion substance. MCA/PMA-induced formation of a transformation focus was strongly suppressed by ERW supplemented with Pt nps but not by ERW or Pt nps individually. ERW supplemented with Pt nps suppressed transformation at the promoter stage, not at initiation, suggesting that ERW supplemented with Pt nps suppressed the PMA-induced augmentation of intracellular ROS. ERW supplemented with Pt nps is a potential new antioxidant against carcinogenesis.

Abbreviations: ASA-2P – ascorbic acid 2-phosphate; ERW – electrolyzed reduced water; FBS – fetal bovine serum; HEPES – 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MCA – 3-methyl cholanthrene; MEM – minimum essential medium; PMA – phorbol-12-myristate-13-acetate; Pt nanoparticles – Pt nps; ROS – reactive oxygen species

Introduction

In vitro cell transformation assay was first reported by Torado and Green (1963). Murine C3H10T1/2, Balb/c 3T3 and NIH/3T3 cells have all been widely used for transformation assays because they lose contact inhibition and form foci when transformed by viruses, X-ray, chemical treatments and gene expression etc. Balb/c 3T3 and C3H10T1/2 cells are sensitive to carcinogens compared with NIH/3T3 cells, which have been used for oncogene research. *In vitro* cell transformation assayed with Balb/c 3T3 and C3H10T1/2 cells, has long been recognized as being directly relevant to carcinogenesis (Kakunaga 1973; Heidelberger 1983; Kennedy 1983) and is regarded as a useful method to screen potential carcinogens (Dunkel et al. 1981; Atchison et al. 1982; Meyer 1983; Fang et al. 2002; Laaksonen et al. 2004). 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 transformation (Tsuchiya and Makoto 1995). Treatment of cells with just an initiator or a promoter is not enough to cause efficient transformation. Phorbol-13-myristate acetate (PMA), a potent promoter, is known to augment intracellular reactive oxygen species (ROS), which also induces phosphorylation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK). MAPKs activate AP-1, NF-κB, iNOS, NOS and finally induce intracellular ROS. This cycle cascade is related to transformation in the promotion stage (Arindam and Mathew 2002).

Intracellular ROS cause irreversible damage to biological macromolecules, especially to DNA, resulting in many diseases (Min and Jingxia 2002). Recently, electrolyzed reduced water (ERW) or electrolyzed alkaline water (Alkali-Ionsui) has received a lot of clinical attention in Japan as drinking water that is beneficial to health. A double-blind controlled-clinical study demonstrated that potable Alkali-Ionsui is safe and effective for improvement of gastrointestinal abnormality syndromes (Fujiyama and Kitahora 2004). ERW contains a high concentration of dissolved hydrogen (Kikuchi et al. 2001a, b) and can scavenge intracellular ROS (Shirahata et al. 1997, 2001; Hanaoka 2001; Li et al. 2002; Hiraoka et al. 2004). ERW has been postulated to improve diabetes (Oda et al. 1999; Li et al. 2002), and has reduced hemodialysis-induced oxidative stress in end-stage renal disease patients (Kuo-Chin and Chiang-Ting 2003). We proposed an active hydrogen reduced water hypothesis that active hydrogen in ERW scavenged ROS (Shirahata et al. 1997, 2001; Shirahata 2002). We also found that ERW contains a small amount of platinum (Pt) nanoparticles (Pt nps) (Shirahata 2004). In general, metal nanoparticles of less than 2 nm exhibit strong catalysis activity because of their high surface reactivity (Kubo et al. 1984). Synthesized Pt nps of 2 nm or less can disperse in aqueous solutions for a long time and convert hydrogen molecules to active hydrogen (atomic hydrogen) on the surface. Many antioxidants are known to prevent two-stage transformation; therefore, ERW supplemented with synthesized Pt nps is expected to be a strong new antioxidant. Here, we present new evidence that ERW supplemented with Pt nps strongly prevents transformation of Balb/c 3T3 cells.

Materials and methods

Cell culture

Murine Balb/c 3T3 A31-1-1 clonal cells were supplied by Japanese Collection of Research Bioresources (JCRB) and cultured in a minimum essential medium (MEM) (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (FBS) (Biowest, France), 2 mM L-glutamine, and 10 mM HEPES (10% FBS/MEM) at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of ERW and ERW-medium supplemented with Pt nps

ERW preparation was prepared as reported previously (Li et al. 2002). Briefly, 0.002 M NaOH solution was electrolyzed for 1 h using a batch-type electrolysis device (TI-200S, Nihon Trim Co., Osaka, Japan). Freshly prepared ERW (pH 11.5; ORP, −800 mV) was neutralized to pH 7.0 with HCl and filtrated with a 0.2-μm filter to dilute 10× MEM. Pt nps of an average 2 nm were purchased from SENEKA Co. Ltd, Tokyo, and added to 10% FBS/MEM. The concentration of Pt nps was

determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500, Agilent Technologies, CA, USA) at the Center for Advanced Instrumental Analysis, Kyushu University.

Two-stage cell transformation assay

BALB/c 3T3 cells (5.0×10^4 cells) were inoculated into a 60-mm dish and cultivated in the presence of 1.0 μg/ml 3-methyl cholanthrene (MCA) from day 1 to day 3 for the initiation phase. After initiation, the cells were cultivated with 300 ng/ml PMA from day 6 to day 21 for the promotion phase. Culture medium was changed every 3 or 4 days. When the cultivation period reached to 25–35 days, cells were fixed using methanol and Giemsa-stained. Transformed foci were found by naked eye and the focus numbers and their sizes determined. ERW and various concentrations of Pt nps were added in the culture medium from days 1 to 21 and their effects on two-stage transformation were examined.

Measurement of intracellular ROS

The amount of intracellular ROS, especially the intracellular H_2O_2 produced by PMA, was determined by using a fluorescent dye, 2′,7′-dichlorofluoresci-diacetate (DCFH-DA) (Li et al. 2002). Briefly, Balb/c 3T3 cells were incubated for 15 min in MEM medium with 300 ng/ml PMA with or without ERW supplemented with Pt nps. After removal of the supernatant, 5 μM DCFH-DA in a Ca^{2+}, Mg^{2+}-free HBSS buffer was added and cells incubated for 10 min. Cells were then harvested by trypsinization, washed with PBS, resuspended in PBS and analyzed immediately using a flow cytometer (Coulter Elite FACSCAN) with excitation and emission wavelengths of 495 and 525 nm, respectively. Gating was performed to remove cellular debris and apoptotic cells before data were collected.

Results

ERW supplemented with Pt nps suppresses two-stage cell transformation of Balb/c 3T3 cells

Balb/c 3T3 cells with no treatment formed a very small number of foci (1.33 ± 0.57 foci/dish [Mean ± SD]). When cells were treated with MCA and PMA together, a number of foci of significantly large sizes were formed in the dishes (52 ± 10.81). The formation of MCA and PMA-induced foci was strongly suppressed by ERW supplemented with Pt nps in a Pt nps concentration-dependent manner. ERW supplemented with 1, 3 and 10 ppm of Pt nps suppressed the MCA/PMA-induced formation of foci by 57.1%, 65.4%, and 100%, respectively. Pt nps alone could not significantly suppress MCA/PMA-induced cell transformation (Figure 1). The lack of the data on the effect of ERW on the MCA/PMA-induced cell transformation in Figure 1 is compensated by that in Figure 2 and Table 1; the experiments were performed under the same conditions.

ERW containing Pt nps suppressed cell transformation at the promotion stage

ERW supplemented with 10 ppm of Pt nps suppressed the transformation at the promotion stage but not at the initiation stage (see Figure 2 and Table 1). Positive control cells treated with MCA and PMA together formed a total 139 foci in 10 dishes. Cells treated with ERW containing 10 ppm of Pt nps at both initiation and promotion stages, in addition to the MCA and PMA treatment, formed only 6 foci, indicating 95.7% suppression of focus formation. Treatment of cells with ERW supplemented with 10 ppm of Pt nps in the initiation stage suppressed only 8.7% of the cell transformation, but the treatment of cells with ERW supplemented with 10 ppm of Pt nps in the promotion stage resulted in 98.6% suppression. The treatment of cells with just ERW at both initiation and promotion stages increased the focus formation by 47.4%. The treatment of cells with ERW at the initiation stage increased the focus formation by 52.5% and at the promotion stage by 17.9%, suggesting that ERW might enhance the action of the initiator.

ERW supplemented with Pt nps suppressed augmentation of intracellular ROS by PMA as well as AsA-2P

ASA-2P is a stable antioxidant which is known to suppress Balb/c 3T3 two-stage cell transformation

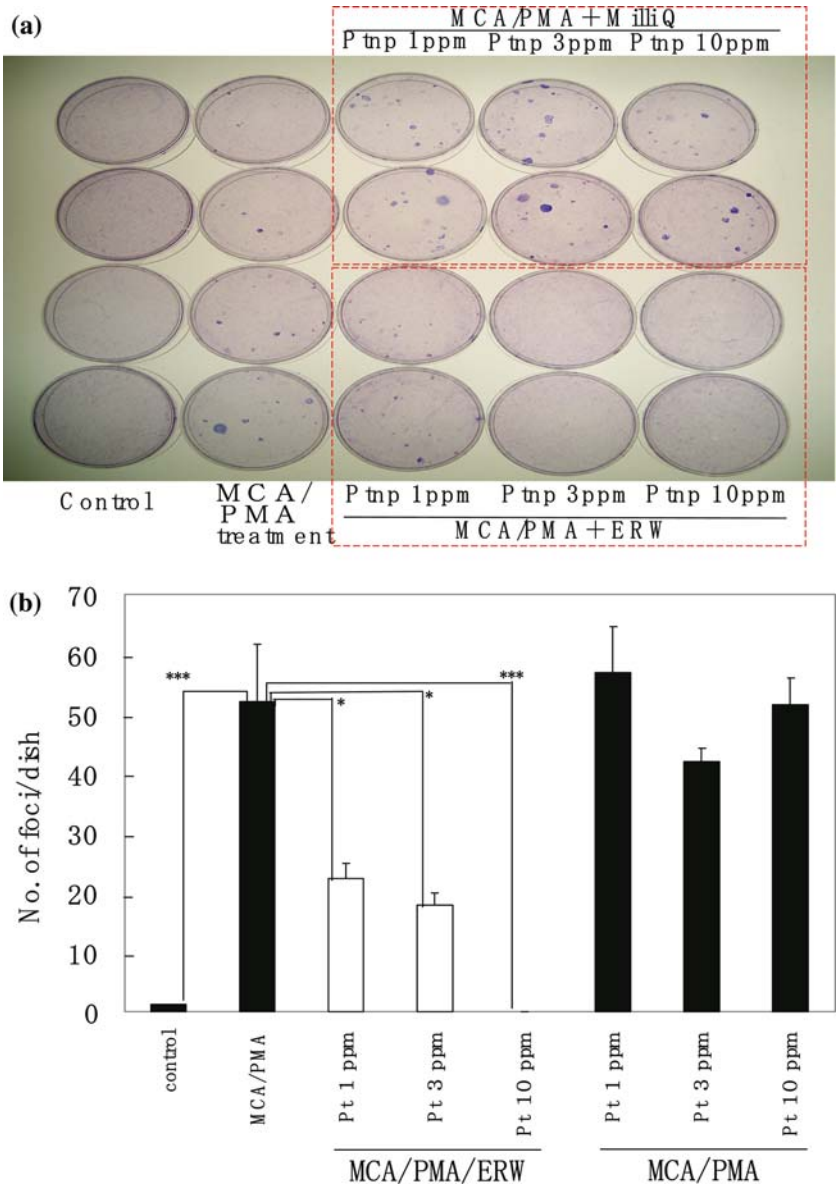

Figure 1. Effect of ERW and Pt nps on two-stage cell transformation of Balb/c 3T3 cells. (a) Photographs of Giemsa-stained dishes showing foci of transformed cells in *in vitro* two-stage transformation assay using Balb/c 3T3 Cells. Representatives of three independent experiments are shown. (b) Comparison of the focus number of each dish in experiment (a). Balb/c 3T3 cells (5.0×10^4 cells) were seeded in 10% FBS/MEM in 60-mm dishes. MCA (1.0 μg/ml) was present for the period from 1 to 3 days (initiation phase), and PMA (300 ng/ml) was present for the period from 6 to 21 days (promotion phase). Cells were cultivated with or without 1, 3 and 10 ppm of Pt nps and ERW from 1 to 25 days. Medium was changed every 3–4 days. After 25 days, cultures were fixed with methanol and stained with Giemsa solution. Transformation foci were counted and compared. The lack of the data on the effect of ERW on MCA/PMA-induced cell transformation in this figure is compensated by data in Figure 2 and Table 1; the experiments were performed under the same conditions. Data are means of three independent experiments. Standard errors were shown with bars. Values were statistically significant at $*p < 0.05$ and $***p < 0.005$ compared with that of positive control cells treated with both MCA and PMA at each time. Pt nps, platinum nanoparticles.

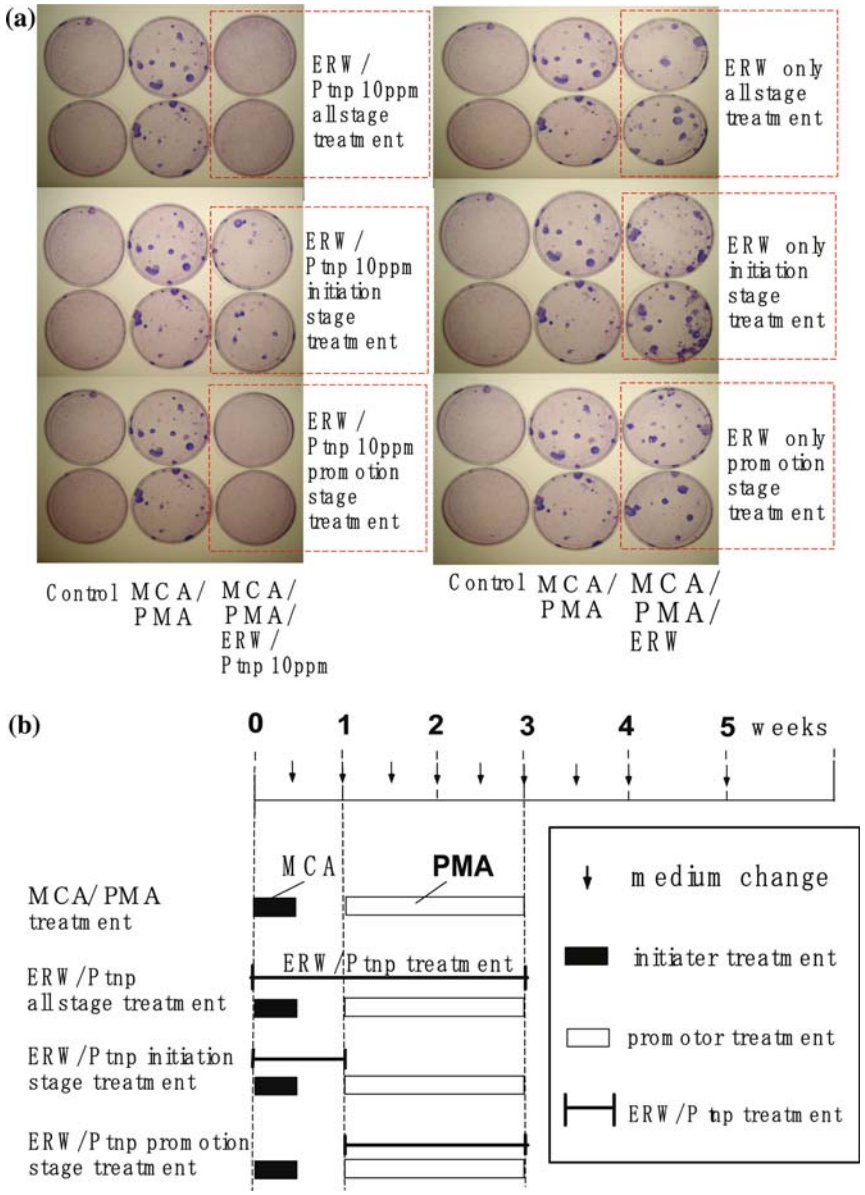

Figure 2. Effects of ERW and Pt nps on the initiation and promotion stages of two-stage cell transformation of Balb/c 3T3 cells. (a) Balb/c 3T3 cells (5.0×10^4 cells) were treated in 10% FBS/MEM in 60-mm dishes with MCA (1.0 μg/ml) for 3 days in 10% FBS/MEM and further cultivated for 3 weeks with or without ERW or ERW supplemented with 10 ppm of Pt nps. The experimental conditions were the same as in Figure 1. PMA (300 ng/ml) was present for the period from 1 to 3 weeks with or without ERW or ERW supplemented with Pt nps in the all stages (Upper). Cells were treated with ERW and Pt nps from 1 to 6 days during the initiation stage (Middle). Cells were treated with ERW and Pt nps from 6 to 21 days for the promotion stage (Low). (b) Time table of the treatment stage.

in the promotion stage (Tsuchiya et al. 2000). Balb/c 3T3 cells were treated with MCA and PMA in the presence of AsA-2P or ERW supplemented with 1, 3 and 10 ppm of Pt nps at both initiation and promotion stages. Since the suppressive effect of AsA-2P on cell transformation was weaker than ERW supplemented with Pt nps, the concentration of MCA was lowered. As shown in Figure 3, 100 μM AsA-2P suppressed the formation of foci by 67.7%. Whereas, ERW supplemented with 1, 3, and 10 ppm of Pt nps suppressed the formation of foci by 90.3%, 96.8% and 98.1%, respectively. Pt

Table 1. Effect of ERW and ERW supplemented with Pt nps on MCA/PMA-induced two-stage cell transformation of Balb/c 3T3 cells.

Initiating treatment	Promoting treatment	No. of dishes with foci/no. of dishes examined	Total no. of foci.	Foci/Dish (Mean ± SD)	% of transformation
–	–	9/10	17	1.7 ± 1.06	12.2***
MCA[a]	PMA[b]	10/10	139	13.9 ± 3.67	100[c]
MCA/ERW/Pt	PMA/ERW/Pt	5/10	6	0.6 ± 0.69	4.3***
MCA/ERW/Pt	PMA	10/10	127	12.7 ± 3.23	91.3
MCA	PMA/ERW/Pt	2/10	2	0.2 ± 0.421	1.4***
MCA/ERW	PMA/ERW	10/10	205	20.5 ± 4.6	147.4**
MCA/ERW	PMA	10/10	212	21.2 ± 3.64	152.5***
MCA	PMA/ERW	10/10	164	16.4 ± 4.01	117.9

[a]The concentration of MCA was 1.0 μg/ml.
[b]The concentration of PMA was 300 ng/ml.
[c]Transformation control.
***$p < 0.005$ vs. transformation from control by the student t-test.
**$p < 0.01$ vs. transformation from control by the student t-test.

nps alone did not significantly inhibit the formation of transformation foci. The effect of ERW on MCA/PMA-induced cell transformation was not examined in this condition, because our focus was on revealing the similarity of action between AsA-2P and ERW supplemented with Pt nps.

To examine whether ERW supplemented with Pt nps can scavenge intracellular ROS, the amount of intracellular ROS, especially intracellular H_2O_2 15 min after PMA treatment, was determined by the DCFH-DA method. PMA increased intracellular ROS in Balb/c 3T3 cells by 11.0%, compared

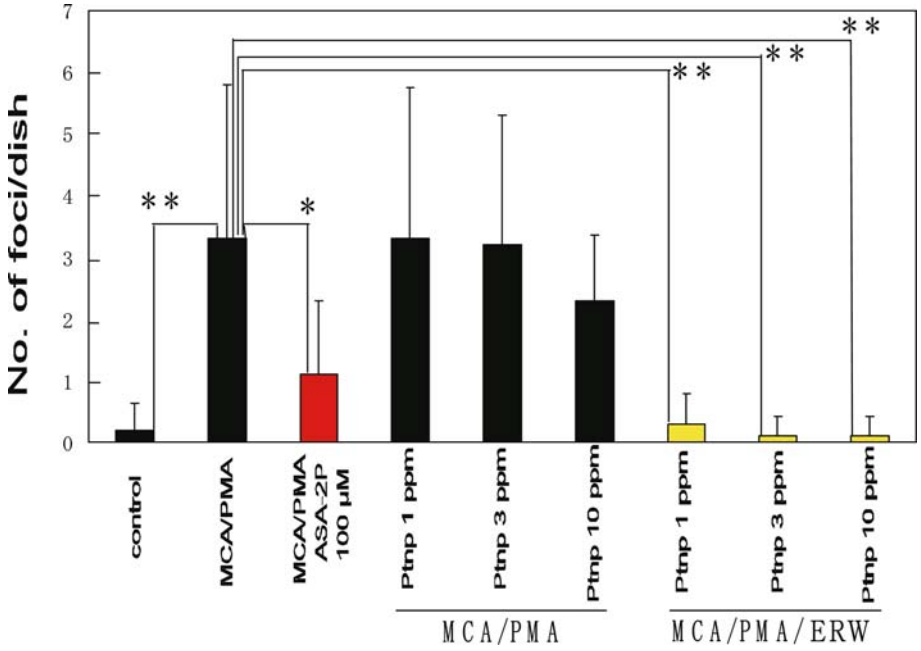

Figure 3. Suppression of two-stage cell transformation by ERW supplemented with Pt nps and ascorbic acid-2-phosphate ester magnesium. Balb/c 3T3 cells (5×10^4 cells) were treated with a reduced amount of MCA (0.2 μg/ml) in 60-mm dishes for 3 days and cultivated for 3 weeks with or without ERW supplemented with 1, 3, and 10 ppm of Pt nps. PMA (100 ng/ml) was present for the promotion period from 1 to 3 weeks with or without ascorbic acid-2-phosphate ester magnesium, ERW and various concentrations of Pt nps. Data are the mean of three independent experiments. Standard deviations were shown as bars. Values were statistically significant at *$p < 0.05$ and **$p < 0.01$ compared with positive control cells treated with both MCA and PMA at the initiation and promotion stages, respectively.

to that of control cells with no PMA treatment (Figure 4). ERW supplemented with 10 ppm of Pt nps significantly decreased the PMA-induced augmentation of intracellular ROS to just 3.5% increase. Pt nps and ERW also significantly suppressed the increase of intracellular ROS level to 5.5% and 4.8% increases, respectively.

Effect of ERW supplemented with Pt nps on the growth of Balb/c 3T3 cells

To examine the effects of ERW and Pt nps on the growth of Balb/c 3T3 cells, cell growth was determined in the same condition as for the focus formation experiments. As shown in Figure 5a, 5×10^4 cells reached a confluence at about 1×10^7 cells in a 60-mm dish after 9 days, and the growth was inhibited by contact inhibition. ERW stimulated cell growth and reached confluence after 5 days. The growth of cells treated with Pt nps or ERW supplemented with Pt nps were suppressed until 5 days, but then they grew rapidly and had nearly reached confluence after 9 days. These results suggested that Pt nps and ERW supplemented with Pt nps do not have much effect on the growth of Balb/c 3T3 cells in the focus formation experiments, because foci were examined after 25–35 days. When the cells were seeded at 1.2×10^6 cells/60-mm dish, cell growth was not affected by ERW, Pt nps and ERW supplemented with Pt nps (Figure 5b). These results indicated that Pt nps and EWR supplemented with Pt nps could suppress cell growth at low cell densities, but not at high cell densities. The suppressive effect of ERW supplemented with Pt nps on foci formation may be due to suppression of transformation, and not to any cytotoxic effect.

Discussion

MCA/PMA-induced transformation focus formation of Balb/c 3T3 cells was strongly suppressed by ERW supplemented with Pt nps, but not by ERW or Pt nps applied individually. Because the treat-

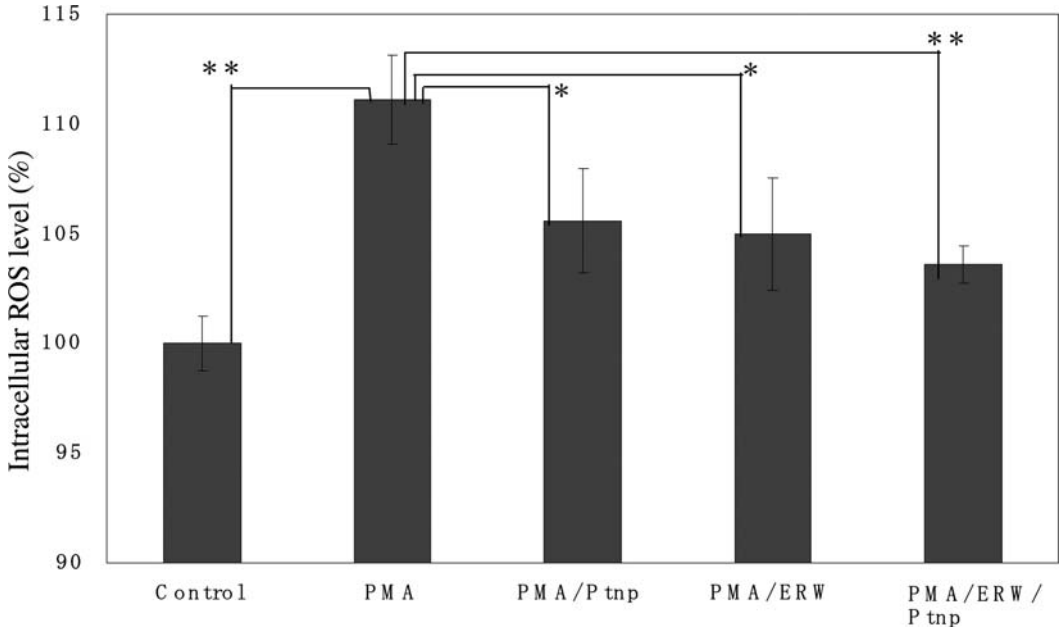

Figure 4. Effects of ERW, Pt nps and ERW supplemented with Pt nps on MCA-induced augmentation of the intracellular ROS level of Balb/c 3T3 cells. The amount of intracellular ROS, especially intracellular H_2O_2, produced by PMA was determined by using DCFH-DA. Cells were incubated for 15 min in MEM containing 300 ng/ml PMA with or without ERW or ERW supplemented with 10 ppm of Pt nps. After removal of the supernatant, 5 μM DCFH-DA in a Ca^{2+}, Mg^{2+}-free HBSS buffer was added and cells were incubated for 10 min. 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.

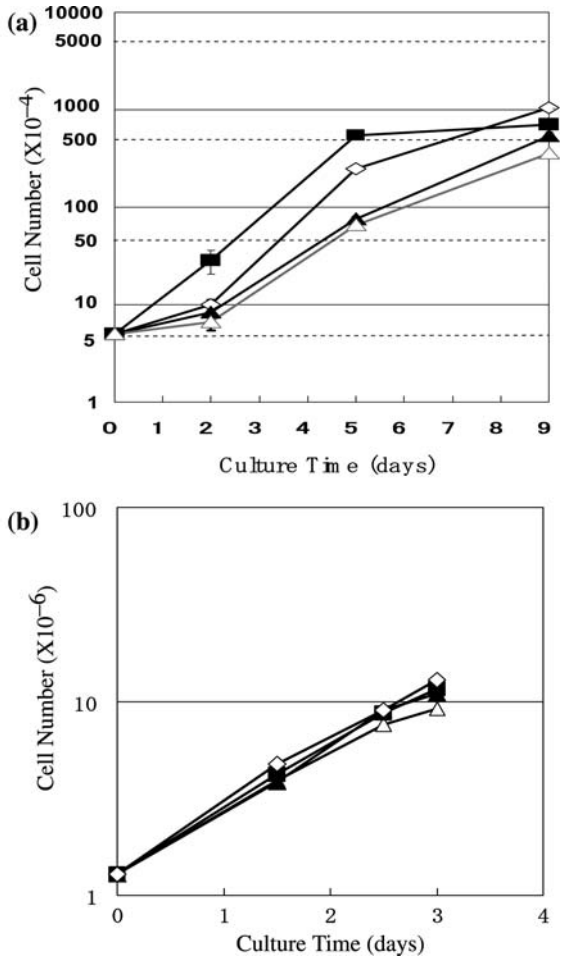

Figure 5. The growth curve of Balb/3T3 cells cultivated in electrolyzed reduced water-medium, medium supplemented with Pt nps and ERW-medium supplemented with Pt nps. Balb/c 3T3 cells were seeded in 10% FBS/MEM, 10% FBS/ERW/MEM, 10% FBS/MEM supplemented with 10 ppm of Pt nps, and 10% FBS/ERW/MEM supplemented with 10 ppm of Pt nps in 60-mm dishes at 5×10^5 cells/dish (a) or 1.3×10^6 cells/dish (b) from 0 to 9 days or 3 days, respectively. Medium was exchanged every 3 days. Cell growth was determined by counting cell numbers using a cell counter. Data are expressed as the mean ± SD of three independent experiments. Standard deviation was calculated with Student's *t*-test and is shown as bars. Opened diamond, control MEM; filled square, ERW/MEM; filled triangle, MEM supplemented with Pt nps; opened triangle, ERW/MEM supplemented with Pt nps.

ment of cells with ERW supplemented with Pt nps at the promotion stage resulted in the strong suppression of transformation, but not at the initiation stage, ERW supplemented with Pt nps may function as a potent inhibitor against the promoter action of PMA (see Table 1). PMA elevated the intracellular ROS level of Balb/c 3T3 cells, and ERW supplemented with Pt nps suppressed the PMA-enhanced elevation of the intracellular ROS level. Intracellular ROS activates MAPK. MAPK activates AP-1, NF-κB, iNOS, NOS, resulting in the induction of intracellular ROS. This cascade may promote cell transformation (Arindam and Mathew 2002). These results suggested that ERW supplemented with Pt nps might function as a ROS scavenger to prevent the ROS induction cascade and suppress MCA/PMA-induced cell transformation. Pt nps are good catalysts to convert hydrogen molecules to active hydrogen on the surface of Pt metal (Mandal et al. 2004). ERW is known to contain high concentrations of dissolved hydrogen (Kikuchi et al., 2001a, b). We revealed that Pt nps are incorporated into cells in a time-dependent manner (data not shown). Hydrogen molecules will be also incorporated into cells because it is gas like NO. Taken together, from these results we can postulate the following mechanism for the strong suppressive effect of ERW supplemented with Pt nps on two-stage cell transformation: (1) Pt nps and hydrogen molecules incorporate into cells; and (2) molecular hydrogen is converted to active hydrogen (atomic hydrogen) on the surface of Pt nps. Active hydrogen or electrons on the surface of Pt nps scavenge intracellular ROS, and then change biochemical signaling pathways via redox-sensitive molecules like AP-1 and NF-κB, inhibiting the cascade pathway for ROS generation. Freshly prepared ascorbic acid could suppress MCA/PMA-induced cell transformation in Balb 10T1/2 cells via regulation of the redox potential, glycoprotein, and lipid in C3H10T1/2 cells but not by ascorbic acid prepared every 3 days (Ibric et al. 1991). Strongly reduced water prepared by supplementation of Pt nps into ERW may be useful as a new, more stable antioxidant than ascorbic acid for prevention of carcinogenesis caused by carcinogens or chemicals in the environment.

On the other hand, ERW significantly enhanced focus formation (Table 1); however, the mechanisms of this phenomenon are unknown. Because treatment of cells with ERW at the promotion stage resulted in no significant stimulation of focus formation, but not so at the promotion stage, there is a possibility that ERW enhances the function of MCA or stimulated the formation rate of foci. Since the safety of ERW or Alkali-Ionsui has been

clinically demonstrated (Fujiyama and Kitahora 2004), more detailed investigation including animal experiments will be needed to better demonstrate this phenomenon. Pt nps and ERW supplemented with Pt nps suppressed the growth of Balb/c 3T3 cells at a low cell density, but not at a high cell density. Further detailed investigations are needed in respect to this mechanism.

References

Arindam D. and Mathew R.Y. 2002. The role of AP-1, NF-κB, and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. Mol. Cell. Biochem. 234/235: 185–193.

Atchison M., Chu C.S., Kakunaga T. and Van Duuren B.L. 1982. Chemical cocarcinogenesis with the use of a subclone derived from Balb/3T3 cells with catechol as cocarcinogen. J. Natl. Cancer Inst. 69: 503–508.

Dunkel V.C., Pienta R.J., Sivak A. and Traul K.A. 1981. Competitive neoplastic transformation responses of Balb/3T3 cells, Syrian hamster embryo cells, and Rescher murine leukemia virus-infected Fischer 344 rat embryo cells to chemical carcinogens. J. Natl. Cancer Inst. 67: 1303–1315.

Fang M.-Z., Mar W. and Cho M.-H. 2002. Cadmium affects genes involved in growth regulation during two-stage transformation of Balb/3T3 cell. Toxicology 177: 253–265.

Fujiyama Y. and Kitahora T. 2004. Alkaline electrolytic water (alkali ions water) for drinking water in medicine. In: Mizu no Tokusei to Atarashii Riyo Gijutsu, Enu-Ti-Esu, Tokyo, pp. 348–357

Hanaoka K. 2001. Antioxidant effects of reduced water produced by electrolysis of sodium chloride. J. Appl. Electrochem. 31: 1307–1313.

Heidelberger C. 1983. Cell transformation by chemical agents – a review and analysis of the literature: a report of the United States Environmental Protection Agency Gene-Tox Program. Mutat. Res. 114: 283–385.

Hiraoka A., Takemoto M., Suzuki T., Shinohara A., Chiba M., Shirao M. and Yoshimura Y. 2004. Studies on the properties and real existence of aqueous solution systems that are assumed to have antioxidant activities by the action of "active hydrogen". J. Health Sci. 50: 456–465.

Ibric L.L.V., Peterson A.R. and Sevanian A. 1991. Mechanisms of ascorbic acid-induced inhibition of chemical transformation in C3H/10T1/2 cells. Am. J. Clin. Nutr. 54: 1236S–1240S.

Kakunaga T. 1973. A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from Balb/3T3. Int. J. Cancer. 12: 463–473.

Kikuchi K., Takeda H., Rabolt B., Okaya T., Ogumi Z., Saihara Y. and Noguchi H. 2001a. Hydrogen concentration in water from an Alkali-Ion-Water electrolyzer having a platinum-electroplated titanium electrode. J. Appl. Electrochem. 31: 1301–1306.

Kikuchi K., Takeda H., Rabolt B., Okaya T., Ogumi Z., Saihara Y. and Noguchi H. 2001b. Hydrogen particles and supersaturation in alkaline water from alkali-ion-water electrolyzer. J. Electroanal. Chem. 506: 22–27.

Kubo R., Kawabata A. and Kobayashi S. 1984. Electronic properties of small particles. Annu. Rev. Mater. Sci. 14: 49–66.

Kuo-Chin H. and Chiang-Ting C. 2003. Reduced hemodialysis-induced oxidative stress in end-stage renal disease patients by electrolyzed reduced water. Kidney Int. 64: 704–714.

Laaksonen M., Maeki-Paakkanen J., Kronberg L. and Komulainen H. 2004. Effects of chlorohydroxyfuranones on 3-methylcholanthrene-induced neoplastic transformation in the two-stage transformation assay in C3H 10T1/2 cells. Arch. Toxicol. 77: 594–600.

Li Y., Nishimura T., Teruya K., Tei M., Komatsu T., Hamasaki T., Kashiwagi T., Kabayama S., Shim S., Katakura Y., Osada K., Kawahara T., Otsubo K., Morisawa S., Ishii Y., Gadek Z. and Shirahata S. 2002. Protective mechanism of reduced water against alloxan-induced pancreatic β-cell damage: Scavenging effect against reactive oxygen species. Cytotechnology 40: 139–149.

Mandal S., Roy D., Chaudhari R.V. and Sastry M. 2004. Pt and Pd nanoparticles immobilized on amine-functionalized zeolite; excellent catalysts for hydrogenation and heck reactions. Chem. Mater. 16: 3714–3724.

Meyer A.L. 1983. *In vitro* transformation assays for chemical carcinogens. Mutat. Res. 115: 323–338.

Min D. and Jingxia L. 2002. Differential role of hydrogen peroxide in UV-induced signal transduction. Mol. Biochem. 234/235: 81–90.

Oda M., Kusumoto K., Teruya K., Hara T., Maki T., Kabayama Y., Katakura Y., Otsubo K., Morisawa S., Hayashi H., Ishii Y. and Shirahata S. 1999. Electrolyzed and natural reduced water exhibit insulin-like activity on glucose uptake into muscle cells and adipocytes. In: Bernard A. et al. (eds), Animal Cell Technology: Products from Cells, Cells as Products. Kluwer Academic Publishers, The Netherlands, pp. 425–427.

Shirahata S. 2002. Reduced water for prevention of diseases. In: Shirahata S. (ed.), Animal Cell Technology: Basic & Applied Aspects, Vol. 12. Kluwer Academic Publishers, pp. 25–30.

Shirahata S. 2004. Reduced water. In: Mizu no Tokusei to Atarashii Riyo Gijutsu (The Characteristics and Advanced Technology of Water), Enu-Ti-Esu, Tokyo, pp. 33–45.

Shirahata S., Kabayama S., Nakano M., Miura T., Kusumoto K., Gotoh M., Hayashi H., Otsubo K., Morisawa S. and Katakura Y. 1997. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem. Biophys. Res. Commun. 234: 269–274.

Shirahata S., Nishimura T., Kabayama S., Aki D., Teruya K., Otsubo K., Morisawa S., Ishii Y., Gadek Z. and Katakura Y. 2001. Anti-oxidative water improves diabetes. In: Linder-Olsson E. et al. (eds), Animal Cell Technology: From Target to Market. Kluwer Academic Publishers, The Netherlands, pp. 574–577.

Torado G.J. and Green H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17: 299–313.

Tsuchiya T. and Makoto U. 1995. Improvement in the efficiency of the *in vitro* transformation assay method using Balb/3T3 A31-1-1 cells. Carcinogenesis 16(8): 1887–1894.

Tsuchiya T., Kato E. and Umeda M. 2000. Anti-transforming nature of ascorbic acid and its derivatives examined by two-stage cell transformation using BALB/c 3T3 cells. Cancer Lett. 160: 51–58.