Exp Oncol 2009
31, 3, 156–162

Experimental ONCOLOGY

PLATINUM NANOCOLLOID-SUPPLEMENTED HYDROGEN-DISSOLVED WATER INHIBITS GROWTH OF HUMAN TONGUE CARCINOMA CELLS PREFERENTIALLY OVER NORMAL CELLS

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Aim: **Hydrogen-dissolved water (HD-water) or platinum nanocolloid (Pt-nc) has been individually expected as a new therapeutic agent for oxidative stress-related diseases, whereas little is known about their combined effects on cancer, which were elucidated in the present study.** ***Methods:*** **HD-water was prepared by microporous gas bubbling, and supplemented with Pt-nc consisting of 0.003–1 ppm Pt and PVP polymers. Antioxidant activities were examined by 1, 1-diphenyl-picrylhydrazyl (DPPH)-radical-scavenging assay. Cytotoxic activities were examined by culturing of tumor and normal cell lines, respectively.** ***Results:*** **HD-water accelerated the Pt-nc-based DPPH-radical scavenging. Pt-nc-supplemented HD-water inhibited either colony formation efficiencies or colony sizes of human tongue carcinoma cells HSC-4, in contrast to no effects of HD-water alone, Pt-nc alone or Pt-absent PVP, but not appreciably inhibit normal human tongue epithelial-like cells DOK. Pt-nc-supplemented HD-water also suppressed cell population growth of HSC-4 cells of near-confluence (at higher cell densities) in view of decreases in either cell numbers or mitochondrial function, although less markedly than colony formation starting from a sparse-cell state (at lower cell densities). Dissolved hydrogen, oxygen concentration or oxido-reduced potentials of HD-water was decreased, rather decreased or increased by Pt-nc addition, respectively.** ***Conclusions:*** **Anti-cancer activity of Pt-nc-supplemented HD-water was shown by its preferential cell-growth inhibition to human tongue carcinoma cells HSC-4 over normal human tongue cells DOK, and might be partly attributed to HD-water-caused enhancement of Pt-nc-relevant antioxidant ability. Pt-nc-supplemented HD-water is expected as a novel agent against human tongue cancers due to its cancer progression-repressive abilities.**

Key Words: **hydrogen-dissolved water, platinum nanocolloid, tumor-preferential repression, human tongue carcinoma, reactive oxygen species, antioxidant.**

It has been reported that human tumor cells produce reactive oxygen species (ROS) more abundantly than non-transformed cell lines [1], and an elevated oxidative stress has been found in many different types of cancer cells [2]. In fact, many evidences suggest that ROS are related to diverse abilities of cancer cells which increase cell proliferation, DNA synthesis [3], survival, cellular migration [4], invasion [5, 6], tumor metastasis [7, 8] and angiogenesis [9]. Furthermore, ROS within cells act as second messengers and play important roles in intracellular signaling cascades which induce and maintain the oncogenic phenotype of cancer cells [1]. On the other hand, it is also known that antioxidants can inhibit tumor cell proliferation, which indicates an important role of ROS in mediating the loss of growth control [10–12]. Thus, these findings suggest the possibility that scavenging of ROS by antioxidant compounds or antioxidant materials can suppress the tumorigenic actions of ROS and the development of certain cancers.

Recently, many researchers have paid attention to hydrogen as a novel antioxidant material, and reported that hydrogen is quite effective for alleviating oxidative stress. Inhalation of hydrogen gas markedly suppressed focal ischemia and reperfusion-induced brain injury in rat [13], reduces myocardial injury by ischemia-reperfusion in rat [14], and suppresses hepatic injury by ischemia-reperfusion in mouse [15]. Furthermore, many reports have been also demonstrated that aqueous solutions containing high concentrations of dissolved hydrogen (hydrogen-dissolved water; HD-water), which were produced by electrolysis or dissolved hydrogen under high pressure, show the antioxidant activities and efficacies in various experimental disease models. It has been showed that HD-water decreased superoxide anion radicals [16, 17], hydrogen peroxide [18], hydroxyl radical [13, 18, 19], and protects DNA [16, 20, 21], RNA [21] and protein [21] from oxidative damages, and HD-water suppressed antimycin A-induced cell death [13], alloxan-derived ROS-induced pancreatic β-cell damage [22], tumor-preferential clonal-growth-inhibition, tumor invasion [19] and tumor angiogenesis [23].

Moreover, it has been demonstrated that drinking HD-water decreased the ROS-induced urinary secretion of 8-hydroxydeoxyguanosine and hepatic formation of peroxidized lipid in rats [24], the hypoxia-reoxygenation-induced ROS formation in vitamin C-depleted mice [25], chronic physical restraint stress-induced increase of oxidative stress markers (malondialdehyde and 4-hydroxy-2-nonenal in the brain) [26] and ameliorate oxidative stress-related diseases such as diabetes mellitus in mice [27, 28]. Furthermore, the efficacy of HD-water has been

Received: June 19, 2009.

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Abbreviations used: HD-water – Hydrogen-dissolved water; DH – dissolved molecular hydrogen; DMEM – Dulbecco's modified Eagle's medium; DO – dissolved oxygen; DPPH – 1, 1-diphenyl-2-picrylhydrazyl; MEM – Eagle's minimum essential medium; ORP – oxido-reduced potential; PR – phenol red; Pt-nc – platinum nanocolloid; PVP – poly (N-vinyl-2-pyrrolidone); ROS – reactive oxygen species.

demonstrated in clinical trials for the patients with type 2 diabetes or impaired glucose tolerance [29] and a hemodialysis-induced oxidative stress in end-stage renal disease patients [30, 31].

On the other hand, platinum nanocolloids (Pt-nc) are generally known to exhibit strong catalysis activity because of their high surface reactivity, and it is also known that Pt-nc have antioxidative effects [32] and the ability to scavenge ROS, superoxide anion radials ($O_2 \cdot -$), and hydrogen peroxide (H_2O_2) [33, 34]. Therefore, HD-water supplemented with Pt-nc is expected to be a strong new antioxidant and a candidate for anti-cancer therapy. Indeed, it has been showed that electrolyzed reduced water supplemented with Pt-nc strongly prevents transformation of murine Balb/c 3T3 cells [35].

In the present study, we investigated the anti-cancer effects of Pt-nc-supplemented HD-water for oral tumor cells and its effects for normal cells.

MATERIALS AND METHODS

Hydrogen-dissolved water (HD-water). HD-water was prepared by microporous gas bubbling into flowing water about 0.1 MPa at hydrogen pressure of more than 0.6 MPa (HYDRO BATH; Hiroshima Kasei, Hiroshima). Concentrations of dissolved molecular hydrogen (DH) in solution were measured by using a DH meter (DH-35A, DKK-Toa, Tokyo), and concentrations of dissolved oxygen (DO) were measured by using a pH/DO meter (D-55, Horiba, Kyoto), and oxido-reduced potential (ORP) was measured by using an ORP meter (RM-20P, DKK-Toa), respectively at 25 °C. In all experiments, we used HD-water, which indicated each parameter at 1.0–1.3 ppm (parts per million) of DH concentrations, -668 to -481 mV of ORP, and 7.0–7.8 of pH. Hydrophilic colloid poly (N-vinyl-2-pyrrolidone) (PVP)-entrapped Pt-nc, containing approximately 4% (wt%) of 2-nm diameter platinum particles, which consist of 8% (wt%) of PVP, was obtained (commercially available) from Tanaka Kikinzoku Kogyo K. (Tokyo). The production method was described in Laid-Open Disclosure Public Patent Bulletin in Japan (333737, 1999) and Japan Patent (P4252151).

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay. DPPH radical-scavenging activity was investigated according to the previous report [36] with minor modification. The solution containing 25 μM DPPH in 50% ethanol mixed with diverse species of waters (purified water (control), purified water + Pt-nc, purified water + PVP, HD-water and HD-water + Pt-nc), and the absorbance of each mixture was scanned at 400–700 nm after 30 s and 10 min, respectively, with a spectrometer (U-2800, Hitachi, Tokyo).

Cell culture. Human tongue squamous carcinoma-derived cell line HSC-4 (RCB1902) was provided by the Health Science Research Resource Bank (Osaka). Cells were cultured in Eagle's minimum essential medium (MEM; Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Corp., CA), and 2 mM L-glutamine [37]. Normal human tongue epithelial-like cells DOK were supplied by DS Pharma Biomedical Co., Ltd. (Osaka). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku) supplemented with 10% heat-inactivated FBS, and 4 mM L-glutamine at 37 °C [38]. Both cell lines were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Culture medium containing HD-water or purified water was prepared by dilution of 10-fold concentrated culture medium with each water, and was supplemented with Pt-nc if necessary.

Colony formation assay. Cells were seeded on a 24-well plate and allowed to attach to the substratum, and then the spent medium was exchanged to fresh cell culture medium that was prepared with HD-water or purified water, which was supplemented with or without Pt-nc. After 3-day incubation, the colonies were stained and photographed, and the total colony numbers per dish and cell numbers per colony were evaluated under a microscope.

Evaluation of cell viability and cell number. Cell viability was assessed based on mitochondrial enzymatic conversion of WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium, sodium salt] (Dojindo, Kumamoto) to yellowish formazan, which is indicative of the number of viable cells. Cells were rinsed with phenol red (PR)-free medium and then incubated for 3 h in PR-free medium containing 10% WST-1 at 37 °C. The absorbance at 450 nm was measured with a microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg). Cell numbers were measured with a particle counter (CDA-500, Sysmex, Kobe).

Statistical Analysis. The unpaired Student's *t*-test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as $P < 0.05$.

RESULTS

Combined effects of HD-water and Pt-nc on DPPH-radical-scavenging activity. Combined effects of HD-water and Pt-nc on antioxidative actions were investigated in a cell-free system by DPPH-radical-scavenging assay (Fig. 1). The treatment with purified water, purified water + PVP or HD-water alone occurred scarce decreases in DPPH radicals both for 30 s and 10 min. On the other hand, purified water + Pt-nc induced an appreciable decrease in the radicals for 30 s, and a marked decrease for 10 min. In contrast, HD-water + Pt-nc dramatically decreased the radicals within 30 s. The antioxidant activity of Pt-nc (1 ppm) is equal to that of Torolox at 2.2 μM for 30 s, and or 5.3 μM for 10 min, respectively. Thus, combination of HD-water and Pt-nc exerted a more rapid radical-scavenging activity than that by treatment with purified water + Pt-nc. Therefore, HD-water can accelerate or enhance the Pt-nc-induced DPPH-radical-scavenging activity.

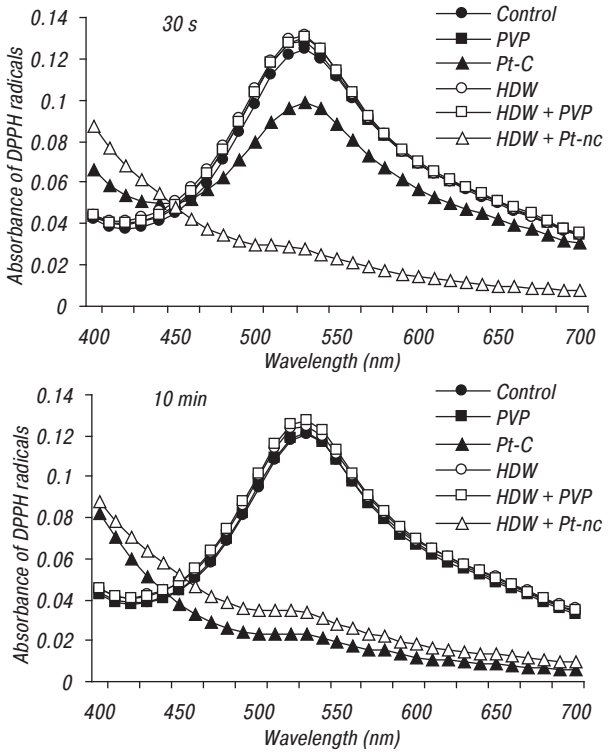

Fig. 1. Combined effects of HD-water and Pt-nc on DPPH-radical-scavenging activity. After mixing a solution of DPPH radicals and diverse species of waters: purified water (control), purified water + PVP (2 ppm), purified water + Pt-nc (1 ppm), HD-water, HD-water + PVP (2 ppm), and HD-water + Pt-nc (1 ppm). The absorbance was measured at 30 s and 10 min. DPPH gives a strong absorption around 520 nm. HPW — HD-water

Repressive effects of concomitant administration with HD-water and Pt-nc on colony formation of tongue carcinoma cells HSC-4. It was investigated whether concomitant administration with HD-water and Pt-nc could repress colony formation of human tongue carcinoma cells HSC-4. The colony number was not affected by purified water at any concentrations of Pt-nc, but it was significantly reduced by concomitant administration with HD-water and Pt-nc increasingly in a manner dependent on Pt-nc doses (Fig. 2, *a*, *b*). The repressive effects were nearly equal to those between two modes for Pt-nc-administration timings ("0 h" and "24 h"). Furthermore, a cell number per colony was substantially reduced upon the concomitant administration with HD-water and Pt-nc as compared with other treatments (Fig. 2, *c*). These results indicate that the concomitant administration with HD-water and Pt-nc exerted repressive effects against both colony formation and cell proliferation in HSC-4 cells.

Effects of PVP on colony formation of HSC-4 cells. The effects of PVP, which is a entrapping and protective polymer agent for Pt to form colloidal particles, on colony formation of HSC-4 cells were examined. The colony number was not affected by any concentrations of PVP regardless of combination with HD-water (Fig. 3). The results suggest that the Pt itself is quite important for the anti-cancer effects of concomitant administration with HD-water and Pt-nc.

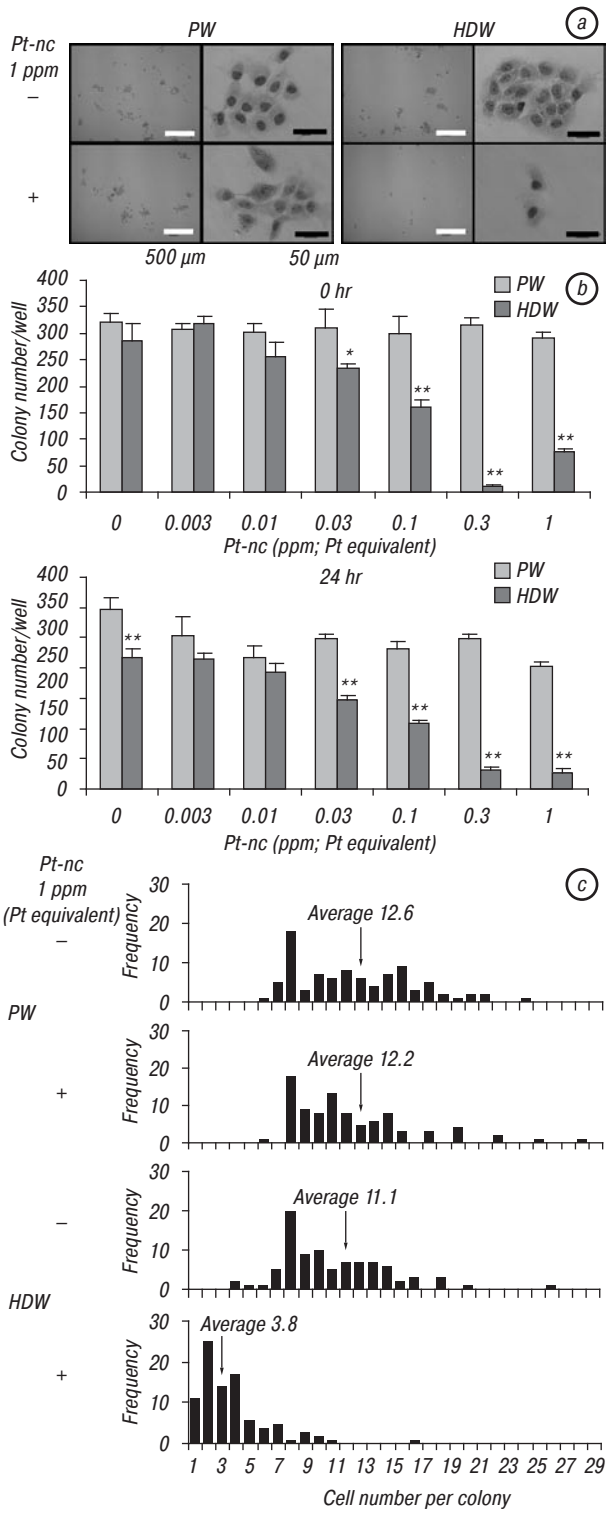

Fig. 2. Effects of concomitant administration with HD-water and Pt-nc on colony formation of human tongue carcinoma cells HSC-4. Cells were seeded into Pt-nc (0–1 ppm)-containing cell culture medium prepared with HD-water or purified water at a density of 500 cells per well. After 3-day incubation, the colonies were stained and photographed. *a*, Morphological aspects of treated cells. The white and black scale bars indicate 500 μm and 50 μm, respectively. *b*, Total colony numbers were evaluated by counting the colonies which consisted of more than 8 cells per colony. Cells were treated with once at only 0 h (when they were seeded) (the upper figure) or twice at 0 h and 24 h after seeding (the lower figure), respectively. Significantly different from purified water: $^{*}P < 0.05$, $^{**}P < 0.01$. *c*, The distribution of cell numbers per colony was also evaluated for 90 colonies under a microscope. PW — purified water; HDW — HD-water; Average — an average cell numbers per colony

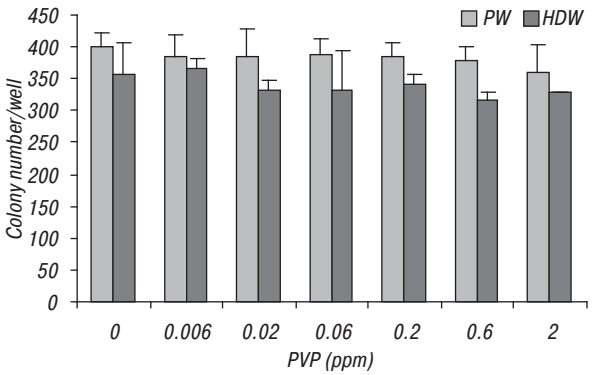

Fig. 3. Effects of PVP on colony formation of human tongue carcinoma cells HSC-4. Cells were seeded into PVP-containing cell culture medium prepared with HD-water or purified water at a density of 500 cells per well. After 3 days of incubation, the colonies were stained, and total colony numbers were evaluated by counting the colonies which consisted of more than 8 cells per colony. PW — purified water; HDW — HD-water

Repressive effects of concomitant administration with HD-water and Pt-nc on colony formation of carcinoma cells preferentially over normal cells. The effects of concomitant administration with HD-water and Pt-nc on colony formation of normal human tongue epithelial-like cells DOK were investigated. As shown in Fig. 4, the colony number was decreased to less than 5% of the maximum for HSC-4 cells by the concomitant administration with HD-water and Pt-nc at 0.3 ppm, whereas the number was scarcely affected for DOK cells. Thus, HD-water did not or scarcely affect the clonal growth of normal cells, but preferentially repressed proliferation of carcinoma cells even regardless of similar cell growth rates of DOK cells (a doubling time of approximately 24 h) and HSC-4 cells (approximately 24 h).

Effects of concomitant administration with HD-water and Pt-nc on cell growth of HSC-4 cells. In addition to effects on clonal growth, the inhibitory effects of HD-water on tumor population growth were also investigated. The cell-growth of HSC-4 cells was significantly inhibited by the concomitant administration with HD-water and Pt-nc as compared with purified water + Pt-nc (Fig. 5). The repressive effects were nearly equal to those between two modes for Pt-nc-administration timings (4 h and 24 h), and these results showed similar tendencies by two methods for evaluation (cell viability estimated by WST-1 assay and cell number by counting with a particle counter).

Temporal changes of DH, ORP and pH in cell culture medium prepared with purified water or HD-water. The temporal changes of DH, ORP, DO and pH in cell culture medium which was prepared with purified water or HD-water were examined, and effects of Pt-nc on their values were also investigated (Fig. 6). Although HD-water showed a high DH and low ORP, the DH value was immediately lowered, by mixing into the culture medium and the subsequent filtration with a 0.10-μm-pore filter, from approximately 1.0 to 0.3 ppm. Moreover, the DH values of culture medium that was produced by HD-water, were much higher than by purified water, and decreased in a time-dependent manner, and they finally reached a level equal to that of purified water at 4 h after preparation. On the other hand, the addition of Pt-nc decreased the DH values in a dose-dependent manner more markedly than for HD-water alone. The addition of Pt-nc increased the ORP values in a dose-dependent manner more markedly than for HD-water alone. The DO values of HD-water were lower than those of purified water, but the effects of Pt-nc addition were not clear. The pH values scarcely changed and kept physiological levels around pH 7.3–7.9 for 4 h.

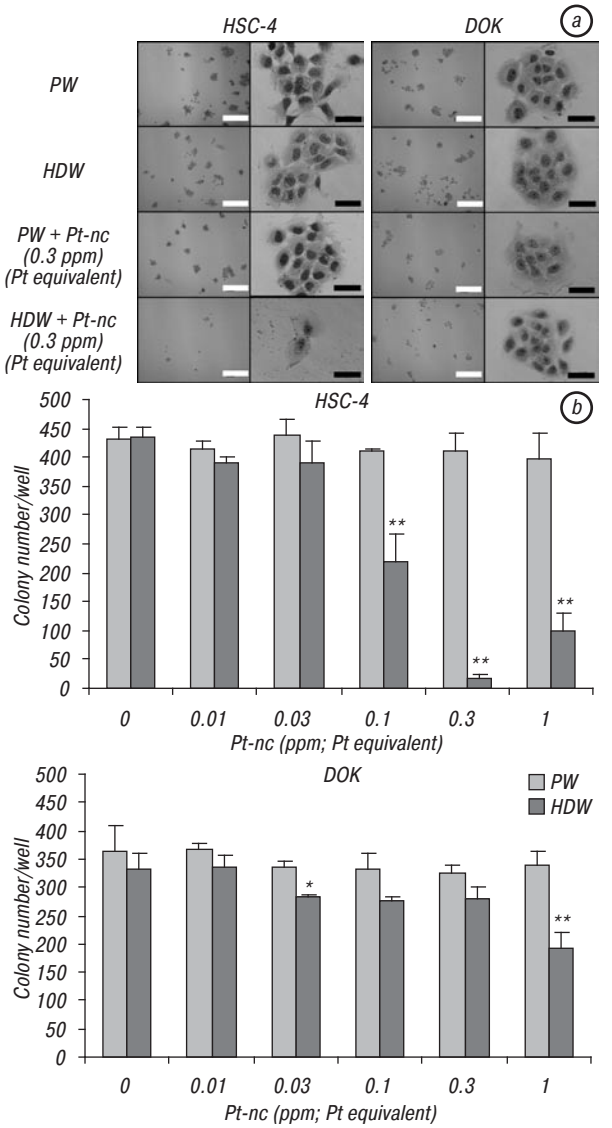

Fig. 4. Differential effects of HD-water on colony formation of human tongue carcinoma cells HSC-4 and normal human tongue epithelial-like cells DOK. Cells were seeded into Pt-nc containing cell culture medium prepared with HD-water or purified water at a density of 500 cells per well. After 3-days of incubation, the colonies were stained, photographed. *a*, Morphological aspects of treated cells. The white and black scale bars indicate 500 μm and 50 μm, respectively. *b*, Total colony numbers were evaluated by counting the colonies which consisted of more than 8 cells per colony. Significantly different from 0 ppm of PW or HDW, respectively: $*P < 0.05$, $**P < 0.01$. PW — purified water; HDW — HD-water

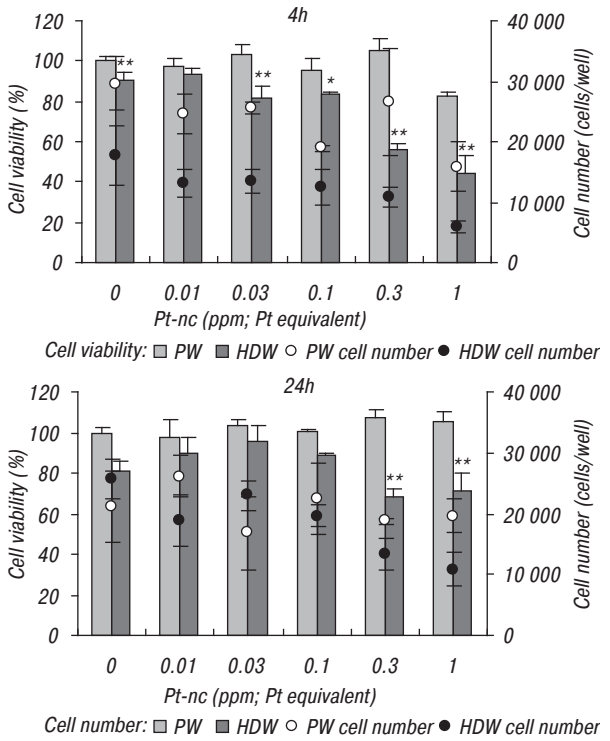

Fig. 5. Effects of concomitant administration with HD-water and Pt-nc on cell growth of human tongue carcinoma cells HSC-4. Cells were seeded at a density of 1.2×10^4 cells per well in a 24-well plate. After 4 or 24-h incubation, the medium was exchanged to Pt-nc containing cell culture medium prepared with HD-water or purified water. After 24-h incubation, the cell-growth ratio was assessed by the WST-1 assay and counting cell number, respectively. Significantly different from purified water: $^*P < 0.05$, $^{**}P < 0.01$. PW — purified water; HDW — HD-water

DISCUSSION

In the present study, we investigated the combined effects of HD-water and Pt-nc on human oral tumor cell growth, and their effects on normal cells derived in common from human tongue. Our data showed the repressive effects against both colony formation and cell proliferation in HSC-4 cells were exerted by the combination of HD-water and Pt-nc, but not purified water alone, purified water + Pt-nc, HD-water alone or HD-water + PVP (see Fig. 2, 3). These results suggest that Pt-nc-supplemented HD-water plays a quite important role for the anti-cancer effects. Furthermore, the treatment of Pt-nc-supplemented HD-water exerted the preferred prevention against colony formation and cell proliferation of carcinoma cells over normal cells (see Fig. 4). In addition to inhibitory effects of Pt-nc-supplemented HD-water on colony formation which of carcinoma cells, correspond to "clonal growth" immediately after formation of a single cancerous or mutated cell, Pt-nc-supplemented HD-water was furthermore demonstrated to inhibit also "massive growth" as a cancer cell population as shown in Fig. 5, which corresponds to an early stage of cancer progression. Though the inhibitory effects on "massive growth" were comparatively weaker than those on "clonal growth" (see Fig. 2–5), these results suggest that the decrease in anti-cancer effects is due to either the enhancement of cellular resistance against hydorogen and Pt-nc or reduction of the accessibilities of hydorogen and Pt-nc because of prevention by the decrease in cellular superificial area accompanied with cellular growth and the subsequent increase in cell density.

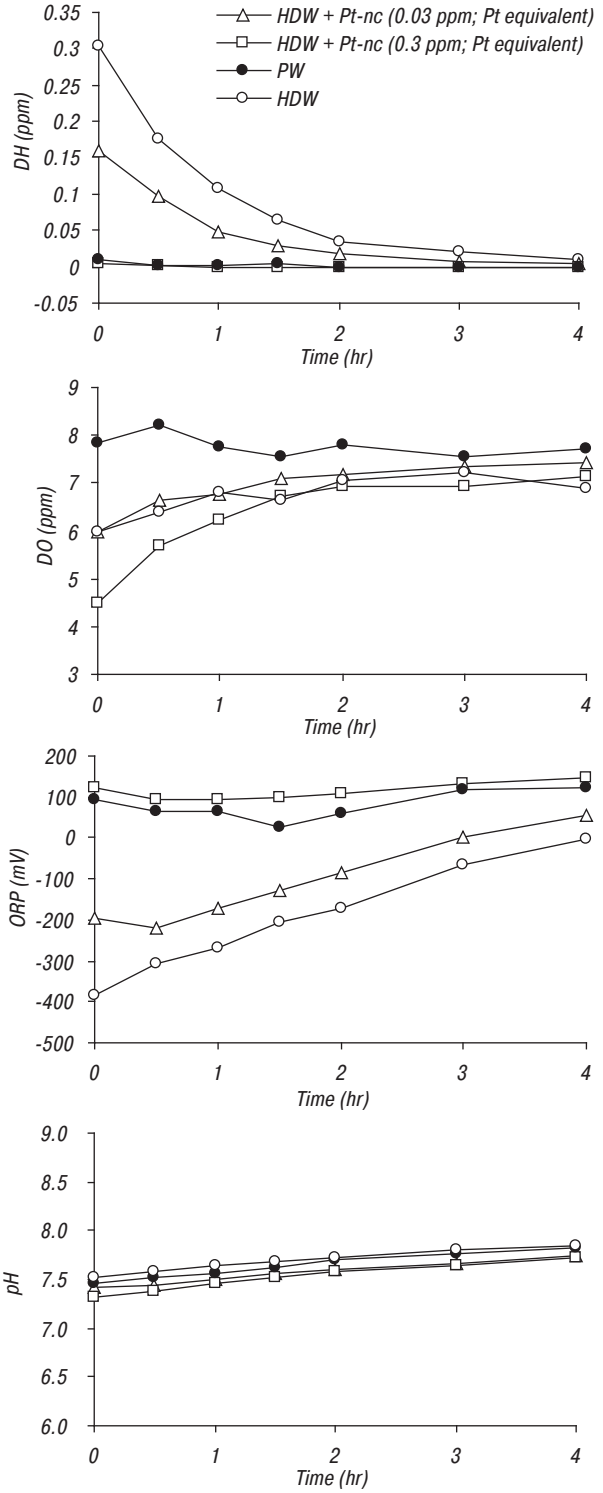

Fig. 6. Temporal changes of DH, ORP, DO and pH in cell culture medium prepared with HD-water or purified water and their dependency on the presence or absence of Pt-nc. After preparation of each medium, they were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Then, the parameters were measured at the indicated time points. PW — purified water; HDW — HD-water

The anti-cancer effects of Pt-nc-supplemented HD-water are suspected to be due to their antioxidant activity, because ROS are related to diverse abilities of cancer cells [1–12]. This is supported by the results that HD-water

could accelerate or enhance the Pt-nc-induced radical scavenging activity (see Fig. 1). Many studies suggested that HD-water possessed the antioxidant activity [13, 16–19], but our results showed that anti-cancer effects were insufficient only with HD-water. Two reasons are able to be considered as the causes of these results. As the first reason, the DH values of HD-water in our experiments were low as compared with those in other reports. In our experiments, the DH values markedly decreased immediately after mixing with cell culture medium, and they are rapidly decreased in a time-dependent manner as shown in Fig. 6. It was demonstrated that DH levels are related with an antioxidant effect [13]. Consequently, the treatment with HD-water alone could not show an excellent antioxidant activity, and could not necessarily exert sufficient anti-cancer effects in our experiments. And the second reason is the co-existence of Pt. There is a possibility that the nature of DH-water may vary according to diverse principle in the methods for the production of HD-water (by electrolysis, dissolved hydrogen under high pressure, or magnesium addition to water). It was assumed that a trace of Pt nanoclusters existed in electrolyzed reduced water by releasing from the Pt-coated electrodes during electrolysis [39]. Since it is well known that metal clusters (such as Pt) can act as catalysts for reductive reactions in aqueous solutions, it is presumed that the HD-water produced by electrolysis contains a trace amount of Pt which helps the antioxidant activity of HD-water. However, there is a few possibility that Pt exists in the HD-water produced by hydrogen bubbling. Therefore, it seems that the HD-water in our experiments could not exert the anti-cancer effects by itself alone. Taken together, although HD-water alone could not exert sufficient anti-cancer effects as compared with previous reports, our results might be able to be explained by the DH values of HD-water or the differences in the methods for the production of HD-water. These hypotheses coincide with our results that the anti-cancer effects were achieved by the co-existence of Pt-nc in spite of low DH values. These results also showed a possibility that the addition of Pt-nc into HD-water can overcome its instability as the weak points, and can enhance its antioxidant activities. Consequently, the DH-water has still potentials for the improvement and augmentation of the anti-cancer activities by finding the way to elevate and keep DH values at higher levels.

Synthesized Pt nanoparticles (nps) of 2 nm or less at a diameter can disperse in aqueous solutions for a long time and function as efficient catalysts to convert hydrogen molecules to atomic hydrogens on the surface of Pt metal [35, 40]. Some reports assumed that the atomic hydrogen scavenge ROS, and therefore, we also postulate that atomic hydrogen, which generated on the surface of Pt, may be related with anti-cancer effects in our experiments. The decrease in DH values by the addition of Pt-nc (see Fig. 6) might be attributed to the catalytic action of Pt. Moreover, we revealed that palladium-PVP, which is another catalyst for hydrogen molecules, also showed the similar anti-cancer effects (data not shown). These assumptions are consistent with our results that the anti-cancer effects were endowed by HD-water supplemented with Pt-nc but not by HD-water or Pt-nc individually.

Recently, it has been showed that electrolyzed reduced water supplemented with Pt nanoparticles strongly prevents transformation of murine Balb/c 3T3 cells [35]. In this report, it was postulated that Pt nanoparticles and hydrogen molecules were incorporated into cells, molecular hydrogen is converted to atomic hydrogen on the surface of Pt nanoparticles, and atomic hydrogen or electrons on the surface of Pt nanoparticles scavenge intracellular ROS. Moreover, they suggested that the decrease in ROS generation changed biochemical signaling pathways via redox-sensitive molecules like AP-1 and NF-κB, interrupting the cascade pathway, which may promote cell transformation [41]. Their hypothesis is considered to be applied to our results that anti-cancer effects were achieved by the addition of Pt-nc into HD-water. Therefore, we presume that the anti-cancer effects of Pt-nc-supplemented HD-water on human cancer cells are achieved through its antioxidant activity. Moreover, augmentation of this antioxidant activity of Pt-nc-supplemented HD-water may become a reinforcing tool useful for certain types of anticancer therapy such as radiotherapy or certain anti-cancer drugs which are concerned with ROS-induced cytotoxicities [42]. On the other hand, although we have not compare HD-water plus Pt-nc with some traditional Pt drugs, we think that the anticancer mechanism of HD-water with Pt-nc is different from the traditional Pt drugs such as DNA synthesis inhibitor cisplatin, because it is reported that cisplatin induces toxic affects via generation of reactive oxygen species [42]. However, we also think that a comparison with traditional Pt drugs is interesting and is worthwhile to try in a future experiment.

The present study demonstrates that Pt-nc-supplemented HD-water exerts a more prompt antioxidant action and preferentially inhibited clonal-growth of human tongue carcinoma cells over that of the corresponding normal cells. Therefore, Pt-nc-supplemented HD-water is expected to become a useful tool for clinical application to anti-cancer therapy, and the further in vivo studies are necessary for clinical applications.

ACKNOWLEDGEMENTS

The authors thank Ms. Haruko Mimura and Ms. Hiroe Masaki for their technical assistance.

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