Antitumor effects of nano-bubble hydrogen-dissolved water are enhanced by coexistent platinum colloid and the combined hyperthermia with apoptosis-like cell death

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Abstract. In order to erase reactive oxygen species (ROS) related with the proliferation of tumor cells by reducing activity of hydrogen, we developed functional water containing nano-bubbles (diameters: <900 nm for 71%/population) hydrogen of 1.1-1.5 ppm (the theoretical maximum: 1.6 ppm) with a reducing ability (an oxidation-reduction potential -650 mV, normal water: +100-200 mV) using a microporous-filter hydrogen-jetting device. We showed that hydrogen water erased ROS indispensable for tumor cell growth by ESR/spin trap, the redox indicator CDCFH-DA assay, and was cytotoxic to Ehrlich ascites tumor cells as assessed by WST-8 assay, crystal violet dye stain and scanning electron microscopy, after 24-h or 48-h incubation sequent to warming at 37˚C or 42˚C. Hydrogen water supplemented with platinum colloid (0.3 ppm Pt in 4% polyvinylpyrrolidone) had more antitumor activity than hydrogen water alone, mineral water alone (15.6%), hydrogen water plus mineral water, or platinum colloid alone as observed by decreased cell numbers, cell shrinkage and pycnosis (nuclear condensation)/karyorrhexis (nuclear fragmentation) indicative of apoptosis, together with cell deformation and disappearance of microvilli on the membrane surface. These antitumor effects were promoted by combination with hyperthermia at 42˚C. Thus, the nano-bubble hydrogen water with platinum colloid is potent as an anti-tumor agent.

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Key words: nano-bubble hydrogen water, microporous-filter hydrogen-jetting device, anti-tumor effect, hyperthermia

Introduction

Tumor cells produce reactive oxygen species (ROS) more abundantly than normal cells (1). Behavior of ROS is suggested to be related to the promotion of cell proliferation, DNA synthesis (2), survival, cellular migration (3), invasion (4), tumor metastasis (5), and angiogenesis (6). Accordingly, anti-oxidants counteract with ROS, and can inhibit tumor cell proliferation (7). The water containing abundant hydrogen, an antioxidant, can erase ROS and is expected to have antitumor activity (8).

An apparatus which can produce the functional water containing nano-bubble hydrogen has been developed (8). The water contains hydrogen nano-bubbles <900-nm diameter, for 71% of total bubbles, and hydrogen of 1.1-1.5 ppm, and has an oxidation-reduction potential (ORP) of -650 mV versus +100-200 mV in normal water. The nano-babble hydrogen-enriched water reduces hydroxyl radicals in normal water to a quarter of the initial amount (8). However, the cell culture medium which was made based on this hydrogen water lost hydrogen during the subsequent adjustment, and heightened the ORP remarkably, and there was little anti-tumor activity of this hydrogen culture medium alone. Therefore, the effect has been acquired by adding the platinum particle (9).

We improved the device and made a compact type of hydrogen water manufacturing device with a microporous-filter hydrogen-jetting nozzle that was able to jet hydrogen gas into the culture medium directly. As a result, the hydrogen dissolved culture medium showed the oxidation-reduction potential of -650 mV even before the experiment. This value was maintained for a period as long as three months when the medium was preserved with a sealed aluminum bottle at 4˚C. The ROS-scavenging ability of the hydrogen culture medium was examined in human skin cells by the redox indicator CDCF-based fluorescence (Fig. 1). Fluorescence exhibiting the existence of abundant ROS is seen in the nucleus of a human skin cell treated without nano-bubble hydrogen medium (Fig. 1A). In the nucleus and adjacent to the

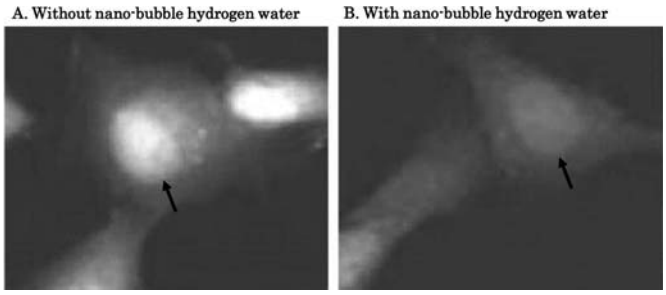

Figure 1. The ROS-erasing ability of the hydrogen culture medium was examined in human skin cells by the redox indicator CDCF-DA-based fluorescence. Fluorescence exhibiting the existence of abundant ROS is seen in the nucleus of a human skin cell treated without nano-bubble hydrogen medium (A). In the nucleus and its adjacent cytoplasm in human skin cells treated with nano-bubble hydrogen medium the fluorescence is weakened (B).

cytoplasm ROS indicated by the fluorescence is erased in human skin cells treated with nano-bubble hydrogen medium (Fig. 1B).

In the present study, anti-tumor effects of new nano-babble hydrogen cell culture medium on Ehrlich ascites tumor (EAT) cells were examined from diverse viewpoints: a) effects of coexistent substances, b) enhanced carcinostatic effects by a combination with hyperthermia which inhibits tumor cell growth (10-12) and effects of subsequent culture period, and c) morphological observation of cellular degeneration.

Carcinostatic effect was evaluated with a decrease in mitochondrial dehydrogenase activity reflecting survival rate of cells as measured by WST-8 assay (13,14) and with a decrease in viable cells observed morphologically by Crystal Violet stain assay (15). Morphological cellular degeneration was observed by scanning electron microscopy (SEM) (16,17).

Materials and methods

Apparatus. A new type of device consisting of a tank and a cover inserted with a jetting nozzle and a guide tube of hydrogen gas was made from the conventional device (8) for manufacturing drinking-hydrogen water (Fig. 2). Main parts are: (a) tank, (b) cover, (c) hydrogen jet nozzle, (d) regulation cock, (e) stop cock, (f) hydrogen gas inlet, (g) hydrogen gas outlet, and (h) culture medium. The device can produce culture medium containing abundant nano-bubble hydrogen.

Production of highly enriched hydrogen culture medium. The inside wall of a tank attached to the apparatus was sterilized by spraying and evaporating of ethanol in a clean bench. Culture medium was added into the tank. A cover was inserted with a guide tube of hydrogen gas, its edge was dipped into the medium and put on the tank. The cover was tightly fixed. Hydrogen gas was supplied to the medium in the tank by jetting at the pressure of 0.55 kg/cm^3. After 20 min, the supply of hydrogen was stopped and the cover was opened. The medium was taken out of the tank and its ORP was measured. The medium was fully added to a 100 ml bottle, and stored in the refrigerator as test stock solutions.

Cells. Ehrlich ascites tumor (EAT) cells (RCB: no. 0142) were purchased from the Institute of Physical and Chemical

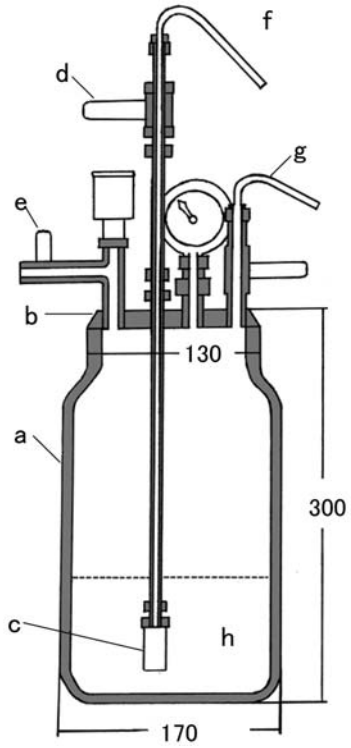

Figure 2. A new type of device consisting of a tank and a cover inserted with a jetting nozzle and a guide tube of hydrogen gas was made by improvement from the conventional apparatus for manufacturing drinking hydrogen water. The device can produce culture medium containing nano-bubble hydrogen abundantly. Main parts are (a) tank, (b) cover, (c) hydrogen jet nozzle, (d) regulation cock, (e) stop cock, (f) hydrogen gas inlet, (g) hydrogen gas outlet, and (h) culture medium.

Research (Tsukuba, Japan). These cells were cultured in minimum essential medium (MEM) (Gibco Labs Life Technologies, Inc., NY) supplemented with 10% fetal bovine serum (FBS) (Gibco Labs Life Technologies, Inc.).

Preparation of diverse culture media. Several kinds of experimental culture media were prepared for examining carcinostatic effects of the nano-bubble hydrogen water and are listed in Table I.

Examination for carcinostatic effects. The carcinostatic effects were examined according to our developed system (12). Briefly, cells were suspended in the culture medium at densities of 2x10^4 cells/ml suitable to cacinostatic effects. The suspension of cells was put in a test tube, the supernatant was removed after centrifugation, and each culture medium pored on the pellet and suspended. The cell suspensions were added to a glass sample bottle (14 mm i.d. x40 mm). Then, the bottle was tightly covered with a plastic cap.

Hyperthermic treatment. The cell suspension was incubated at 37˚C or 42˚C for 30 min in a water bath (Model BT-23, Yamato Scientific Co. Ltd., Tokyo), and maintained by sequential culture in a humidified atmosphere of 5% CO_2 in air at 37˚C for 24 h or 48 h.

Cell viability assay. Viability of cells was measured using the redox indicator dye WST-8 (13,14) (Cell counting kit, Dojin

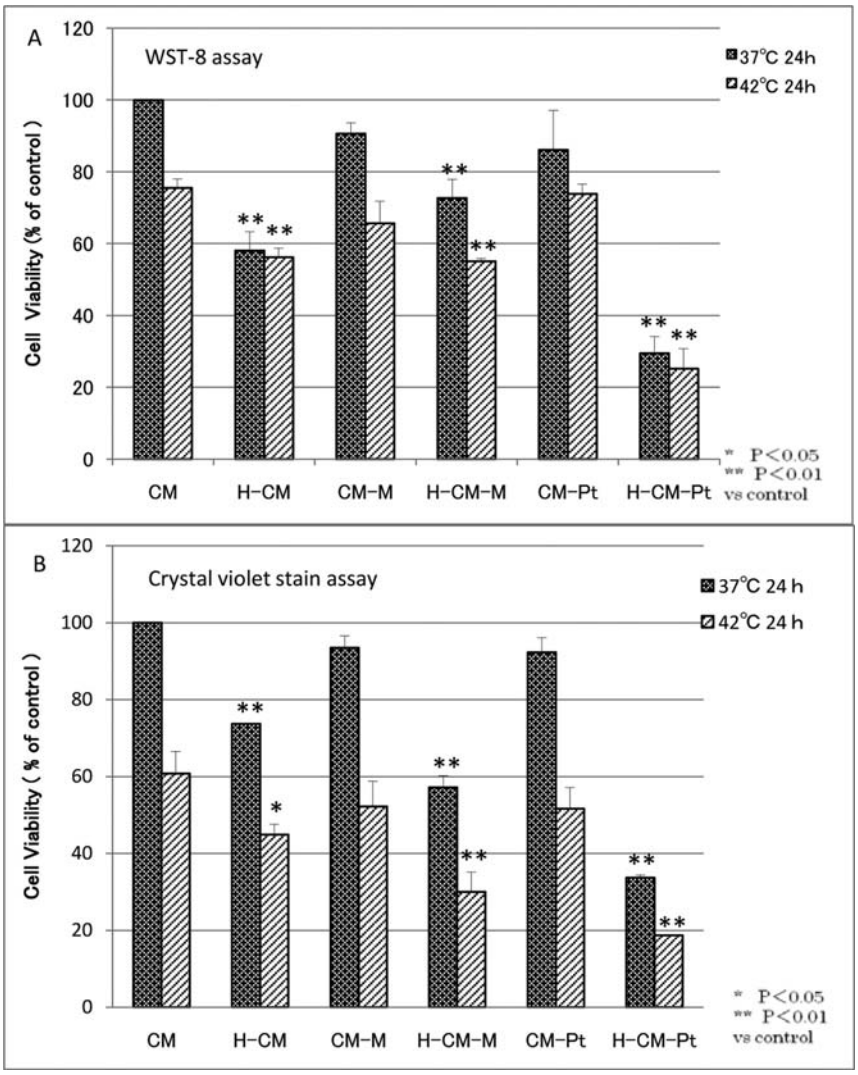

Figure 3. (A) Carcinostatic effects of nano-bubble hydrogen water, coexistent substances and a combination with hyperthermia on Ehrlich ascites tumor (EAT) cells cultured for 24 h after heat treatment as evaluated by WST-8 assay. Cells were seeded at a density of $2x10^4$ cells/ml, heated in the presence of culture medium (CM), hydrogen culture medium (H-CM), culture medium plus mineral water (CM-M), hydrogen culture medium plus mineral water (H-CM-M), culture medium plus platinum colloid plus polyvinylpyrrolidon (PVP, CM-Pt), or hydrogen culture medium plus platinum colloid plus PVP (H-CM-Pt) at 37˚C or 42˚C for 30 min, and further maintained by sequential culture at 37˚C for 24 h. The cell culture was added with WST-8 reagent and incubated at 37˚C for 1 h. Viability of the cells was measured by the absorption at 450 nm. The absorbance of cells treated in the presence of CM at 37˚C or 42˚C was 1.82±0.06 or 2.08±0.02 (the control value), respectively. Data shown represent the means ± SEM for quadruplicate measurements as percentages of the control value. (B) Viability of cells treated in the same manner as described above was separately measured using the crystal violet stain assay. The cultured cells were fixed for 10 min by 25% glutaraldehyde solution, rinsed, dyed for 30 min with crystal violet and rinsed, and microscopic counts of living cells were conducted on a hemocytometer. Data shown represent the means ± SEM for quadruplicate measurements as percentages of the control value.

Chemicals, Kumamoto, Japan). The assay solution becomes more chromic according to mitochondrial dehydrogenase activity. The cultured cell suspension was transferred into a sampling tube and was centrifuged. The supernatant was completely removed from the tube, and 8% WST-8 of 110 μl was added to the cell precipitate. Then the precipitate was suspended and was transferred into each well of a 96-well microplate. After incubation at 37˚C for 1 h, the resultant diformazan formation was determined by measuring the absorption at 450 nm with a plate reader (Benchmark, Bio-Rad Laboratories, CA). Viability of cells was also measured using the crystal violet stain assay (15). The cultured cells were fixed for 10 min with 25% glutaraldehyde solution, rinsed, dyed for 30 min by crystal violet, rinsed, and microscopic counts of living cells were conducted on a hemocytometer.

Morphological observation of cells (crystal violet stain assay). The cells were treated in the crystal violet stain assay and images were taken by electron microscopy.

Morphological observation of cells (SEM). Cells were treated in the culture medium without hydrogen (CM, control) or CM containing nano-bubble hydrogen and platinum colloids (H-CM-Pt) at 37˚C or 42˚C for 30 min and sequentially cultured for 24 h or 48 h in a CO_2 incubator. Specimens were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 h. The specimens were then placed in 0.1 M phosphate buffer overnight, and postfixed with 1% osmium tetroxide for 2 h, then washed in redistilled water (RWD) followed by dehydration through a graded series of ethanol. For the SEM

Table I. Experimental samples.[a]

	Nano-bubble hydrogen	Experimental samples	Abbreviations
10% FBS-M	-	Culture medium	CM
10% FBS-M	+	Hydrogen culture medium	H-CM
+ Mineral water (15.6%)	-	Culture medium plus mineral water	CM-M
+ Mineral water (15.6%)	+	Hydrogen culture medium plus mineral water	H-CM-M
+ Pt (0.3 ppm) -PVP	-	Culture medium plus platinum	CM-Pt
+ Pt (0.3 ppm) -PVP	+	Hydrogen culture medium plus platinum	H-CM-Pt

[a]Platinum in CM-Pt and H-CM-Pt is wrapped with polyvinylpyrrolidone (PVP).

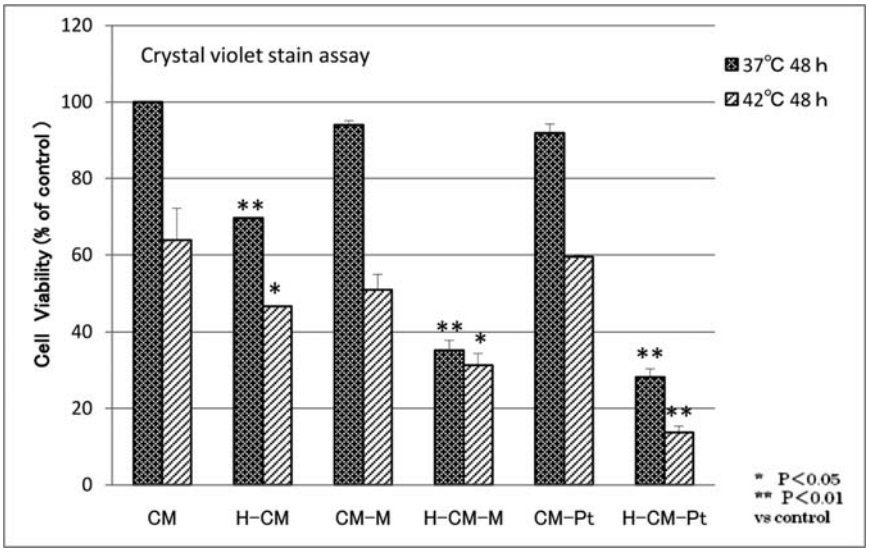

Figure 4. Long-term carcinostatic effects of nano-bubble hydrogen water, coexistent substances and a combination with hyperthermia on EAT cells cultured for 48 h after heat treatment as evaluated by crystal violet stain assay. Cells were seeded at a density as low as $1x10^4$ cells/ml, heated at 37˚C or 42˚C for 30 min in the presence of diverse culture media shown in Fig. 3, and further maintained by sequential culture at 37˚C for a period as long as 48 h. The cultured cells were further manipulated in the same manner as in Fig. 3. Data shown represent the means ± SEM for quadruplicate measurements as percentages of the control value.

study, samples were transferred to tert-butyl alcohol, and dried using a freeze drier (ES-2030, Hitachi, Tokyo), sputter-coated with gold-palladium, and examined with a Hitachi S-2460N scanning electronic microscope operated at 5 kV (17).

Statistics. Statistical differences were analyzed by Student's t-test.

Results

Carcinostatic effects of diverse hydrogen-coexistent substance and hyperthermia on EAT cells cultured for 24 h. Diverse mixture media of hydrogen, mineral water and platinum were added to EAT cells. Then the samples were heated in a water bath at 37˚C or 42˚C for 30 min, and were maintained by sequential culture at 37˚C for 24 h. The carcinostatic effects were measured by WST-8 assay. The effects on the treatment at 37˚C are shown in Fig. 3A. The cell viability of the control in the absence of experimental substances were taken as 100%. H-CM, CM-M, H-CM-M, CM-Pt, and H-CM-Pt gave cell survival rates of 58.1±5.2% (P<0.01), 90.6±3.0%, 72.7±5.2% (P<0.01), 86.1±11.0% and 29.5±4.7 (P<0.01), respectively. Consequently H-CM and mixtures containing H-CM, such as H-CM-M and H-CM-Pt, decreased the cell viability. CM-M and CM-Pt without the hydrogen bubbles gave no effects. At 42˚C, the cell viability diminished to 75.5±2.5% (P<0.01) versus that in the absence of the agent at 37˚C (control). H-CM, CM-M, H-CM-M, CM-Pt, and H-CM-Pt decreased the viability to 56.2 ±2.6% (P<0.01), 65.7±6.1%, 55.1±0.8% (P<0.01), 73.9±2.7% and 25.2±5.6 (P<0.01), respectively.

The carcinostatic effects on EAT cells treated in the same manner as described above were separately measured by crystal violet stain assay (Fig. 3B). Treatment at 37˚C of H-CM, CM-M, H-CM-M, CM-Pt, and H-CM-Pt gave cell

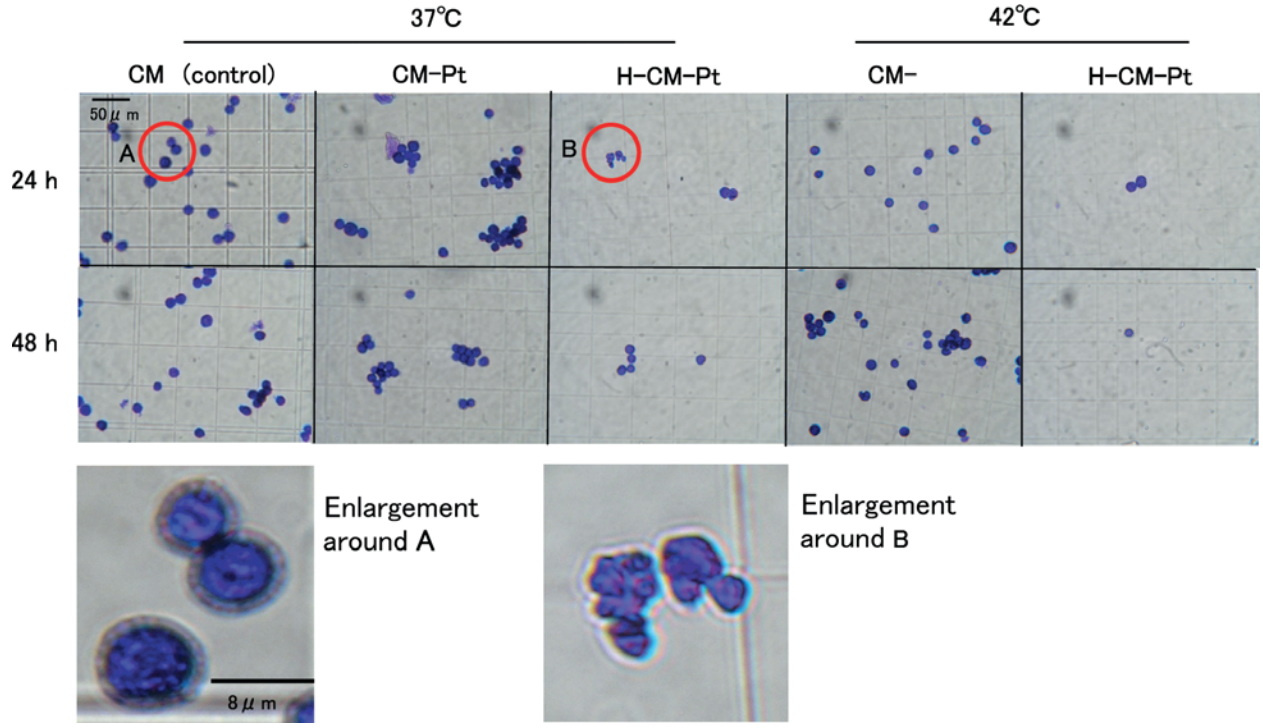

Figure 5. Morphological changes in EAT cells by nano-bubbles hydrogen water, coexistent substances and a combination with hyperthermia as evaluated by crystal violet stain assay. The cells cultured for 24 or 48 h after the treatment at 37˚C or 42˚C and dyed with the crystal violet (Figs. 3B and 4) and images were taken by electron microscopy.

survival rates of 73.7±0% (P<0.01), 93.5±3.0%, 57.1±3.0% (P<0.01), 92.3±3.8% and 33.7±0.7 (P<0.01), respectively. At 42˚C, the heat treatment alone diminished the cell viability to 60.8±5.7% (P<0.01) of the control. They decreased the viability to 44.9±2.7% (P<0.05), 52.5±6.6%, 30.0±5.1% (P<0.01), 51.6±5.5% and 18.7±0.0 (P<0.01), respectively. These values are roughly similar to those obtained by WST-1 assay. H-CM, H-CM-M, and H-CM-Pt increased their carcinostatic activities by hyperthermia.

Carcinostatic effects of diverse hydrogen-coexistent substances and hyperthermia on EAT cells cultured for a long-period. Effect on the EAT cells cultured for 48 h after the heat treatment was measured by crystal violet stain assay (Fig. 4). At 37˚C, H-CM, CM-M, H-CM-M, CM-Pt, and H-CM-Pt diminished the cell viability to 69.6±0.1% (P<0.01), 94.0±1.1%, 35.2±2.6% (P<0.01), 91.9±2.4% and 28.2±2.2% (P<0.01), respectively. The values at the treatment of 42˚C alone was 63.9±8.3% and they were 46.7±0.0%, 51.0±3.9%, 31.3±3.1%, 59.5±0.4% and 13.7±1.7% (P<0.01), respectively. Only the most effective HCM-Pt increased carcinostatic activity by the elongation of culture period, and the others became scarcely more active.

Morphological changes in tumor cells observed by crystal violet stain assay. Morphological observation in tumor cells was performed by crystal violet stain, while the carcinostatic effects were assayed. Morphological changes in EAT cells treated with H-CM-Pt which exhibited the greatest carcinostatic activities are shown in Fig. 5. The changes were observed with decreased cell numbers, cell shrinkage (average diameters: ~8 μm) and pycnosis (nuclear condensation) indicative of apoptosis, together with cell deformation.

Morphological changes in tumor cells observed by SEM. The carcinostatic effects were shown to be the greatest for H-CM-Pt out of diverse samples, and morphological changes in EAT cells were therefore further investigated using H-CM-Pt. SEM elucidated the morphological aspects of the cells treated with H-CM-Pt at 37˚C or 42˚C for 30 min, and subsequently cultured for 24 or 48 h (Fig. 6). At 37˚C, the cells with normal microvilli on the cell membrane surface in the same manner as those of untreated cells (control) were abundantly observed in the experimental sample. In a prolonging culture period of 48 h, the cells with extensive microvilli disappeared to some degree. The cells treated with H-CM-Pt for 24 h produced some cell shrinkage, and in 48-h culture microvilli-disappearing cells and broken cells emerged. Hyperthermia alone at 42˚C induced drastic damage to the cell membrane surface including the disappearance of microvilli (Fig. 7). Furthermore, the treatment with H-CM-Pt induced cell deformation or destruction, and produced cells having slippery membrane surface without microvilli, for 30% of the total cells at 24 h and nearly 100% at 48 h.

Discussion

The nano-bubble hydrogen water production device was initially developed for manufacturing hydrogen water as a drink supplement for health maintenance (8). The cell culture medium was prepared by adding culture materials to the

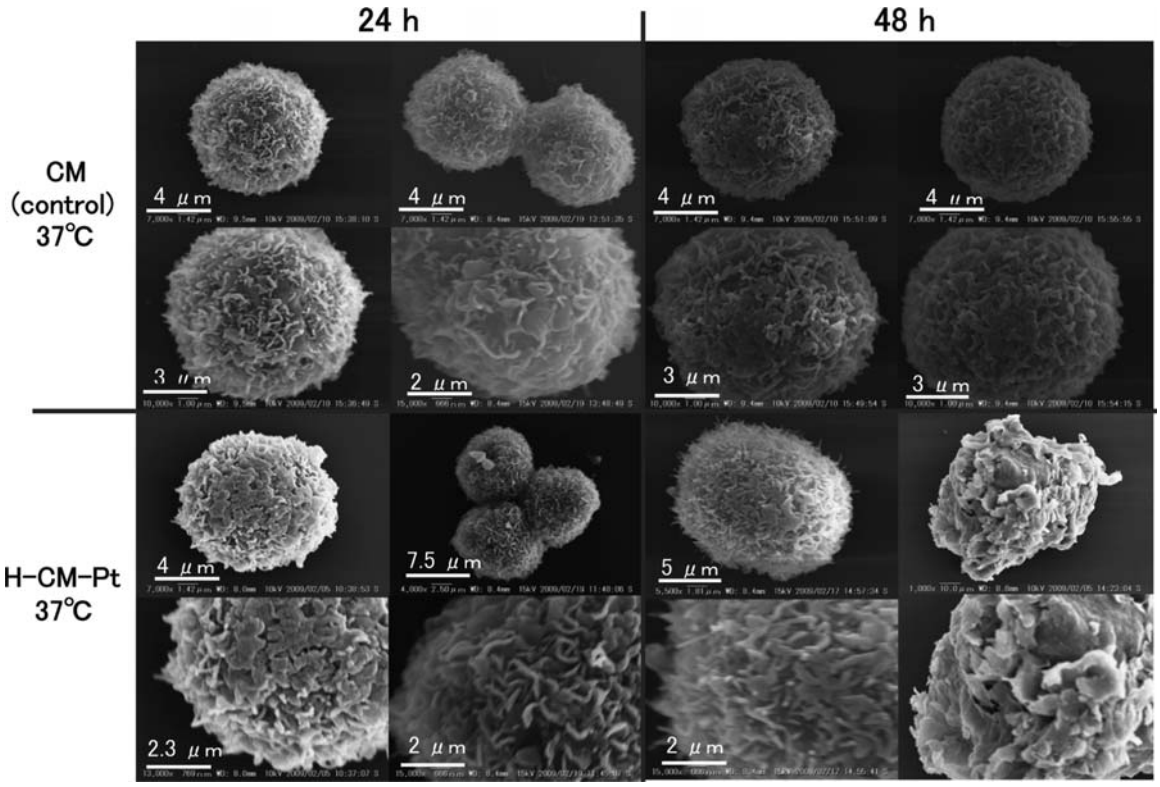

Figure 6. Morphological changes in tumor cells treated with culture medium containing nano-bubble hydrogen and platinum colloids at 37˚C as observed by SEM. Cells were treated in the culture medium without hydrogen (CM, control) or CM containing hydrogen and platinum (H-CM-Pt) at 37˚C for 30 min and sequentially cultured for 24 h or 48 h in a CO_2 incubator. Specimens were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 h. The specimens were then placed in 0.1 M phosphate buffer overnight, and postfixed with 1% osmium tetroxide for 2 h, then washed in redistilled water (RWD) followed by dehydration through a graded series of ethanol. For scanning electronic microscopic (SEM) study, samples were transferred to tert-butyl alcohol, and dried using a freeze drier, sputter-coated with gold-palladium, and examined with a scanning electronic microscope operated at 5 kV.

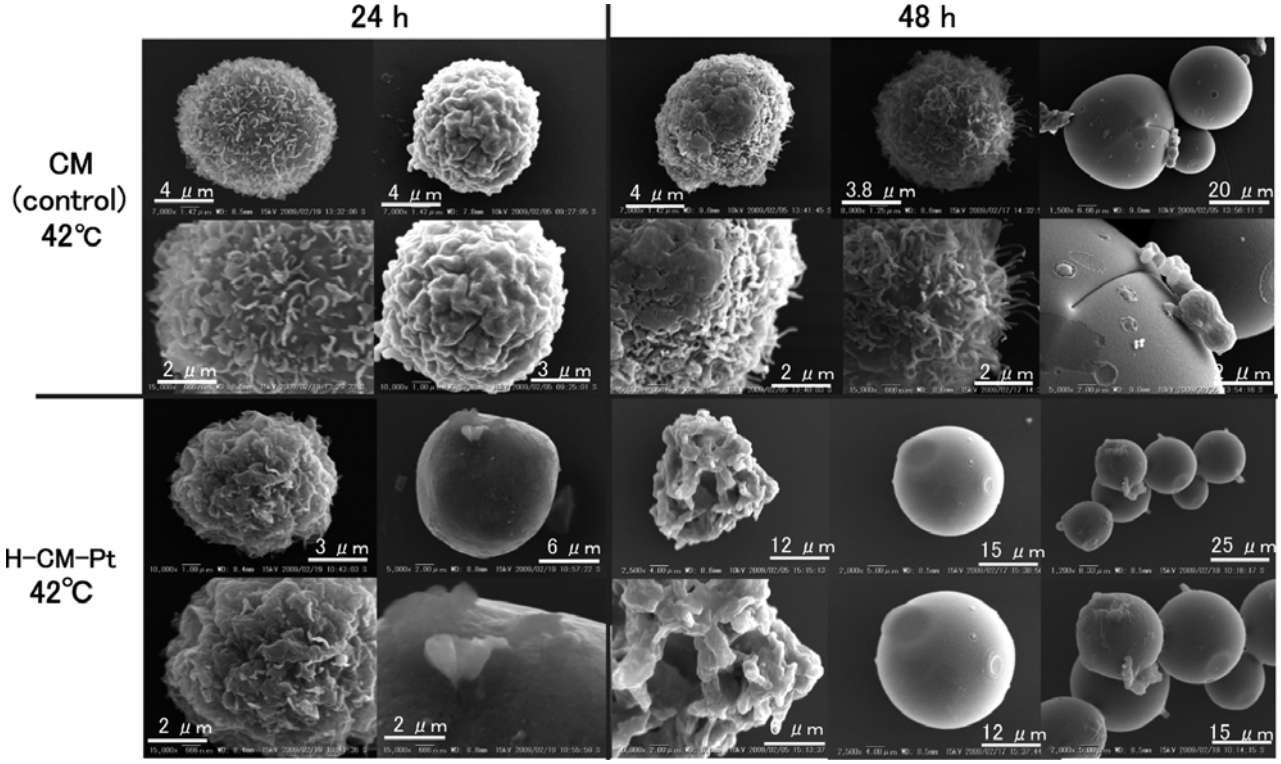

Figure 7. Morphological changes in tumor cells treated with culture medium containing nano-bubble hydrogen and platinum colloids at 42˚C as observed by SEM. Cells were treated as shown in Fig. 6 except at 42˚C. Specimens were further manipulated as in Fig. 6.

hydrogen water produced by jetting hydrogen gas to purified water. Hydrogen was lost in the process of preparation, and oxidation-reduction potential (ORP) decreased remarkably. Therefore, anti-tumor effects were not seen in this hydrogen culture medium (H-CM) alone owing to the decrease in the ability to scavenge ROS (9). We could make H-CM that had a high (-650 mV) level by miniaturizing a new type of the device and jetting the hydrogen gas directly to the culture medium. This ORP value was maintained for three months after preservation at 4˚C under closed conditions. Therefore, even if the H-CM alone was administered, the carcinostatic activity was exhibited.

In the present study, to examine carcinostatic effects of hydrogen we compared those of H-CM alone, culture medium (CM) plus mineral water (CM-M), hydrogen culture medium plus mineral water (H-CM-M), culture medium plus platinum (CM-Pt), and hydrogen culture medium plus platinum plus (H-CM-Pt) against Ehrlich ascites tumor (EAT) cells. Their viability was evaluated with WST-8 assay and crystal violet stain assay. As a result, the effect pattern after 24-h culture consecutive to the treatment at 37˚C or 42˚C is roughly similar (Fig. 3A and B). H-CM, H-CM-M, and H-CM-Pt were effective. As all contained hydrogen, the anti-cancer effect of hydrogen was obvious, and CM-M and CM-Pt were not effective. There was a remarkable effect when platinum (wrapped with PVP) was added to H-CM. This fact suggests that platinum plays an important role in the anti-tumor effect of H-CM.

A mechanism of the promotive effect of H-CM by platinum is suggested to be that the hydrogen molecules are incorporated through phagocytosis into cells in contact on the surface of platinum molecule when the hydrogen molecule is converted into atomic hydrogen by the catalytic action of platinum, the reduction activity enhances, and an ROS-scavenging ability rises (18,19). Effect of a successive culture period after the treatment at 37˚C was examined by crystal violet stain assay, and elongation of culture periods from 24 h to 48 h increased the carcinostatic effect of H-CM. One of the causes of hyperthermic effect is the cytotoxic activity of ROS produced according to the heat treatment (20). Therefore, using H-CM has a contradiction that the anti-tumor effect may be decreased by the depletion of active oxygen dependent on reductive action of hydrogen molecules. However, at 42˚C an additive action of the anti-tumor effect is achieved in this experiment. As reasons for the increase of incorporation of hydrogen-binding platinum colloids into the cells by the heat-induced promotion of the plasma membrane permeability (19,21) and unfolding change of the nucleus made up of megasize protein-nucleic acid complexes that conduct various nuclear functions (DNA packing, repair, replication and transcription) being related to the cell killing are considered (22).

The morphological effects of H-CM-Pt that was the most effective on the EAT cells were observed with SEM. In the cells cultured for 24 h after the treatment at 37˚C, many cells having normal intact microvilli on the cell membrane were seen as well as the control of no additive culture medium, and at 48-h some cells without microvilli were observed. Abnormality of the cells cultured for 24 h after the exposure to CM at 42˚C was not seen, and in 48 h culture microvilli-disappearing cells were significantly seen. In the cells exposed to nano-bubble hydrogen culture medium supplemented by platinum particles (H-CM-Pt), the microvilli-disappearing cells became to occupy 30% of the total cells in 24-h culture and almost 100% upon later 48-h culture.

Moreover, cell shrinkage, pycnosis (nuclear condensation) indicative of apoptosis, and cell distraction were also observed. These deformations are induced by H-CM, platinum colloids, hyperthermia and elongation of the culture period. In morphological observation, if the microvilli-disappearing cells die according to the progress of time, anti-tumor effects of H-CM-Pt may be promoted more markedly than the carcinostatic values measured by WST-8 or crystal violet stain assay. The nano-bubble hydrogen culture medium obtained by jetting hydrogen gas directly to culture medium with the modified production device showed anti-tumor activity, and acquired a more marked activity by adding platinum colloidal particles. In addition, it can be expected that hyperthermia exhibited a combined effect advantageously beyond the decrease in the anti-tumor effects because of the depletion of the hydrogen molecules, by the increase of ROS caused by heat.

Thus, nano-bubble hydrogen water and addition of platinum colloid are more attractive as new anti-tumor regiments from a viewpoint of reduction of the side-effect towards normal tissues.

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