Anticancer effects of 6-*o*-palmitoyl-ascorbate combined with a capacitive-resistive electric transfer hyperthermic apparatus as compared with ascorbate in relation to ascorbyl radical generation

Shinya Kato · Ryoko Asada · Katsuhiko Kageyama ·
Yasukazu Saitoh · Nobuhiko Miwa

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Abstract The aim of the present study is to determine the anti-proliferative activity of 6-*o*-palmitoyl-L-ascorbic acid (Asc6Palm) that is a lipophilic derivative of L-ascorbic acid (Asc), on human tongue squamous carcinoma HSC-4 cells by combined use of hyperthermia

in comparison to Asc. Asc6Palm or Asc were administered to HSC-4 cells for 1 h, to which hyperthermia at 42 °C was applied for initial 15 min. After further 1–72 h incubation at 37 °C, cell proliferation was determined with Crystal Violet staining. Ascorbyl radical (AscR) in HSC-4 cell suspension was measured by electron spin resonance (ESR), and cell morphology was observed with scanning electron microscopy (SEM). At 37 °C, 4 mM Asc or 0.35 mM Asc6Palm were enough to suppress proliferation of HSC-4 cells. By combined use of hyperthermia at 42 °C, cell proliferation was decreased when compared to 37 °C. After Asc of 4 mM was incubated with HSC-4 cell suspensions at 37 °C or 42 °C for 0–180 min, the signal intensity of ascorbyl radical (AscR) by ESR was not different regardless of the presence or absence of cells at 37 °C, whereas AscR signal was enlarged in the presence of HSC-4 cells at 42 °C. It was suggested that oxidation of Asc occurred rapidly in HSC-4 cells by hyperthermia, and thereby enhanced the anti-proliferative activity. By SEM observation, the surface of HSC-4 cells treated with Asc6Palm revealed distinct morphological changes. Thus, the combined regimen of Asc6Palm and hyperthermia is expected to exert a marked antitumor activity.

S. Kato · Y. Saitoh · N. Miwa
Laboratory of Cell-Death Control BioTechnology, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Nanatsuka 562, Shobara, Hiroshima 727-0023, Japan

R. Asada · K. Kageyama
Osaka Butsuryo College, OtoriHigashiMachi 4-410-5, Nishi-ku, Sakai, Osaka 593-8324, Japan

Present Address:
S. Kato
Radioisotope Centre, Osaka City University Graduate School of Medicine, Asahimachi 1-4-3, Abeno-ku, Osaka 545-8585, Japan

Present Address:
Y. Saitoh
Laboratory of Bioscience & Biotechnology for Cell Function Control, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Nanatsuka 562, Shobara, Hiroshima 727-0023, Japan

Present Address:
N. Miwa (✉)
Department of Radiological Technology, Faculty of Health Sciences, Butsuryo College of Osaka, OtoriKitaMachi 3-33, Nishi-ku, Sakai, Osaka 593-8328, Japan
e-mail: miwa@butsuryo.ac.jp

Keywords 6-*o*-Palmitoyl-L-ascorbic acid · Hyperthermia · Human tongue squamous carcinoma cells HSC-4 · Crystal Violet staining · Electron spin resonance (ESR) · Scanning electron microscopy (SEM)

Abbreviations

Asc6Palm	6- <i>o</i> -palmitoyl-L-ascorbic acid
Asc	L-ascorbic acid
AscR	Ascorbyl radical
ESR	Electron spin resonance
SEM	Scanning electron microscopy
CRet system	Capacitive-resistive transfer system

Introduction

Higher doses of L-ascorbic acid (Asc) have been reported to exhibit cytotoxicity and examined to be applied for tumor treatment (Cameron et al. 1979; Pauling et al. 1982; Pauling 1991), but did not exert antitumor activity sufficiently. It might be related to a lower degree of intracellular permeability of Asc owing to its prominent hydrophilicity. To enhance the antitumor activity of Asc, the administration of some transition metal ions with Asc (Stich et al. 1979; Miwa et al. 1986) or diverse derivatives of Asc (Hacker et al. 1985; Miwa et al. 1986) have been studied. We have studied lipophilic derivatives of Asc including 2-*o*-phosphoryl-6-*o*-palmitoyl-L-ascorbic acid (Asc2P6-Palm) and found that Asc2P6Palm was advantageous over Asc in terms of anti-metastatic activity (Liu et al. 2000, 2003) in addition to antitumor effects (Miwa and Yamazaki 1986; Miwa et al. 1988). Hyperthermia is the oldest documented tumor treatment modality (Pajonk et al. 2005), and is applied in combination with chemotherapy and/or radiotherapy in the clinical setting (Takahashi et al. 2002). Though the molecular mechanisms of hyperthermia are not completely understood, it is suggested that inhibition of proteosome function and the relevant signal transduction pathways might be a major factor of heat-induced apoptosis (Pajonk et al. 2005). The p53-inactivated and pRb-inactivated transfectants showed G2 arrest after hyperthermia and the appearance of a sub-G1 population (van Bree et al. 1999). Furthermore, hyperthermia is of clinical interest in the temperature range of 40–43 °C, however higher temperatures of 44–46 °C are not clinically realistic, and may be able to modulate the immune system by inducing the expression of heat-shock proteins (Currie and White 1981; Dietzel 1983; Pennacchioli et al. 2009). Oral squamous carcinoma is one of the most common cancers (Fiaschi et al. 2005), and human tongue

squamous carcinoma cells HSC-4 are multiplied in planar state. Hyperthermia can be especially effective in the treatment of superficially seated malignant tumors (Engin 1996), and enhance the susceptibility of cells to diverse anticancer drugs, especially of alkylating agents (Kampinga 2006). Asc6Palm, which is a lipophilic and autooxidation-resistant derivative of Asc, is expected to exert inhibition of proliferation of HSC-cells through intracellular ascorbate enrichment and the resultant hydrogen-peroxide-generating action (Miwa and Yamazaki 1986) in combination with hyperthermia.

In the present study, we investigated the anti-proliferative activity of Asc6Palm in HSC-4 cells by combined use of hyperthermia as compared to Asc. Cell proliferation on HSC-4 cells was determined with Crystal Violet staining. Ascorbyl radical (AscR) signal intensity in HSC-4 cell homogenate treated with Asc was measured by electron spin resonance (ESR) method. And the surface ultrastructure of HSC-4 cells treated with Asc6Palm was observed by scanning electron microscopy (SEM).

Materials and methods

Cell culture

Human tongue squamous carcinoma HSC-4 cells were cultivated in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (FBS, Biological Industries Ltd., Israel) in a humidified atmosphere of 5% CO₂ in air at 37 °C.

Assay of cell proliferation treated with higher concentration of Asc or Asc6Palm at 37 °C

Sub-confluent HSC-4 cells were seeded by 1.2×10^4 on each well of a 24-well culture plate (Becton, Dickinson and Company, NJ, USA) in 500 µL MEM-10% FBS and incubated for 24 h at 37 °C in a 95% humidified air and 5% CO₂. Asc was initially dissolved at 1,250 mM in reversed-osmosis ultra-pure water with adjustment with NaOH to pH 5.93. Asc6Palm was prepared at 6 mM by sonication for 30 s in MEM-10% FBS. Asc or Asc6Palm were diluted with MEM-10% FBS, and administered at concentrations of 0.4–12 mM or 0.05–1.5 mM, respectively, in HSC-4

cells for 1 h in a CO₂ incubator at 37 °C. Then the medium was exchanged to that without Asc or Asc6Palm, and further incubated. The chronological progress of the cell proliferation was determined at 1, 24, 48 and 72 h after the onset of administration by Crystal Violet staining and observed by phase-contrast microscopy. In these cultures up to 72 h, HSC-4 cells were in the logarithmic growth phase.

Assay of cell proliferation treated with Asc or Asc6Palm in combination of hyperthermia

We applied hyperthermia treatment by a capacitive-resistive electric transfer (CRet) system. CRet system is used for a non-invasive electro-thermal therapy, based on the application of electric currents in the frequency range of 0.45 MHz, which can produce the electric stimulus and thermal effects synergistically in tissues (Hernández-Bule et al. 2007). Sub-confluent HSC-4 cells were seeded at 8,000 cells per well of a 24-well culture plate (Becton, Dickinson and Company, NJ, USA) in 500 µL MEM-10% FBS and incubated for 48 h at 37 °C in a 95% humidified air and 5% CO₂. Asc or Asc6Palm were prepared as mentioned above and administered at concentrations of 2–8 mM or 0.15–0.6 mM, respectively, in HSC-4 cells for 1 h, which were applied to CRet-exposure for initial 15 min at 42 °C or sham-exposed without applying electric current, and subsequently incubated for 45 min in a CO₂ incubator at 37 °C. Then the cells were manipulated in a way similar to the experiment at 37 °C as above mentioned.

Hyperthermia treatment by CRet System

The exposure to CRet electric currents was carried out through pairs of stainless steel electrodes designated ad hoc for in vitro stimulation. The electrodes were inserted in each well (Fig. 1), and connected in series to a CRet-signal generator INDIBA D.H.-308 (INDIBA S.A., Barcelona, Spain). The signal parameters of CRet system were monitored throughout exposure. Temperature of the medium increased to 42 °C approximately for 2 min and could be kept (Fig. 1).

Crystal Violet staining

HSC-4 cells in response to Asc or Asc6Palm were quantitated by the Crystal Violet staining (Gillies et al. 1976), which is based on the inability of staining of dead cells in contrast to ability of staining of adherent viable cells (Flick and Gifford 1984). The medium in a 24-well plate was aspirated, and HSC-4 cells were stained for 10 min with 0.5% Crystal Violet (Wako Pure Chemical Industries, Ltd., Osaka) dissolved in methanol. Subsequently, the stain was aspirated, and the wells were rinsed thoroughly with running water until no residual stain leached from the wells. Three hundred thirty µL of reversed-osmosis ultrapure water was poured in each well, and absorbance was determined immediately using a multi-well plate reader at 595 nm (FLUOstar OPTIMA, BMG Labtech GmbH, Offenburg, Germany), and cell

Fig. 1 The pairs of stainless steel electrodes were inserted in each well and connected to the hyperthermia apparatus with a capacitive-resistive electric transfer (CRet) system (a), and temperature of the culture medium was monitored in the wells

A, B, C (b). A 24-well plate with a pair of parallel stainless steel electrodes was set for CRet-stimulation or sham-stimulation on human tongue squamous carcinoma HSC-4 cells

morphology was observed with a phase-contrast microscope (ECLIPSE Ti-S, Nikon Corp., Tokyo).

Oxidative stress induced by Asc in HSC-4 cell suspension as detected by ESR

Sub-confluent HSC-4 cells of 2.0×10^6 cells were harvested and suspended in MEM-10% FBS medium of 1,400 μL in a Spitz tube. Asc was administered by 4 mM in the cell suspension, which was approximately at pH 7.3. The control was prepared similarly except for the absence of HSC-4 cells. The experimental and control suspensions with 4 mM Asc were incubated at 37 °C or 42 °C in a thermostat bath (Thermo minder 50, Taitec Co., Ltd., Saitama, Japan). At 0, 20, 40, 60, 90 and 180 min, the suspensions of 200 μL were homogenized with a Potter-type Teflon homogenizer (Labo-Stirrer LR-41-B, Yamato Scientific Co., Ltd., Tokyo) at 360 ppm for 10 s on ice and transferred into a quart flat cell to be analyzed with an ESR (FR-30, JEOL Ltd., Tokyo). Oxidative stress induced by Asc is evaluated as an AscR/MnO relative signal intensity. ESR measurements were conducted under the conditions of Power: 4 mW, ModWid: 0.4 mT, C. Field: 336.000 mT, Amp: 250, SwWid ($\pm$): 5 mT, TimeC: 0.3 s., SwTime: 4 min.

Morphological observation by scanning electron microscopy (SEM)

The surface ultrastructure of HSC-4 cells treated with Asc6Palm was observed with a scanning electron microscope (SEM). 0.25 mM Asc6Palm was administered to HSC-4 cells for 1 h, which were applied to CRet-exposure for initial 15 min at 42 °C or sham-exposure using the CRet System (Fig. 1), and subsequently incubated for 45 min in a CO₂ incubator at 37 °C. Then the medium was exchanged to that without Asc6Palm, and incubated for 24 h. HSC-4 cells were prefixed by immersion in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.2) for 1 h, and post-fixed in 1% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer solution (pH 7.2) for 1 h at 4 °C. Then, specimens were treated in a graded series of ethanol and *tert*-butyl alcohol, dried in a vacuum-freeze dryer (ES-3020, Hitachi High-technologies Co., Tokyo),

and gold-coated using an ion coater (IB-2, EIKO Engineering Co. Ltd., Ibaraki, Japan). The surface ultrastructure of HSC-4 cells was observed with a SEM (VE-9800, Keyence Corp., Osaka).

Statistical analysis

Results of ESR and Crystal Violet staining assay were expressed as means $\pm$ SD, for $n = 3$, and evaluated by the Student's *t*-test or Dunnett's test. The *p*-values that are at least 0.05 were regarded to be statistically significant.

Results

Inhibition of cell proliferation by higher doses of Asc or Asc6Palm on HSC-4 cells

Asc and Asc6Palm showed similar dose–response curves of cell proliferation which indicate that Asc and Asc6Palm are significantly effective in dose- and time-dependent manners ($p < 0.05$; Fig. 2). However, Asc6Palm is more sensitive to inhibit cell growth than Asc. When treated with 0.05–1.5 mM Asc6Palm or 0.4–12 mM Asc at 37 °C, Asc6Palm at 0.35 mM or Asc at 4 mM were sufficient to suppress the proliferation of HSC-4 cells (Fig. 2). Namely, Asc6Palm was approximately tenfold superior to Asc in terms of the degree of anti-proliferative activity in HSC-4 cells. The no-additive control at 1–72 h indicated that HSC-4 cells were in the logarithmic growth phase.

Combinational effects of Asc or Asc6Palm and hyperthermia on HSC-4 cells: Cell proliferation and cell morphology

We observed that Asc and Asc6Palm augmented the anti-proliferative effects on HSC-4 cells in combined application with hyperthermia (Figs. 3, 4). When treated with 2 mM Asc, the cell proliferation degree was 0.378 for the sham-exposed control, which decreased to 0.061 for the CRet-exposition experiment at 42 °C. Similarly, when treated with 0.15 mM Asc6Palm, it decreased from 0.380 for the sham-exposed control, to 0.120 for the CRet-exposition experiment. Consequently, in combined use of

Fig. 2 Inhibition of cell proliferation of HSC-4 cells treated with **a** Asc or **b** Asc6Palm for 1 h. Then the medium was changed to that without Asc or Asc6Palm and the cells were further incubated for 24–72 h. Photographs of a 24-well plate exhibiting Asc (**c**) or Asc6Palm-treated (**d**) adherent HSC-4 cells stained with Crystal Violet at 72 h. Values are means $\pm$ SD, $n = 3$. * $p < 0.05$ (At 72 h, each dose of Asc or Asc6Palm vs. 0 mM, Student's *t*-test), # $p < 0.05$ (24, 48 or 72 h vs. 1 h, Dunnett's test)

hyperthermia at 42 °C for 15 min, cell proliferation was inhibited by 83.9% ($p = 0.00426$) at a dose of 2 mM Asc (Fig. 3) and by 68.4% ($p = 0.0216$) at a dose of 0.15 mM Asc6Palm as compared to the sham-exposed control (Fig. 4).

The morphological features of HSC-4 cells were observed using a phase-contrast microscope (ECLIPSE Ti-S, Nikon Corp., Tokyo). HSC-4 cells were allowed to grow for 48 h after onset of 1 h-administration of Asc or Asc6Palm, and stained with Crystal Violet. HSC-4 cells showed a relatively round shape and adhered to the plastic surfaces in the no-additive sham-exposed control (Figs. 3, 4). 2 mM Asc or 0.15 mM Asc6Palm appeared to be most efficient in the combined treatment with hyperthermia. When treated with 2 mM Asc or 0.15 mM Asc6Palm under the sham-exposed condition, HSC-4 cells were inhibited for proliferation, though cell morphology was not altered as compared to the no-additive control. The no-additive CRet-exposition control showed a partially-perforated cell membrane. When treated with combined application of hyperthermia and Asc or Asc6Palm under the CRet-exposed condition, 2 mM Asc produced markedly-condensed nuclei and inhibited cell growth, and 0.15 mM Asc6Palm inhibited proliferation and caused injuries of cell membrane as shown with yellow arrows (Figs. 3, 4). Higher doses of Asc6Palm revealed markedly condensed nuclei similar to 2 mM Asc (Fig. 4).

Ascorbyl radical (AscR) generation in HSC-4 cell suspension

4 mM Asc (pH 7.3) in the presence or absence of HSC-4 cell suspension was incubated in a thermostat bath at 37 °C or 42 °C for 180 min, and then ascorbyl radicals (AscR) were determined using an electron spin resonance (ESR) apparatus. The characteristic doublet signal of AscR (Dodd 1973) was detected. AscR showed an asymmetric doublet signal with a line width of 0.18 mT centered at $g = 2.007$. The no-additive control did not show any detectable free radical signals, but 4 mM Asc in HSC-4 cell-free suspension gave a small asymmetric signal with an AscR/MnO relative signal intensity of 0.078 at 0 min (Fig. 5). Exposure of HSC-4 suspension with 4 mM Asc to hyperthermia at 42 °C enhanced the narrow signal of AscR. The AscR signal intensity increased to the maximum at 60 min, thereafter it decreased to near the initial level at 60–180 min in a time-dependent manner (Fig. 5). As comparison of the AscR/MnO relative signal intensity at 60 min, it was almost the same in HSC-4 cell-free suspension regardless of hyperthermia treatment, namely the value was 0.76 at 37 °C and 0.72 at 42 °C (Fig. 5). Meanwhile in HSC-4 cell suspension, the AscR/MnO ratio was 0.78 at 37 °C and increased significantly to 0.89 at 42 °C ($p = 0.0473$). The AscR signal appeared to be 11.4% greater in HSC-4 cell suspension at 42 °C than at 37 °C. The AscR signal in HSC-4 cell suspension at

Fig. 3 Effect on the cell growth of HSC-4 cells treated with Asc in combined application of hyperthermia at 42 °C shown as **a** cell proliferation (Abs595 nm) and **b** cell morphology under the phase-contrast microscope. HSC-4 cells were treated with sequential concentrations of Asc for 1 h, in which hyperthermia was applied for initial 15 min at 42 °C, and subsequently incubated at 37 °C for 45 min, and then the medium was exchanged to that without Asc. Cell proliferation (Abs595 nm) was determined by Crystal Violet stain at 1, 24 and 48 h after the onset of 1 h-administration of Asc. Values are means $\pm$ SD, $n = 3$. * $p < 0.05$ (Sham-manipulated vs. CRet-manipulated, Student's *t*-test). Photographs exhibit adherent HSC-4 cells stained with Crystal Violet at 48 h. Damages of cell membrane are shown with arrows. Scale bars = 40 μ m, magnification: $\times 200$

42 °C was more outstanding than that incubated at 37 °C or in HSC-4 cell-free suspension throughout the test.

Abnormality of morphology of HSC-4 cell exposed to Asc6Palm in combination with hyperthermia

By scanning electron microscopic observation, the surface ultrastructure of HSC-4 cells treated with Asc6Palm revealed distinct morphological changes (Fig. 6). Untreated cells were flat-shaped with some

metapophysis scattered around the outside of nuclei. Asc6Palm-treated cells lost metapophysis on the cell surface. Upon combined use of Asc6Palm and the hyperthermia, cells exhibited the entire shrinkage and local narrowing of lamellipodia with microvilli.

Discussion

The aim of the present study was to determine the anti-proliferative activity of Asc6Palm in human tongue squamous carcinoma cells HSC-4 by combined use of

Fig. 4 Effects on cell growth of HSC-4 cells treated with Asc6Palm in combined application of hyperthermia at 42 °C shown as **a** cell proliferation (Abs_{595nm}) and **b** cell morphology under the phase-contrast microscope. The procedure is the same as described in Fig. 3. Values are means $\pm$ SD, $n = 3$. * $p < 0.05$ (Sham-manipulated vs. CRet-manipulated, Student's t -test), Photographs exhibit adherent HSC-4 cells stained with Crystal Violet at 48 h. Damages of cell membrane are shown with arrows. Scale bars = 40 μ m, magnification: $\times 200$

hyperthermia as compared to Asc. The results of Crystal Violet stain indicated that Asc and Asc6Palm inhibited cell growth in a dose-dependent manner (Figs. 2, 3). Interestingly, cell growth was markedly reduced when treated with Asc6Palm. We confirmed that, unexpectedly at an Asc concentration of 0.4–2.0 mM cell proliferation was maintained, whereas Asc6Palm exerted an anti-proliferative activity at sensitively lower concentrations than 1.4 mM (Fig. 2). 4 mM Asc or 0.35 mM Asc6Palm were sufficient to almost completely inhibit cell proliferation, thus Asc6Palm was approximately ten times more cytotoxic than Asc. In addition, CRet-exposed hyperthermia at 42 °C on HSC-4 cells augmented their inhibition of cell proliferation induced by Asc or Asc6Palm (Fig. 4). When CRet-exposed at 42 °C for 15 min, cell proliferation was inhibited by 83.9% at a dose of 2 mM Asc (Fig. 3) and by 68.4% at a dose of 0.15 mM Asc6Palm as compared to the sham-exposed

control (Fig. 4). We previously reported that anti-cell proliferation activity of diverse monoacylated Asc was not caused by the fatty acid moiety itself; it is more likely to be caused by the balance in the hydrophobicity and hydrophilicity of the monoacylated Asc (Kageyama et al. 1991). Results of the present study might be explained by the continuous breakdown of cell membrane by Asc6Palm owing to an enhanced intracellular permeability by ester-linked palmitate.

By a phase-contrast microscopic observation, HSC-4 cells treated with 2 mM Asc or 0.15 mM Asc6Palm in sham-exposed hyperthermia were inhibited for cell growth, but cell morphology appeared almost intact (Figs. 3, 4). Whereas Asc or Asc6Palm in combination with hyperthermia exhibited so marked morphological change. When treated with hyperthermia (CRet-exposition), HSC-4 cells showed a partially perforated cell membrane. And the combined application of Asc or Asc6Palm and

Fig. 5 Electron spin resonance spectroscopy (ESR) of 4 mM Asc indicated by an ascorbyl radical/MnO relative signal intensity (**a**) and ESR spectra (**b**). 4 mM Asc at pH 7.3 in HSC-4 cell suspension or HSC-4 cell-free suspension was incubated at 37 °C or 42 °C for 0–180 min and determined with ESR.

The control (reversed-osmosis ultra-pure water) in HSC-4 cell free suspension at 37 °C incubated for 0 min is shown below (**a**). Values are means $\pm$ SD, $n = 3$. * $p < 0.05$ (incubated at 42 °C vs. 37 °C, Student's t -test)

Fig. 6 Surface ultrastructures of HSC-4 cells treated with 0.25 mM Asc6Palm in combination with hyperthermia as compared to the sham-manipulated control, observed using a scanning electron microscope. Scale bars = 20 μ m, magnification: $\times 1,500$. HSC-4 cells were treated with 0.25 mM Asc6Palm for 1 h, for which hyperthermia at 42 °C was applied for initial 15 min using the CRet system and subsequently incubated at 37 °C for 45 min. Then the medium was changed to medium without Asc6Palm and incubated for 24 h

hyperthermia caused a partially perforated cell membrane and markedly condensed nuclei. It is found that the sensitivity to hyperthermia varies with

the types of cell line (Cress et al. 1982) or their malignant potential (Giovannella et al. 1980) in vitro. Hyperthermia led to increased induction of apoptosis

or tumor necrosis (Chen et al. 2008). We also previously reported that Asc or Asc6Palm inhibited DNA synthesis and proliferation of Ehrlich ascites tumor cells, and the inhibition was significantly enhanced by hyperthermia (Kageyama et al. 1995, 1996). The invasion of human fibrosarcoma HT-1080 cells was more markedly inhibited by Asc6Palm than by Asc (Liu et al. 2000). Meanwhile, it was reported that Sarcoma 180 cells exhibited natural resistance to hyperthermia (Chitnis and Adwankar 1986). Results in the present study suggest that Asc6Palm could inhibit cell proliferation more effectively than Asc, and HSC-4 cells were sensitive to hyperthermia by CRet-exposure.

Using ESR spectroscopy, the control (reversed-osmosis ultra-pure water) in HSC-4 cell free suspension at 37 °C incubated for 0 min did not exhibit any signal owing to AscR. And there was an initial rapid rise of AscR in HSC-4 cell suspension incubated in a thermostat bath at 42 °C, to a maximum at 60 min, followed by a decline near to the initial level at 60–180 min. The AscR-signal intensity of HSC-4 cell suspension at 42 °C was higher when compared to HSC-4 cell suspension at 37 °C or in HSC-4 cell-free suspension (Fig. 5). The AscR-signal intensity of HSC-4 cell suspension at 42 °C appeared to be 11.4% higher than at 37 °C. It was reported that AscR is produced from Asc in aqueous phase at physiological pH and temperature (Lohmann and Holz 1984). AscR decays mostly by self-disproportionation and not by other free radical in biological tissues, and the value of AscR is an indicator of oxidative stress (Roginsky and Stegmann 1994). And by ESR spectroscopy upon treatment with hyperthermia, sodium ascorbate produced a higher AscR value and more potent cytotoxicity against human leukemic and glioblastoma cell lines than Asc (Satoh et al. 1996). The ESR signal intensity of the AscR is taken as an indicator of oxidative stress due to oxidation of intracellular ascorbic acid leading to cellular damage (Dodd and Swartz 1984). AscR signal, although not detected in normal muscle, is readily observed in implanted tumors in vivo (Silcock and Dodd 1976). Our results suggest that hyperthermia could accelerate oxidation of Asc in HSC-4 cell suspension, which resulted in anti-proliferative effect and cellular damage. These imply that the AscR signal reflects an oxidative stress on HSC-4 cells, in some way related to inhibition of proliferation. The increase of AscR in HSC-4 cell

suspension treated with hyperthermia could be due to the increased synergic effect of the “drug-tumor reaction” after some of the HSC-4 cells had been destroyed by the cytotoxic action of Asc.

By scanning electron microscopic observation, HSC-4 cells showed a flat-shaped surface ultrastructure with metapophysis scattered on cell membrane (Fig. 6). When treated with Asc6Palm, HSC-4 cells appeared to lose metapophysis, and exhibited numerous microvilli on the cell membrane. Upon combined use of Asc6Palm and hyperthermia, cells exhibited the shrinkage and local narrowing of lamellipodia with microvilli. It was reported that in L1210 leukemic cells treated with hyperthermia, progressive changes in the cell membrane were seen, such as loss of microvilli, folding and corrugation of the cell membrane and development of blebs with SEM (Symonds and Wheldon 1984). Hyperthermic treatment of HeLa cells, which are adherent epidermoid cells, in cell suspension, combined with sonication, produced alterations to the cell surface, that are characterized by the loss of microvilli and smooth appearance of cell surface (Shammari et al. 1990). We also demonstrated that hyperthermia led to increased induction of apoptosis, tumor necrosis, and microvilli disappearance in Ehrlich ascites tumor cells (Tanaka et al. 2007; Kusumoto et al. 2007).

Thus, hyperthermia obviously enhanced the anti-proliferation effects of 0.25 mM Asc6Palm in HSC-4 cells. It was found that the electrically induced hyperthermic stimuli decreased cellular proliferation, possibly due to blocking of the cell cycle in a fraction of the cell population (Hernández-Bule et al. 2007). Correspondingly to this, it is speculated that cell proliferation of HSC-4 cells was inhibited by 0.25 mM Asc6Palm owing to cell cycle arrest, rather than lethal effects on treated cells.

Conclusions

The main conclusion from the present study is that Asc6Palm inhibits growth of human tongue squamous carcinoma cells HSC-4 more effectively than Asc, which is enhanced in combination with hyperthermia. Thus, the combined regimen of Asc6Palm and hyperthermia is expected to exert a marked antitumor activity. Further studies are necessary to explore the clinical significance of our findings in vivo.

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