Free Radical Research, 2003 Vol. 37 (6), pp. 631–643

A Free Radical Initiator, 2,2′-azobis (2-aminopropane) Dihydrochloride Enhances Hyperthermia-induced Apoptosis in Human Uterine Cervical Cancer Cell Lines

HIROYOSHI YUKI[a], TAKASHI KONDO[b,*], QING-LI ZHAO[b], YOSHISADA FUJIWARA[b], KIYOSHI TANABE[b], RYOHEI OGAWA[b], AKITOSHI NAKASHIMA[c], HIROSHI FUSHIKI[c], MASAKI FUJIMURA[d] and SHIGERU SAITO[d]

[a]*Department of Obstetrics and Gynecology, Yukiguniyamato General Hospital, 4415 Urasa, Yamato-machi, Niigata 949-7302, Japan;* [b]*Department of Radiological Sciences, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan;* [c]*Department of Obstetrics and Gynecology, Tonami General Hospital, Toyama 939-1395, Japan;* [d]*Department of Obstetrics and Gynecology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan*

Accepted by Professor N. Taniguchi

(Received 30 September 2002; In revised form 6 January 2003)

Hyperthermia-induced apoptosis and its enhancement in the presence of a temperature-dependent free radical initiator, 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH) were examined in human uterine cervical cancer cell lines, CaSki and HeLa. When both cell lines were treated with hyperthermia at 44°C for 60 min, minimal apoptosis was observed. When combined with nontoxic AAPH (50 mM), significant enhancement of apoptosis was observed, where the initial rate of free radical formation was about twice as high than that at 37°C. Augmentation of the growth delay, lipid peroxidation (LPO), activation of caspase-3 and increase in $[Ca^{2+}]i$ were also observed after the combined treatment. A water-soluble vitamin E, Trolox, blocked the increase in $[Ca^{2+}]i$ and an intracellular Ca^{2+} chelator, BAPTA-AM, prevented the DNA fragmentation induced by the combination. Cytochrome c release was also revealed by fluorescence microscopy. However, no significant change in mitochondrial membrane potential and expression of Bax and Bcl-2 was observed. A slight increase in Fas expression was observed only in CaSki cells after the combined treatment. These results indicate that hyperthermia and AAPH induce enhanced apoptosis and subsequent cell killing via two pathways; a pathway dependent on increase in LPO and $[Ca^{2+}]i$, and a pathway associated with cytochrome c release and subsequent caspase activation without changes of mitochondrial membrane potential and Bax/Bcl-2 expression in these cell lines. Since it is known that cancer cells are generally resistant to physical and chemical stress-induced apoptosis, free radical generators like AAPH appear to be a useful thermosensitizer for hyperthermic cancer therapy.

Keywords: AAPH; Hyperthermia; Uterine cervical cancer cell line; Apoptosis

INTRODUCTION

Hyperthermia and combined treatments with chemotherapy and radiotherapy have been recognized as promising modalities of anticancer treatment. The effectiveness of hyperthermia combined with radiotherapy to various solid tumors has been shown,[1] and recent clinical randomized trials in patients with advanced pelvic tumors clearly indicated, an advantage in the group using hyperthermia combined with radiotherapy compared with that of radiotherapy alone.[2,3] The clinical data from treatment by thermoradiotherapy in stage IIIB cervical carcinoma[4] have also shown the usefulness of hyperthermia for cancer therapy. However, uniform and precise delivery of heat to tumors still remains a challenge. In many circumstances, the temperature is not high enough to eradicate cancer cells completely. Drugs that have been proposed to overcome this difficulty are heat sensitizers. An ideal sensitizer would be nontoxic at normothermia, but would become cytotoxic at hyperthermic temperatures.

A water-soluble azo compound, 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH), has been used as a temperature-dependent free radical generator. Upon activation by hyperthermia, AAPH decomposes to yield two alkyl radicals and

*Corresponding author. Tel.: +81-76-434-7265. Fax: +81-76-434-5190. E-mail: kondot@ms.toyama-mpu.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000088292

a subsequent reaction with oxygen can yield alkoxyl and/or peroxyl radicals to produce excess oxidative stress intracellularly.[5] Although enhancement of hyperthermia-induced cytotoxicity with AAPH in Chinese hamster V79 cells, which are resistant for apoptotic stimuli, has been reported,[6] the detailed mechanisms responsible for the enhanced lethal effect, especially on inducing apoptosis, are not fully understood. Recently, we found that hyperthermia increased intracellular calcium ion concentration ($[Ca^{2+}]i$) arising from increased expression of type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1) and lipid peroxidation (LPO) in human myelomonocytic lymphoma U937 cells, which are sensitive to apoptotic stimuli, while an additional increase in $[Ca^{2+}]i$ due to increased LPO and the activation of a mitochondria-caspase dependent pathway play a major role in thermosensitization by AAPH.[7] However, how AAPH enhances apoptosis induced by hyperthermia in resistant cells for apoptotic stimuli remains to be elucidated.

In this study, we addressed whether AAPH or a combination of AAPH and hyperthermia induced apoptosis, including its mechanism in human uterine cervical adenocarcinoma HeLa cells and squamous cell carcinoma CaSki cells, and showed that the enhancement of apoptosis is due to an increase in $[Ca^{2+}]i$ caused by increased LPO without affecting the typical mitochondria-mediated apoptosis pathway.

MATERIALS AND METHODS

Chemicals

A spin trap, α-phenyl-*N*-tert-butylnitrone (PBN), Trolox, and Hoechst 33258 were obtained from Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO). An intracellular free Ca^{2+} chelator, *O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′,-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) and a fluorescent dye, 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester (Fura 2-AM) were purchased from Dojindo Laboratory (Kumamoto, Japan). Monoclonal antibodies to Bcl-2 and Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A temperature dependent free radical generator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and other reagents were from Wako Pure Chemical Industries Ltd. (Tokyo, Japan).

Cells and Hyperthermic Treatment

A human uterine cervical adenocarcinoma cell line, HeLa, and a squamous cancer cell line CaSki, were obtained from the Japanese Cancer Research Resource Bank. HeLa cells were grown in Eagle's MEM and CaSki cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) and antibiotics (penicillin and streptomycin) in 5% CO_2 and 95% air at 37°C.

Hyperthermic treatments were performed by immersing plastic culture flasks containing culture medium (10 ml) in a water-bath (NTT-1200, Eyela, Tokyo, Japan) at 44.0°C (±0.05°C). The temperature of the solution inside the flask or test tube was monitored with a digital thermometer (#7563, YOKOGAWA, Tokyo, Japan) coupled with a thermocouple 0.8 mm in diameter during heating.

Detection of Free Radicals Induced by AAPH

Electron paramagnetic resonance (EPR) using PBN as a spin trap was employed to detect thermally generated radicals from AAPH in a 50 mM phosphate buffer solution (PB, pH 7.4). One millilitre of PB containing AAPH and a spin trap in a test tube was prepared at ice temperature and the reaction was initiated by immersing the test tube in a water bath at the desired temperature. For EPR measurement, aliquots of the reaction mixture were sampled at different temperatures or incubation times. The EPR measurements were made using a RFR-30 Radical Analyzer System (Radical Research Co., Tokyo, Japan) operating at an X-band frequency of 9.4 GHz.

Counting the Cell Number and Morphological Observation for Apoptosis

The number of intact and live cells after treatments was counted using a Burker-Turk haemocytometer. The medium in the flask was discarded and the attached cells were removed by the treatment with 0.25% trypsin and EDTA for 3 min. In the resultant single cell suspension, dead cells were assessed using the trypan blue dye exclusion test at a concentration of 0.1%.

Morphological changes in nuclei were examined with Hoechst 33258 staining. HeLa and CaSki cells were fixed with 1% glutaraldehyde for 30 min and washed twice with Dulbecco's phosphate-buffered saline (PBS), then stained with 0.2 mM of a fluorescent dye, Hoechst 33258. The samples were observed with a Nikon TMD fluorescent microscope (Tokyo, Japan).

Flow Cytometric Analysis for Apoptosis, Caspase-3 Activity, Transmembrane Potential of Mitochondria and Fas(APO-1/CD95) Expression

Flow cytometry for detection of apoptosis was performed with propidium iodide (PI) and fluorescein

isothiocyanate (FITC)-labeled annexin V in an Annexin V-FITC kit obtained from Immunotech (Marseille, France). Briefly, the samples were washed in cooled PBS at 4°C and centrifuged at 500*g* at 4°C for 5 min. The resulting pellets were adjusted to 10^6 cells/ml with a binding buffer, FITC-labeled annexin V (5 μl) and PI (5 μl) were added to them (490 μl) and they were mixed gently. After incubation for 10 min in the dark, the cells were analyzed with a flow cytometer (Epics XL, Beckman-Coulter, Tokyo, Japan).

The caspase-3 activity was evaluated using a cell permeable fluorigenic substrate (PhiPhiLux-G1D2, OncoImmunin Inc., Geithersburg, MD) in cells 48 h after treatment. Briefly, each sample (10^6 cell/ml) was gently centrifuged and the cell pellet was resuspended with 50 ml of 10 ml PhiPhiLux-G1D2 substrate solution in a growth medium. After incubation for 1 h at 37°C in the dark, the samples were washed once and diluted with 0.5 ml of ice-cold flow cytometry dilution buffer. The fraction of cells showing high caspase-3 activities was measured by flow cytometry.

To measure transmembrane potential of mitochondria after treatments, cells were incubated at 37.0°C for 15 min in the presence of 3,3-deoxycarbocyanene iodide ($DiOC_6(3)$) at a concentration of 40 nM and then analyzed with a flow cytometer.

After adding a FITC-labeled anti-Fas monoclonal antibody (clone:UB2, MBL, Nagoya, Japan), the samples were incubated for 30 min at room temperature. After washing with PBS twice, fluorescent intensity in both cell suspensions was analyzed using a flow cytometer. The procedure for flow cytometry was described in detail in previous reports.[8,9]

Determination of DNA Fragmentation

The amount of DNA extracted from samples of cells that had undergone DNA fragmentation was assayed using a modified method of Sellins and Cohen.[10] Briefly, cells were lysed in a lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) and centrifuged at 13,000*g* for 10 min. Then, each sample of DNA in the supernatant and the resulting pellet was precipitated in 12.5% trichloro acetic acid (TCA) at 4°C, then quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total DNA for that sample (supernatant plus pellet).

Determination of Intracellular Calcium Ion Concentration in Single Cells

The cells grown in a glass-bottom microwell dish (MatTek Corp., Ashland, MA) were washed with HEPES-buffer Ringer solution (HR). The composition of HR was: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.13 mM; Na_2HPO_4, 1.0 mM; glucose, 5.5 mM and HEPES, 10 mM. The buffer was supplemented with 0.2% bovine serum albumin (Sigma, St. Louis, MO), minimal Eagle's essential amino acids (Flow Laboratories, Irvine, UK), and 2 mM L-glutamine. About 3×10^5 cells in 3 ml HR were loaded with 5 mM Fura-2/AM for 30 min at 25°C. The cells loaded with Fura-2/AM were washed once with HR and twice with the growth medium. After hyperthermia and/or AAPH treatment, the cells were washed with HR and HR was added to the dish. Digital imaging of Fura-2 fluorescence was carried out with an inverted microscope and a digital image processor (Argus 50/Ca, Hamamatsu Photonics, Hamamatsu, Japan) as reported previously.[11–13]

Determination of LPO

The product of LPO reacting with 2-thiobarbituric acid, malondialdehyde (MDA), was measured spectrophotometrically at an absorbance of 535 nm, as described by Buege and Aust[14] with modifications.

Western Blotting for Bcl-2 and Bax

Cells were collected, washed and lysed. An aliquot of total protein was separated by SDS 10–20% polyacrylamide gel electrophoresis and transferred to PVDF membranes. After incubation with a blocking buffer, the membrane was incubated with the primary antibody for Bcl-2 and Bax and then with a horseradish peroxidase-linked secondary antibody followed by chromogenic visualization. The detailed procedure was described in a previous report.[7,8]

Immunofluorescence Microscopy

Cells were plated onto glass coverslips. After 24 h, the cells were treated with hyperthermia and /or AAPH for 1 h and then washed. After 24 h incubation cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were sequentially incubated with anti-cytochrome c polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Alexa Fluor 598-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Hoechst 33258 (Molecular Probes, Eugene, OR). Images were acquired using a fluorescence digital imaging system (ARGUS/FISH, Hamamatsu Photopnics, Hamamatsu, Japan) coupled with a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan).

Statistics

Data are expressed as means ± SD. Significance was assessed by two-away or one-away ANOVA

followed by Fisher's PLSD test and the Student's *t*-test. All experiments represent at least three independent replications performed in triplicate.

RESULTS

Free Radical Formation Induced by AAPH

An air-saturated aqueous solution of 10 mM AAPH containing 50 mM of PBN in PB (50 mM, pH 7.4) after heating for 8 min at 44°C showed a 6 line EPR spectrum, which was analyzed as a primary nitrogen triplet ($a_N = 1.53$ mT), further split by a secondary proton ($a_H^\beta = 0.39$ mT). This spectrum is consistent with the spin adduct of alkoxyl or peroxyl radicals formed by the reaction of oxygen and alkyl radicals due to the AAPH thermal decomposition as reported previously.[15] The free radical production increased with increasing incubation time and temperature. The initial increase rate of EPR signal intensity was 36.3 and 15.0 relative units/min at 44 and 37°C, respectively.

Effects of Combination of Hyperthermia and AAPH on Cell Growth

When HeLa and CaSki cells were treated with 44°C hyperthermia and/or, AAPH at a concentration of 50 mM for 60 min, the number of cells was counted

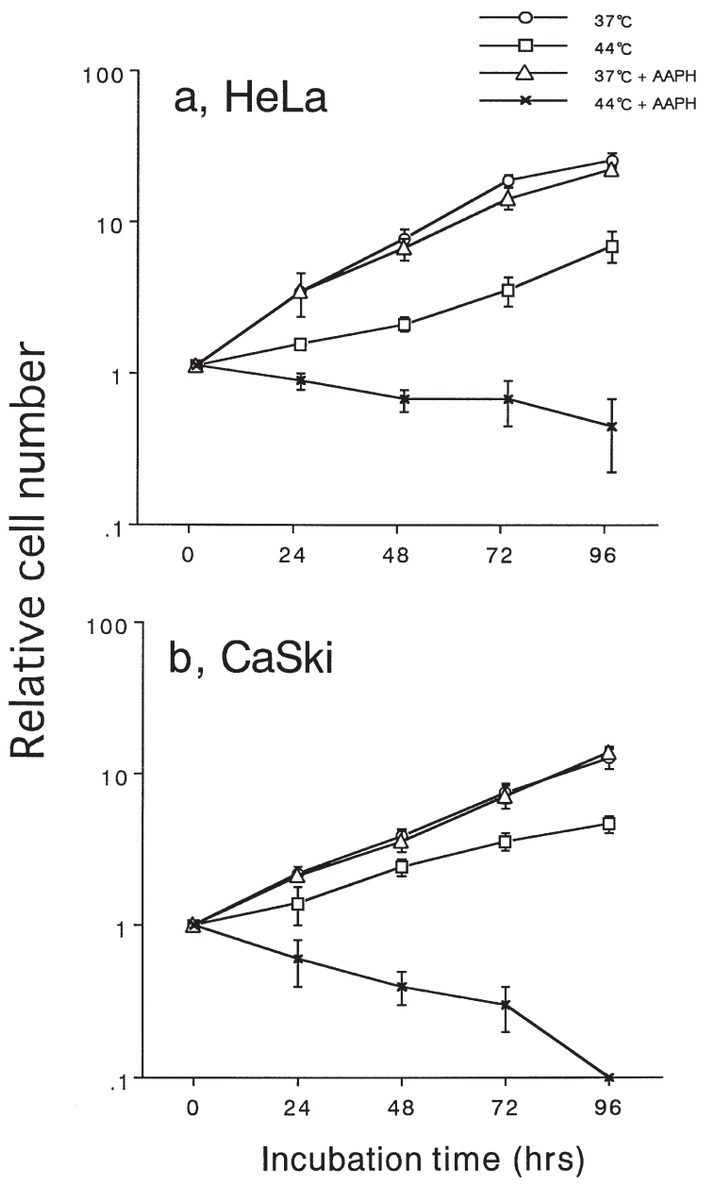

FIGURE 1 Growth curve of HeLa cells (a) and CaSki cells (b) after various treatments for 60 min. The bars in the figure represent means ± SD ($n = 3$).

up to 96 h (Fig. 1). Although AAPH alone did not exert significant cytotoxicity in either cell type, hyperthermia alone induced significant inhibition of cell growth. Moreover, marked growth inhibition was observed after the combined treatment in both cell types.

DNA Fragmentation and Morphological Nuclei Changes

The effects of hyperthermia and/or AAPH on DNA fragmentation after incubation for 24 and 48 h are shown in Fig. 2. A significant increase in DNA fragmentation was observed in both HeLa and CaSki cell lines after combination as compared with hyperthermia alone. The treatment with AAPH alone did not induce significant DNA fragmentation. To verify apoptosis, morphological changes were examined under a fluorescent microscope in HeLa and CaSki cells stained with a fluorescent dye, Hoechst 33258, after 48 h incubation (Fig. 3). In both cases, apoptotic cells showing typical apoptotic features, nuclear chromatin condensation and fragmentation after the combination with hyperthermia and AAPH were observed.

To measure phosphatidylserine (PS) externalization as an endpoint of apoptosis, flow cytometry using annexin-V/FITC and PI double staining revealed that after the combined treatment of hyperthermia and AAPH, cells with externalized PS increased with increasing incubation time, since PS externalization is an early sign of apoptosis. In HeLa cells and CaSki cells, the percentage of early apoptosis, i.e. annexin-V(+)/FITC(−) cells, at 48 h was slightly enhanced by the combined treatments of hyperthermia and AAPH, and the percentage of secondary necrosis i.e. annexin-V(+)/FITC(+) was markedly enhanced by the combination (Table I).

Caspase-3 Activity

When activation of caspase-3, one of the main caspases in apoptosis, was measured by flow cytometry using a PhiPhiLux-G1D2 Kit at 48 h, the fraction of HeLa and CaSki cells treated with the combination showed significantly increased caspase-3 activity (Table I). These results indicate that caspase-3 is involved in enhanced apoptosis in both cells treated with hyperthermia and AAPH.

Determination of LPO

LPO is a free radical-mediated chain reaction occurring in unsaturated fatty acids of membrane phospholipds which form lipid peroxides. When HeLa and CaSki cells were treated with 44°C hyperthermia and/or AAPH (50 mM) for 60 min, LPO was monitored immediately after treatments

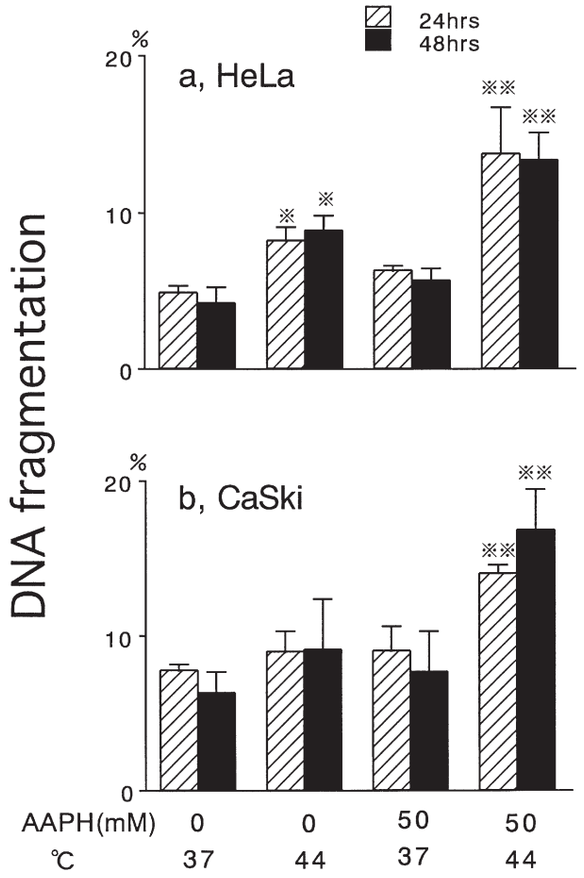

FIGURE 2 The effects of hyperthermia (44°C, 60 min) combined with AAPH (50 mM) on DNA fragmentation. The bars in the figure represent means ± SD ($n = 3$). *$p < 0.05$ vs. 37°C treatment, **$p < 0.05$ vs. 44°C treatment.

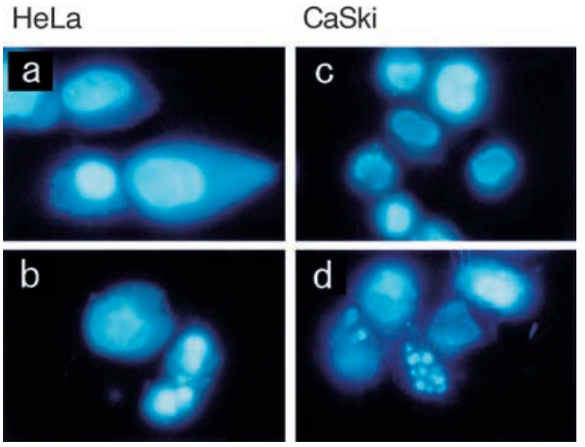

FIGURE 3 Apoptotic features induced by the combination of hyperthermia (44°C, 60 min) and AAPH (50 mM). Fluorescent image of cells stained with fluorescent dye, Hoechst 33258. (a) Control HeLa cells at 37°C. (b) HeLa cells after combined treatment with hyperthermia and AAPH. (c) Control CaSki cells at 37°C. (d) CaSki cells after combined treatment with hyperthermia and AAPH.

TABLE I Measurement of endpoints on apoptosis induced by hyperthermia and/or AAPH in HeLa cells and CaSki cells

	37°C	44°C	37°C+AAPH	44°C+AAPH
HeLa cells				
Early apoptosis	0.5 ± 0.2	2.1 ± 1.0*	0.6 ± 0.3	3.7 ± 1.0*
Secondary necrosis	1.0 ± 0.6	4.6 ± 2.1*	0.9 ± 0.8	22.4 ± 4.0**
Caspase-3 activity	0.5 ± 0.1	5.0 ± 3.3*	0.6 ± 0.1	29.3 ± 1.4**
Low MMP†	6.5 ± 1.0	2.5 ± 1.7	3.0 ± 2.5	0.3 ± 0.2
Fas expression	0.3 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	0.9 ± 0.1
CaSki cells				
Early apoptosis	1.3 ± 0.8	2.2 ± 1.1	1.7 ± 1.1	13.1 ± 1.0*
Secondary necrosis	2.7 ± 1.0	8.9 ± 3.2*	3.9 ± 1.5	33.8 ± 5.5**
Caspase-3 activity	1.9 ± 0.5	2.3 ± 0.7	3.7 ± 0.3	16.3 ± 3.5**
Low MMP	10.0 ± 4.3	5.9 ± 2.7	6.9 ± 1.0	0.9 ± 0.4
Fas expression	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	3.4 ± 0.5**

Cells were treated with 44°C hyperthermia with or without AAPH (50 mM), and after incubation for 48 h cells were collected and analyzed with flow cytometry (see "Materials and methods" section).Values are the mean ± SD ($n = 3$). †MMP; mitochondrial membrane potential. *$p < 0.05$ vs. 37°C treatment. **$p < 0.05$ vs. 44°C treatment.

since rapid facilitation of LPO after hyperthermia has been observed.[7] Figure 4 shows the malondialdehyde (MDA) levels in samples treated with hyperthermia and/or AAPH. The combination with hyperthermia and AAPH was significantly higher than those of the control in both cell types.

Change of $[Ca^{2+}]i$ and Effects of Trolox and BAPTA-AM

The $[Ca^{2+}]i$ in single cells immediately after treatment of hyperthermia with or without AAPH is displayed in pseudocolor in Figs. 5 and 6. After hyperthermia, the number of cells showing much higher $[Ca^{2+}]i$ increased, and increasing $[Ca^{2+}]i$ became more prominent in cells treated with the combination with AAPH. Histograms of $[Ca^{2+}]i$ in 100 randomly selected cells are shown in Figs. 7 and 8. The mean $[Ca^{2+}]i$ was 142 ± 37 nM (mean ± SD) in HeLa cells and 102 ± 28 nM in CaSki cells. When cells were treated with hyperthermia, the number of cells with higher $[Ca^{2+}]i$ increased; the mean $[Ca^{2+}]i$ was 286 ± 42 nM in HeLa cells and 251 ± 97 nM in CaSki cells. When cells were treated with the combination, the number of cells with higher $[Ca^{2+}]i$ was greatly increased; the mean $[Ca^{2+}]i$ was 460 ± 76 nM in HeLa cells and 458 ± 115 nM in CaSki cells. These results indicate that hyperthermia initiated the increase of $[Ca^{2+}]i$, while the combination further enhanced it.

When the effect of an antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), on the increase of $[Ca^{2+}]i$ induced by the combination was examined, Trolox at a concentration of 1 mM significantly reduced the $[Ca^{2+}]i$. The value was 160 ± 31 nM (mean ± SD) in HeLa cells and 112 ± 29 nM in CaSki cells), while the mean $[Ca^{2+}]i$ was 135 ± 28 nM in HeLa cells and 95 ± 22 nM in CaSki cells after treatment with Trolox alone. The results revealed that Trolox inhibited in part the elevation of $[Ca^{2+}]i$ induced by hyperthermia and AAPH.

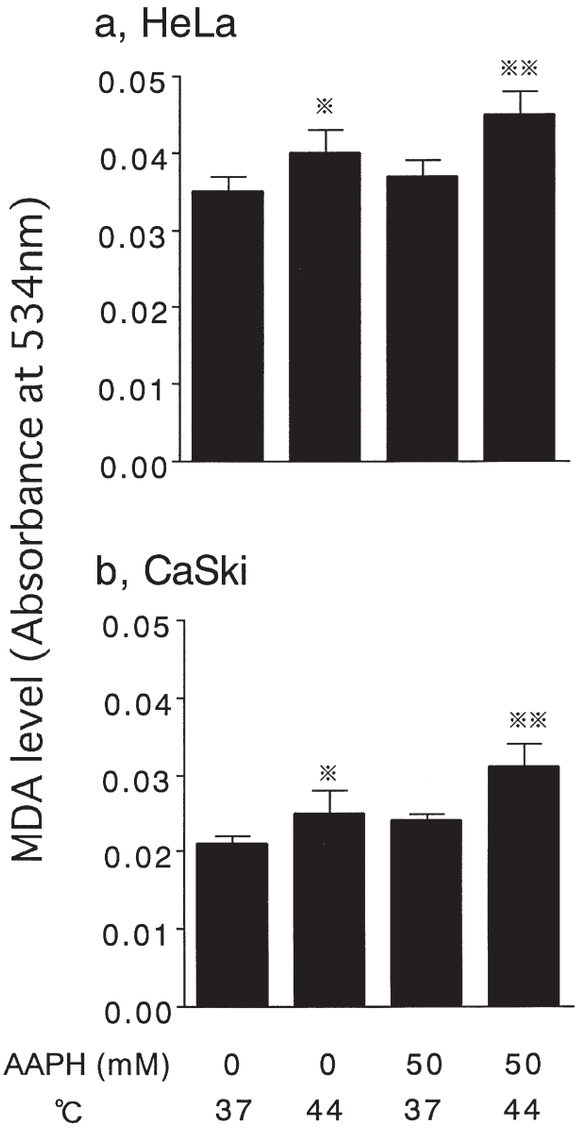

FIGURE 4 MDA level of HeLa cells (a) and CaSki cells (b) after various treatments for 60 min. The bars in the figure represent means ± SD ($n = 3$). *$p < 0.05$ vs. 37°C treatment, **$p < 0.05$ vs. 44°C treatment.

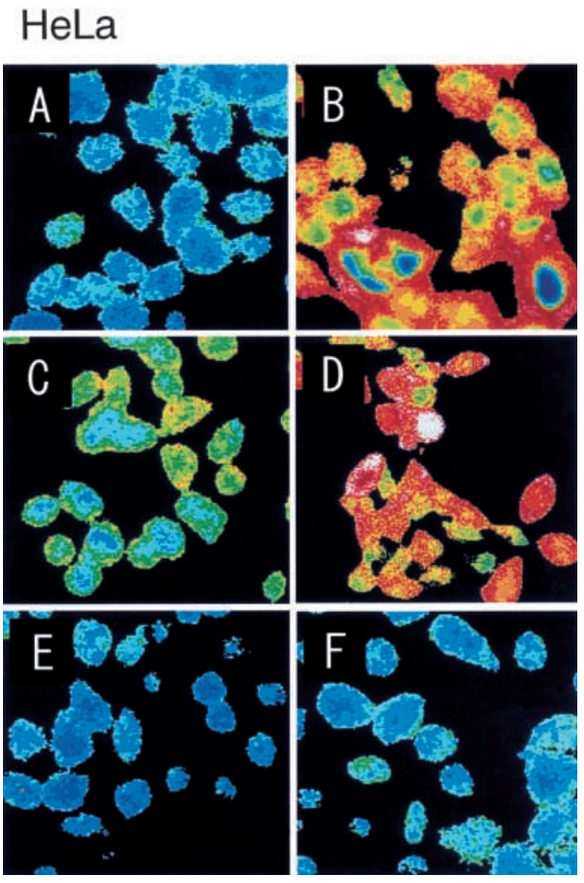

FIGURE 5 Pseudo color image of $[Ca^{2+}]i$ in HeLa cells after hyperthermia combined with AAPH. (A) 37°C, (B) 44°C (60 min), (C) 37°C + AAPH (50 mM), (D) 44°C + AAPH, (E) 37°C + Trolox (1 mM), (F) the combined treatment + Trolox.

Effects of BAPTA-AM on DNA Fragmentation Induced by Hyperthermia and AAPH

To elucidate the role of $[Ca^{2+}]i$ in hyperthermia-induced apoptosis, an intracellular Ca^{2+} chelator, BAPTA-AM, was employed. When the cells were incubated in a culture medium containing graded concentrations of BAPTA-AM during the combined treatments and for 48 h, the DNA fragmentation decreased with increasing concentrations of BAPTA-AM to almost the control level (Fig. 9). These results clearly demonstrate that the increase in $[Ca^{2+}]i$ induced by hyperthermia plays a critical role in the enhancement of apoptosis induced by hyperthermia and AAPH.

Fas Expression

To determine the correlation between hyperthermia combined with AAPH and the level of Fas expression, the expression was examined by flow cytometry in both cell types. The fraction of cells showing increased Fas expression in HeLa cells and CaSki cells is listed in Table I. After 48 h, the combination with hyperthermia and AAPH induced a slight, but significant, increase in the expression of Fas in CaSki cells, whereas it did not in HeLa cells.

Transmembrane Potential of Mitochondria and Bcl-2 and Bax Expression

When HeLa and CaSki cells were treated with 44°C hyperthermia and/or AAPH (50 mM) for 60 min and incubated for 48 h, changes in mitochondrial function was examined. The change in transmembrane potential of mitochondria was measured by flow cytometry using $DiOC_6(3)$ as a fluorescent probe, the fraction of cells showing low transmembrane potential in HeLa cells and CaSki cells is listed in Fig. 1. These results indicate that no increase in the fraction of cells showing low transmembrane potential was observed in both cell types after hyperthermia and AAPH. In addition, the expressions of Bcl-2 and Bax indicating involvement of mitochondria in apoptosis were examined after 24 h; the results of Western blotting are shown in

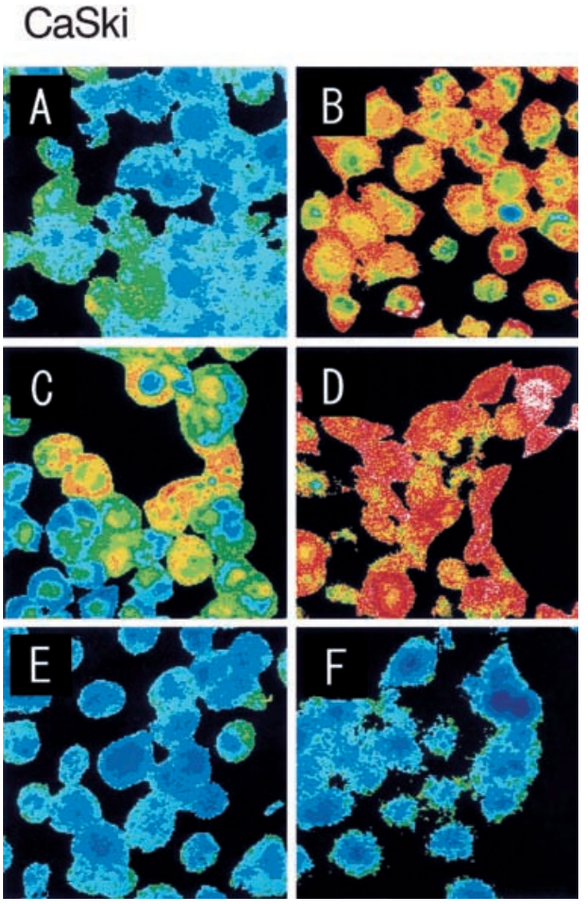

FIGURE 6 Pseudo color image of $[Ca^{2+}]i$ in CaSki cells after hyperthermia combined with AAPH. (A) 37°C, (B) 44°C (60 min), (C) 37°C + AAPH (50 mM), (D) 44°C + AAPH, (E) 37°C + Trolox (1 mM), (F) the combined treatment + Trolox.

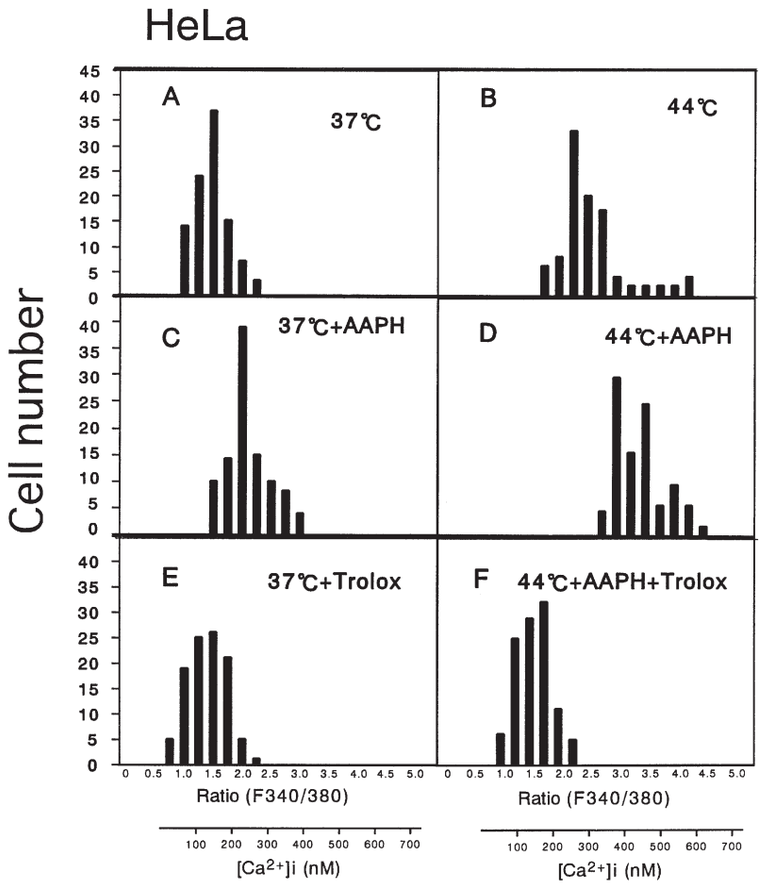

FIGURE 7 Histogram analysis of $[Ca^{2+}]i$ in HeLa cells after hyperthermia combined with AAPH. (A) 37°C, (B) 44°C (60 min), (C) 37°C + AAPH (50 mM), (D) 44°C + AAPH, (E) 37°C + Trolox (1 mM), (F) the combined treatment + Trolox.

Fig. 10. No substantial change in Bcl-2 or Bax expression in HeLa cells and CaSki cells after the combined treatment was observed.

Fluorescence Microscopy for Observation of Cytochrome C release

When HeLa and CaSki cells were treated with 44°C hyperthermia and/or AAPH (50 mM) for 60 min and incubated for 24 h, intracellular distribution of cytochrome c was examined by fluorescence microscopy. Immunocytochemistry with cytochrome c revealed a punctate staining pattern in nontreated HeLa and CaSki cells (Fig. 11A, 1). This mitochondrial staining changed into diffuse pattern in most of the cells after the combined treatment, suggesting that cytochrome c was released from mitochondria (Fig. 11D, L). Partial diffuse pattern of cytochrome c staining in cells treated with hyperthermia or AAPH was also observed in apoptotic cells showing nuclear morphological changes as revealed by Hoechst 33258 staining (Fig. 11B, F, J, K, N, O). These results indicate that cytochrome c was released from mitochondria in the cells treated with hyperthermia and AAPH.

DISCUSSION

The present results demonstrate that hyperthermia at 44°C combined with a temperature-dependent free radical generator, AAPH, at a nontoxic concentration (50 mM), causes the enhancement of apoptosis induced by hyperthermia in human cervical cancer HeLa and CaSki cells, which are generally resistant to apoptotic stimuli. In addition, these results indicate that the combination induced enhanced apoptosis and subsequent cell killing via an increase in LPO and $[Ca^{2+}]i$, but not via the activation of a mitochondria-mediated apoptosis pathway. Previously, we reported that hyperthermia combined with AAPH enhanced apoptosis by increasing $[Ca^{2+}]i$ due to increased LPO and the activation of a mitochondria-caspase dependent apoptotic pathway in human myelomonocytic

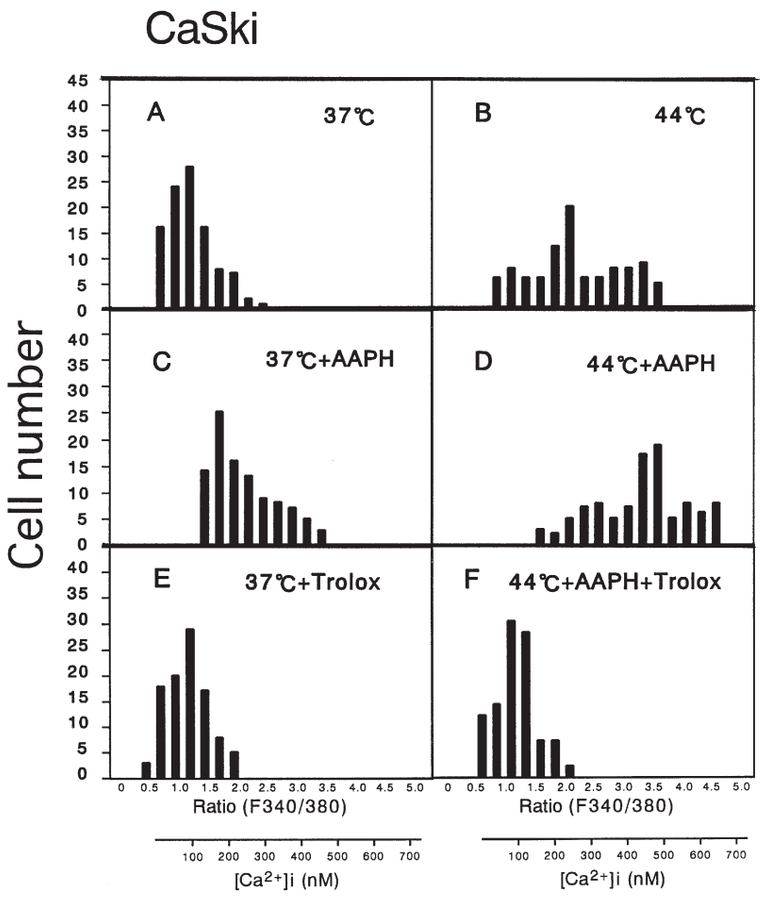

FIGURE 8 Histogram analysis of $[Ca^{2+}]i$ in CaSki cells after hyperthermia combined with AAPH. (A) 37°C, (B) 44°C (60 min), (C) 37°C + AAPH (50 mM), (D) 44°C + AAPH, (E) 37°C + Trolox (1 mM), (F) the combined treatment + Trolox.

lymphoma U937 cells, which are generally sensitive to various apoptotic stimuli.[7] The present results are in accord with the previous evidence in terms of AAPH enhancement of apoptosis induced by hyperthermia, although the mechanism of enhancement (i.e. the former is due to additional activation of a mitochondria-pathway while the latter is not), the duration of hyperthermia treatment (i.e. in the former; 10 min, in this study; 60 min), the degree of apoptotic induction (i.e. in the former; up to about 45%, in this study; up to about 15%) and the incubation time for apoptosis induction (i.e. the former; 6 h, in this study; 24 or 48 h) are different. We have shown caspase-3 activation and cytochrome c release in cells after the combined treatment of hyperthermia and AAPH in HeLa and CaSki cells. However, no significant change in mitochondrial membrane potential and expression of Bax and Bcl-2 were observed. Cytochrome c release appears to be due to the disruption of mitochondrial outer membrane without opening the permeability transition pore. Flow cytometric analysis for apoptosis after the combined treatment of hyperthermia and AAPH revealed that fractions of secondary necrosis showing plasma membrane damages were higher than those of early apoptosis in HeLa and CaSki cells, whereas, fractions of early apoptosis was predominant in U937 cells.[7] In secondary necrotic cells, mitocondrial membranes appear to be disrupted. It has been shown that excess oxidants or Ca^{2+} overload can trigger mitochondria to release cytochrome c by two general mechanisms; one involves osmotic disequilibrium leading to an expansion of the matrix space, organellar swelling, and subsequent rupture of the outer membrane of the mitochondria, the other involves the opening of the channel in the outer membrane which is mostly intact.[16] The present results appear to reflect the first mechanism, since cells with ruptured mitochondria are at risk of cell death through a slow non-apoptotic mechanism. These differences appear to be due to intrinsic sensitivity based on the involvement of a typical mitochondria-pathway for apoptosis in the two cell lines, HeLa and CaSki.

A possible mechanism of hyperthermia enhancement induced apoptosis by free radical generators has already been stated in our previous paper.[17] As we reported previously, hydrophilic AAPH

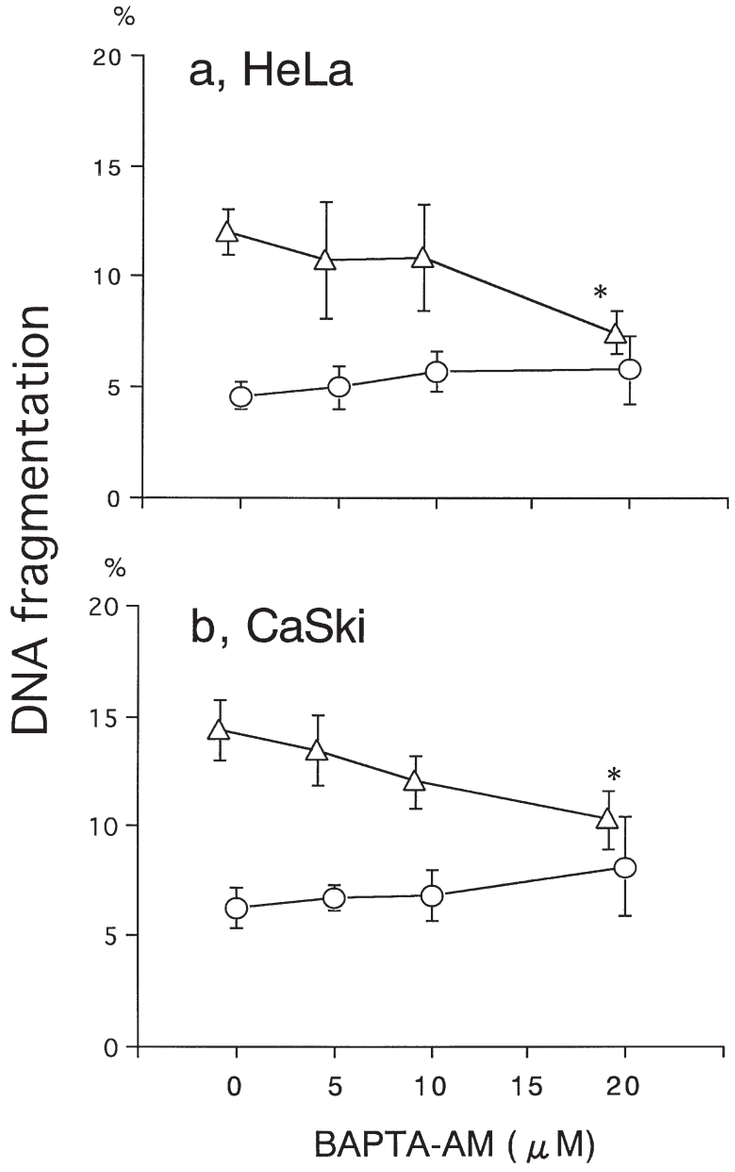

FIGURE 9 Effects of BAPTA-AM on DNA fragmentation induced by hyperthermia (44°C, 60 min) combined with AAPH (50 mM) in HeLa cells (a) and CaSki cells (b). Open triangles show the DNA fragmentation induced by hyperthermia (44°C, 60 min) combined with AAPH (50 mM) in the presence of BAPTA-AM, and open circles show the DNA fragmentation induced by BAPTA-AM alone. (*) indicates a statistically significant difference ($p < 0.05$) compared to the value obtained from cells treated with hyperthermia and AAPH without BAPTA-AM.

enhanced apoptosis only in hyperthermic conditions. Since it is known that AAPH generates free radicals more at hyperthermic conditions, more free radicals could enter the cells enhancing apoptosis. In addition, this enhanced apoptosis might be due to increase in permeability and fluidity of the cell membrane due to hyperthermia, which may facilitate influx of AAPH and/or the generated free radicals. At 37°C such enhancement was not observed. On the other hand, if the lipophilicity of free radical generators is high, they could enter the cell even at 37°C, inducing apoptosis by a small number of free radicals. At hyperthermic conditions, increased influx of the substances and increased generation of free radicals intracellularly and extracellulary may all contribute to enhance hyperthemia-induced apoptosis.

As for resistance to apoptosis, interesting evidence has been found. Chinese hamster V79 cells originated from lung fibroblasts were resistant to the induction of apoptosis not only by ionizing radiation, but also by H_2O_2 treatment. However, treatment of cells with a high concentration of H_2O_2 (i.e. excess oxidative stress) could induce apoptosis which has inhibited by an antioxidant, a-phenyl *N*-tert-butylnitrone, and BAPTA-AM. From this

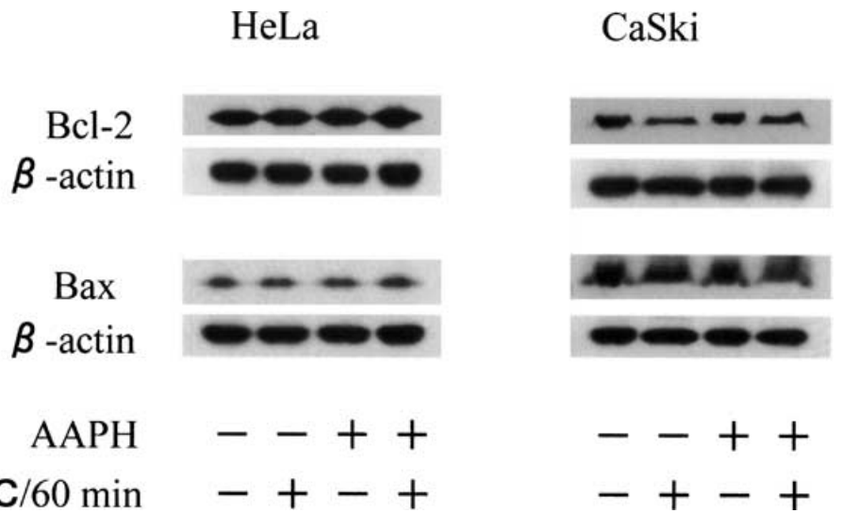

FIGURE 10 Western blot analysis of Bcl-2 and Bax protein expression in HeLa cells (a) and CaSki cells (b)treated with hyperthermia (44°C, 60 min) and combined with AAPH (50 mM).

evidence, accumulation of damage in the cytoplasm induced preferentially by a high concentration of H_2O_2 has been suggested to cause apoptotic processes even in apoptosis-resistant cell lines.[18]

As similar evidence that LPO and $[Ca^{2+}]i$ play a major role in the enhancement of apoptosis in HeLa and CaSki cells has been found, it is probable that the antioxidant Trolox inhibited Ca^{2+} release from intracellular Ca^{2+} stores by protecting against LPO in the organellar membrane. From these results, it was inferred that Ca^{2+}, which was released from Ca^{2+} store sites due to hyperthermia- or hyperthermia-mediated free radicals-induced membrane denaturation, triggered the signal transduction pathways of apoptosis, and a further increase due to excess free radical stress enhanced the apoptosis. The *in vivo*

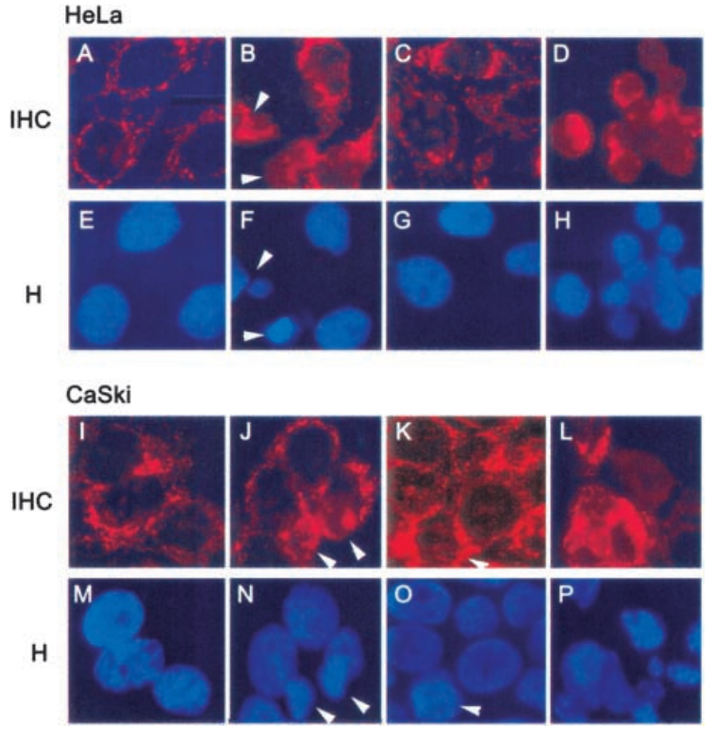

FIGURE 11 Fluorescent microgram on release of cytochrome c from mitochondria in HeLa cells (A–H) and CaSki cells (I ~ P). Immunohistochemical images with anti-cytochrome c antibody (IHC) and images with Hoechst 33258 staining (H) were shown. A, E, I, M; 37°C, B, F, J, N; 44°C (60 min), C, G, K, O; 37°C + AAPH (50 mM), D, H, L, P; 44°C + AAPH. White arrows indicate partial diffuse pattern of cytochrome c staining in cells showing nuclear morphological changes as revealed by Hoechst 33258 staining.

antitumor effect of 43°C hyperthermia combined with AAPH has been shown in hepatocellular carcinoma in the rat, and the concentration of thiobarbituric acid-reactive substances (TBARS), an index of LPO, is increased in cancerous tissue, but not in normal tissue.[19] The efficacy of AAPH as a thermosensitizer has been evaluated in both hypoxic and thermotolerant V79 cells in mild hyperthermia at 42°C, where no thermo-cytotoxicities was observed even after 2 h of treatment. Therefore, free radical generators appear to be useful for enhancing apoptosis in hyperthermic cancer therapy. Of course, AAPH is a proto-type thermosensitizer and *in vivo* toxicities, especially in normal tissues, should be reduced before clinical application. Further development of heat sensitizers to produce free radicals at hyperthermic temperatures greater than 42°C, not 37°C, is needed.

Recently, the usefulness of apoptosis induction after radiation therapy in uterine cervical cancer as a predictor of not only short-term prognosis, but also long term survival of patients has been shown.[20–24] Therefore, apoptosis is an important factor for radiation therapy and prognosis in uterine cervical cancer, although the real apoptotic index in tissues from patients determined by immunohistological staining is not high. In contrast, HeLa and CaSki cell lines established from uterine cervical cancer are not sensitive to apoptotic stimuli because E6 proteins of infected human papiloma virus interfere with the function of p53, which positively regulates apoptosis induction.[25,26] Interestingly, changes in expression of mitochondria apoptotic proteins were not evident in either cultured cell line, which similarly respond to the combination of hyperthermia and AAPH. A subtle difference in Fas expression may explain the characteristics of the apoptotic pathway between HeLa cells and CaSki cells, since the role of Fas expression in apoptosis induced by oxidative stress has been reported.[27,28]

In summary, combination with a temperature dependent free radical generator enhanced the apoptosis and cell killing induced by hyperthermia alone in cultured cancer cell lines resistant to apoptosis by two pathways; a pathway dependent on increase in LPO and $[Ca^{2+}]i$, and a pathway associated with cytochrome c release and subsequent caspase activation without changes of mitochondrial membrane potential and Bax/Bcl-2 expression. Free radical generators appear to be a useful candidate for enhancing apoptosis in hyperthermic cancer therapy.

Acknowledgements

This study was supported in part by a Grant in Aid for Scientific Research on Priority Areas (C)(12217049) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

[1] Overgaard, J. (1989) "The current and potential role of hyperthermia in radiotherapy", *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 535–549.
[2] van der Zee, J., Gonzalez-Gonzalez, D., van Rhoon, G.C., van Dijk, J.D.P., van Putten, W.L.J. and Hart, A.A.M. (2000) "Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumors: a prospective, randomized, multicentre trial", *Lancet* **355**, 1119–1125.
[3] van der Zee, J. and Gonzalez-Gonzalez, D. (2002) "The Dutch deep hyperthermia trial: results in cervical cancer", *Int. J. Hyperther.* **18**, 1–12.
[4] Harima, Y., Nagata, K., Harima, K., Ostapenko, V.V., Tanaka, Y. and Sawada, S. (2001) "A randomized clinical trial of radiation therapy versus thermoradiotherapy in stage IIIB cervical carcinoma", *Int. J. Hyperther.* **17**, 97–105.
[5] Terao, K. and Niki, E. (1986) "Damage to biological tissues induced by radical initiator 2,2′-azobis (2-amidinopropane) dihydrochloride and its inhibition by chain-breaking antioxidants", *Free Radic. Biol. Med.* **2**, 193–201.
[6] Krishna, M.C., Dewhirst, M.W., Friedman, H.S., Cook, J.A., DeGraff, W., Samuni, A., Russo, A. and Mitchell, J.B. (1994) "Hyperthermic sensitization by the radical initiator 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). I. *In vitro* studies", *Int. J. Hyperther.* **10**, 271–278.
[7] Li, F.-J., Kondo, T., Zhao, Q.-L., Tanabe, K., Ogawa, R., Li, M. and Arai, Y. (2001) "Enhancement of hyperthermia-induced apoptosis by a free radical initiator 2,2′-azobis (2-amidinopropane) dihydrochloride, in human histiocytic lymphoma U937 cells", *Free Radic. Res.* **35**, 281–299.
[8] Li, M., Kondo, T., Zhao, Q.-L., Li, F.-J., Tanabe, K., Arai, Y., Zhou, Z.-C. and Kasuya, M. (2000) "Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochondria-dependent pathways", *J. Biol. Chem.* **275**, 39702–39709.
[9] Arai, Y., Kondo, T., Zhao, Q.-L., Li, F.-J., Ogawa, R., Li, M. and Kasuya, M. (2002) "Enhancement of hyperthermia-induced apoptosis by local anesthetics on human histiocytic lymphoma U937 cells", *J. Biol. Chem.* **277**, 18986–18993.
[10] Sellins, K.S. and Cohen, J.J. (1987) "Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes", *J. Immunol.* **139**, 3199–3206.
[11] Kondo, T., Kano, E., Habara, Y. and Kanno, T. (1993) "Enhancement of cell killing and increase in cytosolic calcium concentration by combined treatments with hyperthermia and TMB-8 in mouse mammary carcinoma FM3A cells", *Cell Calcium* **14**, 621–629.
[12] Kondo, T., Habara, Y., Kanno, T. and Kano, E. (1994) "Thermosensitization and modification of cytosolic calcium concentration by verapamil and diltiazem in mouse mammary carcinoma FM3A cells", *Int. J. Radiat. Oncol. Biol. Phys.* **25**, 511–517.
[13] Kameda, K., Kondo, T., Tanabe, K., Zhao, Q.-L. and Seto, H. (2001) "The role of intracellular Ca^{2+} in apoptosis induced by hyperthermia and its enhancement by verapamil in U937 cells", *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 1369–1379.
[14] Buege, J.A. and Aust, S.D. (1978) "Microsomal lipid peroxidation", *Methods Enzymol.* **52**, 302–310.
[15] Buettner, G. (1987) "Spin trapping: EPR parameters of spin adducts", *Free Radic. Biol. Med.* **3**, 259–303.
[16] Green, D.R. and Reed, J.C. (1998) "Mitochondria and apoptosis", *Science* **281**, 1309–1312.
[17] Li, F.-J., Kondo, T., Zhao, Q.-L., Hayashi, Y., Ogawa, R., Cui, Z.-G. and Feril, Jr., L.B. (2002) "A lipophilic free radical initiator, 2,2′-azobis (2,4-dimethylvaleronitrile) (AMVN) enhances caspase-dependent apoptosis induced by hyperthermia", *Int. J. Hyperther.*, In press.
[18] Hiraoka, W., Fuma, K. and Kuwabara, M. (1997) "Concentration-dependent modes of cell dath in Chinese hamster V79 cells after treatments with H_2O_2", *J. Radiat. Res.* **38**, 95–102.
[19] Nakagawa, S. (1998) "Antitumor effect of hyperthermia combined with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)", *J. Kyoto Pref. Univ. Med.* **107**, 323–331.
[20] Levine, E.L., Davidson, S.E., Roberts, S.A., Chadwick, C., Potten, C.S. and West, C.M.L. (1994) "Apoptosis as predictor

of response to radiotherapy in cervical carcinoma", *Lancet* **344**, 472.
[21] Wheeler, J.A., Stephens, L.C., Tornos, C., Eifel, P.J., Kian Ang, K., Milas, L., Allen, P.K. and Meyn, R.E., Jr. (1995) "Apoptosis as a predictor of tumor response to radiation in stage Ib cervical carcinoma", *Int. J. Radiat. Oncol. Biol. Phys.* **32**, 1487–1493.
[22] Ohno, T., Nakano, T., Niibe, Y., Tsuji, H. and Oka, K. (1998) "Bax protein expression correlates with radiation-induced apoptosis in radiation therapy for cervical carcinoma", *Cancer* **83**, 103–110.
[23] Harima, Y., Harima, K., Shikata, N., Oka, A., Ohnishi, T. and Tanaka, Y. (1998) "Bax and Bcl-2 expression predict response to radiotherapy in human cervical cancer", *Cancer Res. Clin. Oncol.* **124**, 503–510.
[24] Yuki, H., Fujimura, M., Yamakawa, Y., Hidaka, T. and Saito, S. (2000) "Detection of apoptosis and expression of apoptosis-associated proteins as early predictors of prognosis after irradiation therapy in stage IIIb uterine cervical cancer", *Jpn. J. Cancer Res.* **91**, 127–134.
[25] Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. and Howley, P.M. (1990) "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53", *Cell* **63**, 1129–1136.
[26] Seedorf, K., Oltersdorf, T., Krammer, G. and Rowekamp, W. (1987) "Identification of early proteins of the human papilloma viruses type 16 (HPV16) and type 18 (HPV 18) in cervical carcinoma cells", *EMBO J.* **6**, 139–144.
[27] Takahashi, K., Inanami, O., Hayashi, M. and Kuwabara, M. (2002) "Protein synthesis-dependent apoptotic signalling pathway in X-irradiated Molt-4 human leukaemia cell line", *Int. J. Radiat. Biol.* **78**, 115–124.
[28] Kwon, D., Choi, C., Lee, J., Kim, K.O., Kim, J.D., Kim, S.J. and Choi, I.H. (2001) "Hydrogen peroxide triggers the expression of Fas/FasL in astrocytoma cell lines and augments apoptosis", *J. Neuroimmunol.* **113**, 1–9.