Thermosensitization by parthenolide in human lung adenocarcinoma A549 cells and p53- and hsp72-independent apoptosis induction via the nuclear factor-κB signal pathway

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Received December 5, 2007; Accepted January 25, 2008

Abstract. The thermo-enhancement effects of the sesquiterpene lactone parthenolide (PTL), which targets the transcription factor nuclear factor-κB (NF-κB), and hyperthermia at 40, 42 and 44˚C on the human lung adenocarcinoma A549 cell line were investigated *in vitro*. Thermotherapy using a combined treatment with PTL (0.02 μM) prior to hyperthermia showed synergistic thermo-enhancement effects towards A549 cells. The expression of p53 and hsp72 proteins following the application of PTL and hyperthermia at 44˚C, both alone and in combination, were examined to investigate whether p53 and hsp72 participated in apoptosis induction via the NF-κB signal pathway. After treatment with PTL alone, Hsp72 was only slightly induced, which was the same as for the control, while the level following the combination treatment was not significantly different as compared with hyperthermia alone. In addition, the level of p53 after the combination treatment was only slightly increased in comparison with hyperthermia alone. The kinetics of apoptosis and necrosis induction during the incubation periods following PTL exposure and hyperthermia, and the combination of both were also determined. The incidence of apoptosis following hyperthermia alone was approximately 0.6% on average after 12, 24 and 48 h of incubation, while that of PTL alone was approximately 1.7%, and that with the combination treatment was around 2.3%. Thus, induction of apoptosis following the combination treatment was increased as compared to each treatment alone. With regard to the kinetics of necrosis, the incidence of necrosis after treatment with hyperthermia alone was approximately 2.7%, while that with the combination treatment was lower, at around 2.2%. We hypothesized that cells treated with PTL had an altered arrangement of stressed cells undergoing the transformation from necrotic cell death to apoptotic cell death via another mechanism. Our results suggested that the PTL-induced apoptosis of A549 cells was due to the direct suppression of NF-κB activity in a p53- and hsp72-independent manner based on NF-κB signaling.

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Key words: parthenolide, nuclear factor-κB, hyperthermia, thermosensitivity, A549 cells, p53, hsp72, apoptosis, necrosis

Introduction

Parthenolide (PTL), a sesquiterpene lactone isolated from feverfew (*Tanacetum parthenium*), is the principal ingredient of the medicinal herb *Magnolia grandiflora* L (1) and it has been reported that PTL, extracted from its leaves and stems has shown antitumor activity (2-4). Furthermore PTL is conventionally used in the west to treat such conditions as migraine and inflammation (5-7).

Patel *et al* reported that the transcription factor nuclear factor-κB (NF-κB) regulates genes that are important for tumor invasion, metastasis, and chemoresistance, and that PTL mimicked the effects of IκBα by inhibiting NF-κB DNA binding activity (8). The antitumor activity of PTL is due to its ability to inhibit DNA binding of NF-κB and STAT-3, reduce MAP kinase activity, and induce the production of reactive oxygen species (9). Walmsley *et al* reported that hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity and that the inhibition of NF-κB by PTL resulted in the abrogation of hypoxic survival (10). Furthermore, Sweeney *et al* showed that PTL has an ability to inhibit DNA binding of the antiapoptotic transcription factor NF-κB and activation of JNK, and that it was effective in reducing the expression of the pro-metastatic gene IL-8 and the antiapoptotic gene GADD45ß1 (11).

The effects of PTL in combination chemotherapy have been reported (12-15), however, there is no known study of its effects in combination with hyperthermia. When cancer cells are exposed to hyperthermia, they usually acquire thermotolerance during heating or afterwards, which is one of the problems associated with thermotherapy (16-18). In an attempt to overcome the problems of thermotolerance or drug resistance, thermo-chemotherapy, a combined treatment with hyperthermia and chemotherapeutic agents or chemical substances, has become widely adopted as an interdisciplinary cancer treatment strategy to obtain greater therapeutic benefits by sensitizing the cytotoxicity of the chemotherapeutic drug utilized. We previously reported that anthracycline derivatives, amrubicin (AMR) and its active metabolite amrubicinol (AMROH), showed significant thermo-enhancement effects *in vitro* towards human lung adenocarcinoma A549 cells (19). When A549 cells were treated with AMR or AMROH prior to heating at 44˚C , the sublethal thermal damage repair (SLTDR) elicited during the early stages of hyperthermia was inhibited, while the thermosensitivity of A549 cells to 44˚C was enhanced in an additive manner. Comparable results were obtained in about one-third of the treatment times performed by combining hyperthermia with AMR or AMROH (19).

p53, a tumor suppressor protein, has been reported to induce apoptosis (20,21) and cell growth arrest (22) after external and transitional stimuli, such as heating (23), ionizing radiation (24), ultraviolet irradiation (25), and hypoxia (26,27), as well as the following application of internal stimuli including activation of an oncogene (28), and abnormal nucleotide (29) and cytokine (30) metabolism. Lee reported that wild-type p53 (wtp53) induced apoptosis for those cells which received transitional physiological stress, so that wtp53 protein resulted in increased cellular responsiveness to the stress, and the stressed cells going to apoptosis (31). However, these findings are controversial, because some studies have shown relationships between the p53 and NF-κB signaling pathways. For example, apoptosis was induced by the inhibition of NF-κB activity through p53-independent mechanisms (32,33), while the induction of apoptosis was mediated through p53-dependent mechanisms, which regulated NF-κB activity (34-36). We also reported that the effects of cellular thermo- and radio-sensitivity in A549 cells with the wtp53 gene (37) and with the muted K-ras gene (38) were significantly enhanced by AMR or AMROH, which was due to the induction of a greater number of necrotic cells than apoptotic cells in a p53-independent manner (19,39). In the present study, we examined the thermo-enhancement effects of PTL on the thermosensitivity of A549 cells, with the above-mentioned genetic backgrounds, in a kinetic manner, and also analyzed the relationship of p53 with the inhibition of NF-κB activity by PTL in combination with hyperthermia at 44˚C. Using Western blot analysis, the expression of p53 protein was determined at various time points following the application of PTL and hyperthermia, and a combination of both.

Cells exposed to nonlethal elevated temperatures develop a resistance to subsequent severe heat stress, which is called acquired thermotolerance. We previously reported, i) the enhancement effect of preheating at 42˚C on the lethal chemosensitivity of thermotolerant Chinese hamster V79 cells to adriamycin (ADM), ii) that the effect of ADM on survival rate reduction of step-up (42-44˚C) thermotolerant V79 cells, has been shown to have an interactive effect, and iii) that the thermosensitivity of V79 cells at 42˚C after simultaneous treatment with ADM, showed marked thermo-enhancement within the short term of less than 1 h, but over a longer period than this, the cells showed reduced thermosensitivity (40). Hsp72, stress-inducible hsp70, was markedly accumulated after application of hyperthermia alone at 42 or 44˚C, but it was reduced by ADM combined with hyperthermia at 42˚C (40). This protection has mainly been attributed to members of the hsp70 family, which are induced by mild heat shock (41,42). In the present study, the kinetics of hsp72 expression following treatment with hyperthermia at 44˚C and PTL, and a combination of both, was analyzed to determine the role of heat-induced hsp72 in the inhibition of NF-κB activation by PTL.

Murine L cells with wtp53 showed high thermosensitivity and hyperthermia effectively induced apoptosis (16), though in A549 cells with wtp53 apoptosis was not increased significantly by hyperthermia and AMR, as mentioned above (19,39). In the present study, we examined the induction of apoptosis and necrosis to elucidate the possible mechanism for sensitization by PTL in A549 cells, which had the wtp53 gene and were thermotolerant, over various time periods following PTL treatment combined with hyperthermia at 44˚C.

We analyzed the effects of PTL on the sensitivity of A549 cells to these treatments in regard to apoptosis and necrosis at the kinetic and molecular levels, dependent on p53 or hsp72 levels in the NF-κB signal pathway.

Materials and methods

Cells and culture medium. A549 cells, from a human non-small-cell lung adenocarcinoma cell line, were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing NCTC-135 (Gibco Laboratories, Grand Island, NY), lactalbumin hydrolysate solution (Difco Laboratories, Detroit, MI), and 15% newborn calf serum (Gibco) (hereinafter referred to as MLN-15) under conventional conditions at 37˚C in a humidified incubator with 5% CO_2 in 95% air.

PTL, hyperthermia and combined treatments. PTL (MP Biomedicals LLC, OH) was dissolved in culture medium to an appropriate final concentration (0.02 μM) prior to being used for treatment. Adhered cells on the inner face in the bottom of the flasks were exposed with PTL by replacement with 6 ml of PTL solution in MLN-15 for graded time periods, the PTL solution was chased, and the adhered cells were gently rinsed twice with culture medium containing 3% serum and re-fed with 6 ml of the culture medium, MLN-15 at 37˚C. The treated flasks were incubated in a stationary manner to determine visible colony formation.

Hyperthermia was performed by immersion of the culture flasks fixed with tightened screw tops in a temperature-regulated water bath (Model EPS-47, Tokyo Seisakusho Co., Tokyo, Japan) pre-set at 40, 42 or 44˚C, with the temperature

maintained within ±0.05˚C as measured with a thermistor (Model D116-1251, Takara Thermistor Instruments Co., Yokohama, Japan). For the combined treatment, applications of PTL and hyperthermia were carried out sequentially. Cells were treated with PTL for 3 h, then rinsed twice with culture medium containing 3% serum, placed in MLN-15, and subjected to hyperthermia. Kinetic assessments of the sensitivity of A549 cells to PTL and hyperthermia were carried out using colony forming assays and the results were corrected based on the plating efficiency of the control cells (i.e. 80-90%). The average colony multiplicity was <1.1. The T_0 value, adopted as the criterion of cellular thermo- and herbal medicine sensitivity, represented the treatment period required to reduce survival by 1/e in the exponentially regressing portion of the survival curves, i.e. the linear portions of the treatment period shown in semilogarithmic survival curves.

Assays of p53 and hsp72 proteins. The accumulation of p53 and hsp72 proteins in cells incubated at 37˚C for various periods after the application of hyperthermia, PTL, and a combination of both were examined by Western blot analysis. The treated cells were rinsed with PBS (-), then centrifuged and pelleted at 4˚C. A series of the cell pellets thus obtained was dissolved in RIPA lysis buffer (43), and treated by freezing and thawing 3 times. The proteins obtained from supernatants after centrifugation (10,000 x g, 15 min) were quantified using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Proteins in 5- and 20-μg aliquots were used for Western blot analyses of p53 and hsp72, respectively. After electrophoresis on 10% w/v polyacrylamide gels containing 0.1% w/v SDS, the proteins were transferred to Poly-Screen PVDF membranes (DuPont/Biotechnology Systems NEN Research Products, Boston, MA), then the proteins on each membrane were incubated with anti-p53 (Pab421; Oncogene Sci. Inc., Uniondale, NY) and anti-hsp72 (C92F3A5; Stress-Gen, Biotechnologies Corp., Victoria, BC, Canada) monoclonal antibodies. The bands were visualized using horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Zymed Laboratories Inc., San Francisco, CA) and amplified with the BLAST-R Blotting Amplification System (DuPont/Biotechnology Systems NEN Research Products). The relative amounts of p53 and hsp72 as compared to the controls were calculated from the scanning profiles of the bands obtained using a Windows XP-equipped computer with the public domain NIH Image Program, written by Wayne Rasband of the US NIH and available by anonymous FTP from zippy.nimh.nih.gov. or on floppy disk from NTIS (Springfield, VA, part no. PB93-504868). Two-fold serial dilutions of the samples were analyzed to verify the linearity of the detection system.

Analysis of apoptosis and necrosis. Induction of apoptosis was analyzed at 12, 24, and 48 h after treatment with 0.02 μM of PTL for 3 h, hyperthermia at 44˚C for 15 min, or a combination of both. Trypsinized cells were placed in MLN-15 to stop trypsinization, then rinsed with PBS (-), fixed overnight in 1% glutaraldehyde (Nacalai Tesque Inc., Kyoto, Japan), and placed in PBS (-). For observation, cells were stained with 0.2 mM Höchst 33342; Bisbenzimide H 33342 Fluorochrome, Trihydrochloride (Calbiochem, Merck KgoA, Dormstodt, Germany) and analyzed under a fluorescence microscope (Nikon Eclipse E600, Nikon Co. Ltd, Tokyo, Japan). A total of 2000 cells were identified, and the percentage of typical apoptotic cells was calculated. The induction of necrosis was also determined following the above-described treatments. After the cells were double-stained using acridine orange (Sigma-Aldrich Co., St. Louis, MO) and ethidium bromide (Nacalai Tesque Inc., Kyoto, Japan) (AO/EB), they were observed in real time under a fluorescence microscope, and the percentage of typical necrotic cells was calculated.

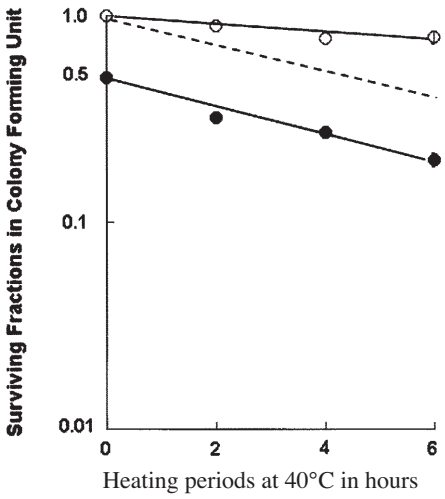

Figure 1. The 40˚C heating period and survival relationships of A549 cells. The thermo-enhancement effect of PTL (0.02 μM) in A549 cells was examined. Ordinate, log surviving fractions (SFs) in colony forming unit; abscissa, heating periods at 40˚C in hours. Open circles represent SFs of cells treated with 40˚C hyperthermia alone. Closed circles represent SFs of cells treated with PTL for 3 h (LD_{50}) followed by hyperthermia at 40˚C for graded periods. Symbols with vertical bars represent means with standard deviation (SD) obtained from at least three data sets. Symbols without a bar represent the SD within the symbols. The broken line represents the curve normalized with combination treatments.

Results

Heating period and survival relationships of A549 cells at 40, 42 and 44˚C . The survival curve at 40˚C to 6 h was drawn as a straight slope which showed slight heat-induced cell death as shown in Fig. 1. Cellular lethal thermosensitivity was estimated as the T_0 value. As for the thermosensitivity of A549 cells, the T_0 value (heating time required to reduce survival by 1/e), which was reciprocal to the straight slope of the survival curve in the exponential phase, was 27.2 h for hyperthermia at 40˚C alone, and 6.5 h for the combination therapy with PTL and hyperthermia at 40˚C as shown in Table I.

When A549 cells were heated alone at 42˚C for graded periods up to 4 h, the thermosensitivity was moderate, as shown in Fig. 2. In the initial part of the survival curve, SLTDR was shown in the 'shoulder' profile followed by exponential regression appearing within the 42˚C heating periods of 1.5-3 h in the survival curve. With regard to heating periods >3 h, surviving fractions further regressed exponentially in a gentler slope of the survival curve as

Table I. T_0 values of A549 cells in survivals from treatment with hyperthermia at 40, 42 or 44˚C and with hyperthermia in combination with PTL.

	Hyperthermia alone T_{01}	PTL (3 h) and hyperthermia T_{02}	Enhancement ratio T_{01}/T_{02}
40˚C	27.14 h	6.54 h	4.15
42˚C (1)	1.74 h	0.70 h	2.49
42˚C (2)	2.96 h	2.37 h	1.25
44˚C	15.5 min	11.5 min	1.35

The thermo-enhancement effects of thermotherapy with PTL in NF-κB inhibitor were compared by T_0 value. A549 cells were treated with PTL (0.02 μM) for 3 h and followed by hyperthermia at 40, 42 or 44˚C in the combined treatment. (1), before the induction of thermotolerance; (2), after the induction of thermotolerance.

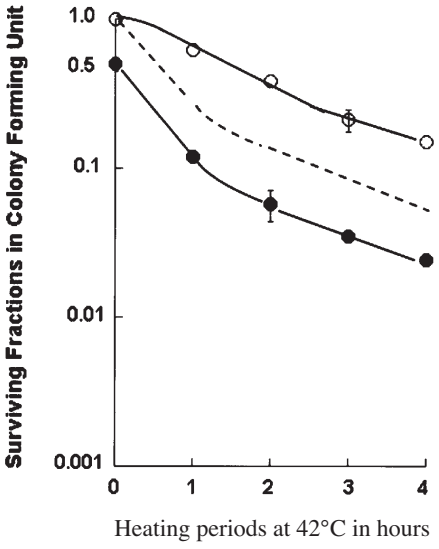

Figure 2. The 42˚C heating period and survival relationships of A549 cells. The thermo-enhancement effect of PTL (0.02 μM) in A549 cells was examined. Ordinate, log surviving fractions (SFs) in colony forming unit; abscissa, heating periods at 42˚C in hours. Open circles represent SFs of cells treated with 42˚C hyperthermia alone. Closed circles represent SFs of cells treated with PTL for 3 h (LD_{50}) followed by hyperthermia at 42˚C for graded periods. Symbols with vertical bars represent means with standard deviation (SD) obtained from at least three data sets. Symbols without a bar represent the SD within the symbols. The broken line represents the curve normalized with combination treatments.

thermotolerance developed, as shown with open circles in Fig. 2. The T_0 value of the cells heated at 42˚C alone was 1.7 h before the induction of thermotolerance and 3.0 h after the induction of thermotolerance as shown in Table I. When A549 cells were treated at 44˚C for up to 40 min, lower levels of thermosensitivity were seen, as shown in Fig. 3. The survival curve with hyperthermia at 44˚C also showed SLTDR as well as that at 42˚C, followed by an exponential regression curve. The T_0 value of cells heated at 44˚C alone was 15.5 min, as shown in Table I.

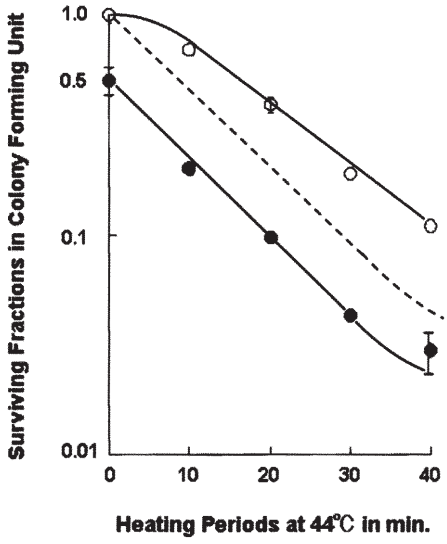

Figure 3. The 44˚C heating period and survival relationships of A549 cells. The thermo-enhancement effect of PTL (0.02 μM) in A549 cells was examined. Ordinate, log surviving fractions (SFs) in colony forming unit; abscissa, heating periods at 44˚C in min. Open circles represent SFs of cells treated with 44˚C hyperthermia alone. Closed circles represent SFs of cells treated with PTL for 3 h (LD_{50}) followed by hyperthermia at 44˚C for graded periods. Symbols with vertical bars represent means with standard deviation (SD) obtained from at least three data sets. Symbols without a bar represent the SD within the symbols. The broken line represents the curve normalized with combination treatments.

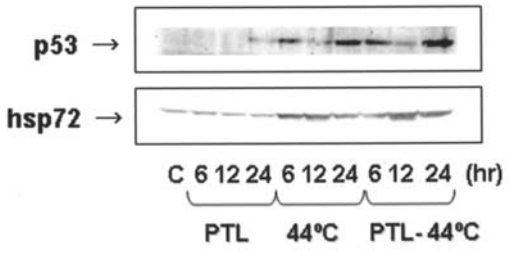

Figure 4. Western blot analysis of cellular amounts of p53 (upper panel) and hsp72 (lower panel) at graded periods of incubation intervals after treatment of A549 cells with PTL (0.02 μM) for 3 h in LD_{50}, hyperthermia at 44˚C for 15 min in LD_{50} and a sequential combination of both. Numerals on the horizontal line indicate 37˚C-incubation intervals in h between these treatments and the blotting, respectively, and the 'C' on the left indicates the lane for p53 or hsp72 in the non-treated control A549 cells.

Effects of PTL on hyperthermia. Using A549 cells, the thermo-enhancement effects of PTL were investigated based on the survival curves drawn after combination therapy with PTL and hyperthermia, as shown in Figs. 1-3. When the cells were treated with 0.02 μM of PTL for 3 h, the 50% surviving fraction was determined (LD_{50}) (closed circle on the ordinate). The survival curve of the cells that received thermotherapy alone at 42˚C or 44˚C elicited a shoulder, indicating SLTDR, whereas the curve for those cells receiving treatment with PTL prior to hyperthermia was reduced synergistically. In other words, SLTDR associated with short periods of heating was blocked by PTL, as shown in Figs. 2 and 3. Furthermore, thermotolerance induced by heating at 42˚C alone for 1.5-3 h was not reduced by PTL

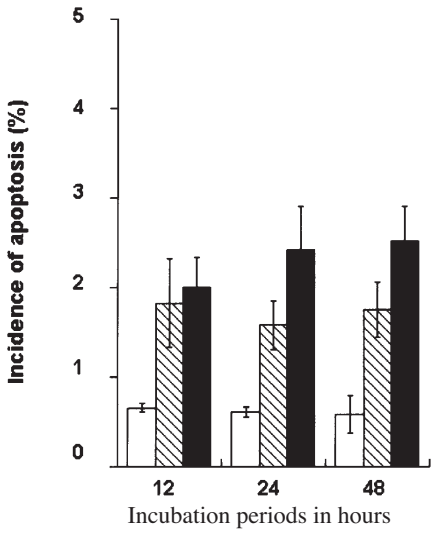

Figure 5. Kinetics of apoptosis induction after 12-, 24- and 48-h incubation periods following exposure to 44˚C hyperthermia or PTL or a combination of both. At the indicated times, A549 cells stained with Höchst 33342 were observed under a fluorescence microscope. The cells of DNA-fragmented nuclei with typical morphological features of apoptosis were counted as apoptotic cells. Ordinate, percentage of apoptosis in the total cell count; abscissa, 37˚C-incubation periods in h after treatment. Columns represent 44˚C hyperthermia for 15 min, PTL (0.02 μM) exposure for 3 h and the combined treatments, respectively from left. The incidence of apoptosis following the combined treatment was induced additively compared to treatment with hyperthermia or PTL alone.

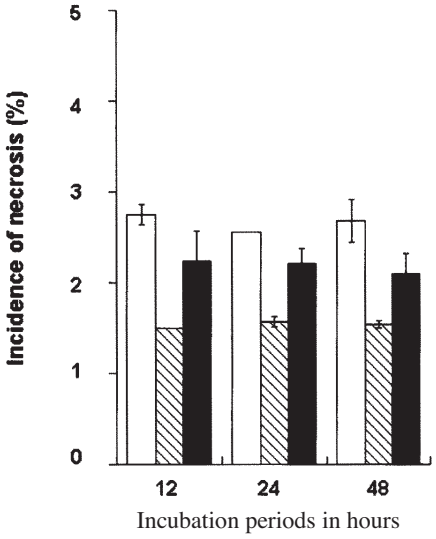

Figure 6. Kinetics of necrosis induction after 12-, 24- and 48-h incubation periods following exposure to 44˚C hyperthermia or PTL or a combination of both. At the indicated times, A549 cells double stained with AO/EB were observed under a fluorescence microscope. Rubious cells were counted as necrotic. Ordinate, percentage of necrosis in the total cell count; abscissa, 37˚C-incubation periods in h after treatment. Columns represent 44˚C hyperthermia for 15 min, PTL (0.02 μM) exposure for 3 h and the combined treatments respectively from left. The incidence of necrosis following the combined treatment with both hyperthermia and PTL was less than with hyperthermia alone.

treatment (Fig. 2). The T_0 values and enhancement ratios determined by the survival curves are shown in Table I. The thermosensitivity of A549 cells subjected to combination therapy showed synergistic enhancement effects of ~4.2-fold at 40˚C, ~2.5-fold before thermotolerance induction at 42˚C and 1.3-fold after the induction of thermotolerance, and ~1.3-fold at 44˚C, respectively.

Western blot analysis following treatment with PTL and hyperthermia, and a combination of both. The increases in amounts of intracellular accumulation of p53 and hsp72 proteins over time were qualitatively analyzed by Western blotting, as shown in Fig. 4. Qualitative observations for p53 indicated that its accumulation was increased slightly by PTL treatment after 24 h of incubation, while there was very little change in quantity after 6 and 12 h. In contrast p53 accumulation was significantly induced by hyperthermia at 44˚C from 6 to 24 h after treatment, while it was also induced by the combination treatment after 24 h, whereas no significant changes were seen after 6 and 12 h. As for hsp72, qualitative observations indicated that its accumulation was low and not appreciably changed by PTL at 6, 12 and 24 h after treatment. However, the accumulation of hsp72 was increased by hyperthermia alone at 44˚C in comparison with the control, whereas no significant increase in accumulation of hsp72 was seen after the combination treatment.

Kinetics of apoptosis induction following exposure to PTL and hyperthermia, and a combination of both. The thermo-enhancement effects of PTL on A549 cells were investigated on the basis of apoptosis induction, as shown in Fig. 5. Following incubation for 12, 24 and 48 h at 37˚C, the cells were analyzed for morphologic changes by observation under a fluorescence microscope at different time points. The number of typical apoptosis cells stained with Höchst 33342 was counted and the ratio of apoptotic cells to the total among 2000 cells was determined. The incidence of apoptosis after treatment with hyperthermia alone at 44˚C averaged ~0.6% after 12, 24 and 48 h. In the same way, the incidence of apoptosis by PTL exposure averaged ~1.8%, while that with the combination therapy averaged ~2.4%. Thus, when the cells were treated with PTL prior to exposure to hyperthermia at 44˚C, the incidence of apoptosis increased ~4-fold as compared with hyperthermia alone.

Kinetics of necrosis induction following exposure to PTL and hyperthermia, and a combination of both. The thermo-enhancement effects of PTL on A549 cells were also investigated in regard to the induction of necrosis as shown in Fig. 6. The incidence of necrosis following exposure to PTL or hyperthermia at 44˚C, and a combination of both was investigated by AO/EB double staining at intervals of 12, 24 and 48 h after treatment. The incidence of necrosis after treatment with PTL alone was ~1.5, 1.6 and 1.5%, respectively, while it was ~2.8, 2.6 and 2.7%, respectively, following hyperthermia alone at 44˚C, and ~2.2, 2.2 and 2.1%, respectively, following the combination treatment, (lower than with hyperthermia alone).

Discussion

Previously, we investigated the abilities of various chemicals, including adriamycin (doxorubicin), bleomycin, cisplatin,

and amrubicin and its metabolite amrubicinol, to modify the effects of hyperthermia at kinetic and molecular levels (40,44-46). In 1997, Bork *et al* reported that the sesquiterpene lactone parthenolide (PTL), found in medicinal herbs, inhibited the activation of transcription factor NF-κB (47). Later, Markovic *et al* reported that short-term hyperthermia prevented the activation of proinflammatory genes in fibroblast-like synoviocytes by blocking the activation of NF-κB (48). In the present study, we investigated the modification of thermosensitivity by PTL in a human lung cell line, A549, as well as the relationship of p53 and hsp72 via the NF-κB signal pathway. In addition, the induction of apoptosis and necrosis was investigated following treatment with PTL or hyperthermia at 44˚C, alone, and treatment with these in sequential combination.

Thermo-enhancement effects of PTL on hyperthermia. Clinically, hyperthermia is usually performed in combination with another treatment modality, such as radio- or chemotherapy. In the present study, PTL was shown to dynamically enhance the thermosensitivity of A549 cells, as shown in Figs. 1-3. Thermosensitivity was synergistically enhanced by treatment with PTL followed by exposure to hyperthermia at 40˚C, as shown in Fig. 1. The T_0 results were 27.2 h for hyperthermia at 40˚C alone, and 6.5 h for hyperthermia at 40˚C combined with PTL exposure (Table I). Thus, combination therapy with PTL and hyperthermia at 40˚C had thermo-enhancement effects 4.2-fold greater than hyperthermia at 40˚C alone. We have previously found that the SLTDR seen during the early stages of hyperthermia (19), and repair of sublethal damage, associated with fractionated X-ray irradiation (39), were blocked by the topoisomerase II inhibitors AMR and AMROH, respectively.

In this study, it was clarified that SLTDR elicited by hyperthermia in A549 cells was blocked by PTL, which targets the transcription factor NF-κB. When A549 cells were treated with PTL prior to hyperthermia at 42˚C for various periods, SLTDR was inhibited, though the thermotolerance that appeared before and after 2 h of heating was not prevented (Fig. 2). Similarly, SLTDR shown by the survival curve of cells treated with PTL and hyperthermia at 44˚C was also inhibited and showed slight synergistic enhancement (Fig. 3). In comparison with the enhancement ratio shown in Table I, combined treatments with PTL and hyperthermia at 40, 42, and 44˚C showed a greater enhancement effect with mild hyperthermia as compared to the higher temperature.

Expression of p53 and hsp72 proteins following treatment. A number of different cellular stimuli result in the induction of the tumor suppressors p53 and NF-κB. However in contrast to the activation of p53, which is associated with the induction of apoptosis, the stimulation of NF-κB has been shown to promote resistance to programmed cell death. Webster *et al* reported transcriptional crosstalk between NF-κB and p53 (49). Furthermore, Yang *et al* characterized the mechanisms of NF-κB DNA binding activity and p53 activation following ionizing radiation (50), while Ryan *et al* reported that the inhibition of NF-κB abrogates p53-mediated apoptosis (51). In the present study, the cellular content of p53 protein in A549 cells with the wild-type p53 gene was not induced by PTL exposure after 6 and 12 h, and was only slightly evident after 24 h (Fig. 4). Following sequential treatment with PTL and hyperthermia at 44˚C, p53 was increased to the same degree as with hyperthermia alone. Thus, in A549 cells, p53 protein was induced by treatment with heating alone, but not by PTL alone. These results suggest that following combined treatment with PTL and hyperthermia at 44˚C, heat-induced p53 did not play a significant role in the inhibition of NF-κB activation by PTL.

Tumor cells develop tolerance to thermo-, radio-, and chemotherapy due to induction of the heat shock protein hsp72, which leads to problems clinically. We previously showed that an initial combined short-term treatment of hyperthermia and adriamycin (ADM) resulted in markedly enhanced cytocidal effects. Furthermore, Western blot analysis following this combined treatment showed a markedly reduced accumulation of hsp72, lower than that estimated to occur after hyperthermia alone, which suggested the inhibition of hsp72 accumulation by ADM (40). Meldrum *et al* reported that liposomal delivery of hsp72 inhibited ischemia-induced renal tubular cell apoptosis by preventing NF-κB activation and subsequent TNF-α production (52). On the other hand, Chase *et al* reported that hsp72 induced inflammation and regulated cytokine production in airway epithelia through a TLR4- and NF-κB-dependent mechanism and that hsp72 induced a dose-dependent increase in IL-8 expression, which was inhibited by the NF-κB inhibitor parthenolide (53).

In the present study, accumulation of hsp72 following treatment with hyperthermia alone at 44˚C was induced in an ordinary manner, while treatment with PTL did not induce this accumulation (Fig. 4). With regard to the quantity of heat-induced hsp72 following combination therapy with PTL and hyperthermia at 44˚C, there was little difference as compared with hyperthermia alone. These results suggest that heat-induced hsp72 had no relationship with NF-κB signaling in regard to the thermotolerance of cells.

Kinetics of apoptosis and necrosis. Mayo *et al* reported that NF-κB activation suppressed the induction of p53-independent apoptosis (32), while Kalra *et al* found that apoptosis in human prostate cells was mediated through two related pathways: the up-regulation of p53 and down-regulation of NF-κB activity (54). We investigated the kinetics of apoptosis over various time periods following exposure to PTL and hyperthermia, and a combination of these, as shown in Fig. 5. The incidence of apoptosis following treatment with hyperthermia at 44˚C alone was ~0.6% on average at the 3 different time points, while that with 0.02 μM of PTL alone was ~1.8%. Furthermore, the induction of apoptosis following hyperthermia at 44˚C in combination with exposure to 0.02 μM of PTL increased significantly, with an additive increase of ~2.4% on average.

As in the case of apoptosis, the kinetics of necrosis induction following treatment with PTL and hyperthermia, or a combination of these were examined, as shown in Fig. 6. The induction of necrosis following hyperthermia at 44˚C was increased by ~2.7% on average at 3 time points, while after 3 h of PTL exposure followed by hyperthermia at 44˚C it was only increased by ~2.2%, which was less than the

increase with thermotherapy alone (2.7%). These results showed that cell death by heating was caused more by the induction of necrosis than apoptosis. Necrosis diminished with a combination of PTL exposure followed by heating, because the cells programmed for necrosis altered their arrangements to undergo apoptosis. These results also indicate that there is no involvement of p53 and hsp72 in the NF-κB signaling pathway, though apoptotic cell death is favorable for tumor cell death. Cory and Cory investigated the molecular basis of the differences in cells that lead to apoptotic or necrotic responses to drugs, and reported that drug-resistant p53-deficient cells showed much greater apoptotic than necrotic responses to an inhibitor of IκBα kinase (55).

Cellular thermosensitivity was provided by combined treatment with PTL, an NF-κB inhibitor, and subsequent hyperthermia in human non-small-cell lung adenocarcinoma A549 cells with the wild-type *p53* gene. The mechanisms involved appear to induce apoptosis due to direct suppression of the activation of the transcription factor NF-κB in a p53-independent and heat-induced hsp72-independent manner via the NF-κB signaling pathway. We concluded that the use of medicinal herbs containing parthenolide was beneficial for patients undergoing cancer thermotherapy as a treatment option without side effects. Furthermore, in multidisciplinary anticancer therapy, the therapeutic value of hyperthermia can be improved by administering PTL prior to hyperthermia.

Acknowledgements

The present work was supported in part by Grant-in Aid of the Ministry of Education, Science and Culture, Japan, for Scientific Research (C), No. 19591456, Analysis of mechanisms on apoptosis induction by NF-κB inhibitor parthenolide, 2007-2009.

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