Inhibition of NF-κB by combination therapy with parthenolide and hyperthermia and kinetics of apoptosis induction and cell cycle arrest in human lung adenocarcinoma cells

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Received August 12, 2009; Accepted October 6, 2009

DOI: 10.3892/ijmm_00000316

Abstract. We investigated the mechanisms of thermosensitization related to combination therapy with sesquiterpene lactone parthenolide (PTL), a nuclear factor-κB (NF-κB) inhibitor, and hyperthermia using human lung adenocarcinoma cells A549. The kinetics of apoptosis induction and cell cycle of cells treated with PTL, heating, and combined treatment were examined by flow cytometric analysis. The flow cytometric distribution was calculated and expressed as a percentage. The ratios of the sub-G_1 division, used to determine the induction of apoptosis, increased significantly with the combination therapy. Furthermore, the ratios of G_2/M division increased and the ratios of G_0/G_1 division decreased, indicating cell cycle arrest in G_2/M. The cell phase response to PTL by A549 cells synchronized in the G_1/S border with hydroxyurea was also analyzed. PTL showed remarkable cytotoxicity at the S phase of the cell cycle in A549 cells at all concentrations as well as with hyperthermia, thus PTL reduced the number of cells in the proliferation phase. Inhibition of intracellular transcription factor NF-κB activation in A549 cells with various incubation periods after treatments with PTL, heating and combined treatment was examined by Western blot analysis. Unexpectedly, PTL alone did not inhibit NF-κB activation in cells stimulated with TNF-α, while heating alone inhibited NF-κB early after treatment and that effect faded over time. In contrast, PTL combined with heating completely inhibited NF-κB activation. Our results demonstrated that PTL and heating in combination cause significant thermosensitization of A549 cells via induction of apoptosis or cell cycle arrest in G_2/M by inhibiting NF-κB activation in a synergistic manner.

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Key words: parthenolide, hyperthermia, nuclear factor-κB, apoptosis, G_2/M arrest, cell-phase response, A549 cells

Introduction

Parthenolide (PTL), a sesquiterpene lactone isolated from feverfew (*Tanacetum parthenium*) by extraction from its leaves and stems, is the principal ingredient of the medicinal herb *Magnolia grandiflora* L (1) and has also been reported to have antitumor activity (2-4). We previously found that combination therapy with PTL and heating at 40, 42 or 44˚C showed synergistic thermosensitization effects on human lung adenocarcinoma A549 cells with *wtp*53, which resulted in p53- and hsp72-independent apoptosis induction via the nuclear factor-κB (NF-κB) signal pathway (5). We continued to investigate this issue by investigating the cell phase response to PTL by cells synchronized with hydroxyurea (HU), and cell cycle distribution by flow cytometric analysis, and we also assessed the inhibition of NF-κB activation in regard to the mechanisms of thermosensitization by combined treatment with PTL and hyperthermia using human lung adenocarcinoma A549 cells.

In our previous report of Murine L cell survival, treatment with bleomycin prior to low hyperthermia showed appreciable thermal enhancement effects, which were caused by a blockade of the repair of sublethal damage, while it was also clarified that bleomycin did not elicit cell phase responses in the cell cycle (6). Thereafter in a study of A549 cell sensitivity, amrubicin or its active metabolite amrubicinol, both topoisomerase II inhibitors, in combination with hyperthermia showed significant thermosensitization, whereas cell phase

responses to those agents when administered separately showed no changes in the cell cycle (7). It is well known that the cell phase response of cells to hyperthermia has high sensitivity in the S phase of the cell cycle (8,9), while X-ray irradiation causes sensitivity in the G_2/M phase in a manner that is opposite to hyperthermia, which is similar to a mirror image in regard to the curves on a graph (10,11). With respect to the cell cycle, it is understandable that combination treatments of hyperthermia (high sensitivity in the S phase) with radiation (high sensitivity in the G_2/M phase), or another agent, exhibit cell phase response, such as that seen with radiation, and produce appreciable antitumor effects because when the two agents are given, each survival curve causes mirror-image cell phase responses (12-14). In the present study, we examined the cell phase responses of A549 cells synchronized into G_1/S phase with HU to PTL at various concentrations (0.02, 0.2, and 2.0 μM).

A previous study reported that the cytostatic effects of low concentrations of PTL inhibit the growth of tumor lines by labeling the S-phase of BrdU cells, a mouse fibrosarcoma cell line, though the results were unclear (15). Ralstin *et al* found that PTL functions in cooperation with NS398 to inhibit the growth of human hepatocellular carcinoma cells by effects on apoptosis and G_0/G_1 cell cycle arrest (16). In the present study, we investigated the kinetics of apoptosis induction and cell cycle distribution after 0, 6, 12, and 24 h of incubation with PTL, treatment with hyperthermia and combination treatment using flow cytometric analysis.

NF-κB is a dimeric transcription factor that activates the expression of many genes involved in the inflammatory process. In unstimulated cells, NF-κB is retained in the cytoplasm via interaction with its inhibitor IκB (17). In response to various pro-inflammatory stimuli, IκB is rapidly phosphorylated by the IκB kinase complex. This leads to the ubiquitination and subsequent proteasome-mediated degradation of IκB, allowing NF-κB to enter the nucleus (18). The NF-κB p65 subunit at Ser-536 in the C-terminal transactivation domain is also phosphorylated by the IKK complex during the process of IκB degradation (19). A number of reports have noted that PTL suppresses growth of tumor cells by inhibiting the activation of NF-κB. Patel *et al* reported that the transcription factor NF-κB regulates genes that are important for tumor invasion, metastasis, and chemoresistance, and that PTL mimicked the effects of IκB by inhibiting NF-κB DNA binding activity (20). In addition, various studies of PTL have shown cross-talk of dependent and independent genes via the NF-κB signal pathway. Antitumor activity of PTL is due to its ability to inhibit DNA binding of NF-κB and STAT-3, a reactive oxygen species (21). 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 (22). Furthermore, Sweeney *et al* showed that PTL has the ability to inhibit DNA binding of NF-κB, an anti-apoptotic transcription factor, and activation of JNK, and that it was effective in reducing the expression of the pro-metastatic gene IL-8 and the anti-apoptotic gene GADD45ß1 (23). In addition, studies of hyperthermia regarding cross-talk of NF-κB have also been reported. It was reported that activation of NF-κB was delayed in the presence of hyperthermia, whereas IκBα was stabilized in the cytoplasm (24), while another report showed that hyperthermia inhibited the TNF-α-induced an increase in binding activity of NF-κB to DNA (25). It was also reported that hyperthermia induced NF-κB- or TNF-α-mediated apoptosis in normal human gastric cancer cells and human monocytes (25,26). Furthermore, short-term hyperthermia prevented activation of pro-inflammatory genes in fibroblast-like synoviocytes by blocking the activation of NF-κB (27). In the present study, we investigated whether thermosensitization by a combination of PTL and hyperthermia was due to apoptosis induction or cell cycle arrest caused by effective inhibiting of NF-κB activation.

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 (28,29).

PTL, hyperthermia, and combined treatment. Sesquiterpene lactone parthenolide (PTL) (MP Biomedicals LLC, OH, USA) was dissolved in culture medium (MLN-15) to an appropriate final concentration (0.02, 0.2, and 2.0 μM) prior to being used for treatment as PTL solution. Cells adhered to the inner side of the bottom of the flasks and were exposed to PTL by replacement with 6 ml of PTL solution for various time periods. Then the PTL solution was chased, and adhered cells were gently rinsed twice with culture medium containing 3% serum and re-fed to 6 ml of MLN-15 at 37˚C. Hyperthermia was performed by immersing culture flasks equipped with tightened screw tops in a temperature-regulated water bath (Model EPS-47, Tokyo Seisakusho Co., Tokyo, Japan) pre-set at 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 heating were carried out sequentially, during which the cells were exposed to PTL for 4 h, then rinsed twice with culture medium containing 3% serum, placed in MLN-15, and subjected to hyperthermia.

Cell phase responses. Kinetic assessments of cell phase responses to PTL by A549 cells were carried out using colony forming assays. A549 cells were treated with 4 mM HU (Sigma Chemical, St. Louis, MO, USA) (30,31) for 5 h to synchronize them at the G_1-S border, which was followed by treatment with PTL for 4 h after an interval incubation of 0-12 h. The treated flasks were incubated in a stationary manner at 37˚C for ~10 days to determine visible colony formation. The results were corrected based on the plating efficiency of control cells (i.e. 80-90%). The average of colony multiplicity was <1.1.

Analysis of apoptosis and cell cycle distribution. Quantifications of the sub-G_1 fraction for the induction of apoptosis, and the G_0/G_1 and G_2/M fractions for cell cycle arrest at 0, 6, 12, and

24 h of incubation at 37˚C after treatment with 0.2 μM of PTL for 4 h, 44˚C hyperthermia for 30 min, and those in combination were analyzed by flow cytometry. Following treatment, cells were harvested by trypsinization, resuspended in culture medium, rinsed twice with ice-cold PBS(-), and fixed in ice-cold ethanol at the rate of 7 after PBS(-) was added at the rate of 3. Then they were stored at 4˚C for at least 24 h. Next, the cells were collected by centrifugation, rinsed twice with ice-cold PBS(-), and treated with 1 mg/ml of RNase A (Type II-A, Sigma-Aldrich Corp.) at room temperature for 30 min. Cells were then stained with 100 μg/ml of propidium iodide (Sigma-Aldrich Corp.) for at least 30 min on ice in the dark. The cell cycle distribution of the cells was analyzed using a flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Immediately before analysis, cell suspensions were filtered through a 40-μm diameter nylon mesh to remove the cell aggregate and debris. Ten thousand events per determination were analyzed for each sample, and quantification of the cell cycle distribution was performed using software provided by the manufacturer.

Analysis of NF-κB activation. The effects of inhibition of intracellular NF-κB activation after application of PTL, heating and combined treatment on A549 cells ($1x10^6$) were examined by Western blotting. To investigate the phosphorylation of NF-κB p65, cells were stimulated with TNF-α (Genzyme Techne, Boston, MA) at 20 ng/ml for 5 min immediately following incubation at 37˚C for various periods after treatment, while the control was not stimulated with TNF-α (32). Cells were harvested by trypsinization and resuspended in MLN-15. After rinsing with ice-cold PBS(-) twice, they were solubilized in RIPA lysis buffer, then treated by freezing at -20˚C and thawing on ice 3 times. Cell lysates were centrifuged at 14,000 rpm at 4˚C for 10 min to remove cell debris. Next, supernatants were diluted by half with SDS-PAGE and subjected to a block incubator at 95˚C for 3 min after stirring with a vortex and transformed. The protein content of the supernatants was quantified using a protein assay kit (BioRad Laboratories, Richmond, CA). Aliquots of protein (10 μg) were subjected to Western blotting for NF-κB. After electrophoresis on 10% polyacrylamide gels containing 0.1% solution dodecyl sulfate (SDS) and electrophoretic transfer to Immobilon-P PVDF-membranes (Millipore Corp., Medford, MA), membranes were incubated with phospho-p65 (Ser536) (Cell Signaling Technology Inc., Boston, MA, USA), p65 (C20-G) and PCNA (PC-10) (Santa Cruz Biotechnologies, CA, USA) antibodies, as a loading control (33). The antibodies were detected using a horseradish peroxidase-conjugated anti-rabbit antibody.

Results

Cell phase responses to PTL. A549 cells were synchronized into the G_1-S border by treatment with 4 mM HU for 5 h and then incubated for various periods of time, after which they were exposed to PTL at 37˚C for 4 h in order to analyze the cell phase responses, as shown in Fig. 1. As a control, A549 cells were synchronized under equal conditions and incubated for various periods of time, then treated with 4 mM HU for 2 h (data not shown). Cell survival was plotted in order to compare

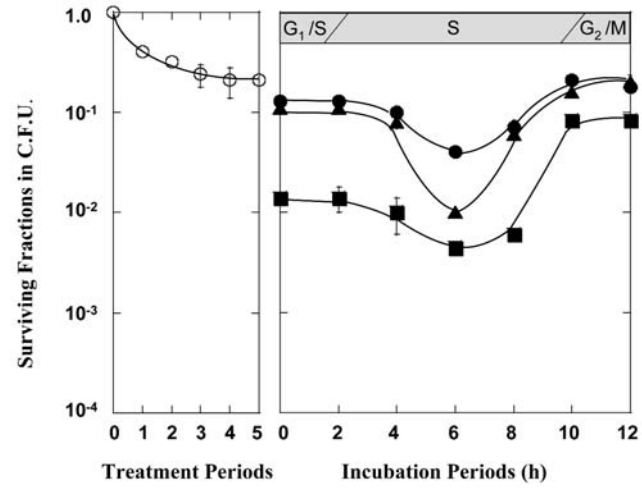

Figure 1. Left panel shows the HU treatment period-survival relationship of cells. Right panel shows the cell phase responses to PTL in gradational concentration for A549 cells synchronized by the exposure to HU for 5 h. Ordinates, log surviving fractions (SFs) in colony forming unit. Abscissa in left panel, treatment periods with 4 mM HU in hours. Abscissa in right panel, incubation periods at 37˚C between the pre-treatment with HU for 5 h and the post-treatment with PTL for 4 h. Closed circles, triangles and squares represent SFs from post-treatment with PTL in 0.02, 0.2 and 2.0 μM, respectively. Symbols with vertical bars represent means with standard errors obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

results with the standard cell cycle phase. After a specified interval, synchronized A549 cells were treated with 3 concentrations (0.02, 0.2, or 2.0 μM) of PTL for 4 h. Assessments of survival curves were primarily sensitive in the S-phase as well as heating at all concentrations.

Analysis of apoptosis and cell cycle distribution. In order to evaluate induced apoptosis and cell cycle distribution at various periods after treatment with PTL, heating, and combined treatment, we used a flow cytometric method. A representative histogram of population distribution in regard to cell cycle status based on DNA content as measured by PI is shown in Fig. 2. The sub-G_1 divisions indicated the distribution of apoptotic cells, which was represented as percentage of the coefficient of variation (CV) value (Table I). Fig. 3 shows induction of apoptosis after treatment with PTL, heating, and combined treatment on a graph using the ratios presented in Table I. PTL-induced apoptosis, which was calculated based on the percentage of cells located in the landing corresponding to the sub-G_1 phase, was 3.3±0.62% after treatment. Heat-induced apoptosis was significantly lower than that induced by PTL (2.23±0.38%). Apoptosis was remarkably increased to 4.8±0.9% following combined treatment. The G_2/M phase distribution showed a significant increase following treatment with PTL (16.4±3.0%), heating (18.6±7.8%), and combined treatment (34.1±2.2%) in comparison with the control (9.9%) (Fig. 4). In contrast, G_0/G_1 phase distribution was decreased by treatment with PTL, heating, and combined treatment to 51.9±3.9%, 52.85±5.8%, and 36.1±4.6%, respectively, as compared to the control (69.2%) (Fig. 5). These results indicate that PTL and heating, and notably in combination induced cell cycle arrest in the G_2/M phase.

Table I. Distribution of cell population in the phase of cell cycle after treatments.

	Interval periods (h)	Control	PTL	44˚C	PTL-44˚C
sub-G_1	0		4.40±2.8	2.37±1.49	6.43±0
	6	0.38±0.02	2.98±2.67	2.60±0.04	4.57±0.01
	12		2.87±2.72	1.70±0.78	4.16±0.05
	24		3.03±2.02	1.43±0.01	4.21±0.01
G_2/M	0		21.60±0.6	13.4±1.7	35.4±0
	6	9.90±0.60	15.20±2.9	11.6±0.1	33.4±0.03
	12		14.90±1.4	17.8±6.9	36.8±0.05
	24		13.90±1.5	31.6±0.05	30.9±19.3
G_0/G_1	0		47.00±1.6	55.5±0.05	32.2±0
	6	69.2±2.20	49.30±4.2	59.9±4.5	36.2±0.03
	12		54.50±2.0	52.1±0.8	32.3±0.02
	24		56.80±1.3	43.9±0.9	43.5±18.0

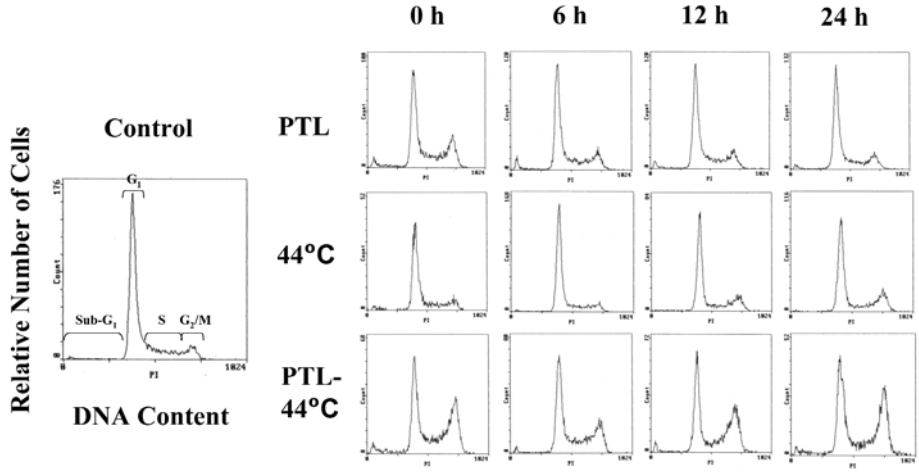

Figure 2. Flow cytometric determination of cell cycle distribution at 0, 6, 12, and 24 h of incubation at 37˚C after treatment of A549 cells with PTL (0.2 μM) at 37˚C for 4 h, hyperthermia at 44˚C for 30 min, or a sequential combination of both. Ordinates, relative number cells. Abscissae, DNA contents through fluorescence intensity with propidium iodide staining.

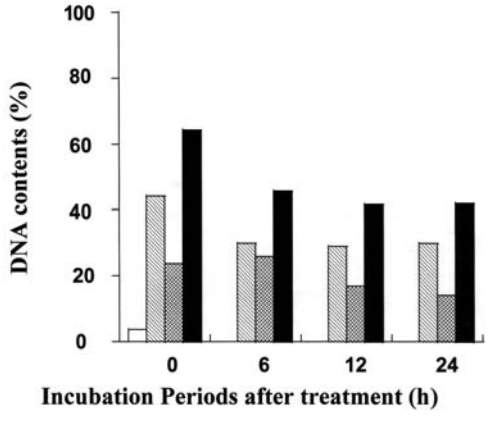

Figure 3. Estimated quantifications of the sub-G_1 fractions of cell-cycle, indicate induced apoptosis, obtained by flow cytometric analysis. Ordinate, relative number apoptotic cells in the totals were shown by percentage. Abscissa, 37˚C incubation periods in hours after treatment. Columns represent, blank, control; slanting stripes, treatment with PTL (0.2 μM) 4 h; checkers, treatment with hyperthermia at 44˚C for 30 min; and black treatment with combination of both, respectively from left.

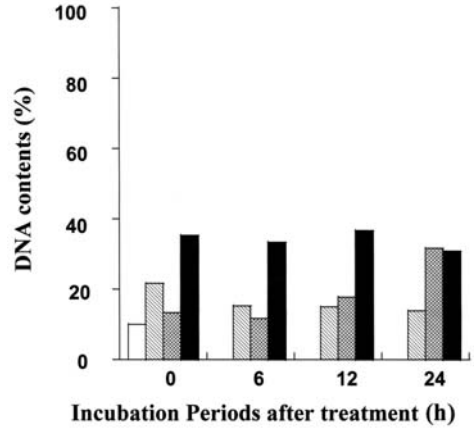

Figure 4. Estimated quantifications of the G_0/G_1 fractions for cell-cycle arrest obtained by flow cytometric analysis. Ordinate, relative number G_0/G_1 arrest cells were shown by percentage. Abscissa, 37˚C incubation periods in hours after treatment. Columns represent, blank, control; slanting stripes, treatment with PTL (0.2 μM) 4 h; checkers, treatment with hyperthermia at 44˚C for 30 min; and black, treatment with combination of both, respectively from left.

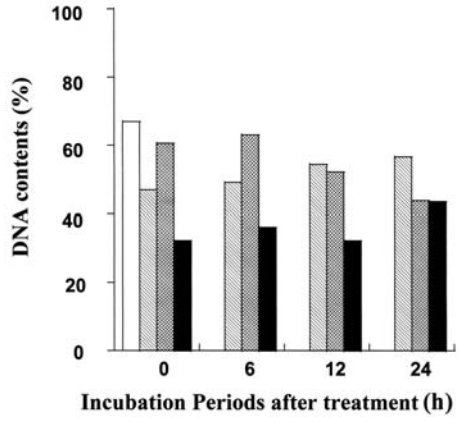

Figure 5. Quantifications of the G_2/M fractions for cell-cycle arrest obtained by flow cytometric analysis were estimated. Relative number G_2/M arrest cells were shown by percentage. Ordinate, relative number G_2/M arrest cells were shown by percentage. Abscissa, 37˚C incubation periods in hours after treatment. Columns represent, blank, control; slanting stripes, treatment with PTL (0.2 μM) 4 h; checkers, treatment with hyperthermia at 44˚C for 30 min; and black, treatment with combination of both, respectively from the left.

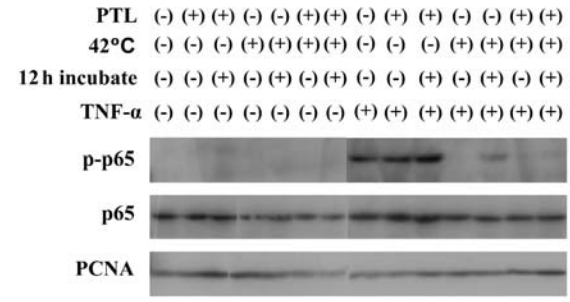

Figure 6. Western blotting of TNF-induced phosphorylation of NF-κB p65 on serine 536 was examined. Inhibiting effects of NF-κB activation at various periods after treatments with PTL (0.2 μM) 4 h, hyperthermia at 42˚C for 4 h, and a sequentially combination of both were analyzed. At 12 h and immediately after incubation after treatment, cells were stimulated with or without TNF-α (20 ng/ml) for 5 min. Whole cell lysates were immunoblotted with the anti-phospho-p65 (Ser536P) (upper panel), anti-p65 (middle panel) and anti-PCNA (lower panel) antibodies. PCNA was used as indicator for transition to nuclear of activated NF-κB since binding a region of DNA replication.

Western blot analysis of NF-κB activation with PTL and heating. The inhibition of intracellular NF-κB activation in A549 cells treated with PTL, heating, and combined treatment were investigated by Western blotting, as shown in Fig. 6. The lanes on the right side of the figure show results of cells stimulated with TNF-α after treatment with PTL, heating at 42˚C, and combined treatment, which were performed to activate intracellular p65. The left side shows results of cells after those treatments that were not exposed to TNF-α, to demonstrate the contrast in elicitation of NF-κB activation. Phosphorylation of p65 was not inhibited by PTL alone, whereas it was inhibited immediately after treatment with heating alone. Incubation for 12 h after heating recovered the TNF-α-induced NF-κB activation partially. However, treatment with PTL blocked this recovery from heating. Thus, inactivation of NF-κB was prolonged by the combination of PTL and heating.

Discussion

Plant extracts that contain sesquiterpenes, including PTL are frequently used to treat such conditions as migraine headaches and inflammation (34-36), while hyperthermia as cancer therapy is usually performed in some combination with another treatment modality, such as radio- or chemotherapy. Reports of experiments that utilized a combination of PTL and radiation are few (37,38), while results of hyperthermia have only been presented in *in vitro* experiments (5). In addition, there is only a single report of PTL used for clinical cancer therapy in a phase I dose escalation trial (39). Therefore, we think that additional experimental evidence is important before clinical application. We previously demonstrated that thermotherapy, using a combined treatment of PTL (0.02 μM) prior to hyperthermia at 40, 42, or 44˚C, had synergistic thermo-enhancement effects toward the A549 human lung adenocarcinoma cell line, and concluded that the effects of thermosensitization by PTL were due to p53- and hsp72-independent apoptosis induction via the NF-κB signal pathway (5).

Cell phase responses to PTL. In order to clarify the mechanisms of thermal enhancement by PTL, we examined the cell phase responses of A549 cells to PTL doses of 0.02, 0.2 and 2.0 μg/ml by determining survival curves for cell phase progression, as shown in Fig. 1. A549 cells were synchronized by HU into the G_1-S border, then treated with PTL for 4 h after an interval incubation time of 0-12 h. Cell phase responses to PTL by the A549 cells were highly sensitive during the S phase at all concentrations as well as with heating (8). Other studies have been conducted to determine the target of hyperthermia, though no conclusive evidence has been presented. We previously reported that bleomycin (BLM), which inhibits DNA synthesis, as well as amrubicin (AMR) and amrubicinol (AMROH), which inhibit topoisomerase II activity, were cell cycle nonspecific regardless of concentration (6,7). Therefore, more effective synergistic effects may be obtainable by combining hyperthermia with the target drug to obtain a cell phase similar to that caused by irradiation in order to enhance and improve antitumor effects. The reason why agents such as AMR, AMROH, and BLM cause no cell phase responses may be due to the fact that they target different types of DNA damage (40-42). As for the mechanisms of those agents, it has been reported that BLM predominantly co-localizes with 3 proteins that bind directly to BLM, telomerase-associated protein 1, heat shock protein 90, and topoisomerase II α, in foci actively synthesizing DNA during the late S and G_2/M phases of the cell cycle in L cells (43), while AMR and AMROH inhibit cell growth prior to DNA-protein complex formation followed by double-strand DNA breaks, which are mediated by topoisomerase II (44). Heat induced apoptosis was also significantly increased by combinations of those agents.

Assessment of sub-G_1 fraction with flow cytometric distribution. In the present study, the sub-G_1 fraction of cell cycle distribution was used to indicate the induction of apoptosis, which was examined by flow cytometric analysis. Our results showed that PTL had a stronger effect than heating, while those in combination had the greatest effect. That is, blocking the

progress to DNA synthesis (Fig. 2). In a previous report regarding the mechanisms of PTL on cancer cell differentiation and apoptosis, PTL treatment presented a novel strategy for chemotherapy, as it inhibited DNA methyltransferase 1 possibly through alkylation of the proximal thiolate of Cys (1226) of the catalytic domain by γ-methylene lactone (45).

Assessments of G_2/M and G_0/G_1 fractions with flow cytometric distribution. The DNA content of the G_2/M fraction in flow cytometric distribution indicates cell cycle arrest. The rate of DNA content in the G_2/M fraction of cells treated with a combination of PTL and heating was markedly high in comparison to cells with separate administrations, whereas that in the G_0/G_1 fraction was low (Fig. 3). PTL may also be effective in combination with COX-2 inhibitors for the treatment of hepatocellular carcinoma, for example, combination treatment with PTL and NS398 altered the cell cycle distribution resulting in greater G_0/G_1 accumulation, which increased apoptosis only in PLC cells, 1 of 3 human hepatocellular carcinoma lines, which may decrease the apoptotic threshold in cells (16). As for the cell cycle distribution of HL-60 and Jurkat cells with inactive NF-κB, it was demonstrated that PTL induced transient cell arrest in the G_2 and M phases followed by apoptosis (46). Transcriptional regulation of cyclin as a cell cycle checkpoint to control cell growth and differentiation was reported to be conducted via NF-κB (47).

Inhibition of NF-κB by combination of PTL and hyperthermia. It is known that transcription factor NF-κB is activated by various stimuli along with TNF-α, such as inflammation related cytokines, UV irradiation, and oxidative stress, while NF-κB regulates the induction of many genes that participate in immune and inflammation reactions (48-50). NF-κB p65 forms a DNA-binding complex with NF-κB p50, while the binding of this complex can be specifically inhibited by IκB (51). When tumor intracellular NF-κB activation is inhibited by some agent, cell proliferation or tolerance is suppressed and anticancer effects increase. When IκBα is binding to the NF-κB complex in a steady state it is also stimulated by TNF-α, its serine residue is phosphorylated and ubiquitination and proteasomal degradation are facilitated (52). In this study, we examined whether PTL, heating, or combined treatment effectively inhibit the phosphorylation of TNF-α-induced NF-κB using A549 cells (Fig. 6). PTL alone did not inhibit the phosphorylation of NF-κB, which was an unexpected finding, and heating alone inhibited that immediately after treatment, though the effects decreased for many hours. In contrast, those treatments in combination continuously inhibited NF-κB activation. It has been suggested that NF-κB activity is delayed in the presence of hyperthermia, which is caused by hsp70 expression (24). In our previous study, though hsp72 was induced by heating at 44˚C for 15 min, it was not induced by PTL (5). There is another report that PTL caused rapid apoptosis without suppression of NF-κB activity in glioblastoma cells (53). We believe the reason why hyperthermia decreased the inhibition of NF-κB phosphorylation over time was because heating delays NF-κB activity, which may be due to heat-induced hsp72.

In conclusion, we demonstrated that sequential combination therapy with PTL and hyperthermia caused a significant anti-cancer effect in A549 cells by targeting cells in the S-phase. The mechanisms were due to the induction of NF-κB-dependent or independent apoptosis and G_2/M cell cycle arrest. Furthermore, combination therapy blocked the revival of NF-κB activation by heat-induced hsp72. Based on these results, it is considered that PTL contributes to the thermosensitization via another pathway on NF-κB signaling or by cross-talk with another mediated gene, but the interrelations are unclear.

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.

References

1. Schinella GR, Giner RM, Recio MC, Mordujovich de Buschiazzo P, Rios JL and Manez S: Anti-inflammatory effects of South American *Tanacetum vulgare*. J Pharm Pharmacol 50: 1069-1074, 1998.
2. Wiedhopf RM, Young M, Bianchi E and Cole JR: Tumor inhibitory agent from *Magnolia grandiflora* (*Magnoliaceae*). I. Parthenolide. J Pharm Sci 62: 345, 1973.
3. Hoffmann JJ, Torrance SJ, Widehopf RM and Cole JR: Cytotoxic Agents from *Michelia champaca* and *Talauma ovata*: Parthenolide and costunolide. J Pharm Sci 66: 883-884, 1977.
4. El-Feraly FS and Chan YM: Isolation and characterization of the sesquiterpene lactones costunolide, parthenolide, costunolide diepoxide, santamarine, and reynosin from *Magnolia grandiflora* L. J Pharm Sci 67: 347-350, 1978.
5. Hayashi S, Hatashita M, Hayashi A, *et al*: Thermosensitization by parthenolide in human lung adenocarcinoma A549 cells and p53- and hsp72-independent apoptosis induction via the nuclear factor-κB signal pathway. Int J Mol Med 21: 585-592, 2008.
6. Shioura H, Hayashi S, Matsumoto H, Kitai R, Ohtsubo T, Nishida T, Zhang SW, Yoshida M, Ishii Y and Kano E: The effects of combined treatments with low hyperthermia and bleomycin on survivals of murine L cells. J Exp Clin Cancer Res 16: 147-152, 1997.
7. Hayashi S, Hatashita M, Matsumoto H, Jin ZH, Shioura H and Kano E: Modification of thermosensitivity by amrubicin or amrubicinol in human lung adenocarcinoma A549 cells and the kinetics of apoptosis and necrosis induction. Int J Mol Med 16: 381-387, 2005.
8. Westra A and Dewey WC: Variation in sensitivity to heat shock during the cell cycle of Chinese hamster cells in vitro. Int J Radiat Biol Relat Stud Phys Chem Med 19: 467-477, 1971.
9. Bhuyan BK, Day KJ, Edgerton CE and Ogunbase O: Sensitivity of different cell lines and of different phases in the cell cycle to hyperthermia. Cancer Res 37: 3780-3784, 1977.
10. Sinclair WK and Morton RA: X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. Radiat Res 29: 450-474, 1966.
11. Kano E: Fundamentals of thermochemotherapy of cancer. Gan No Rinsho 33: 1657-1663, 1987.
12. Cao J, Wells RL and Elkind MM: Enhanced sensitivity to neoplastic transformation by ^{137}Cs gamma-rays of cells in the G_2-/M-phase age interval. Int J Radiat Biol 62: 191-199, 1992.
13. Miller RC, Geard CR, Geard MJ and Hall EJ: cell cycle-dependent radiation-induced oncogenic transformation of C3H 10T1/2 cells. Radiat Res 130: 129-133, 1992.
14. Smith JW, Jackson WE, Clark EP and Raaphorst GP: Oncogenic potential of hyperthermia in combination with radiation. Int J Hyperthermia 10: 309-313, 1994.
15. Ross JJ, Arnason JT and Birnboim HC: Low concentrations of the feverfew component parthenolide inhibit in vitro growth of tumor lines in a cytostatic fashion. Planta Med 65: 126-129, 1999.
16. Ralstin MC, Gage EA, Yip-Schneider MT, Klein PJ, Wiebke EA and Schmidt CM: Parthenolide cooperates with NS398 to inhibit growth of human hepatocellular carcinoma cells through effects on apoptosis and G_0-G_1 cell cycle arrest. Mol Cancer Res 4: 387-399, 2006.

17. Ghosh S, May MJ and Kopp EB: NF-kappaB and Rel proteins: evolutionarily concerved mediators of immune responses. Annu Rev Immunol 16: 225-260, 1998.
18. Chen ZJ, Parent L and Maniatis T: Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell 84: 853-862, 1996.
19. Sakurai H, Chiba H, Miyoshi H, Sugita T and Toriumi W: IκB kinases phosphorylate NF-κB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 274: 30353-30356, 1999.
20. Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelfanov V, Boswell SH, Goulet RJ Jr, Sledge GW Jr and Nakshatri H: Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by Ikappa-Balpha super-repressor and parthenolide. Oncogene 19: 4159-4169, 2000.
21. Nakshatri H, Rice SE and Bhat-Nakshatri P: Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. Oncogene 23: 7330-7344, 2004.
22. Walmsley SR, Print C, Farahi N, Peyssonnaux C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N and Chilvers ER: Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J Exp Med 201: 105-115, 2005.
23. Sweeney CJ, Mehrotra S, Sadaria MR, Kumar S, Shortle NH, Roman Y, Sheridan C, Campbell RA, Murry DJ, Badve S and Nakshatri H: The sesquiterpene lactone parthenolide in combination with docetaxel reduces metastasis and improves survival in a xenograft model of breast cancer. Mol Cancer Ther 4: 1004-1012, 2005.
24. Frossard JL, Pastor CM and Hadengue A: Effect of hyperthermia on NF-κB binding activity in cerulein-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 280: G1157-G1162, 2001.
25. Kokura S, Yoshida N, Ueda M, Imamoto E, Ishikawa T, Takagi T, Naito Y, Okanoue T and Yoshikawa T: Hyperthermia enhances tumor necrosis factor alpha-induced apoptosis of a human gastric cancer cell line. Cancer Lett 201: 89-96, 2003.
26. Aravindan N, Shanmugasundaram K and Natarajan M: Hyperthermia induced NFkappaB mediated apoptosis in normal human monocytes. Mol Cell Biochem 327: 29-37, 2009.
27. Markovic M and Stuhlmeier KM: Short-term hyperthermia prevents activation of proinflammatory genes in fibroblast-like synoviocytes by blocking the activation of the transcription factor NF-κB. J Mol Med 84: 821-832, 2006.
28. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H and Parks WP: In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 51: 1417-1423, 1973.
29. Lieber M, Smith B, Szakal A, Nelson-Rees W and Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17: 62-70, 1976.
30. Young CW and Hodas S: Hydroxyurea: Inhibitory effect on DNA metabolism. Science 146: 1172-1174, 1964.
31. Sinclair WK: Hydroxyurea: differential lethal effects on cultured mammalian cells during the cell cycle. Science 150: 1729-1731, 1965.
32. Sakurai H, Suzuki S, Kawasaki N, *et al*: Tumor necrosis factor-α-induced IKK phosphorylation of NF-κB p65 on Serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. The J Bio Chem 278: 36916-36923, 2003.
33. Sakurai H, Nishi A, Sato N, Mizukami J, Miyoshi H and Sugita T: TAK1-TAB1 fusion protein: a novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF-κB signaling pathways. Biochem Biophys Res Commun 297: 1277-1281, 2002.
34. Jain NK and Kulkarni SK: Antinociceptive and anti-inflammatory effects of *Tanacetum parthenium* L. extract in mice and rats. J Ethnopharmacol 68: 251-259, 1999.
35. Johnson ES, Kadam NP, Hylands DM and Hylands PJ: Efficacy of feverfew as prophylactic treatment of migraine. Br Med J (Clin Res Ed) 291: 569-573, 1985.
36. Smolinski AT and Pestka JJ: Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb(1) (ginseng) and parthenolide (feverfew). Food Chem Toxicol 41: 1381-1390, 2003.
37. Curry EA III, Murry DJ, Yoder C, Fife K, Armstrong V, Nakshatri H, O'Connell M and Sweeney CJ: Phase I dose escalation trial of feverfew with standardized doses of parthenolide in patients with cancer. Invest New Drugs 22: 299-305, 2004.
38. Sun Y, St Clair DK, Fang F, Warren GW, Rangnekar VM, Crooks PA and St Clair WH: The radiosensitization effect of parthenolide in prostate cancer cells is mediated by nuclear factor-kappaB inhibition and enhanced by the presence of PTEN. Mol Cancer Ther 6: 2477-2486, 2007.
39. Mendonca MS, Chin-Sinex H, Gomez-millan J, Datzman N, Hardacre M, Comerford K, Nakshatri H, Nye M, Benjamin L, Mehta S, Patino F and Sweeney C: Parthenolide sensitizes cells to X-ray-induced cell killing through inhibition of NF-kappaB and split-dose repair. Radiat Res 168: 689-697, 2007.
40. Kobayashi E, Yamagishi M, Kamamoto T, Yoshida Y and Uchino H: Cell cycle-dependent heat sensitization of murine granulocyte-macrophage progenitor cells in regenerating marrow. Cancer Res 45: 1459-1463, 1985.
41. Kanno S, Hyodo M, Suzuki K and Ohkido M: Effect of DNA-damaging agents on DNA replication and cell cycle progression of cultured mouse mammary carcinoma cells. Jpn J Cancer Res 76: 289-296, 1985.
42. Olive PL and Banath JP: Detection of DNA double-strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and 125IdUrd. Int J Radiat Biol 64: 349-358, 1993.
43. Bhattacharyya S, Keirsey J, Russell B, Kavecansky J, Lillard-Wetherell K, Tahmaseb K, Turchi JJ and Groden J: Telomerase-associated protein 1, HSP90, and topoisomerase II alpha associate directly with the BLM helicase in immortalized cells using ALT and modulate its helicase activity using telomeric DNA substrates. J Biol Chem 284: 14966-14977, 2009.
44. Hanada M, Mizuno S, Fukushima A, Saito Y, Noguchi T and Yamaoka T: A new antitumor agent amrubicin induces cell growth inhibition by stabilizing topoisomerase II-DNA complex. Jpn J Cancer Res 89: 1229-1238, 1998.
45. Liu Z, Liu S, Xie Z, *et al*: Modulation of DNA methylation by a sesquiterpene lactone parthenolide. J Pharmacol Exp Ther 329: 505-514, 2009.
46. Pozarowski P, Halicka DH and Darzynkiewicz Z: Cell cycle effects and caspase-dependent and independent death of HL-60 and Jurkat cells treated with the inhibitor of NF-kappaB parthenolide. Cell cycle 2: 377-383, 2003.
47. Guttridge DC, Albanese C, Reuther JY, Pestell RG and Baldwin AS Jr: NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 19: 5785-5799, 1999.
48. Szlosarek PW and Balkwill FR: Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. Lancet Oncol 4: 565-573, 2003.
49. Bender K, Göttlicher M, Whiteside S, Rahmsdorf HJ and Herrlich P: Sequential DNA damage-independent and -dependent activation of NF-kappaB by UV. EMBO J 17: 5170-5181, 1998.
50. Bowie A and O'Neill LA: Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59: 13-23, 2000.
51. Nolan GP, Ghosh S, Liou HC, Tempst P and Baltimore D: DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. Cell 64: 961-969, 1991.
52. Senftleben U, Cao Y, Xiao G, Greten FR, Krähn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC and Karin M: Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science 293: 1495-1499, 2001.
53. Anderson KN and Bejcek BE: Parthenolide induces apoptosis in glioblastomas without affecting NF-kappaB. J Pharmacol Sci 106: 318-320, 2008.