LY294002, an inhibitor of PI-3K, enhances heat sensitivity independently of *p53* status in human lung cancer cells

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Abstract. The aim of this study was to ascertain whether LY294002, an inhibitor of PI-3K, enhances heat sensitivity in human cancer cells regardless of their *p53* status. Colony formation assays showed that LY294002 enhanced heat sensitivity in two human lung cancer cell lines; H1299/wild-type *p53* (wtp*53*) and H1299/mutated *p53* (m*p53*) cells. These cell lines have identical genetic backgrounds except for their *p53* status. LY294002 suppressed the heat-induced accumulation of heat shock protein 27 (hsp27) and heat shock protein 72 (hsp72) in these cell lines. Heat-induced apoptosis was observed more frequently in H1299/wt*p53* cells than in H1299/m*p53* cells, and was enhanced by LY294002 in both cell lines. In addition, both the heat-induced phosphorylation of Akt and the accumulation of survivin were suppressed by LY294002. These results suggest that LY294002 inhibits anti-apoptosis signaling through hsp27 and hsp72 as well as cell survival signaling through Akt and survivin. LY294002 appears to be an attractive candidate for a p53-independent heat sensitizer in hyperthermic cancer therapy.

Introduction

Hyperthermia can be an effective cancer therapy (1,2). However, to develop more efficient regimens for using heat to treat various malignant tumors, agents are required which can sensitize cancer cells to heat. Apoptosis is a beneficial physiological response resulting in cell death without inflammation. Molecular biological studies have examined the heat-induced signal transduction pathway for apoptosis operating through p53 (3) and/or c-Jun N-terminal kinase (JNK) (4,5). However, heat also activates signal transduction pathways for anti-apoptosis/cellular proliferation activity. Several signaling factors such as Akt, p38, extracellular signal-regulated kinase (ERK) and heat shock protein (hsp) play important roles in the anti-apoptosis/cellular proliferation pathway (4,6-15). Since active signaling pathways mediated by such factors can reduce the efficacy of hyperthermic cancer therapy, the targeted inhibition of anti-apoptosis/cellular proliferation signaling factors could be useful for the development of potent hyperthermic cancer therapies.

A serine/threonine kinase, Akt, is known to mediate many biological processes which affect anti-apoptotic/cell survival responses (14,15). Amplification of the *Akt* gene is frequently observed in various types of cancer cells. The activity of Akt is high in cancer cells defective in the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) which down-regulates Akt activity. These abnormalities of Akt expression are considered to be closely related to tumorigenesis. Thus, Akt is now becoming a promising and attractive molecular target for enhancing apoptosis (16) and for cancer therapy (17). Akt is activated by heat as well as radiation through a phosphatidylinositol-3-kinase (PI3-K) mediated phosphorylation pathway (18). LY294002 is known to be a specific inhibitor of PI-3K (19), and radio-sensitization caused by LY294002 has been reported in *in vitro* (20) and *in vivo* experiments (21). In contrast to this, the effects of LY294002 on heat sensitization have not been examined. This study examined the effects of LY294002 on heat sensitivity in two human lung cancer cell lines which differed only in their *p53* status.

This study also examined the effects of LY294002 on the heat-induced accumulation of hsp27 and hsp72 which are involved in anti-apoptosis signaling pathways (6-12). The effects of LY294002 on heat sensitivity are discussed in relation to the functions of hsp27 and hsp72 in anti-apoptosis signaling pathways.

Materials and methods

Cell culture. Human lung cancer H1299 cells (*p53*-null) were a gift from Dr M. Oren, Weizmann Institute of Science, Rehovot, Israel. These cells were transfected with either pC53-SN3 containing the wt*p53* gene, or with pC53-248 containing the mp53 gene (Arg to Trp at codon 248). The transcription of the wt*p53* or the m*p53* gene was confirmed in stable clones of these cells using RT-PCR RFLP, and the translation of the p53 protein was confirmed using Western blot analysis (22). The cell lines were cultured at 37˚C in Dulbecco's modified Eagle's medium (ICN Biomedicals, Aurora, OH) containing 10% (v/v)

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fetal bovine serum (ICN Biomedicals), penicillin (50 U/ml; Sigma, St. Louis, MO), streptomycin (50 μg/ml; Meiji, Tokyo, Japan) and kanamycin (50 μg/ml; Meiji) (DMEM-10). Heating was performed by immersing 25-cm^2 flasks containing the cells into a circulating water bath (TAITEC, Saitama, Japan) at 44˚C and then incubating them for the indicated periods at 37˚C. X-ray irradiation was performed with a 150-kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan).

LY294002 treatment. LY294002 (at a final concentration of 40 μM) was added to the culture media one hour before heat or X-ray treatment.

Colony formation assay. The fraction of surviving cells after heat exposure or X-ray irradiation was determined using a clonogenic assay; the number of colonies for each treatment point was corrected using the plating efficiency (~75%) of the non-treated cells as a control. A total of 5x10^2 or 1x10^3 cells were plated in each of two T25 flasks per experiment, and three independent experiments were performed for each survival point. Cells were heated or irradiated and, after 14 days of incubation, visible colonies composed of more than 50 cells were counted. Colonies were fixed with 100% methanol for a few minutes and stained with a 2% Giemsa solution (Merck & Co., Inc., Rahway, NJ). Each colony was scored as having grown from a single surviving cell. The values obtained were expressed as the means of three independent experiments; bars (standard errors) are shown when the standard errors exceed the values indicated by the symbols.

Western blot analysis. A total of 5x10^5 cells was resuspended in 50 μl RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.05% SDS) and then subjected to freezing at -30˚C and thawing (on ice) successively three times. Total protein extracts were prepared from non-irradiated control cells or heated cells incubated at 37˚C for 0, 12, or 24 h after heating according to experimental procedures. The sample at 0 h was prepared from heated cells immediately after heating. The protein levels in the supernatants obtained after centrifugation (15,000 x g) were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of proteins (20 μg) were analyzed using Western blotting. After electrophoresis on 10 or 15% polyacrylamide gels containing 0.1% SDS, the proteins were transferred onto Poly Screen PVDF membranes using an electro-blot BLAST® blotting amplification system (DuPont/Biotechnology Systems, NEN Research Products). The membranes were then incubated with an appropriate antibody: anti-Akt phosphorylated at threonine 308 polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA), anti-survivin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-hsp27 monoclonal antibody (Ab-1, Oncogene Science, Inc., Uniondale, NY), anti-hsp72 monoclonal antibody (SPA-810, StressGen, Victoria, Canada), or heat shock factor 1 (HSF1) polyclonal antibody (SPA-901, StressGen). For visualization of the bands, a horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used for anti-hsp27 and anti-hsp72, and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) was used for anti-phospho-Akt, anti-survivin and anti-HSF1.

Gel mobility-shift assay. Cells (~2x10^7 cells) were heated at 44˚C for 50 min in the presence or absence of LY294002 (40 μM). Nuclear extracts were prepared at appropriate times after heating as previously described (23). The binding activity of HSF1 to heat shock elements (HSE) was measured by a gel mobility-shift assay using a synthetic double-stranded DNA fragment encoding the hsp72 HSE (5'-CTAGAAGCTT CTAGAAGCTTCTAG-3', Japan Bioservice, Niiza, Saitama, Japan) based on a specific sequence located upstream of the *hsp* genes (24,25).

Analysis of apoptosis. For detection of apoptotic bodies, cells were fixed with 1% glutaraldehyde (Nakalai Tesque, Kyoto, Japan) in PBS at 4˚C, washed with PBS, stained with 0.2 mM Hoechst 33342 (Sigma Chemical Co.), and then observed under a fluorescence microscope.

Results

Effect of LY294002 on heat sensitivity. LY294002 treatment alone showed almost no toxicity in a concentration range from 0 to 50 μM in H1299/wt*p53* and H1299/m*p53* cells. In view of this, LY294002 was used at a non-toxic concentration of 40 μM in all subsequent experiments. The sensitivity of H1299/wt*p53* and H1299/m*p53* cells to heat or X-rays was measured using colony formation assays. The surviving fractions of both cell lines treated with LY294002 decreased as the heating period or dose of X-rays increased. Fig. 1 shows the sensitization rate in response to heat or X-rays. The sensitization rates were calculated from doses producing a 10% survival rate in H1299/wt*p53* cells (1.40 in heat, 1.67 in X-rays) and H1299/m*p53* cells (1.88 in heat, 1.71 in X-rays). LY294002 thus increased heat sensitivity as well as X-ray sensitivity regardless of *p53* status.

Effect of LY294002 on expression of Hsp27, Hsp72 and survivin, and the phosphorylation of Akt. Western blotting was used to examine the effects of LY294002 on the heat-induced accumulation of hsp27 and hsp72 after heating. As shown in Fig. 2, an accumulation of hsp27 and 72 proteins was observed at 12 h after heating in H1299/wt*p53* and H1299/m*p53* cells. In both cell lines treated with LY294002, the accumulation of these proteins was suppressed. The suppressive effect of LY294002 on heat-induced hsp accumulation was somewhat stronger for hsp27 than for hsp72 in both cell lines. After LY294002 treatment alone, there was no sign of increased hsp27 and hsp72 levels at 12 and 24 h after treatment (data not shown). The effects of LY294002 on the heat-induced phosphorylation of Akt and expression of the downstream factor survivin were also examined using Western blotting. As shown in Fig. 2, Akt phosphorylated at threonine 308 was observed at 12 h after heating, and constitutive expression of survivin was apparent in both H1299/wt*p53* and H1299/m*p53* cells. In both cell lines, after treatment with LY294002, the phosphorylation of Akt and the expression of survivin were suppressed. However, after LY294002 treatment alone, there was no sign of increased phosphorylated Akt and survivin 12 and 24 h after treatment (data not shown).

Effect of LY294002 on DNA binding activity of HSF1. To examine the effect of LY294002 on *hsp* gene expression, the

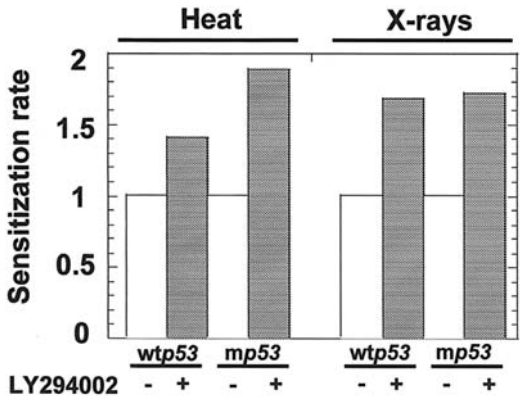

Figure 1. Effects of LY294002 on cellular sensitivity to heat or X-rays. The sensitization rate to heat or X-rays by LY294002 was calculated from doses producing a 10% survival rate in H1299/wt*p53* and H1299/m*p53* cells which are labeled as wt*p53* and m*p53*, respectively.

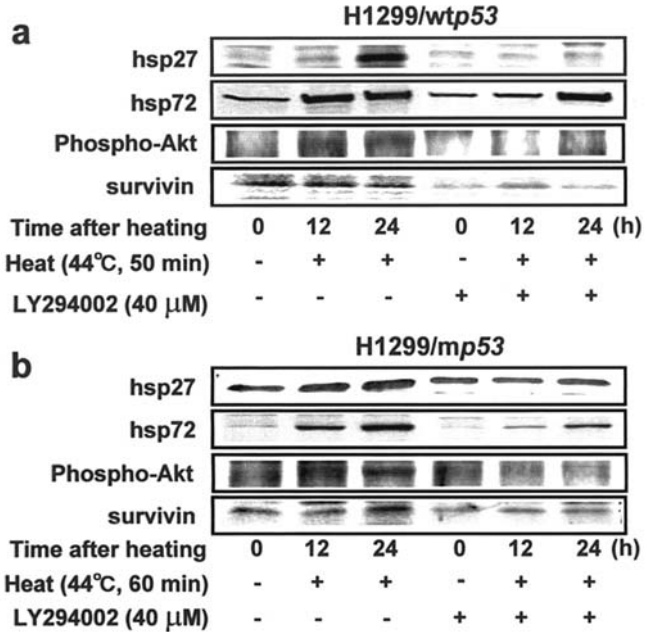

Figure 2. Effects of LY294002 on the expression of hsp27, hsp72 and survivin, and phosphorylation of Akt after heating. The cells were heated under conditions which resulted in a D_{10} survival rate: 44˚C for 50 min (H1299/wt*p53* cells) and 44˚C for 60 min (H1299/m*p53* cells). Whole cell proteins were extracted 0, 12 or 24 h after heating with or without LY294002 (40 μM) and analyzed using Western blotting.

DNA binding activity of HSF1 for HSE sequences of hsp72 was measured in nuclear proteins extracted from H1299/wt*p53* and H1299/m*p53* cells using gel mobility-shift assay (Fig. 3). The amount of HSF1/hsp72HSE complex increased in both cell lines 6 h after heating. When the cells from both lines were treated with LY294002, the amount of HSF1/hsp72HSE complex decreased after heating. When cold probes for hsp72HSE were added to a mixture of [^{32}P]-labeled hsp72HSE and extracts from both cell lines, HSF1/hsp72HSE complexes were not detected (data not shown). The cellular amounts of HSF1 in both cell lines did not change after heating and were not affected by LY294002 (Fig. 3, lower row).

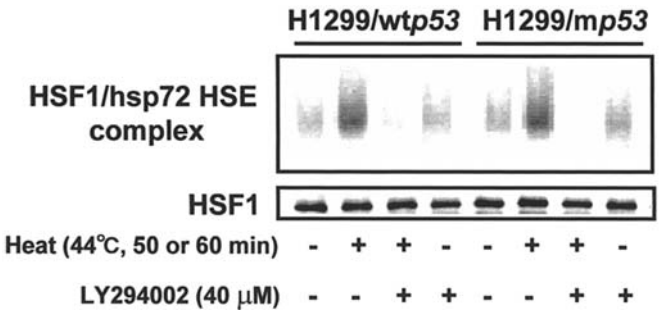

Figure 3. Effects of LY294002 on the binding activity of HSF1 to hsp72HSE before or after heating. The cells were heated (H1299/wt*p53* cells, 44˚C for 50 min; H1299/m*p53* cells, 44˚C for 60 min) with or without LY294002 (40 μM). Nuclear fractions were extracted 6 h after heating and analyzed with gel mobility-shift assay.

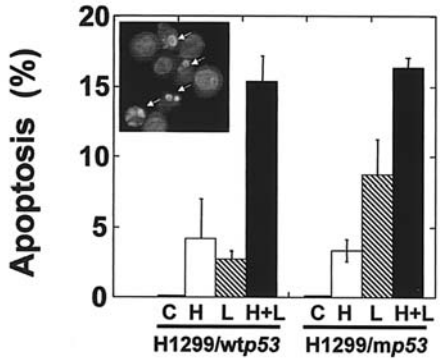

Figure 4. Effects of LY294002 on heat-induced apoptosis. H1299/wt*p53* and H1299/m*p53* cells were heated for 50 min and 60 min at 44˚C, respectively and incubated for 24 h at 37˚C. After incubation, apoptotic bodies were detected by Hoechst 33342 staining. Open bars, heat (H); shaded bars, LY294002 (40 μM) (L); closed bars, heat + LY294002 (H + L). C is unheated control. Inset photograph shows apoptotic bodies indicated by arrows.

Effect of LY294002 on heat-induced apoptosis. We examined the induction of apoptosis in order to elucidate the mechanism of enhancement of heat sensitivity by LY294002 in H1299/wt*p53* and H1299/m*p53* cells. Nuclear morphology was analyzed with Hoechst 33342 staining and the number of apoptotic bodies, such as those indicated by arrows in the insert in Fig. 4, was scored. The incidence of apoptosis 24 h after treatment with either heat or LY294002 alone was approximately 3% in H1299/wt*p53* cells, and approximately 3% (heat alone) and 8% (LY294002 alone) in H1299/m*p53* cells, respectively. The combined treatment with heat and LY294002 enhanced the incidence of apoptosis to approximately 15% in H1299/wt*p53* cells and to approximately 17% in H1299/m*p53* cells.

Discussion

In hyperthermic cancer therapy, it is helpful to inhibit signaling factors related to the survival of cancer cells. Although LY294002 has been reported to be a radiation sensitizer which interferes with PI-3K and PI-3K-regulated Akt signaling pathways (20,21), it has not been reported to be a heat

sensitizer. It was shown here that LY294002 sensitizes cancer cells to hyperthermia as well as to radiation (Fig. 1). LY294002 inhibited heat-induced hsp27 and hsp72 accumulation as well as heat-induced activation of Akt (Fig. 2). These results suggest that LY294002 interferes with heat-activated anti-apoptosis signaling pathways via hsps and/or heat-activated cellular survival signaling pathways via Akt, and consequently enhances the sensitivity of H1299/wt*p53* and H1299/m*p53* cells to heat.

It is known that hsp27 and hsp72 play inhibitory roles in various signal transduction pathways involved in apoptosis. Hsps bind to cytochrome c released from mitochondria, and block the binding of cytochrome c to apoptosis protease-activating factor-1 (Apaf-1) (6,7). Consequently, the apoptosome, a complex of Apaf-1, caspase-9 and cytochrome c is not formed. Since the apoptosome activates caspase-3 (26,27), interference with apoptosome formation could inhibit caspase-3 activation. Other anti-apoptotic functions of hsp27 are to regulate the activity of Akt (8) and to modulate the metabolism of glutathione, resulting in radioresistance (12). Hsp72 also suppresses heat-induced apoptosis by inactivating JNK (4,9,10) or by antagonizing apoptosis-inducing factor (AIF) (28). The work reported here shows that LY294002 decreased the cellular amounts of hsp27 and hsp72 after heating and increased heat-induced apoptosis in H1299/wt*p53* and H1299/m*p53* cells (Figs. 2 and 4). In agreement with the increased heat-induced apoptosis observed after exposure to LY294002, the phosphorylation of Akt and expression of survivin were inhibited by LY294002. The LY294002-induced defect in cell proliferation signaling via Akt and survivin appears likely to be a another possible cause of the observed enhanced heat sensitivity.

The results from the gel mobility-shift assay (Fig. 3) correlated well with those from Western blotting. Since HSF1 binding to DNA up-regulates the transcription of *hsp* gene expression, the suppression of heat-induced accumulation of hsp72 by LY294002 appears to be due to the suppression of HSF1 DNA binding activity. The suppression of HSF1 binding activity to HSE does not result from a decreased amount of HSF1 present in response to LY294002 because the level of HSF1 is not affected by LY294002 (lower row of Fig. 3). HSF1 is activated through phosphorylation and then forms trimers and binds to the HSE sequences located on *hsp* genes (29-32). A possible explanation of the effect of LY294002 is that it may inhibit the phosphorylation of HSF1 after heating, or block the trimerization of HSF1.

p53 is a tumor suppressor gene that exerts multiple effects via the regulation of numerous genes which are closely related to apoptosis or growth arrest in cells. It has been suggested that *p53* status is a predictive indicator of the success of radiation therapy for patients with squamous cell carcinoma of the cervix (33). *p53*-dependent heat sensitivity has also been reported in human squamous cell carcinoma cells (3). Heat activates p53 and elevates its DNA-binding potential (3). This, together with other reports (34,35), suggests that the effectiveness of hyperthermic cancer therapy could be closely related to *p53* staus (36). In this study, the question examined was whether the effects of LY294002 on heat sensitivity depend on *p53* status in H1299 cells transfected with wt*p53* or m*p53*; it was found that LY294002 enhanced heat sensitivity in human cancer cell lines in a manner which did not depend on cellular *p53* status. From this result, LY294002 would appear to be a good candidate for heat sensitization in hyperthermic cancer therapy, regardless of cellular *p53* gene status.

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