Journal of Cellular Biochemistry 101:619–630 (2007)

Effect of Hyperthermia on TRAIL-Induced Apoptotic Death in Human Colon Cancer Cells: Development of a Novel Strategy for Regional Therapy

Jinsang Yoo and Yong J. Lee*

Department of Surgery and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Abstract Approximately 25% of patients with colorectal cancer will develop metastatic disease exclusively or largely confined to the liver, and the vast majority of these cases are not amenable to surgical resection. These unresectable cases of liver metastatic disease can be treated with isolated hepatic perfusion (IHP), which involves a method of complete vascular isolation of the liver to allow treatment of liver tumors with toxic systemic doses of chemotherapeutic agents. To improve the efficacy of IHP, hyperthermia and biological agents have been applied along with the chemotherapeutic agents. In this study, we investigated whether hyperthermia in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) enhances mortality in human colorectal carcinoma CX-1 cells. Cells were treated with various concentrations of TRAIL (0–200 ng/ml) at various temperatures (40–46°C) for 1 h and further incubated at 37°C in the presence of TRAIL. We observed that hyperthermia at 42–43°C effectively promoted TRAIL-induced apoptosis, as indicated by cell death, poly (ADP-ribose) polymerase (PARP) cleavage, and activation of caspase-8, -9, and -3. In contrast, hyperthermia at 45–46°C suppressed TRAIL-induced apoptosis. We also observed that mild hyperthermia, but not acute hyperthermia, promoted cytochrome *c* release during treatment with TRAIL. Our data suggest that promotion of cytochrome *c* release during mild hyperthermia is responsible for the enhancement of TRAIL cytotoxicity. J. Cell. Biochem. 101: 619–630, 2007.

Key words: hyperthermia; TRAIL; apoptosis; caspase; cytochrome *c*

Colorectal cancer in the United States is the second leading cause of cancer-related mortality, and death usually results from uncontrolled metastatic disease. The liver is the most common site of metastasis for colorectal cancer. Isolated hepatic perfusion (IHP) involves a method of complete vascular isolation of the liver to enable treatment of liver tumors with high doses of chemotherapeutic agents, biologic agents, and mild hyperthermia. In this study, we examined whether tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo-2L) in combination with mild hyperthermia effectively kills colorectal cancer cells.

More than 5,000 years ago, the Egyptians described the medical use of hyperthermia on a patient with a tumor in the breast [Overgaard, 1985]. Also, the ancient Greeks used hyperthermia to treat many diseases, including cancer [Hahn, 1982]. The effects of hyperthermia were observed in the last decades of the 19th century, when spontaneous tumor regression occurred in patients with high fever after bacterial infections [Corey, 1893]. In the past 20 years, the biological effects of hyperthermia as well as its use as adjuvant in cancer therapy have been

Abbreviations used: DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; IHP, isolated hepatic perfusion; PAGE, polyacrylamide gel electrophoresis; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal dUTP nick-end labeling.

Grant sponsor: NCI; Grant numbers: CA95191, CA96989, CA121395; Grant sponsor: Department of Defense Prostate Cancer Research Program Fund; Grant number: PC020530; Grant sponsor: Susan G. Komen Breast Cancer Foundation.

*Correspondence to: Dr. Yong J. Lee, Department of Surgery, University of Pittsburgh, The Hillman Cancer Center 1.46C, 5117 Centre Ave., Pittsburgh, PA 15213.
E-mail: leeyj@upmc.edu

Received 29 August 2006; Accepted 16 October 2006

DOI 10.1002/jcb.21203

extensively investigated. The hyperthermic temperatures of interest for cancer therapy are limited to the temperature range of 40–46°C to maximize the damage to tumors while preserving the surrounding normal tissues. Besides the cell killing effect of heat alone, it was found that heat acts synergistically with ionizing radiation [Dewey et al., 1978; Holahan et al., 1984; Kampinga and Dikomey, 2001], with a number of chemotherapeutic agents [Herman et al., 1982; Haas et al., 1984; Ko et al., 2006], and with various cytokines [Srinivasan et al., 1990; Klostergaard et al., 1992; Lee et al., 1993].

TRAIL/Apo-2L has been shown to induce apoptosis in a broad range of cancer cell types but not in normal cells and tissues [Ashkenazi and Dixit, 1999; Walczak et al., 1999]. TRAIL is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family, which includes Fas ligand (FasL) and TNF. The C-terminal extracellular region of TRAIL (amino acids 114–281) exhibits a homotrimeric subunit structure [Pitti et al., 1996] and its function is to induce apoptosis of tumor cells. The mechanism of TRAIL-induced apoptosis is initiated by binding to death receptors such as TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and induces the apoptotic signal. Both DR4 and DR5 contain a cytoplasmic death domain, which is required for TRAIL receptor-induced apoptosis. TRAIL also binds to decoy receptors (DcR1 and DcR2), which results in inhibition of TRAIL signaling [Degli-Esposti et al., 1997a,b; Marsters et al., 1997; Pan et al., 1997a,b; Sheridan et al., 1997; Walczak et al., 1997]. TRAIL-induced cytotoxicity can be modulated by various agents such as chemotherapeutic drugs [Griffith et al., 1998; Keane et al., 1999; Nagane et al., 2000], ionizing radiation [Chinnaiyan et al., 2000], cytokines [Park et al., 2002], and matrix metalloprotease inhibitors [Nyormoi et al., 2003].

For IHP, mild hyperthermia (41–42°C) has been successfully used to enhance the effectiveness of chemotherapeutic agents. In this study, we investigated whether hyperthermia can enhance TRAIL-induced cytotoxicity in human colorectal cancer cells. We observed that mild hyperthermia, but not acute hyperthermia (44–46°C), promotes TRAIL-induced cytotoxicity by facilitating activation of caspase through the mitochondria-dependent cytochrome *c* release.

MATERIALS AND METHODS

Cell Culture and Survival Assay

Human colorectal carcinoma CX-1 cells were cultured in RPMI-1640 medium (Gibco BRL) containing 10% fetal bovine serum (HyClone, Logan, UT) and 26 mM sodium bicarbonate for monolayer cell culture. Human prostate adenocarcinoma DU-145 and human pancreatic carcinoma MIA PaCa-2 cells were cultured in DMEM medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum. The dishes containing cells were kept in a 37°C humidified incubator with 5% CO_2. One or 2 days prior to the experiment, cells were plated into 60-mm dishes. For trypan blue exclusion assay [Burow et al., 1998], trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination.

Production of Recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114–281) obtained by RT-PCR was cloned into a pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the Qiagen express protein purification system (Qiagen, Valencia, CA).

Hyperthermia Treatment

Cells cultured in 60-mm dishes were sealed with parafilm and were placed in a circulating water bath (Heto, Thomas Scientific, Denmark) which was maintained within ± 0.02°C of the desired temperature.

Morphological Evaluation

Approximately 5×10^5 cells were plated into 60-mm dishes overnight. Cells were treated with TRAIL and/or hyperthermia and then analyzed by phase-contrast microscopy for signs of apoptosis.

TUNEL Assay

For detection of apoptosis by the TUNEL method, cells were plated in slide chambers. After treatment, cells were fixed with 4% paraformaldehyde in PBS. Cells were washed

once, permeabilized by incubating with 100 μl of 0.1% Triton X-100 and 0.1% sodium citrate, and then washed twice in PBS. The TUNEL reaction was carried out with 50 μl of TUNEL reaction mixture (450 μl of label solution/50 μl of enzyme solution; AP cell death detection kit, Roche, Germany). After washing three times with PBS, 50 μl of converter-AP was added on a sample. Thirty minutes after incubation in a humidified chamber at 37°C, each 100 μl of substrate (NBT/BCIP, Roche) was added. After 10 min incubation in the dark, cells were subjected to washing and were examined under a microscope.

Antibodies

Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz (Santa Cruz, CA). Anti-DR4, anti-DR5, anti-DcR1, and anti-DcR2 antibodies were from ProSci (Poway, CA). Anti-cIAP-1 and anti-cIAP-2 antibodies were from R&D Systems (Minneapolis, MN). Anti-caspase-8, anti-FLIP (C-term: 447–464), anti-FLIP γ/δ, and anti-FLIP-L antibodies were from Cell Signaling (Beverly, MA). Monoclonal antibodies were purchased from each of the following companies: anti-Bcl-2 and anti-Bcl-XL antibodies from Santa Cruz, anti-caspase-9 antibody from Upstate Biotechnology (Lake Placid, NY), anti-PARP antibody from Biomol Research Laboratory (Plymouth Meeting, PA), anti-cytochrome *c* from PharMingen (San Diego, CA), and anti-actin antibody from ICN (Costa Mesa, CA).

Protein Extracts and Polyacrylamide Gel Electrophoresis (PAGE)

Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate (SDS), 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–polyacrylamide gels. SDS–PAGE analysis was performed according to Laemmli [1970] using a Hoefer gel apparatus.

Immunoblot Analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% non-fat dry milk in PBS-Tween-20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL).

In Vitro Caspase Activity Assays

Activities of caspase-8, -9, and -3 were measured by spectrophotometric detection of the chromophore p-nitroalanine (pNA) at 405 nm (Chemicon, Temecula, CA). The assay was performed by manufacturer's instruction. Briefly, CX-1 cells, treated or untreated with TRAIL, were harvested and lysed with 1× caspase lysis buffer. The cell lysates were chilled in ice for 10 min and the insoluble fraction was removed by centrifugation of 10,000*g*, 5 min at 4°C. Supernatants were used for further caspase assay. Total protein was measured by BCA protein assay kit (Pierce). To measure caspase activity, 50 μg of total protein was used for each assay.

Cytochrome *c* Release

To measure the release of cytochrome *c* from mitochondria, subconfluent cells growing in 100-mm dishes were treated as indicated in Figure 9. After treatment, cells were scraped, washed in PBS, washed in buffer H (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin), and resuspended in 200 μl buffer H. After 1 h incubation on ice, cells were lysed by forcing them through a 27-gauge needle 15–20 times. The lysate was centrifuged at 20,000*g* for 15 min and the supernatant was collected. Cytochrome *c* levels in the resulting supernatant were analyzed by immunoblotting.

Transfection

In order to generate Bcl-2 overexpressing CX-1 cells, cells were transfected with pcDNA-3-Bcl-2 or control plasmid pcDNA3-neo using LipofectAMINE Plus (Gibco-BRL, Grand Island, NY). Transfected cells were selected with G418. The clone expressing the highest level of Bcl-2 was used for this study.

RESULTS

Effect of Hyperthermia on TRAIL-Induced Cytotoxicity

The technique of IHP employs a single hyperthermic 1 h treatment via an isolated vascular recirculating perfusion circuit. To examine the effect of hyperthermia on TRAIL-induced cytotoxicity, human colorectal carcinoma CX-1 cells were treated with 50 ng/ml TRAIL for 1 h at various temperatures (37–46°C) and then incubated at 37°C for 3 h. Data from morphological analysis in Figure 1A show that no or minimal morphological alterations were observed during mild hyperthermia (40–42°C) alone, but severe changes were observed during acute hyperthermia (43–46°C) alone. During treatment with TRAIL, cells undergoing apoptosis showed cell surface blebbing and formation of apoptotic bodies (Fig. 1A). An increase in the number of rounded cells and detached cells was observed during treatment with TRAIL in combination with hyperthermia. However, Figure 1A clearly shows that the augmentation of TRAIL-induced cytotoxicity was dependent upon heating temperatures. A maximal augmentation occurred at 43°C and the augmentative effect markedly disappeared at higher temperatures. Similar results were observed with TUNEL staining (Fig. 1B). Data from TUNEL assays show that apoptotic death occurred during treatment with TRAIL, and hyperthermia at 42°C, but not at 46°C, increased TRAIL-induced apoptotic death. Similar results were observed with biochemical analysis (Fig. 2A). Data from Figure 2A show that the combination of hyperthermia (40–43°C) and TRAIL treatment enhances poly (ADP-ribose) polymerase (PARP) cleavage, the hallmark feature of apoptosis. PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of 50 ng/ml TRAIL. This cleavage was enhanced by hyperthermia, in particular heating at 42–43°C. However, the cleavage of PARP was reduced and even suppressed at 45–46°C (Fig. 2A). It is well known that TRAIL-induced apoptosis is mediated through a caspase cascade. To examine whether hyperthermia alters TRAIL-induced apoptosis through activation/inhibition of caspases, several caspases known to be involved in TRAIL-induced apoptosis were examined. Figure 2A shows that hyperthermia at 40–43°C enhanced TRAIL-induced caspase-8 activation. Western blot analysis shows that procaspase-8 (55/54 kDa) cleavage to intermediate (43/41 kDa) forms was enhanced with increasing temperature in the presence of TRAIL. Hyperthermia at 40–43°C enhanced the proteolytic processing of procaspase-9 (48 kDa) into its active form

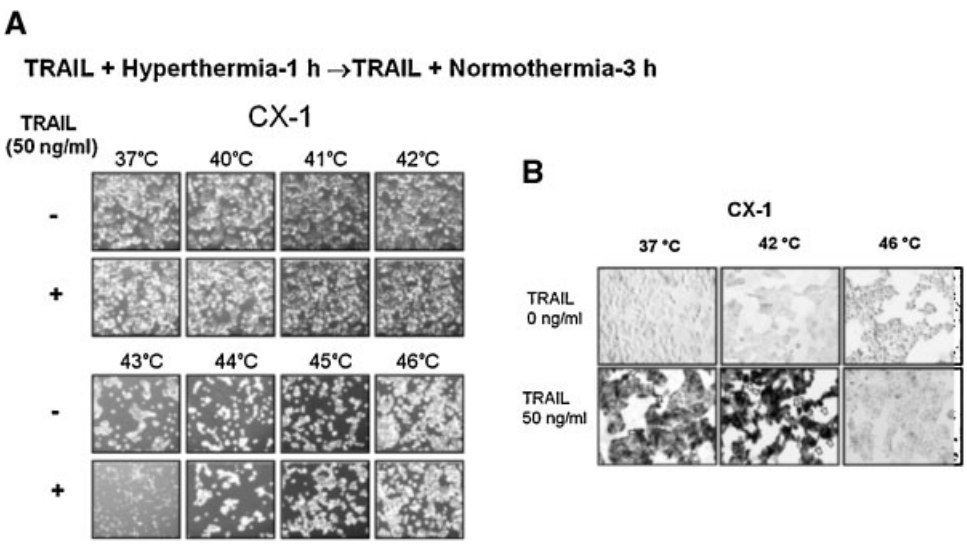

Fig. 1. Effect of hyperthermia (40–46°C) on TRAIL-induced apoptosis in human colorectal carcinoma CX-1 cells. **A**: Cells were exposed to hyperthermia (40–46°C) or normothermia in the presence of 50 ng/ml TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of 50 ng/ml TRAIL. The morphological features were analyzed with a phase-contrast microscope. **B**: Cells were exposed to hyperthermia (42°C or 46°C) or normothermia in the presence of 50 ng/ml TRAIL for 1 h and then incubated at 37°C for 3 h. After treatment, apoptosis was detected by the TUNEL assay.

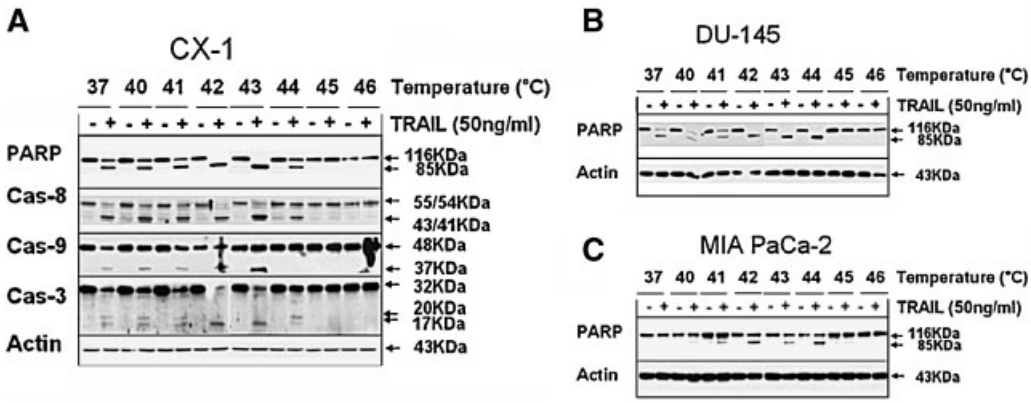

Fig. 2. Effect of hyperthermia (40–46°C) on TRAIL-induced proteolytic cleavage of PARP and activation of caspases in CX-1 (**A**), DU-145 (**B**), or MIA PaCa-2 (**C**) cells. Cells were exposed to hyperthermia (40–46°C) or normothermia in the presence of 50 ng/ml TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of 50 ng/ml TRAIL. Cell lysates were subjected to immunoblotting for caspase-8, -9, -3, or PARP. Antibody against caspase-8 detects inactive form (55/54 kDa) and cleaved intermediates (41, 43 kDa). Anti-caspase-9 antibody detects both inactive form (48 kDa) and cleaved intermediate (37 kDa). Anti-caspase-3 antibody detects inactive form (32 kDa) and cleaved active form (17 kDa). Immunoblots of PARP show the 116 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the equal amount of proteins loaded in each lane.

(37 kDa). Hyperthermia at 40–43°C also increased TRAIL-induced caspase-3 activation. Western blot analysis shows that procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (20 and 17 kDa) in the presence of TRAIL. The combined treatment with TRAIL and hyperthermia increased the level of active form, in particular 17 kDa. However, unlike hyperthermia at 40–43°C, hyperthermia at 45–46°C suppressed TRAIL-induced activation of caspases. These results clearly revealed that hyperthermia-enhanced TRAIL cytotoxicity was dependent upon heating temperatures. To examine whether our observations are unique to CX-1 cells, human prostate adenocarcinoma DU-145 and human pancreatic carcinoma MIA PaCa-2 cells were also employed. Figure 2B,C shows that the combined treatment of TRAIL and hyperthermia at 42–44°C, but not at 45–46°C, resulted in an increase in cell death and PARP cleavage in these tumor cells. These results were similar to the observations in CX-1 cells.

We extended our studies to investigate the dose response of apoptotic death during treatment with various concentrations of TRAIL (10–50 ng/ml) in combination with hyperthermia (40–43°C). Figure 3A,B shows that cytotoxicity gradually increased when the dose was increased. Similar results were observed with biochemical analysis (Fig. 3C). Data from Figure 3C show that the combination of hyperthermia (40–43°C) and TRAIL treatment enhances PARP cleavage as well as activation of caspases. The augmentation of TRAIL-induced apoptosis was dependent upon heating temperatures and TRAIL doses.

We also investigated the effect of pretreatment with TRAIL in combination with hyperthermia (42°C) for 1 h on the time course of apoptotic death which occurred subsequently during various periods of incubation at 37°C. Figure 4 shows that apoptotic death gradually increased when the incubation time was increased. Combined pretreatment with TRAIL and hyperthermia (42°C) for 1 h facilitated apoptotic death.

Effect of Hyperthermia on TRAIL Protein

Figure 1 clearly demonstrated that hyperthermia at 40–43°C, but not at 45–46°C, promotes TRAIL-induced PARP cleavage. We hypothesized that these differential effects were due to inactivation of TRAIL protein during hyperthermic treatment at 45–46°C. To examine this possibility, TRAIL protein was heated at various temperatures (40–45°C) for 1 h prior to treatment of CX-1 cells with TRAIL. Figure 5 shows that there was no significant difference in TRAIL-induced cytotoxicity resulting from different prior heating temperatures. These results suggest that TRAIL protein is not altered during hyperthermic treatment.

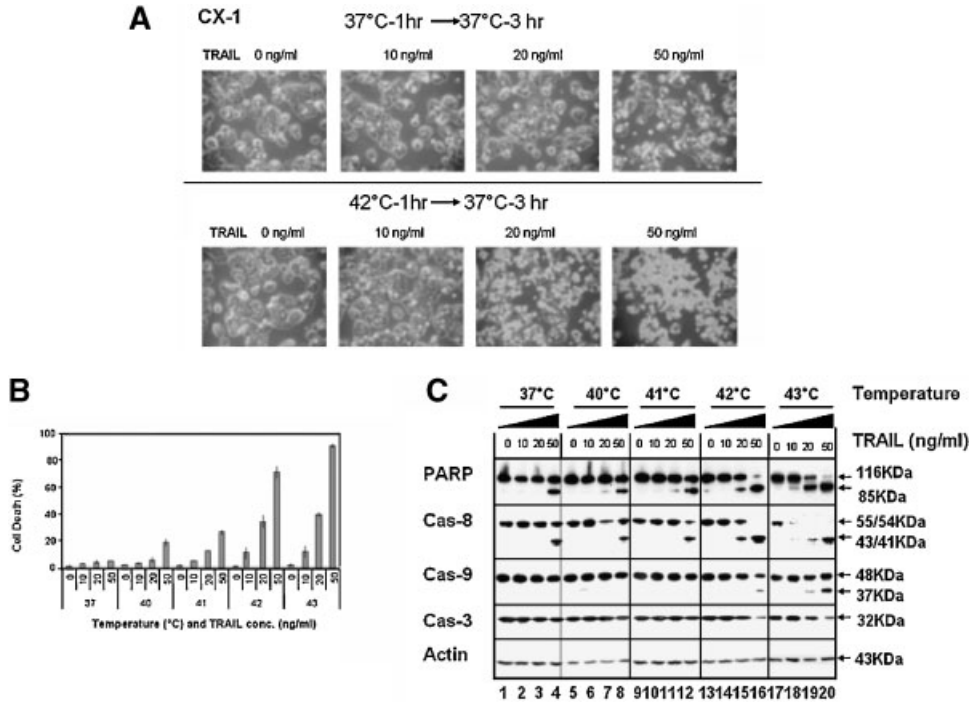

Fig. 3. Effect of hyperthermia (40–43°C) on various concentrations of TRAIL-induced apoptosis in human colorectal carcinoma CX-1 cells. **A**: Cells were treated with various concentrations of TRAIL (10–50 ng/ml) for 1 h at 37°C or 42°C and then incubated for 3 h at 37°C in the presence of TRAIL. The morphological features were analyzed with a phase-contrast microscope. **B**: Cells were exposed to hyperthermia (40–43°C) or normothermia in the presence of various concentrations of TRAIL (10–50 ng/ml) for 1 h and then treated with TRAIL for 3 h at 37°C. Cell survival was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. **C**: Cells were harvested from (B) and cell lysates were subjected to immunoblotting for caspase-8, -9, -3, or PARP as described in Figure 2. Actin was used to confirm the amount of proteins loaded in each lane.

Effect of Post-Hyperthermic Treatment on TRAIL-Induced Apoptosis

We examined whether hyperthermia modulates the initiation or the process of TRAIL-induced apoptosis. CX-1 cells were treated with 50 ng/ml TRAIL for 1 h and heated or unheated for 1 h. Cells were then further incubated at 37°C for 3 h. Results from Figure 6A show that hyperthermia at 42°C promoted TRAIL-induced PARP cleavage as well as activation of caspases. In contrast, hyperthermia at 46°C

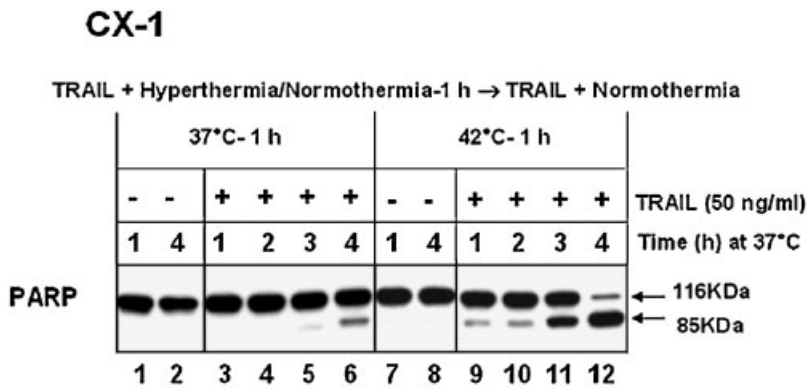

Fig. 4. Time course of apoptosis after treatment with TRAIL in the conditions of normothermia and hyperthermia. CX-1 cells were treated with 50 ng/ml TRAIL at 42°C or 37°C for 1 h and then incubated for various times (1–4 h) at 37°C in the presence of TRAIL. The time course of PARP cleavage was assayed by Western blot analyses as described in Figure 2.

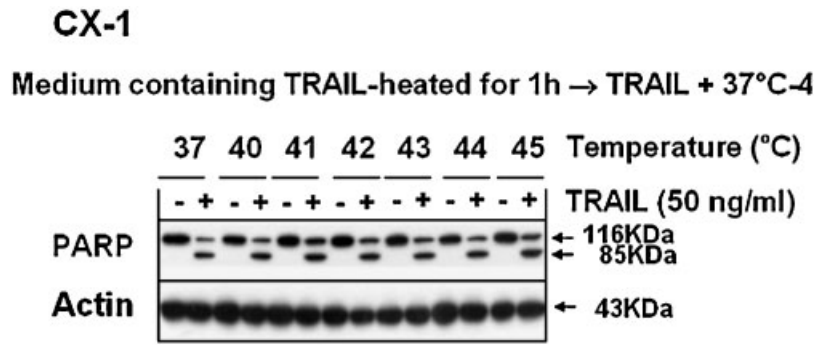

Fig. 5. Heated TRAIL protein-induced apoptosis in CX-1 cells. TRAIL was heated for 1 h at various temperatures (40–45°C). Cells were treated with heated TRAIL (50 ng/ml) for 4 h and then cell lysates were subjected to immunoblotting for PARP as described in Figure 2. Actin was used to confirm the amount of proteins loaded in each lane.

suppressed TRAIL-induced PARP cleavage and activation of caspases. These results are similar to Figures 1 and 2, in which TRAIL treatment and heat were applied simultaneously. Interestingly, when cells were washed after TRAIL application but prior to hyperthermia, hyperthermia at 42°C, but not at 46°C, still augmented TRAIL-induced PARP cleavage (Fig. 6B). These results suggest that hyperthermia modulates the process of TRAIL-induced apoptosis.

Effect of Hyperthermia and TRAIL on Anti-Apoptotic Proteins and the TRAIL Receptor Family

It is well known that the reduction of intracellular anti-apoptotic molecules such as FLIP, IAP-1, IAP-2, Bcl-2, and Bcl-X_L sensitizes TRAIL-resistant cancer cells to TRAIL. Thus, we examined whether changes in the amounts of anti-apoptotic proteins are associated with

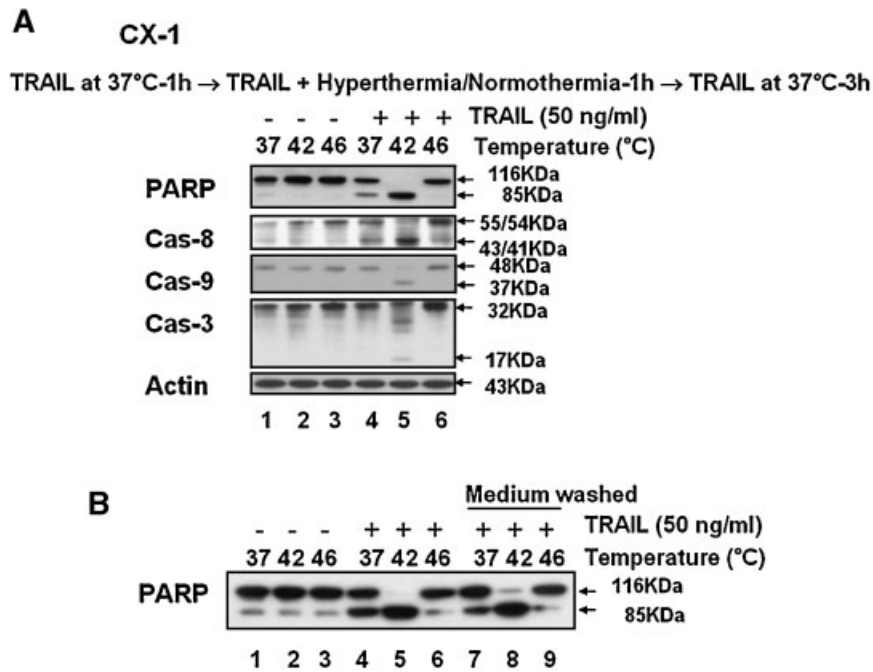

Fig. 6. Effect of post-hyperthermic treatment on TRAIL-induced apoptosis in CX-1 cells. **A**: Cells were treated with or without TRAIL at 37°C for 1 h and heated at 42°C or 46°C for 1 h and then incubated at 37°C for 3 h. Cell lysates were subjected to immunoblotting for caspase-8, -9, -3, or PARP as described in Figure 2. Actin was used to confirm the amount of proteins loaded in each lane. **B**: Experiments were repeated (**lanes 1–6**) except that cells were washed prior to hyperthermic or normothermic treatment (**lanes 7–9**). PARP cleavage was detected as described in Figure 2.

the promotion by hyperthermia of apoptosis by TRAIL. Data from Western blot analysis reveal that the combined treatment did not significantly alter the levels of FLIPL, FLIPS, IAP-1, IAP-2, and Bcl-X_L (Fig. 7A). Interestingly, there is no detectable level of Bcl-2 in CX-1 cells.

It is also well known that TRAIL-induced apoptotic signals are triggered by interaction with two death receptors (DR4 and DR5). Such signals may be blocked by antagonistic decoy receptors (DcR1 and DcR2). Previous studies also demonstrate that increased DR5 levels induced by genotoxic agents are responsible for increasing TRAIL cytotoxicity. Thus, we examined whether treatment with TRAIL in combination with hyperthermia affects the level of TRAIL receptors. Data from Western blot analysis revealed that the combined treatment did not significantly alter the total cellular levels of death receptors DR4 and DR5 as well as decoy receptors DcR1 and DcR2 (Fig. 7B).

Effect of Hyperthermia on Caspase Activity

TRAIL-induced cell death is mediated through caspase cascades. So we hypothesized that promotion of caspase enzyme activities by hyperthermia is responsible for enhancement of TRAIL-induced apoptotic death. To test this possibility, CX-1 cells were treated with TRAIL, cell lysates were harvested, and then caspase activity was measured during hyperthermia (42°C or 46°C). Data from in vitro enzyme kinetics assay demonstrated that hyperthermia at both temperatures significantly promoted caspase enzyme activity (Fig. 8A–C). These results from in vitro enzyme kinetics assay

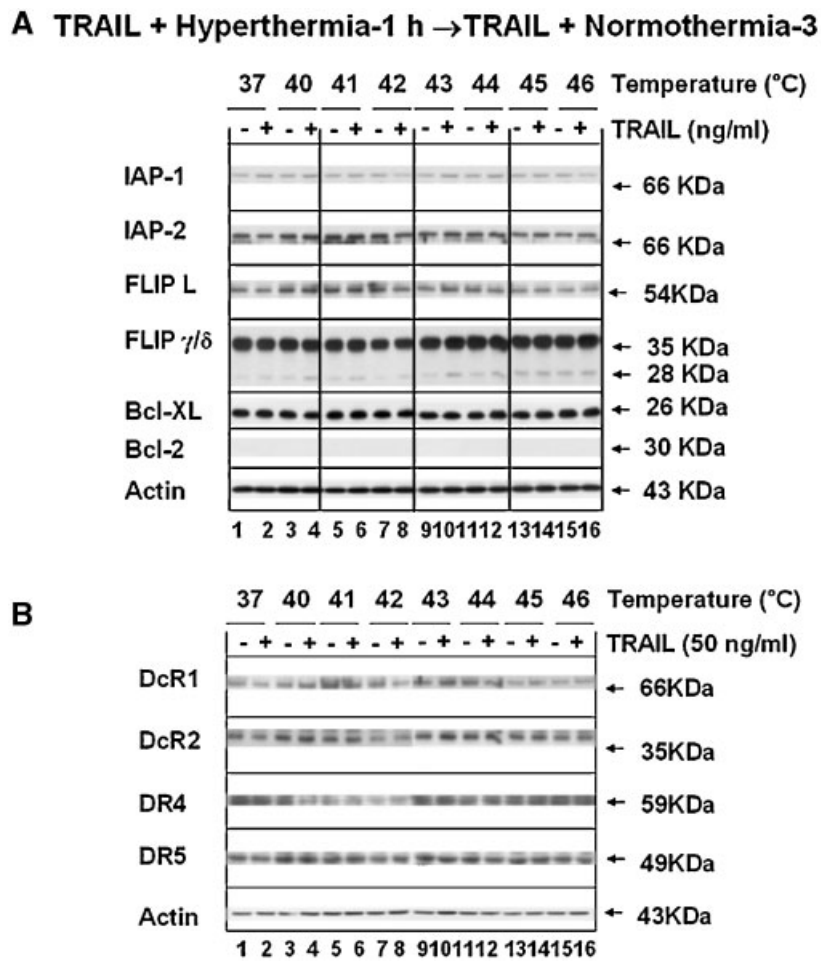

Fig. 7. Effect of hyperthermia on intracellular levels of anti-apoptotic proteins (**A**) or TRAIL receptors (**B**) during treatment with TRAIL. CX-1 cells were exposed to hyperthermia (40–46°C) or normothermia in the presence of 50 ng/ml TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of 50 ng/ml TRAIL. Equal amounts of protein (20 μg) were separated and immunoblotted as described in Materials and Methods. Actin was shown as an internal standard.

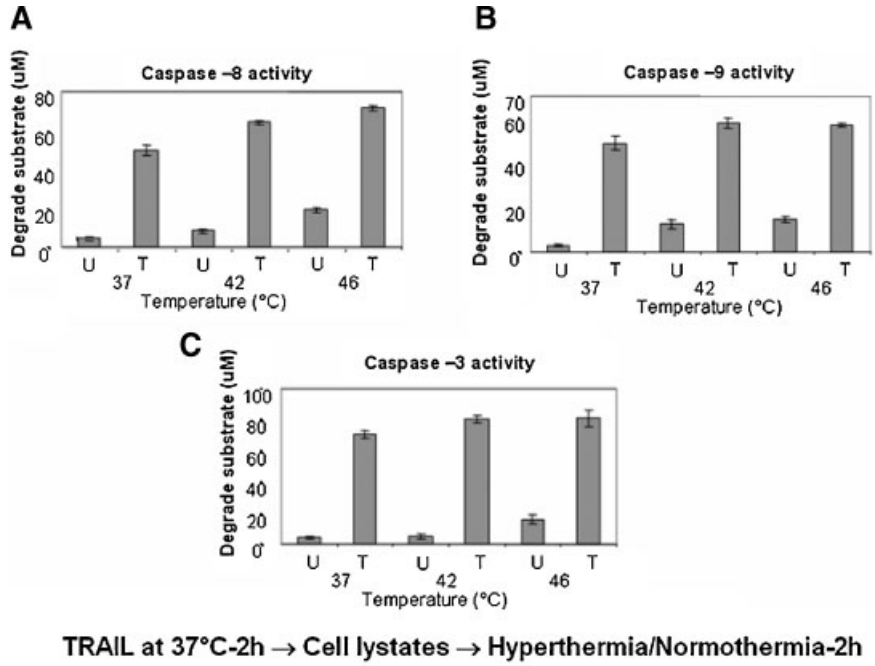

Fig. 8. Effects of hyperthermia on caspase activity. CX-1 cells were treated with or without 200 ng/ml of TRAIL for 2 h, and the cell lysates were prepared for caspase activity measurement. In order to measure the activity of caspase-8 (**A**), caspase-9 (**B**), and caspase-3 (**C**), cell lysates were subjected to reaction at 37°C, 42°C, or 46°C for 2 h. U, untreated cell lysates; T, TRAIL-treated cell lysates. Error bars represent the mean ± SE from three separate experiments.

somewhat contradicted our previous observations from cells that hyperthermia at 46°C inhibits rather than promotes TRAIL-induced apoptosis (Figs. 1 and 2). These results suggest that even though caspase enzyme activity is thermodynamically enhanced during hyperthermia, biological responses are more complicated than biochemical reactions.

Differential Effect of Hyperthermia on TRAIL-Induced Apoptosis Is Mediated Through Mitochondria

Previous studies demonstrated that apoptotic signaling via a mitochondria-dependent pathway plays an important role in TRAIL-induced activation of caspases. We hypothesized that the differential effect of hyperthermia is due to differences in cytochrome *c* release from mitochondria. It is also well known that the cytochrome *c* release from mitochondria activates various caspases during apoptosis (31–33). Figure 9A shows that hyperthermia at 42°C promoted cytochrome *c* release. In contrast, hyperthermia at 46°C inhibited cytochrome *c* release. We examined whether overexpression of Bcl-2 inhibits cytochrome *c* release. This is based on findings that overexpression of the Bcl-2 gene inhibits mitochondria-dependent apoptosis pathways and that overexpression of the Bcl-2 gene also inhibits the enhancing effect of hyperthermia, PARP cleavage, and caspase activation [Li et al., 1997; Yang et al., 1997; Pastorino et al., 1998]. Overexpression of Bcl-2 inhibited the promotive effect of hyperthermia on TRAIL-induced cytochrome *c* release (Fig. 9B). These results suggest that hyperthermia promotes TRAIL-induced cytochrome *c* release from mitochondria and the inclusion of the involvement of the mitochondria-dependent pathway may elucidate the enhancement of TRAIL-induced cytotoxicity by hyperthermia.

DISCUSSION

IHP therapy as a treatment for liver metastatic disease from colon cancer has been developed since 1970 when the totally implantable hepatic artery pump became available. Isolated perfusion delivering high doses of chemotherapeutic agents, biological agents, and hyperthermia via an isolated vascular recirculating perfusion circuit effectively controls local disease. In this study, we demonstrate that TRAIL in combination with mild

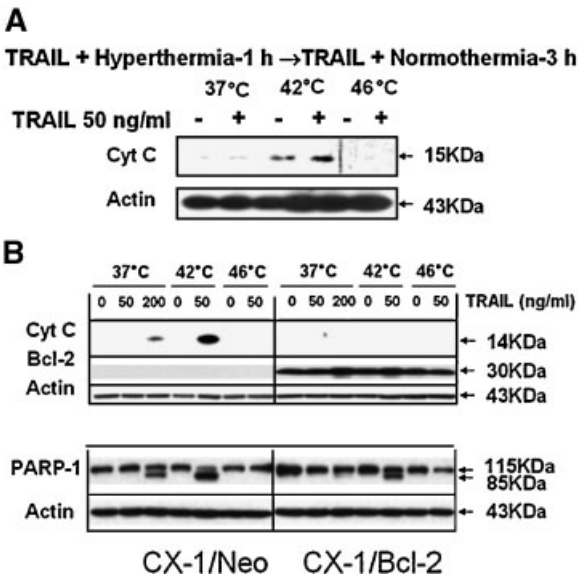

Fig. 9. Effect of Bcl-2 on the hyperthermia-TRAIL combined treatment of CX-1 cells. **A**: Cells were exposed to hyperthermia (42°C or 46°C) or normothermia in the presence of 50 ng/ml TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of 50 ng/ml TRAIL. Cytosolic fractions were used for detecting cytosolic cytochrome *c*. **B**: Control vector transfected (CX-1/Neo) or Bcl-2 transfected (CX-1/Bcl-2) cells were exposed to hyperthermia (42°C or 46°C) or normothermia in the presence of 50–200 ng/ml TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of 50–200 ng/ml TRAIL. Cytosolic fractions were used for detecting cytosolic cytochrome *c*. Actin was shown as an internal standard.

hyperthermia, which is clinically applicable, effectively kills human colorectal carcinoma CX-1 cells.

Previous studies reveal the synergistic effect of hyperthermia on TNF- and/or interferon-γ-induced cytotoxicity [Srinivasan et al., 1990; Klostergaard et al., 1992]. However, our studies have demonstrated differential effects of hyperthermia on TRAIL-induced apoptosis. Hyperthermia at 41–44°C promotes TRAIL-induced apoptosis by facilitating caspase activity; in contrast, hyperthermia at 45–46°C inhibits TRAIL-induced apoptosis (Fig. 2). The latter observation is similar to a previous report that heat shock protects cells from TRAIL-induced apoptosis [Ozoren and El-Deiry, 2002]. However, in that case, cells were heated prior to TRAIL treatment, so the resistance to TRAIL may be due to the synthesis of anti-apoptotic molecules such as HSP70 and HSP90. It is well known that HSP70 and HSP90 interact with Apaf-1, preventing efficient assembly of the apoptosome [Beere et al., 2000; Pandey et al., 2000; Saleh et al., 2000; Ran et al., 2004], or antagonize the caspase-independent death effector apoptosis inducing factor (AIF) [Ravagnan et al., 2001]. However, since heat shock at 45–46°C inhibits protein synthesis for several hours [Lee and Dewey, 1987], HSP70 and HSP90 are not likely to be involved in inhibiting TRAIL-induced apoptosis.

Data from Figure 9 show that hyperthermia at 42°C, but not at 46°C, enhances TRAIL-induced apoptotic death through a mitochondria-dependent pathway. A fundamental question is how hyperthermia has a dual effect on TRAIL-induced apoptosis. Previous studies suggest that, during the process leading to cytochrome *c* release and consequent apoptosis, truncated Bid is associated with Bax and subsequently induces oligomerization of Bax. The oligomerized Bax translocates from the cytosol to the mitochondria and then integrates into the outer mitochondrial membrane, where it triggers cytochrome *c* release [Eskes et al., 2000]. Hashimoto et al. [2003] reported that an increase in intracellular Ca^{2+} is the important initiating factor in hyperthermia-induced apoptosis. The generation of reactive oxygen species and an increase in intracellular Ca^{2+} may lead to an elevation of Bax level and produce Bax-mediated mitochondrial apoptosis [Salah-Eldin et al., 2003]. Hyperthermia also induces gradual Bax translocation from the cytoplasm to the nucleus [Zhao et al., 2006]. It is possible that hyperthermia promotes or inhibits cytochrome *c* release, dependent upon heating temperatures, by differentially altering intracellular Ca^{2+} concentrations and the translocation of Bax. We believe that this model provides a framework for future studies.

REFERENCES

Ashkenazi A, Dixit VM. 1999. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 11:255–260.

Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM, Green DR. 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475.

Burow ME, Weldon CB, Tang Y, Navar GL, Krajewski S, Reed JC, Hammond TG, Clejan S, Beckman BS. 1998. Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res 58:4940–4946.

Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A.

2000. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci USA 97: 1754–1759.

Corey WB. 1893. The treatment of malignant tumours by repeated inoculation of erysipelas—With a report of ten original cases. Am J Med Sci 105:487–511.

Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG. 1997a. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity 7:813–820.

Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, Goodwin RG, Smith CA. 1997b. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J Exp Med 186:1165–1170.

Dewey WC, Sapareto SA, Betten DA. 1978. Hyperthermic radiosensitization of synchronous Chinese hamster cells: Relationship between lethality and chromosomal aberrations. Radiat Res 76:48–59.

Eskes R, Desagher S, Antonsson B, Martinou JC. 2000. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol 20:929–935.

Griffith TS, Chin WA, Jackson GC, Lynch DH, Kubin MZ. 1998. Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J Immunol 161:2833–2840.

Haas GP, Klugo RC, Hetzel FW, Barton EE, Cerny JC. 1984. The synergistic effect of hyperthermia and chemotherapy on murine transitional cell carcinoma. J Urol 132:828–833.

Hahn GM. 1982. Hyperthermia and cancer. New York: Plenum Press.

Hashimoto T, Shibata MA, Ito Y, Nakao KI, Sasaki S, Otsuki Y. 2003. Elevated levels of intracellular Ca^{2+} and apoptosis in human lung cancer cells given heat-shock. Int J Hyperthermia 19:178–192.

Herman TS, Sweets CC, White DM, Gerner EW. 1982. Effect of heating on lethality due to hyperthermia and selected chemotherapeutic drugs. J Natl Cancer Inst 68: 487–491.

Holahan EV, Highfield DP, Holahan PK, Dewey WC. 1984. Hyperthermic killing and hyperthermic radiosensitization in Chinese hamster ovary cells: Effects of pH and thermal tolerance. Radiat Res 97:108–131.

Kampinga HH, Dikomey E. 2001. Hyperthermic radiosensitization: Mode of action and clinical relevance. Int J Radiat Biol 77:399–408.

Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. 1999. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 59: 734–741.

Klostergaard J, Leroux E, Siddik ZH, Khodadadian M, Tomasovic SP. 1992. Enhanced sensitivity of human colon tumor cell lines in vitro in response to thermochemoimmunotherapy. Cancer Res 52:5271–5277.

Ko SH, Ueno T, Yoshimoto Y, Yoo JS, Abdel-Wahab OI, Abdel-Wahab Z, Chu E, Pruitt SK, Friedman HS, Dewhirst MW, Tyler DS. 2006. Optimizing a novel regional chemotherapeutic agent against melanoma: Hyperthermia-induced enhancement of temozolomide cytotoxicity. Clin Cancer Res 12:289–297.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685.

Lee YJ, Dewey WC. 1987. Effect of cycloheximide or puromycin on induction of thermotolerance by heat in Chinese hamster ovary cells: Dose fractionation at 45.5°C. Cancer Res 47:5960–5966.

Lee YJ, Hou Z, Curetty L, Cho JM, Corry PM. 1993. Synergistic effects of cytokine and hyperthermia on cytotoxicity in HT-29 cells are not mediated by alteration of induced protein levels. J Cell Physiol 155: 27–35.

Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz LC. 1997. Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. J Biol Chem 272: 30299–30305.

Marsters SA, Sheridan JP, Pitti RM, Brush J, Goddard A, Ashkenazi A. 1997. Identification of a ligand for the death-domain-containing receptor Apo3. Curr Biol 7:1003–1006.

Nagane M, Pan G, Weddle JJ, Dixit VM, Cavenee WK, Huang HJ. 2000. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. Cancer Res 60:847–853.

Nyormoi O, Mills L, Bar-Eli M. 2003. An MMP-2/MMP-9 inhibitor, 5a, enhances apoptosis induced by ligands of the TNF receptor superfamily in cancer cells. Cell Death Differ 10:558–569.

Overgaard J. 1985. History and heritage—An introduction. In: Overgaard J, editor. Hyperthermic oncology 1984, Vol. 2. London and Philadelphia: Taylor & Francis. pp 3–8.

Ozoren N, El-Deiry W. 2002. Heat shock protects HCT116 and H460 cells from TRAIL-induced apoptosis. Exp Cell Res 281(2):175–181.

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. 1997a. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science 277:815–818.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM. 1997b. The receptor for the cytotoxic ligand TRAIL. Science 276:111–113.

Pandey P, Saleh A, Nakazawa A, Kumar S, Srinivasula SM, Kumar V, Weichselbaum R, Nalin C, Alnemri ES, Kufe D, Kharbanda S. 2000. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. EMBO J 19:4310–4322.

Park SY, Billiar TR, Seol DW. 2002. IFN-gamma inhibition of TRAIL-induced IAP-2 upregulation, a possible mechanism of IFN-gamma-enhanced TRAIL-induced apoptosis. Biochem Biophys Res Commun 291:233–236.

Pastorino JG, Chen ST, Tafani M, Snyder JW, Farber JL. 1998. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. J Biol Chem 273:7770–7775.

Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 271(22):12687–12690.

Ran R, Zhou G, Lu A, Zhang L, Tang Y, Rigby AC, Sharp FR. 2004. Hsp70 mutant proteins modulate additional

apoptotic pathways and improve cell survival. Cell Stress Chaperones 9:229–242.

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, Kroemer G. 2001. Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3:839–843.

Salah-Eldin AE, Inoue S, Tsukamoto S, Aoi H, Tsuda M. 2003. An association of Bcl-2 phosphorylation and Bax localization with their functions after hyperthermia and paclitaxel treatment. Int J Cancer 103:53–60.

Saleh A, Srinivasula SM, Balkir L, Robbins PD, Alnemri ES. 2000. Negative regulation of the Apaf-1 apoptosome by Hsp70. Nat Cell Biol 2(8):476–483.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277:818–821.

Srinivasan JM, Fajardo LF, Hahn GM. 1990. Mechanism of antitumor activity of tumor necrosis factor alpha with hyperthermia in a tumor necrosis factor alpha-resistant tumor. J Natl Cancer Inst 82:1904–1910.

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, Goodwin RG, Rauch CT. 1997. TRAIL-R2: A novel apoptosis-mediating receptor for TRAIL. EMBO J 16:5386–5397.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JCL, Lynch DH. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5: 157–163.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. 1997. Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science 275:1129–1132.

Zhao QL, Fujiwara Y, Kondo T. 2006. Mechanism of cell death induction by nitroxide and hyperthermia. Free Radic Biol Med 40:1131–1143.