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Effect of Hyperthermia and Chemotherapeutic Agents on TRAIL-Induced Cell Death in Human Colon Cancer Cells

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Abstract Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent because of its tumor selectivity. TRAIL is known to induce apoptosis in cancer cells but spare most normal cells. In the previous study [Yoo and Lee, 2007], we have reported that hyperthermia could enhance the cytotoxicity of TRAIL-induced apoptosis. We observed in human colorectal cancer cell line CX-1 that TRAIL-induced apoptotic death and also that mild hyperthermia promoted TRAIL-induced apoptotic death through caspase activation and cytochrome-*c* release. Although its effects in vivo are not clear, hyperthermia has been used as an adjunctive therapy for cancer. Hyperthermia is often accompanied by chemotherapy to enhance its effect. In this study, CX-1 colorectal adenocarcinoma cells were treated with TRAIL concurrently with hyperthermia and oxaliplatin or melphalan. To evaluate the cell death effects on tumor cells via hyperthermia and TRAIL and chemotherapeutic agents, FACS analysis, DNA fragmentation, and immunoblottings for PARP-1 and several caspases and antiapoptotic proteins were performed. Activities of casapse-8, caspase-9, and caspase-3 were also measured in hyperthermic condition. Interestingly, when analyzed with Western blot, we detected little change in the intracellular levels of proteins related to apoptosis. Clonogenic assay shows, however, that chemotherapeutic agents will trigger cancer cell death, either apoptotic or non-apoptotic, more efficiently. We demonstrate here that CX-1 cells exposed to 42°C and chemotherapeutic agents were sensitized and died by apoptotic and non-apoptotic cell death even in low concentration (10 ng/ml) of TRAIL. J. Cell. Biochem. 103: 98–109, 2008.

Key words: colon cancer; hyperthermia; isolated hepatic perfusion (IHP); oxaliplatin; melphalan; chemotherapeutic agents; TRAIL; apoptosis; caspase-8; caspase-9; caspase-3

INTRODUCTION

Colorectal cancer remains the second leading cause of cancer-related mortality in Europe and in the United States, responsible for approximately 0.4 million deaths annually worldwide [Boyle and Langman, 2000]. The main cause of death of patients with colorectal cancer is hepatic metastases [Ruers and Bleichrodt, 2002]. Although there have been occasional applications of immunotherapy, the primary treatment for colorectal cancer at this stage is surgical resection.

Approximately 25% of patients with colorectal cancer will develop metastatic disease exclusively or largely confined to the liver [de Gramont et al., 2000]. In 25% of metastatic patients, hepatic metastases become clinically evident during postoperative follow-up, usually within 2 years after detection of primary colorectal cancer [Finlay and McArdle, 1986] but 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 maximized systemic doses of chemotherapeutic agents, biologic agents, and hyperthermia [Ridge et al., 1987a; Ridge et al., 1987b;

Abbreviations used: IHP, isolated hepatic perfusion; DcR1, decoy receptor 1; DcR2, decoy receptor 2; DR4, death receptor 4; DR5, death receptor 5; FADD, Fas-associated death domain; 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.

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Vahrmeijer et al., 2000]. IHP was first performed more than 40 years ago by Ausman [1961] and, in the past 20 years, the biological effects of hyperthermia as well as its use as adjuvant in cancer therapy have been extensively investigated. Alexander et al. [2000a,b] yielded promising results with IHP for liver metastases from uveal melanoma showing a response rate of 62% and a median survival of up to 12 months. After IHP treatment for liver metastases from ocular melanoma, Noter and his colleagues also reported a response rate of 50% and an overall median survival of 9.9 months with a 1-year survival of 50% and a 2-year survival of 37.5% after IHP [Noter et al., 2004].

Although IHP results in considerable tumor response and in high survival rates in a selective group of patients, novel strategy for regional therapies is needed to improve its efficacy. We previously reported that mild hyperthermia (42°C) promotes tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo-2L)-induced cytotoxicity by facilitating activation of caspases through mitochondria-dependent cytochrome-*c* release in colorectal cancer cells [Yoo and Lee, 2007]. In CX-1 human colorectal cancer cells, we observed that TRAIL-induced apoptotic death can be enhanced by mild hyperthermia through caspase activation and cytochrome-*c* release.

TRAIL, a type II integral membrane protein belonging to the TNF family, induces apoptosis in a broad range of cancer cells types but spares normal cells and tissues [Ashkenazi and Dixit, 1999; Walczak et al., 1999]. TRAIL initiates the death signal by binding to death receptors such as TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and inducing the apoptotic signal. Both DR4 and DR5 contain a cytoplasmic death domain, which is required for TRAIL receptor-induced apoptosis. However, in normal cells TRAIL also binds to highly expressed 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]. We are reporting here how hyperthermia increases the efficacy of chemotherapeutic agents like oxaliplatin or melphalan used in combination with TRAIL killing the colorectal cancer cells more effectively.

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. The dishes containing cells were kept in a 37°C humidified incubator with 5% CO2. One or two days prior to the experiment, cells were plated into 60-mm dishes. For the morphological evaluation of cell death, approximately 5×10^5 cells were plated into 60-mm dishes overnight. Cells were treated with TRAIL and/or hyperthermia and/or chemotherapeutic agents (oxaliplatin, melphalan, Sigma, St. Louis, MO) then analyzed by phase contrast microscopy for signs of apoptosis.

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.

DNA Fragmentation Assay

For detection of apoptosis by the DNA fragmentation method, 1 day prior to treatment, 5×10^5 cells were plated to 60 mm dishes. After treatment, cells were trypsinized, washed with 1X PBS and harvested. Cells were resuspended in 0.5 ml of lysis buffer (20 mM EDTA, 10 mM Tris pH 8.0, 200 mM NaCl, 0.2% Triton X-100, 100 μg/ml Proteinase K) and incubated 1.5 h in a 37°C incubator. And then the samples were centrifuged (12,000 rpm) in room temperature 5 min. The supernatant was transferred to a new Eppendorf tube and equal

volumes of isopropanol and 25 μl 4M NaCl (100 mM final concentration) were added, followed by overnight incubation of the samples at −20°C. DNA was acquired by centrifugation of the samples and washed and dried and dissolved in 30 μl TE (10 mM Tris-1 mM EDTA, pH 8.0) buffer. DNA (5 μg) were loaded on each lane of 1.5% agarose gel.

Antibodies

Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz (Santa Cruz, CA). Monoclonal antibodies were purchased from the each of following companies: anti-caspase-8 antibody from Cell Signaling (Beverly, MA), 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 lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–PAGE. 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% nonfat 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).

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 8. 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.

Colony Formation Assay

For colony formation assay, CX-1 cells treated with hyperthermia and TRAIL with or without chemicals were trypsinized, counted, and plated at appropriate dilutions ($200 - 2 \times 10^6$ cells/plate). After 10 days of culture at 37°C, colonies were fixed by 10% formalin and stained with 2% crystal violet. After the staining, all of the colonies were counted.

Flow Cytometry

Cells were pelleted, washed with PBS, and resuspended in 200 μl of staining buffer containing fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). After 15 min of incubation, 300 μl of sorting buffer was added and analysis was performed using the FACScan flow cytometer (Beckman Coulter, Inc., Hialeah, FL). Results were analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

Effect of Oxaliplatin and Melphalan on CX-1 Colorectal Adenocarcinoma Cells

To study the effect of chemotherapeutic agents on TRAIL-induced cytotoxicity, first, we studied whether oxaliplatin or malphalan caused apoptotic cell death effects in CX-1 cells. Human colorectal carcinoma CX-1 cells were treated with various concentrations of oxaliplatin and melphalan (0–50 μg/ml) for 6–72 h at 37°C. Figure 1A,B shows that cytotoxicity was observed during the treatment with high dose of oxaliplatin. When treated with oxaliplatin,

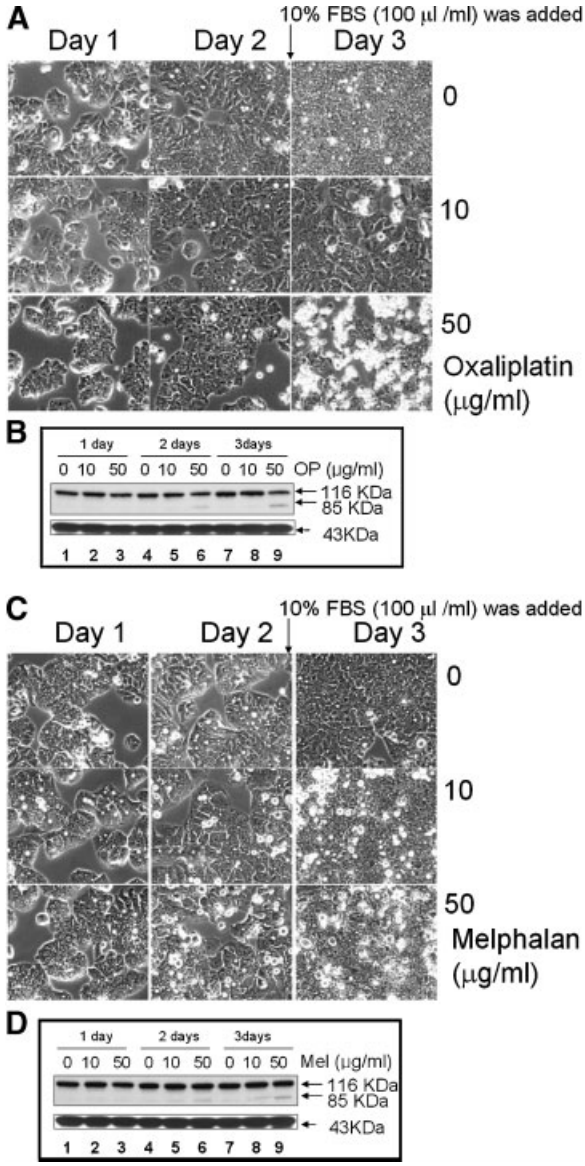

Fig. 1. Effect of oxaliplatin (OP) and melphalan (Mel) on apoptotic cell death in human colorectal carcinoma CX-1 cells. Cells were treated by OP (0, 10, 50 μg/ml) in normothermic condition for 3 days. To prevent serum deprivation, 100 μg/ml of FBS was added after 48 h incubation (**A**, **B**). Cells were treated by Mel (0, 10, 50 μg/ml) in normothermic condition for 3 days (**C**, **D**). The morphological features were analyzed with a phase-contrast microscope (200×) (A, C). 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 (B, D).

50 μg/ml (high dose for treatment), morphologically severe cell death was observed in normothermic condition. In addition, cells didnot seem to grow well with oxaliplatin (Fig. 1A). TRAIL-induced apoptosis was assessed by poly (ADP-ribose) polymerase (PARP-1) cleavage. The TRAIL-induced PARP-1 cleavage appeared 2 or 3 days after treatment of oxaliplatin at the concentration of 50 μg/ml (Fig. 1B). Figure 1C shows that during treatment with melphalan, cells undergoing apoptosis showed cell surface blebbing and formation of apoptotic bodies. There was an increase in the number of cell deaths with the high dose (50 μg/ml) of melphalan similar to oxaliplatin. PARP-1 cleavage also could be detected 2 or 3 days after the melphalan treatment (Fig. 1D).

Although apoptotic cell death was observed during treatment with the chemotherapeutic agents, as the time went by, there was little cleavage of PARP-1 (Fig. 1B,D). With the treatment of low dose (1–5 μg/ml) of oxaliplatin/melphalan we could not observe apoptotic events (data not shown). High dose of oxaloplatin/melphalan was needed to induce apoptotic cell death. Using CX-1 cells, it is not clear whether apoptosis could be induced with the clinically approachable dose of up to 1 μg /ml and 10 μg/ml of oxaliplatin and melphalan, respectively. Moderate dose of oxaliplatin or malphalan (10 μg/ml) did not induce PARP-1 cleavage, but there was morphological damage to the cells (Fig. 1A,C).

Effect of Hyperthermia on the Cells That Were Treated With Oxaliplatin or Melphalan

The technique of IHP employs a single hyperthermic 1 h treatment via an isolated vascular recirculating perfusion circuit. In the course of hyperthermic hepatic perfusion, some alkylating agents have been used for tumor eradication. To examine the effect of hyperthermia on cells that were treated with chemotherapeutic agents, human colorectal carcinoma CX-1 cells were treated with oxaliplatin or melphalan and subjected to hyperthermia at 37°C or 42°C. Data from Western blot analysis show that PARP-1 cleavage occurred within 1 day of treatment of high dose (50 μg/ml) oxaliplatin and melphalan (Fig. 2A,B). These data mean that apoptotic cell death by chemotherapeutic alkylating agents can be enhanced by hyperthermia.

TRAIL Induced Apoptosis With Hyperthermia and Oxaliplatin or Melphalan

Measurement of tumor eradication and inhibition of tumor recurrency by IHP requires new protocols. Additional studies were designed to

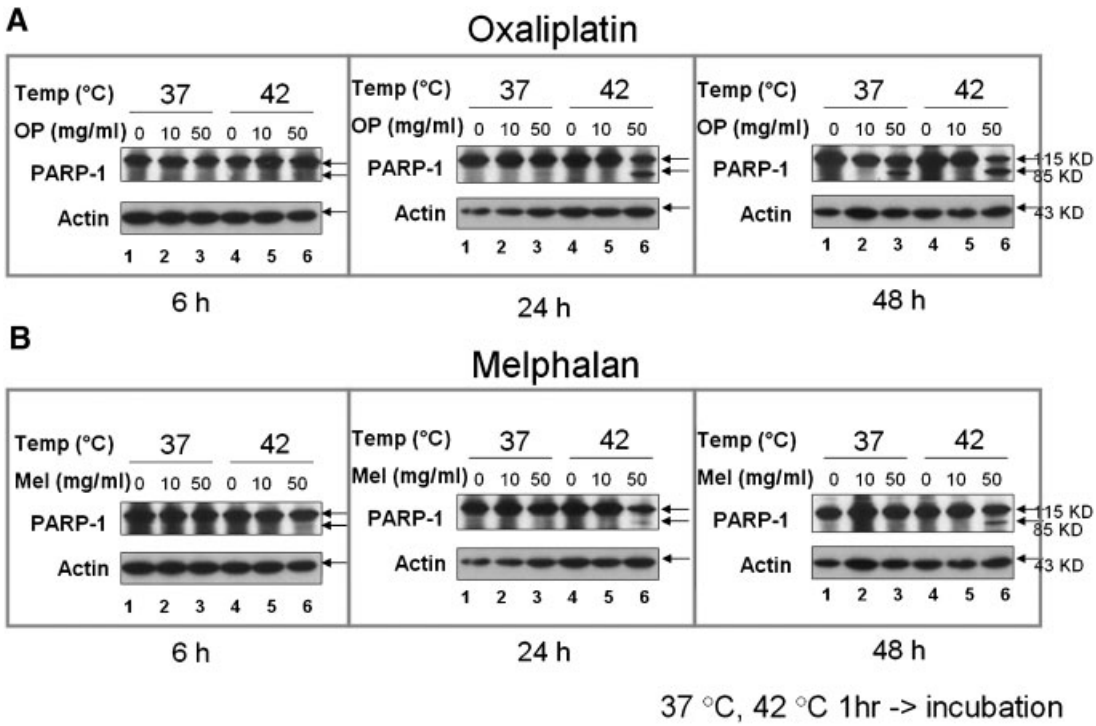

Fig. 2. PARP-1 cleavages in CX-1 cells induced by treatment of oxaliplatin and melphalan combined with hyperthermia. Cells were treated by OP (0, 10, 50 μg/ml) (**A**) or Mel (**B**) for 1 day and exposed to 1 h normothermia (37°C) or hyperthermia (42°C) in a temperature controlled water bath. After additional 3 h incubation in 37°C, 5% CO_2, cells were harvested and Western blots for PARP-1 and actin were taken.

examine whether the combination of hyperthermia and TRAIL treatment enhances cell death of oxaliplatin or melphalan pre-treated CX-1 cells.

Oxaliplatin or melphalan were treated 24 h before TRAIL and/or hyperthermia treatment. One hour of normothermia or hyperthermia treatment was followed in accurately temperature controlled water bath. Data from morphological analysis in Figure 3A,B show that no or minimal morphological alterations were observed during treatment of TRAIL or chemotherapeutic agents alone, and even during co-treatment of TRAIL with the chemotherapeutic agent. However, severe damage was observed during introduction of hyperthermia to the conditions mentioned above (42°C) (Fig. 3A,B). We recently reported that hyperthermia enhances TRAIL-induced apoptosis even in low concentrations of TRAIL (10 ng/ml) [*J Cell Biochem*]. The enhancement of apoptosis is due to the increased activities of caspases and cytochrome-*c* release from the mitochondria. In the current experiment, we observed that TRAIL treatment with hyperthermia and high dose of oxaliplatin or melphalan to CX-1 cells enhances PARP-1 cleavage, the hallmark feature of apoptosis. PARP-1 (115 kDa) was cleaved yielding a characteristic 85 kDa fragment in the presence of 3 ng/ml TRAIL with oxaliplatin or melphalan. Similar results were observed with biochemical analysis of other caspases (Fig. 4A,B).

TRAIL-induced apoptosis is mediated through a caspase cascade. To examine whether hyperthermia plus chemotherapeutic agents alter TRAIL-induced apoptosis through activation/inhibition of caspases, several caspases known to be involved in TRAIL-induced apoptosis were examined. Figure 4A,B shows that hyperthermia at 42°C enhanced TRAIL-induced caspase-8 activation in the presence of high concentration (50 μg/ml) of oxaliplatin or melphalan. Thus, hyperthermia-induced apoptotic cell death was enhanced by oxaliplatin or melphalan. Western blot analysis shows that procaspase-8 (55/54 kDa) cleavage to intermediate (43/41 kDa) forms was enhanced with increasing concentration of TRAIL (3–10 ng/ml) at 42°C. High concentration of oxaliplatin and melphalan enhanced formation of active form of caspase-9 (37 kDa) from procaspase-9

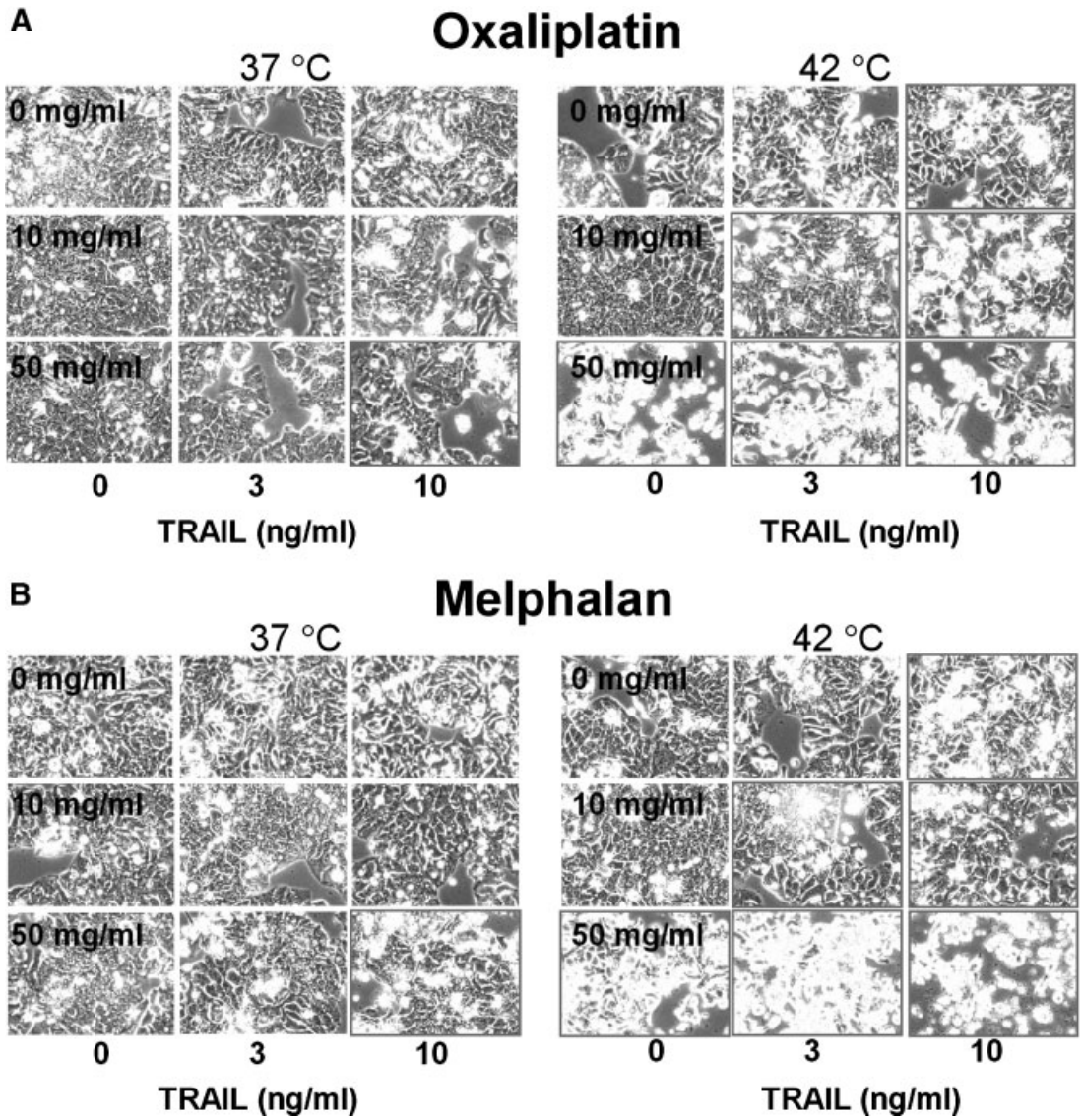

Fig. 3. Morphological evaluations of CX-1 cells treated with hyperthermia, TRAIL and oxaliplatin or melphalan. Cells were incubated with OP (0, 10, 50 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL and then incubated for 3 h at 37°C in the presence of TRAIL. Morphological features were analyzed with a phase-contrast microscope (200×) (**A**). CX-1 cells were incubated with Mel (0, 10, 50 μg/ml); the same treatment was followed as above (**B**).

(48 kDa) at 42°C. Hyperthermia plus 50 μg/ml of oxaliplatin or melphalan at 42°C also increased TRAIL-induced caspase-3 activation (Fig. 4A,B). The combined treatment with TRAIL and hyperthermia and alkylating agents increased the levels of active forms of caspases. These results clearly reveal that hyperthermia-enhanced TRAIL cytotoxicity is related to the heating temperature and dose of oxaliplatin and melphalan.

Data from DNA fragmentation assays show that no apoptotic cell death occurred with oxaliplatin or melphalan treatment without TRAIL, even in hyperthermia at 42°C. However TRAIL treatment of cells pretreated with oxaliplatin or melphalan increased TRAIL-induced apoptotic death (Fig. 5A,B). We also investigated that the dose response of apoptotic death during treatment with various concentrations of TRAIL (0–10 ng/ml) in combination with oxaliplatin and melphalan (0–50 μg/ml). We observed DNA ladders of nucleosome size, which is critical evidence of apoptosis in the hyperthermia and TRAIL-treated cells.

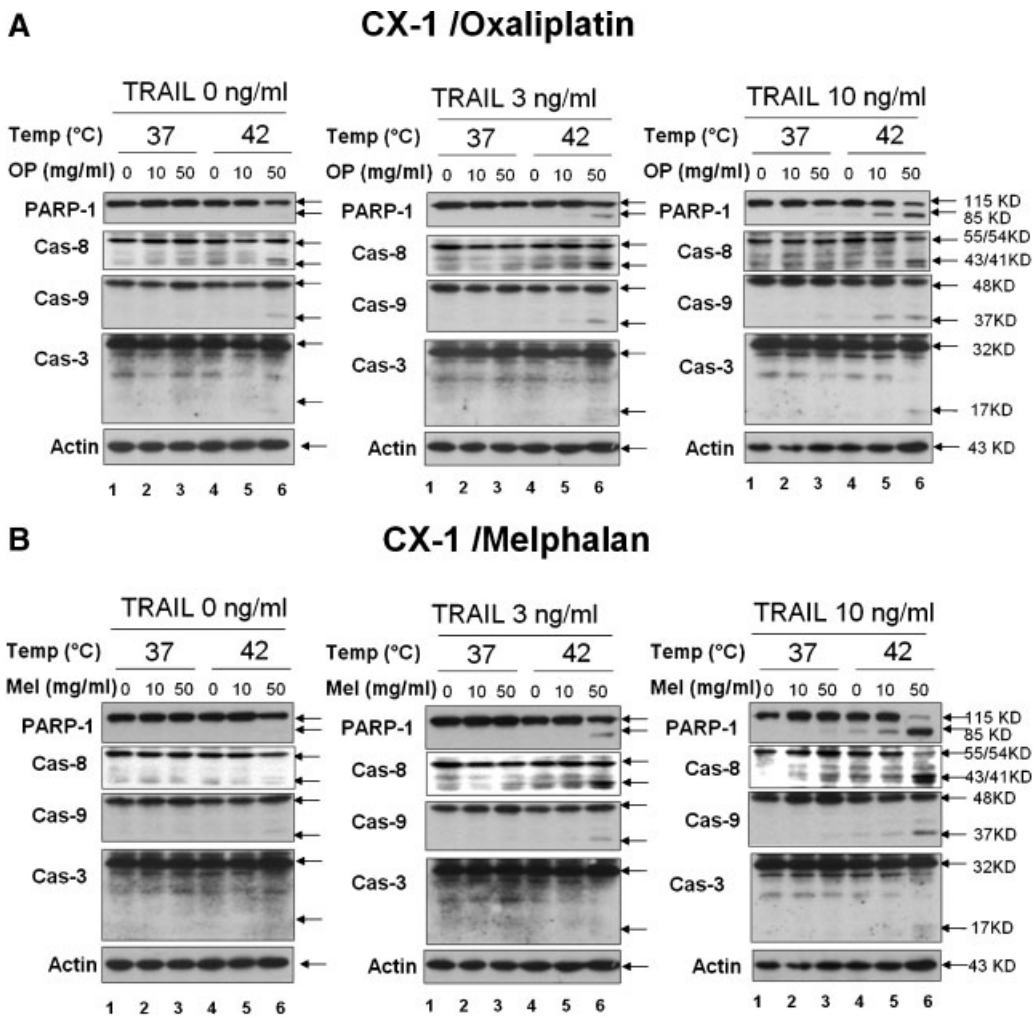

Fig. 4. Western blot analysis of proteins related to apoptosis in CX-1 cells which were treated with hyperthermia, TRAIL and oxaliplatin or melphalan. Cells were incubated with OP (0, 10, 50 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of TRAIL (**A**). CX-1 cells were incubated with Mel (0, 10, 50 μg/ml); the same treatment was followed as above (**B**). Cell lysates were subjected to immunoblotting for caspase-8, caspase-9, caspase-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). Anticaspase-3 antibody detects inactive form (32 kDa), and cleaved active form (17 kDa). Immunoblots of PARP show the 115 kDa PARP and the 85 kDa apoptosis-related cleavage fragment. Actin was used to confirm the equal amount of proteins loaded in each lane.

Heat and TRAIL treatment to CX-1 cells induced apoptotic cell death. FACS analysis shows that there was little cell death without hyperthermia. Oxaliplatin and melphalan did not promote apoptotic or non-apoptotic cell death without hyperthermia (Fig. 6A,B). We hypothesized that the effects of chemotherapeutic agents, when combined with hyperthermia and TRAIL treatment, are not immediate, but instead, are long term because the alkylating agents bind to the DNA and block DNA replication or transcription. And in fact, cells treated with oxaliplatin or melphalan had abnormal morphologies, did not grow well, and eventually died a few weeks after the treatment. So, it is nessessary to analyze reproductive cell death. Colony formation assay confirms how alkylating agents work with a high concentration of oxaliplatin or melphalan; cells did not grow, however they are attached to the plate and lived 1–2 weeks, and died out 3 weeks after treatment and plating. After the plating, colonies were stained with crystal violet and counted (Fig. 7A,B). Less than 10% of CX-1 cells

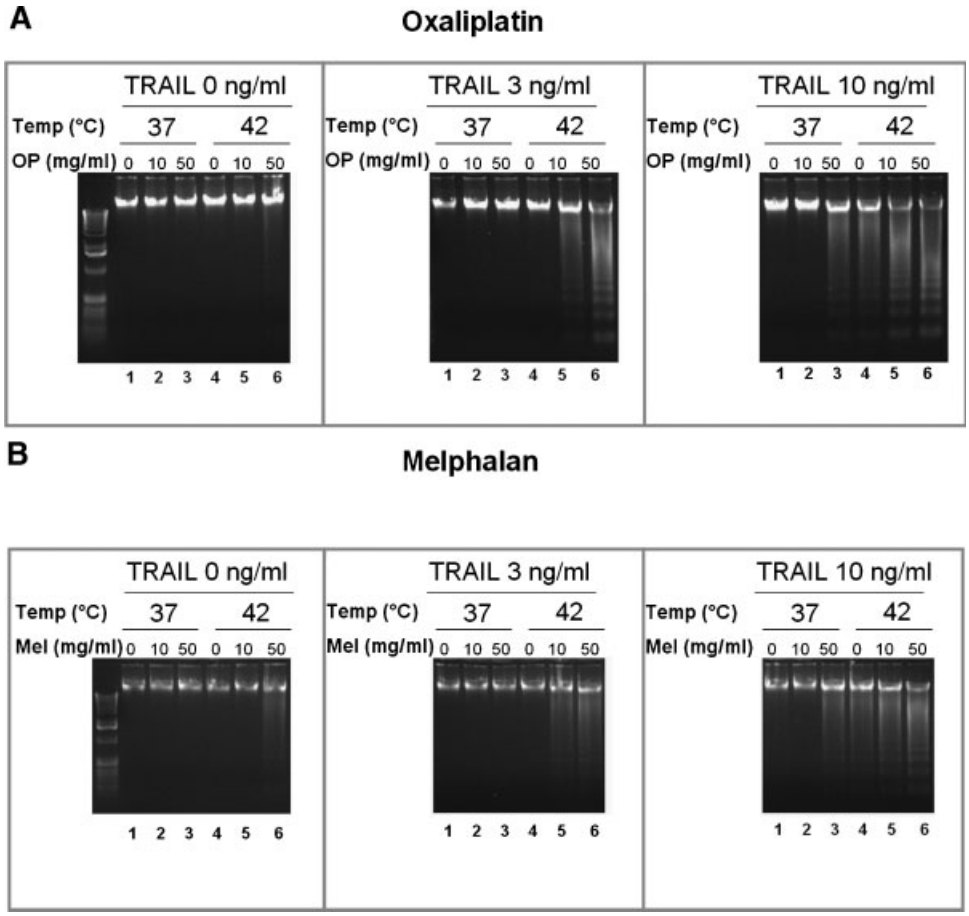

Fig. 5. DNA fragmentation in CX-1 cells treated with hyperthermia, TRAIL and oxaliplatin, or melphalan. Cells were incubated with OP (0, 10, 50 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL and then incubated for 3 h at 37°C in the presence of TRAIL (**A**). CX-1 cells were incubated with Mel (0, 10, 50 μg/ml); the same treatment was followed as above (**B**). For detection of apoptosis by the DNA fragmentation method, 5×10^5 cells were plated to 60 mm dishes. After treatment, cells were trypsinized, washed with PBS and harvested. Cells were resuspended in lysis buffer and treated with proteinase K. DNA was acquired by centrifugation of the samples and washed and dried and dissolved in RNase A containing TE (10 mM Tris-1 mM EDTA, pH 8.0) buffer. DNA (5 μg) were loaded each lane of 1.5% agarose gel.

could live reproductively in as low a concentration as 1 μg/ml of oxaliplatin and 5 μg/ml of melphalan. At these concentrations, cells were highly sensitive to hyperthermia and TRAIL. Hyperthermia and TRAIL, at these concentrations, promoted reproductive cell death. These results suggest that the combination of hyperthermia and alkylating agents in the presence of TRAIL greatly increases reproductive cell death as a result of DNA damage. Clonogenic survival assay indicated that with the addition of TRAIL and hyperthermia treatment, significantly low numbers of colonies survived compared to the number of colonies treated with chemotherapeutic agents only (Fig. 7A,B).

Differential Effect of Hyperthermia on TRAIL-Induced Apoptosis Is Mediated Through Mitochondria-Induced Apoptosis With Hyperthermia and Melphalan or Oxaliplatin

Previous studies demonstrated that apoptotic signaling via a mitochondria-dependent pathway plays an important role in the TRAIL-induced activation of caspases. We hypothesized that the differential effects of hyperthermia and chemotherapeutic agents are 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. Figure 8 shows that hyperthermia at 42°C

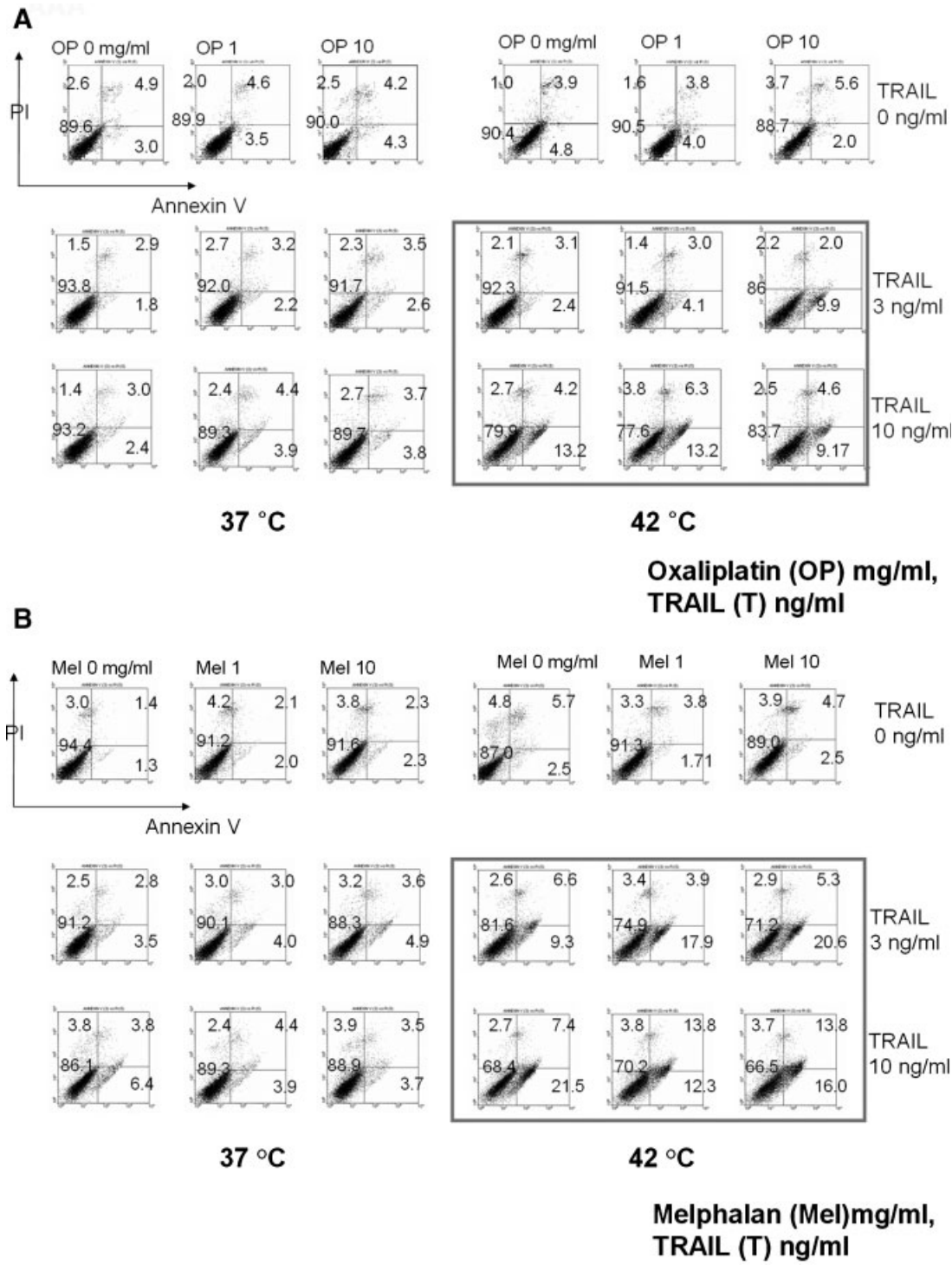

Fig. 6. FACS analysis with CX-1 cells treated with hyperthermia, TRAIL and oxaliplatin, or melphalan. Cells were incubated with OP (0, 1, 10 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL and then incubated for 3 h at 37°C in the presence of TRAIL (**A**). CX-1 cells were incubated with Mel (0, 1, 10 μg/ml); the same treatment was followed as above (**B**). Cells were trypsinized, harvested, and washed with 1X PBS. Fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) were incubated with the harvested cells. After treatment, apoptosis was detected by the flow cytometric assay.

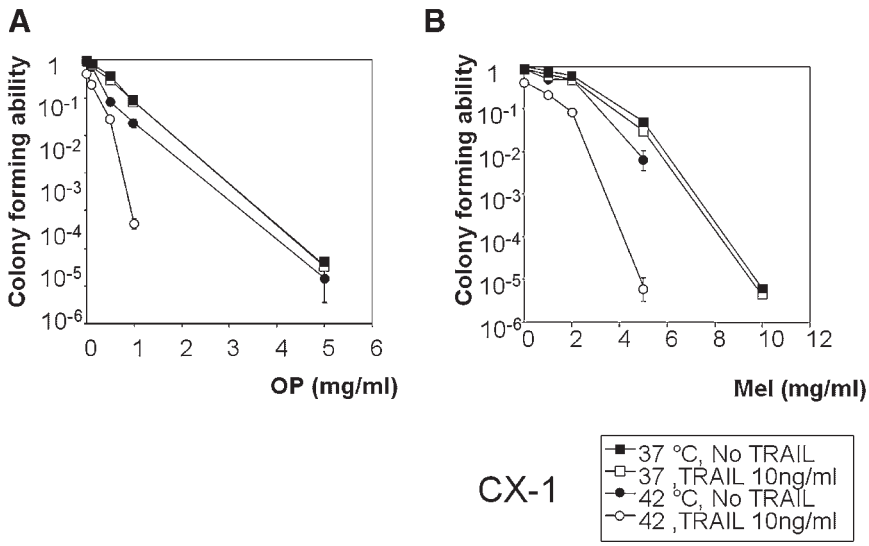

Fig. 7. Clonogenic assay with CX-1 cells treated with hyperthermia, TRAIL and oxaliplatin, or melphalan. Cells were incubated with OP (0, 0.1, 0.5, 1, 5 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL and then incubated for 3 h at 37°C in the presence of TRAIL After all of the treatments CX-1 cells were trypsinized, counted, and diluted. Then the cells (up to 2×10^6 cells) were plated to 100 mm dishes. 2–3 weeks after plating, colonies were stained with crystal violet and counted (**A**). We defined a colony as cell division clumps that have 50 or more cells. Cells were incubated with Mel (0, 1, 2, 5, 10 μg/ml) for 24 h and exposed to normothermic or hyperthermic conditions for 1 h in the presence of 0, 3, 10 ng/ml of TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of TRAIL (**B**). Error bars represent standard error of the mean (SEM) from three separate experiments. One asterisk (*) indicates little difference by the *t*-test ($P > 0.05$). Two asterisks (**) indicate values, which are different from the respective control (*t*-test, $P < 0.05$).

without TRAIL or chemotherapeutic agents promoted only a small amount of cytochrome-*c* release. However, chemotherapeutic treatment without hyperthermia or TRAIL did not promoted the cytochrome-*c* release. Previously, we examined whether overexpression of Bcl-2 inhibits cytochrome-*c* release [Yoo and Lee, 2007]. As mentioned in that report, hyperthermia promotes TRAIL-induced cytochrome-*c* release from mitochondria and enhances TRAIL-induced cytotoxicity. However, as seen in the current experiment, chemotherapeutic agents have no effect on cytochrome-*c* release. The chemicals are related to apoptotic cell death only when used in high dose, and at the doses used here cause primarily non-apoptotic cell death, through DNA damage which blocks the DNA replication, or transcription.

DISCUSSION

Hyperthermia is a simple protocol for the induction of tumor cell death. Results from our study indicate that treating cells with both TRAIL and hyperthermia (42°C, 1 h) enhanced the cytotoxicity when compared to that of single

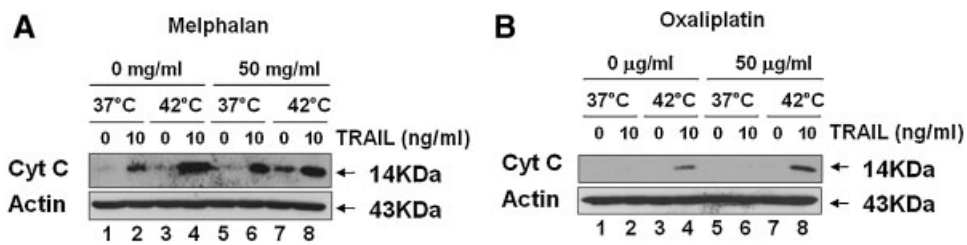

Fig. 8. Effect on cytochrome-*c* release from mitochondria by the treatment with hyperthermia, TRAIL and oxaliplatin, or melphalan. Cells were exposed to hyperthermia (42°C) or normothermia in the presence of 50 μg/ml OP (**A**) or Mel (**B**) and 0, 3, 10 ng/ml of TRAIL for 1 h and then incubated for 3 h at 37°C in the presence of TRAIL. Cytosolic fractions were used for detecting cytosolic cytochrome-*c*. Actin was shown as an internal standard.

treatments. In the previous study, we showed that hyperthermia did not induce apoptosis in colorectal cancer CX-1 cells; however, combination of TRAIL and hyperthermia caused apoptotic cell death. Activation of caspases and enhancement of the release of cytochrome-*c* in high temperatures elicited apoptotic cell death. Our results are in good agreement with the findings that DNA fragmentation, a hallmark of apoptosis, was found in the same cell line after TRAIL and hyperthermia treatment. In that study, we examined whether TRAIL in combination with mild hyperthermia effectively kills cancer cells. We believe that investigating the cooperative interaction between two modalities will provide information to improve the efficacy of IHP [Yoo and Lee, 2007].

Besides TRAIL, there are other agents that act synergistically when applied with hyperthermia. This includes ionizing radiation [Dewey et al., 1978; Holahan et al., 1984; Kampinga and Dikomey, 2001], as well as a number of chemotherapeutic agents [Herman et al., 1982; Haas et al., 1984; Ko et al., 2006], and various cytokines [Srinivasan et al., 1990; Klostergaard et al., 1992; Lee et al., 1993].

In our study, we applied hyperthermia and TRAIL with chemotherapeutic agents like oxaliplatin and melphalan to boost cancer cell death. Oxaliplatin and melphalan are clinically applicable DNA damaging chemicals. We observed that those chemicals can induce apoptotic cell death when treated long term with high concentration (>10 μg/ml, 2 or 3 days) combined with hyperthermia and TRAIL.

We still have many unanswered questions regarding regional hyperthermia therapy. Can we apply cytokines like TRAIL or TNFα and hyperthermia? How can we optimize regional therapy considering pharmacokinetic consequences of different techniques? How can we develop of regional therapies, to be less invasive and easily repeatable? Are there new agents available for application in regional hyperthermia therapy? Finally, will we be able to predict treatment outcome by assessment of patients' tumor characteristics?

For isolated hepatic perfusion (IHP), hyperthermia (41–42°C) has been successfully used to enhance the effectiveness of chemotherapeutic agents. In this study, we investigated whether the chemotherapeutic agents like oxaliplatin and melphalan can enhance hyperthermia in combination with TRAIL-induced cytotoxicity in human colorectal cancer cells. We observed that hyperthermia (42°C) and TRAIL-induced apoptotic cancer cell death was enhanced by the co-treatment with oxaliplatin or melphalan. Observing colony formation, we also concluded that besides apoptotic cell death, reproductive cell death by TRAIL and hyperthermia could be enhanced with the chemotherapeutic agents (Fig. 8).

In conclusion, our data indicate that chemotherapeutic agents amplify TRAIL and hyperthermia-induced apoptosis in CX-1 cells through cytochrome *c* release from mitochondria. When the chemicals are treated long-term (<2 days), DNA damage is the main cause of cell death.

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