Hyperthermia Induces Endoplasmic Reticulum-Mediated Apoptosis in Melanoma and Non-Melanoma Skin Cancer Cells

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Hyperthermia has been revived as a promising approach for cancer treatment. To understand the underlying mechanisms of hyperthermic killing of cancer cells, we examined the cytotoxic effects of hyperthermia on various skin cancer cell lines using cell viability, morphological analyses, and caspase activation assays. Hyperthermia induced cytotoxicity in a time- and temperature-dependent manner. At middle dose/time combinations, heat-induced apoptosis, whereas at higher doses, necrosis was the mechanism of cell death. To investigate the mechanisms of hyperthermia-induced apoptosis, we examined the activation of extrinsic (Caspase 8) and intrinsic (Caspase 9) apoptotic pathways. Hyperthermia did not activate Caspases 8 or 9, but did activate Caspase 3/7, suggesting a non-conventional apoptotic pathway. Last, analysis of Grp78 expression and Caspase 12 or 4 activation indicated that hyperthermia induced endoplasmic reticulum-mediated apoptosis. Thus, hyperthermia induced apoptosis in two types of skin cancer cells through endoplasmic reticulum-mediated apoptosis and not through the classical intrinsic or extrinsic apoptosis pathways. Hyperthermia may be a promising treatment for basal cell carcinoma and melanoma, bypassing the antiapoptotic defenses concentrated in the intrinsic and extrinsic apoptosis pathways. These results also raise the possibility that heat may be combined with other approaches for induction of apoptosis to achieve synergistic killing of skin cancers.

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INTRODUCTION

There is a renewed interest in hyperthermia as a cancer treatment since new technology provides precise control and measurement of heat delivery (Jones *et al.*, 2005; Coffey *et al.*, 2006). Promising results from recent clinical trials indicate the effectiveness of hyperthermia treatment as an adjunct to radiotherapy or chemotherapy in treating numerous cancers, including superficial cutaneous tumors, recurrent breast cancer, recurrent malignant melanoma, head and neck squamous cell carcinoma, lymph node metastases, glioblastoma, and cervical carcinoma (Jones *et al.*, 2005; Coffey *et al.*, 2006).

Hildebrandt *et al.* (2002) provided a thorough summary of the current understanding of hyperthermia effects at cellular and molecular levels, and highlighted the limitation of hyperthermia studies performed in the 1970s and early 1980s when techniques were insufficient. They also pointed out that further basic research is needed to fully utilize and optimize hyperthermia treatments. A detailed understanding of the mechanisms through which hyperthermia kills tumor cells would provide useful information and allow engineers to develop more efficacious applicators while minimizing adverse events and bystander tissue damage.

The mechanisms through which hyperthermia induces apoptosis in skin tumor cells is one of the major aims of this study. Apoptosis has been recognized as the major programmed cell death mechanism since the seminal article of Kerr *et al.* (1972). Conventionally, it is believed that induction of apoptosis involves the activation of a complex array of caspases through two major apoptotic pathways: extrinsic or intrinsic apoptotic pathways.

The extrinsic pathway involves death receptor binding to proapoptotic death ligands such as tumor necrosis factor-α, Fas, and tumor necrosis factor related apoptosis including

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Abbreviations: BCC, basal cell carcinoma; EB/AO, ethidium bromide and acridine orange; ER, endoplasmic reticulum; HRP, horseradish peroxidase; MTS, a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; PBS, phosphate-buffered saline; TRAIL, tumor necrosis factor related apoptosis including ligand

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ligand (TRAIL) and activation of *Caspase 8*. Subsequent direct activation of effector caspases, such as Caspase 3 in a mitochondria-independent fashion, leads to cell death (Chinnaiyan *et al.*, 1996; Tschopp *et al.*, 1998).

In contrast, the intrinsic pathway involves mitochondrial-dependent cell death induced by cytotoxic, genotoxic, or stress-inducing agents. These agents induce mitochondria damage and result in the release of several death-promoting factors and activation of *Caspase 9* (Li *et al.*, 1997; Zou *et al.*, 1997), which then activates Caspase 3 and other effector caspases that eventually lead to cell death.

In addition to these two major apoptotic pathways, recent studies implicate the importance of a non-conventional apoptotic pathway that can be induced by some apoptotic triggers: the endoplasmic reticulum (ER)-mediated pathway (see review Boyce and Yuan, 2006).

The ER is responsible for the synthesis, initial post-translational modification, and proper folding of proteins, as well as their sorting and export for delivery to appropriate cellular destinations. Disruption in Ca^{2+} homeostasis, inhibition of protein glycosylation, and accumulation of misfolded proteins may all induce ER stress, which is associated with a range of diseases, including ischemia/reperfusion injury, neurodegeneration, and diabetes (Kaufman, 2002; Boyce and Yuan, 2006; Zhang and Kaufman, 2006).

The proper functioning of the ER is critical for numerous aspects of cell physiology. Accordingly, to ensure that cells maintain proper ER homeostasis, the ER in eukaryotic cells has evolved highly specific signaling pathways including the unfolded protein response (Kaufman, 2002; Boyce and Yuan, 2006; Zhang and Kaufman, 2006). Normally, unfolded protein response is activated to protect cells that are experiencing ER stress. However, sustained or intense ER stress can lead to apoptosis (see review Boyce and Yuan, 2006; Zhang and Kaufman, 2006).

Grp78, also referred to as BiP, is an ER chaperone protein. It has been indicated as the master unfolded protein response regulator and is significantly upregulated by ER stress (see review Boyce and Yuan, 2006; Zhang and Kaufman, 2006). Grp78 is also a central regulator of ER function due to its roles in protein folding and assembly, targeting misfolded proteins for degradation, ER Ca^{2+} binding, and controlling the activation of transmembrane ER stress sensors. Thus, upregulation of Grp78 is a good indicator of ER stress (Li and Lee, 2006; Zhang and Kaufman, 2006).

When an overload of unfolded or misfolded proteins in the ER is not resolved, prolonged or severe unfolded protein response activation will lead to apoptosis (see review Boyce and Yuan, 2006; Zhang and Kaufman, 2006). Although the detailed mechanisms by which ER stress leads to cell death remain enigmatic (see review Xu *et al.*, 2005), Caspase 12 in mice has been revealed as a major ER-associated proximal caspase of the caspase activation cascade, and activation of Caspase 12 eventually leads to activation of Caspase 3/7 and apoptosis (Nakagawa *et al.*, 2000). The loss of full-length pro-Caspase 12 has been used in many studies to detect activation of Caspase 12 and thus ER-mediated apoptosis. However, human Caspase 12 contains several mutations, which renders it non-functional (Fischer *et al.*, 2002). It has been reported that human Caspase 4, which is also a resident of the ER, might be the counterpart of murine Caspase 12 and is similarly activated by ER stress (Kim *et al.*, 2006).

Owing to their complexity, new aspects of apoptotic pathways are still being investigated for more than 30 years after the seminal article about apoptosis was published (Kerr *et al.*, 1972). ER-mediated apoptosis is one of the newly recognized components of apoptotic pathways (see review Boyce and Yuan, 2006). However, to our knowledge, there are no current studies that investigate whether hyperthermia induces ER-mediated apoptosis in melanoma and non-melanoma skin cancer cells.

In this study, we examined the cytotoxicity of hyperthermia treatments on several cutaneous cell lines and investigated the apoptotic cell death pathways induced by these hyperthermia treatments.

RESULTS

Hyperthermia induced cytotoxicity in multiple cutaneous cell lines

We examined the cytotoxic effects of hyperthermia treatments on various cutaneous cell lines by a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays. We found that hyperthermia reduced cell viability/proliferation in a time- and temperature-dependent manner in all the cells tested (Figure 1 and data not shown), and Figure 1 shows the examples of a few cell lines we tested. Cell sensitivity to hyperthermia treatment varied among different cell lines, and the basal cell carcinoma (BCC) cell line ASZ001 was quite sensitive (Figure 1 and data not shown).

Hyperthermia stimulated Caspase 3/7 activity under certain conditions

To examine whether these hyperthermia treatments induce apoptosis, we analyzed the activation of Caspase 3/7 (the apoptotic effector caspases) using the Caspase 3/7 Glo assay in several different cell lines. We found that at 45 °C for 60–120 minutes and at 48 °C for 15–30 minutes treatments induced caspase 3/7 activity, suggesting that apoptosis occurred under limited time/temperature conditions (Figure 2). We have observed similar results in multiple cell lines including ASZ001, A375, HS294T, and WM35 cells (Figure 2 and data not shown).

Hyperthermia induced apoptosis at mild temperature (45 °C), but necrosis at high temperature (48 °C)

To examine the kinetics of hyperthermia-induced cytotoxicity, we performed MTS viability and Caspase 3/7 activity assays at indicated post-treatment time points of both 45 and 48 °C for 90 minutes. We found that hyperthermia at either 45 or 48 °C decreased cell viability starting 3 hours post-treatment in ASZ001 cells (Figure 3). Although both treatments decreased cell viability, only the 45 °C treatment activated Caspases 3/7. Caspase 3/7 activation appreciated 6 hours after treatment and peaked at 12 hours post-treatment

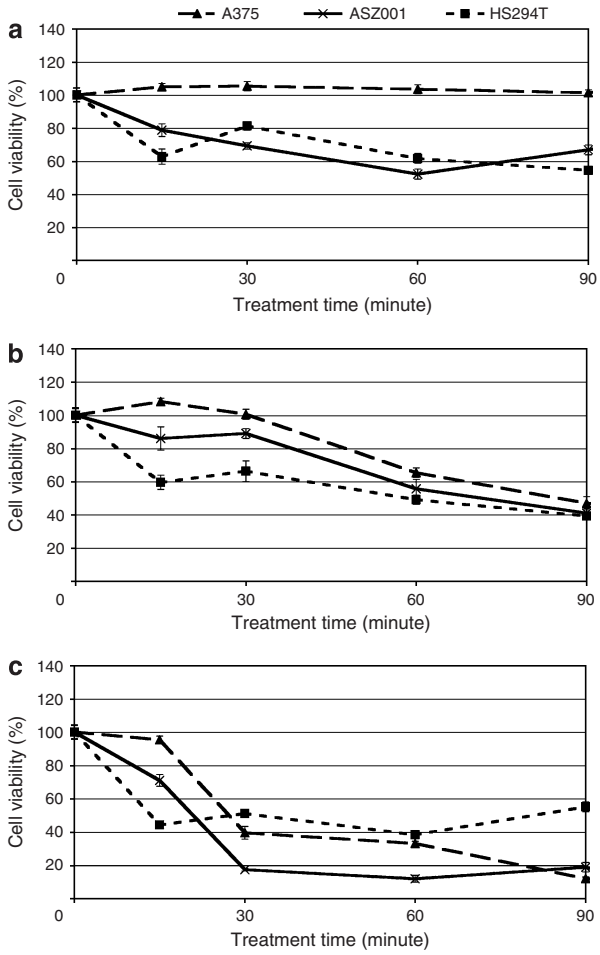

Figure 1. Hyperthermia induced cytotoxicity in multiple cutaneous cell lines. Cells were seeded in 96-well plates overnight before being treated at the indicated temperature (°C) for the indicated amount of time. MTS assays were performed 24 hours after treatment. Viability at 37 °C of each cell line was set as 100%. Percentages of cell viability at other temperatures were compared to the cell viability of 37 °C. (**a**) Viability at 41 °C. (**b**) Viability at 45 °C. (**c**) Viability at 48 °C. Error bars were defined as "mean ± SEM," and there were 6–8 samples for each point.

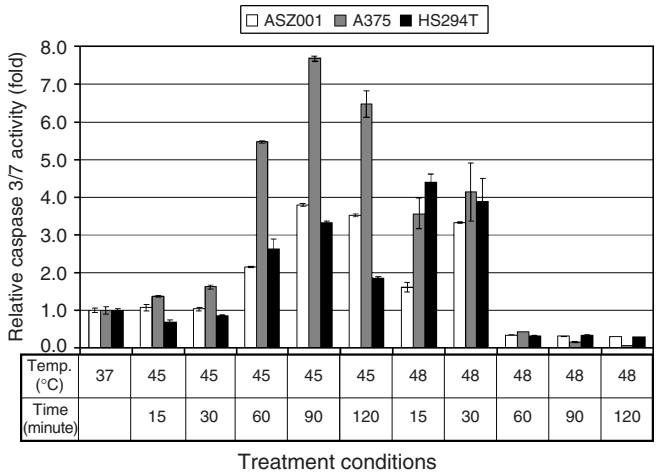

Figure 2. Hyperthermia stimulated Caspase 3/7 activity under certain conditions. Cells were seeded in 96-well plates overnight before being treated at the indicated temperature (°C) for the indicated amount of time (minutes). Caspase 3/7 Glo activity assays were performed 24 hours after treatment. Caspase 3/7 activity of 37 °C of each cell line was set as 1. Activities of other temperatures were compared to that of 37 °C. Error bars were defined as "mean ± SEM," and $n = 3$.

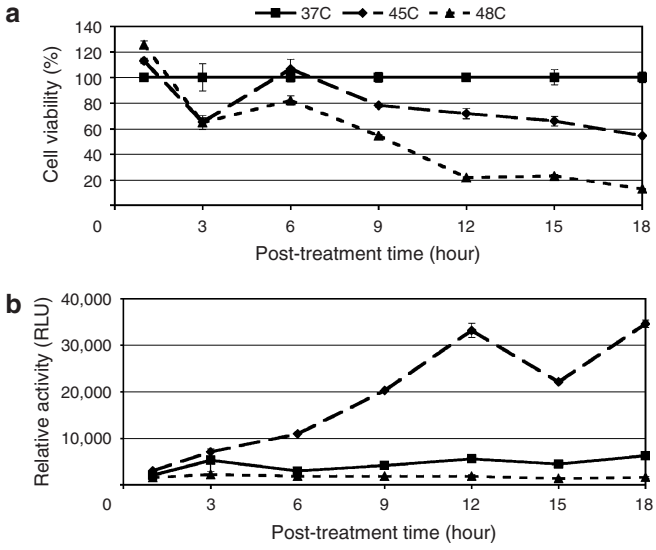

Figure 3. Time-course experiments with hyperthermia treatment of ASZ001 cells. Cells were seeded in 96-well plates overnight before being treated at the indicated temperature for indicated amount of time. MTS cell viability assays and Caspase 3/7 activity Glo assays were performed according to the manufacturer's instructions at the indicated amount of post-treatment time. (**a**) Cell viability assays. Viabilities of cells in the 37 °C incubator at each time point were set at 100%. Error bars were defined as "mean ± SEM," and there were 6–8 samples for each point. (**b**) Caspase 3/7 activity assay. Error bars were defined as "mean ± SEM," and $n = 3$.

in the ASZ001 cells (Figure 3). We also observed similar results in A375 cells (data not shown).

To examine further whether hyperthermia induced apoptosis or necrosis, we performed ethidium bromide and acridine orange (EB/AO) morphologic assays with ASZ001 cells (Figure 4). This assay is based on the detection of changes in nuclear morphology that are characteristically specific for apoptosis and necrosis. Apoptosis induces characteristic nuclear condensation and fragmentation, whereas necrosis is characterized by the inability to exclude vital dye, leading to orange staining nuclei. The staining characteristics are: *live cells* have normal nuclei staining, which present *green* chromatin with organized structures; *apoptotic cells* contain condensed or fragmented chromatin (green or orange); and *necrotic cells* have similar normal nuclei staining as live cells except the chromatin is orange instead of green.

The untreated cells in the left panel show few apoptotic cells. The majority of the cells show large intact green nuclei. The panels of cells exposed to either 45 or 48 °C show progressive cell death increasing to 12 hours post-hyperthermia treatment. Differences between the two temperature treatments are apparent at 12 hours, in the far right panels. In

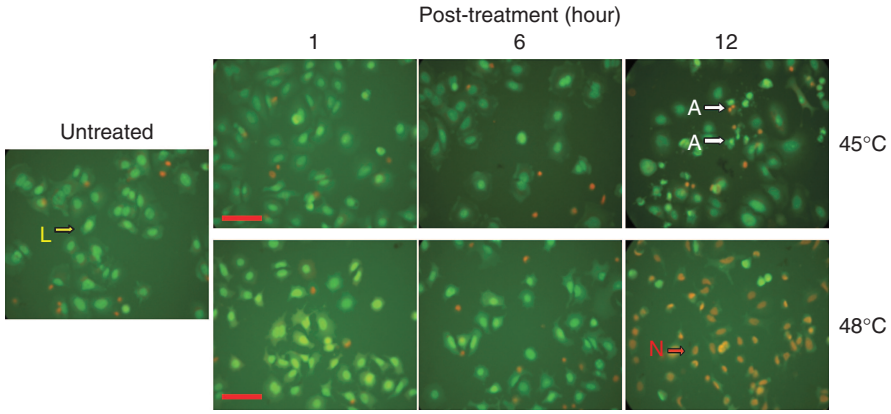

Figure 4. Representative pictures of EB/AO staining at indicated time points. The same experimental settings were used as in Figure 3. The EB/AO staining method was used to assess the cell status at the indicated amount of post-treatment time (hours). Arrows point to the cells representing certain cell viable status: L, is the live cells; A, the apoptotic cells; and N, the necrotic cells. Bar = 200 μm.

the cells exposed to 45 °C, multiple apoptotic cells are seen. These cells are characterized by condensed and/or fragmented nuclei, stained either green or red. In the cells exposed to 48 °C, dead cells are largely necrotic, showing intact nuclei stained orange indicating the inability to exclude vital dye. Thus, Figure 4 shows that both hyperthermia treatments (45 and 48 °C) induced cell death, decreasing live cells, and increasing cells showing characteristics of apoptosis or necrosis. However, treatment at 45 °C induced primarily apoptotic cells, whereas treatment of 48 °C induced predominantly necrotic cells. Similar results were observed with A375 cells (data not shown).

Hyperthermia induced activation of Caspase 3, but not Caspase 8 or 9

To investigate whether hyperthermia activated intrinsic or extrinsic apoptotic pathways, we examined the activation of Caspase 8 (extrinsic) or Caspase 9 (intrinsic) in addition to Caspase 3 using Caspase Glo assays. Lysates from Jurkat cells treated with anti-Fas antibodies for 5 hours were used as a positive control, and both Caspase 8 and 9 assays worked properly (Figure 5a). In ASZ001 cells, even though a high level of Caspase 3 activation was observed with the 45 °C hyperthermia treatment, surprisingly neither 45 nor 48 °C hyperthermia treatments activated Caspase 8 or 9 (Figure 5b–d). Similar results have been observed in A375 cells (Figure 6). These data suggest that hyperthermia induced caspase 3-dependent apoptosis, independent of the intrinsic or extrinsic pathways.

Hyperthermia induced ER stress and ER-mediated apoptosis

ER-mediated apoptosis might be induced by hyperthermia as neither intrinsic nor extrinsic pathways were activated by hyperthermia in these cells. As stated earlier, induction of Grp78 expression is a good indicator of ER stress, and disappearance of full-length Caspase 4 or 12 signifies ER-mediated apoptosis. Thus, to examine the effects of hyperthermia on ER stress and ER-mediated apoptosis, we performed western blot to measure the expressions of Grp78 and full-length Caspase 12 or 4 on hyperthermia treatment under the apoptosis-inducing conditions (Figure 7).

Hyperthermia significantly upregulated Grp78 expression in both A375 (Figure 7a) and ASZ001 cells indicating that ER stress was activated at multiple time points (Figure 7b). In addition, hyperthermia decreased full length Caspase 4 in the human cell line A375 and full length Caspase 12 in the mouse cell line ASZ001 (Figure 7a and b), suggesting that activation of these caspases occurred on these hyperthermia treatments.

DISCUSSION

Methods of treating cancer are as varied as the forms of the disease. Treatment techniques include naturally occurring (Bai *et al.*, 2003; Wolf *et al.*, 2007) or synthetic (Oltersdorf *et al.*, 2005) chemotherapeutic agents and radiation therapy. In addition, hyperthermia treatment is a new promising cancer therapy. ER-mediated apoptosis has only recently been recognized as one of the important components of apoptotic pathways (Boyce and Yuan, 2006). Thus, this report attempts to determine if hyperthermia induces ER-mediated apoptosis in melanoma and non-melanoma skin cancer cells.

We examined whether hyperthermia treatments induce apoptosis in various skin cancer cell lines, and aimed to clarify the mechanism through which the apoptotic pathway is induced. We found that hyperthermia killed most cells in a temperature- and time-dependent manner (Figure 1). Further, these cells seem to have a wide range of sensitivity to hyperthermia (Figure 1 and data not shown). Our results from MTS assays are consistent with many studies that used clonogenic assays previously (Sapareto *et al.*, 1978; Hahn, 1984), which validate our approaches. Our study also suggests that the BCC cell line ASZ001 was quite sensitive to hyperthermia treatments (Figure 1), and thus hyperthermia may be worthy of further investigation for treating BCCs. BCC is a malignancy arising in the superficial epidermis, which would allow for sufficient and accurate delivery of heat doses to the tumors with little collateral tissue damage.

The Caspase 3/7 activation assays and EB/AO morphologic assays collectively indicate that hyperthermia induces apoptosis under mild conditions (Figures 2–4). In contrast to the wide-spread destructive inflammatory features seen in

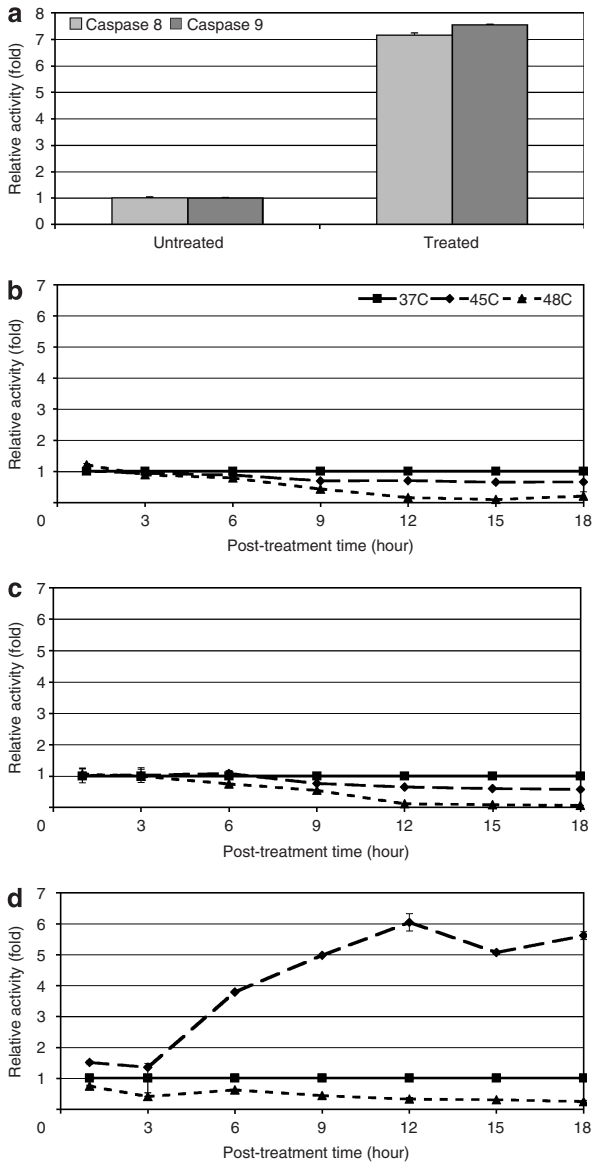

Figure 5. Hyperthermia induced activation of Caspase 3, but not Caspase 8 or 9 in ASZ001 cells. Cell lysates from Jurkat treated with PBS or 100 ng ml^{-1} of anti-Fas antibody for 5 hours were used as positive controls for (**a**) Caspases 8 and 9 activity assays. The same experimental settings were used as in Figure 3 for (**b**) Caspase 8, (**c**) 9, or (**d**) 3/7 activity. Glo assays were performed according to the manufacturer's instructions at the indicated post-treatment time. Error bars were defined as "mean ± SEM," and $n = 3$.

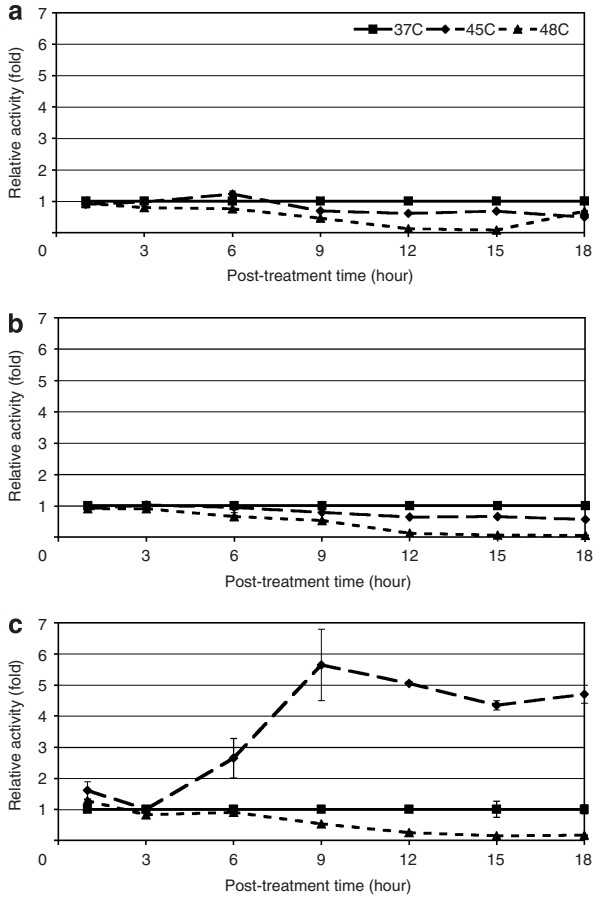

Figure 6. Hyperthermia induced activation of Caspase 3, but not Caspase 8 or 9 in A375 cells. The same experimental settings were used as in Figure 5, except that A375 cells were used here instead of ASZ001. Caspase G10 activity assays were performed for (**a**) Caspase 8, (**b**) Caspase 9, and (**c**) Caspase 3/7 at the indicated post-treatment time. Error bars were defined as "mean ± SEM," and $n = 3$.

cell death induced by necrosis, apoptotic cell death does not induce inflammation and results in targeted discrete cell death with fewer local side effects, such as reduced scarring. We propose that identifying the conditions that favor apoptosis in hyperthermia-treated BCCs in the future will maximize tumor killing and minimize bystander tissue damage and scarring.

When we investigated the apoptotic pathways which hyperthermia induces, surprisingly, we found that neither Caspase 8 nor 9 were activated in these cells, even though the same conditions did activate effector Caspase 3/7 very efficiently (Figures 4–6). However, hyperthermia activated Caspase 12/4 (Figure 7). These data imply that hyperthermia treatments induce ER-mediated apoptosis but not through the extrinsic or intrinsic pathways.

It has long been speculated that the destruction of cancer cells at elevated temperatures is probably due to damage in the plasma membrane, the cytoskeleton organization and the cell nucleus, or inhibition of DNA repair enzyme (Romer, 1999; Hildebrandt *et al.*, 2002). However, most of these claims have not been proved, and some of the results might also be explained by unspecific protein inactivation (Hildebrandt *et al.*, 2002).

The idea that hyperthermia induces ER-mediated apoptosis does not contradict the earlier studies mentioned above, and it might even help explain some of the early results. The ER encompasses about one-half of the total membrane area and one-third of the newly translated proteins in a typical eukaryotic cell (Boyce and Yuan, 2006). In fact, hyperthermia has been reported to change cytoskeleton organization that includes the ER (Hildebrandt *et al.*, 2002) and has been shown to disrupt Ca^{2+} levels in human lung cancers (Hashimoto *et al.*, 2003).

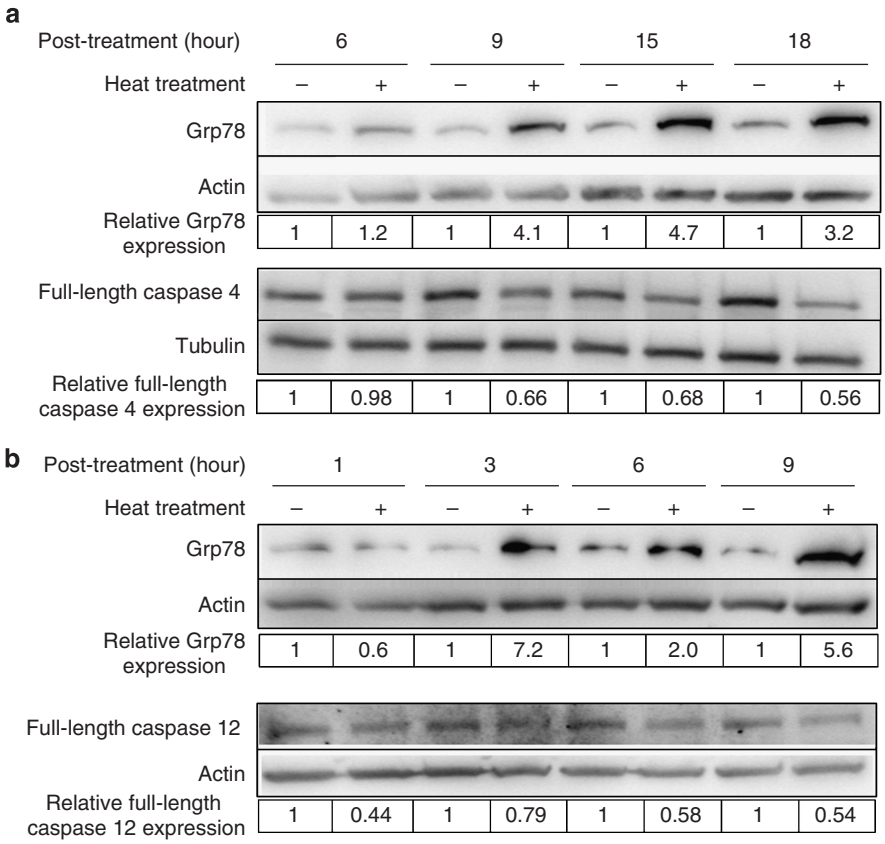

Figure 7. Hyperthermia induced ER stress and ER-mediated apoptosis. Cells were grown overnight in 10 cm dishes before being subjected to hyperthermia treatment. The cell lysates were prepared at indicated time points post-treatment before being subjected to western blot. (**a**) Grp78 and full-length Caspase 4 expression in A375 cells. (**b**) Grp78 and full-length Caspase 12 expression in ASZ001 cells.

It is well known that heat induces heat-shock proteins as a stress response. Interestingly, heat-shock proteins are protein chaperones in the cytoplasm and are upregulated by various stress stimuli that damage proteins and promote accumulation of misfolded proteins probably in the cytoplasm (Brostrom and Brostrom, 1998). In comparison, Grp78 is a protein chaperone in the ER. Our data show that hyperthermia induced Grp78, and suggest that heat also denatures proteins and causes accumulation of misfolded proteins in the ER. Thus, it makes sense that hyperthermia can induce ER stress and ER-mediated apoptosis.

Since Grp78 is a protein chaperone, it can help to resolve ER stress and protect cells from apoptosis under certain conditions. Further investigation is necessary to determine whether agents that inhibit Grp78 will synergize with hyperthermia treatment.

Induction of apoptosis involves the activation of cascade caspases through three major apoptotic pathways (Figure 8): extrinsic, intrinsic, and ER-mediated pathways. Many conventional anticancer agents act through intrinsic and/or extrinsic apoptotic signaling mechanisms, which are impaired in many tumor cells, leading to therapy resistance (Linder and Shoshan, 2005).

Both melanoma and BCC have been shown to be resistant to extrinsic and/or intrinsic apoptotic pathways, either due to the activation of Ras/Raf/Mek/Erk pathways and phosphatidylinositol 3-kinase/Akt pathways in melanomas (Hersey, 2006) or inappropriate activation of the Sonic Hedgehog signaling pathway and its downstream targets in BCC (Athar *et al.*, 2004; Tang *et al.*, 2006).

It is biologically plausible that when melanoma and BCC cells are treated with hyperthermia, their ability to inhibit intrinsic and extrinsic apoptotic pathways force these cells to die through ER-mediated apoptosis.

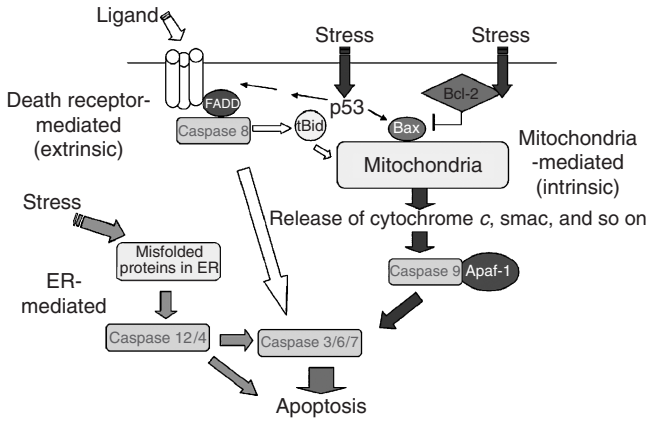

Figure 8. Simplified apoptotic pathways.

Recently, the ER has been proposed as a promising anticancer target and mediator of apoptosis, which may be less affected by intrinsic or chemotherapy-induced resistance mechanisms (Linder and Shoshan, 2005). Some emerging evidence indicates the anticancer potential of ER stress-inducing agents. Thapsigargin sensitizes tumor cells to TRAIL-induced cell death (Huang *et al.*, 2004), and tunicamycin increased the sensitivity of head-and-neck tumor cell lines to cisplatin *in vitro* and *in vivo* (Noda *et al.*, 1999). The idea that hyperthermia induces ER-mediated apoptosis might help explain why hyperthermia is a very promising treatment when combined with radiation or chemotherapies (Wust *et al.*, 2002; Jones *et al.*, 2005). Hyperthermia plus other apoptosis inducers such as radiotherapy or chemotherapy may synergize by simultaneously activating multiple apoptotic pathways.

In summary, our data suggest that hyperthermia treatment induces apoptosis in a non-conventional way that is not through the extrinsic or intrinsic pathways, but rather through ER-mediated apoptosis in melanoma and non-melanoma skin cancer cells. These results point to a new perspective of hyperthermia cytotoxicity, and might provide the scientific basis for developing cancer therapy with hyperthermia treatment as a single agent or as a part of rational combinations especially in cancer cells resistant to intrinsic or extrinsic apoptotic pathways.

MATERIALS AND METHODS

Cell lines and culture conditions

A human melanoma cell line, A375, a squamous carcinoma cell line HaCaT, and an epidermoid carcinoma A431 were obtained from ATCC (Manassas, VA). A primary human melanoma cell line, WM35, and a metastatic melanoma cell line, HS294T, were kindly provided by Dr Meenhard Herlyn (Wistar Institute, Philadelphia, PA). A mouse BCC cell line (ASZ001) (Xie *et al.*, 2001) was kindly provided by Dr Ervin Epstein Jr. at UCSF. The mouse BCC cell line ASZ001 was maintained in 254CF medium (Cascade Biologics, Portland, OR) with 0.05 mM $CaCl_2$ and 10% Chelexed fetal bovine serum (Chelex 100 Resin; Bio-Rad, Hercules, CA). Other cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Gemini Bio-Products Inc., West Sacramento, CA). All cells were grown as monolayer culture in an incubator set at 37 °C with 5% CO_2. The medical ethical committee of UCDHSC at Anschutz Medical Campus approved all described studies.

Hyperthermia treatments

Cells were treated using methods we described previously (Shellman *et al.*, 2004). Briefly, cells were grown in 96-well plates with 100 µl medium using standard culture conditions overnight before treatment. Plates were then transferred onto pre-heated copper blocks in treatment incubators set at the indicated temperatures for the indicated amounts of time before being transferred back to the 37 °C incubator. We have shown that contact between copper blocks and the lower surface of 96-well plates in an incubator provides a highly reproducible heating method (Shellman *et al.*, 2004). To treat cells for use in western blot, cells were grown overnight in 10 cm dishes and then heat-treated using the same methods at 45 °C for 60 or 90 minutes before being subjected to sample preparation for western blot at indicated post-treatment time points.

Cell Titer 96 Aqueous One solution cell proliferation assay for quantification of cell viability (MTS assay)

The reagents were obtained from Promega (Madison, WI), and procedures were followed according to the manufacturer's instructions (Cory *et al.*, 1991; Kaliberov *et al.*, 2004). Briefly, assays were performed by adding 20 µl of the reagent to each well containing cells with 100 µl medium for 1–4 hours, and OD readings at 490 nm were recorded with a microplate reader BioKinetics Reader EL312e (Bio-tek Instruments Inc., Winooski, VT). The optical density readings for growth media alone were used as background readings.

Caspase Glo 3/7, Caspase Glo 8, or Caspase Glo 9 activity assays

The reagents were obtained from Promega, and procedures were followed according to the manufacturer's instructions. The Caspase-Glo assays are homogeneous luminescent assays that measure specific caspase activities by providing proluminescent caspase-specific substrates linked to aminoluciferin and a proprietary thermostable luciferase in a reagent optimized for caspase activity, luciferase activity, and cell lysis. Caspase 3, 8, or 9 assays contain DEVD, LETD, or LEHD aminoluciferin substrates, respectively. The relative luminescent unit readings of luminescence are proportional to caspase activity and were recorded with a microplate reader BioKinetics Reader EL312e (Bio-tek Instruments Inc.). The readings for growth media alone were used as background readings.

EB/AO morphologic assay for quantifying apoptosis and necrosis

To determine the percentage of live, apoptotic, or necrotic cells, the assays were performed as we described previously (Ribble *et al.*, 2005). AO and EB were purchased from Sigma (St Louis, MO). The dye mix for the EB/AO staining was 100 µg ml^{-1} AO and 100 µg ml^{-1} EB in phosphate-buffered saline (PBS). This assay is based on the fact that apoptosis induces characteristic nuclear condensation and fragmentation, whereas necrosis is characterized by the inability to exclude vital dye, leading to orange staining of nuclei.

Briefly, plates were centrifuged, dye mixtures were added to individual wells, cells were viewed under an inverted fluorescent microscope, and images were obtained at the indicated post-treatment times.

Immunoblot. Cells, floating and adherent, were harvested with 1 × Laemmli sample buffer (Bio-Rad) at indicated post-treatment time points. Lysates were heated at 95 °C for 5–10 minutes and then clarified by centrifugation at 13,200 *g* for 5 minutes. Samples were run on SDS-PAGE 4–20% acrylamide gradient gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes using a Trans-blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad) at 15 V for 30 minutes. Membranes were probed with primary antibodies in PBS with 5% non-fat milk (or Tris-buffered saline/0.1% Tween with 5% non-fat milk if specified by the manufacturer), then washed with PBS for 5 minutes, Tris-buffered saline/Tween for 2 × 5 minutes, then PBS for 5 minutes, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in PBS with 5% non-fat milk. Blots were developed with HRP substrate (West Pico or Femto developing

solutions; Pierce, Rockford, IL) for 5 minutes at room temperature and analyzed using a Chemi-doc chemiluminescence detector (Bio-Rad). The following antibodies were used at the suggested dilution from manufacturer: Caspase 3, Caspase 12, and α/β-tubulin from Cell Signaling Technology (Danvers, MA); mouse anti-Caspase 4 mAb from Stressgen Bioreagents (Ann Arbor, MI); anti-actin mouse mAb and HRP-conjugated goat anti-mouse IgM from EMD Biosciences Inc. (San Diego, CA); BiP/Grp78 and HRP-conjugated goat anti-rabbit IgG from BD Transduction Laboratories (San Jose, CA); and HRP-conjugated goat anti-mouse IgG from Jackson Immuno-Research (West Grove, PA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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