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Original article

The effects of hyperthermia on human hepatocellular carcinoma stem and mature cancer cells

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ABSTRACT

Introduction and objectives: Hepatocellular carcinoma (HCC) can recur following radiofrequency ablation and other hyperthermic treatment modalities. Cancer stem cells (CSCs) are a subpopulation of HCC cells that are difficult to eradicate and largely responsible for tumor recurrences. Thus, the principal objective of this study was to determine whether human HCC CSCs are relatively thermal-resistant compared to non-stem or mature cancer cells (MCCs).
Materials and methods: Epithelial cell adhesion molecule (EpCAM) positive enriched CSCs and EpCAM$^-$ MCCs were derived from a human HCC cell line using fluorescence activated cell sorting. Each cell population was exposed to 65 °C heat for 0–16 min and survival documented at various time points.
Results: Cell survival curves were similar in CSC and MCCs throughout the 16 min heat exposure period. Maximum killing was obtained after 12–14 min of heat exposure. Cytoprotective, heat shock proteins-70 (HSP70) and -90 (HSP90) mRNA expression were not disproportionately increased in CSCs.
Conclusions: These results suggest that human HCC CSCs are not more thermal resistant than MCCs and therefore, do not support the hypothesis that HCC recurrences following hyperthermic treatment reflect CSC thermal-resistance.

Abbreviations

HCC	hepatocellular carcinoma
CSC	scancer stem cells
MCC	smature cancer cells
EpCAM	epithelial cell adhesion molecule
HSP	heat shock proteins
HSP70	heat shock proteins-70
HSP90	heat shock proteins-90
RFA	radiofrequency ablation
CT	cycle threshold

1. Introduction and objectives

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer related death in the world today [1]. Unfortunately, potentially curative therapeutic options for HCC continue to be limited to liver transplantation, surgical resection and for small tumors; hyperthermic ablation.

There are various types of hyperthermic techniques presently available including radiofrequency (RFA), microwave and laser ablations [2]. Each technique is designed to achieve the same goals; induce irreversible cellular damage and create sufficient intra-tumor heat to coagulate tumor-feeding blood vessels. The heat generated by each modality varies between 60–100 °C. Typical application times are relatively short (5–15 min) and depend on the ablative technique employed, patient tolerance and operator experience [2]. However, despite suggested application times, tumor-site recurrences continue to occur in 5–20% of treated HCC patients [3].

Cancer stem cells (CSCs) are a small, subpopulation of tumor cells that possess the ability to self-replicate and differentiate into various cellular lineages [4]. They can be identified by the expression of certain stem cell surface markers including epithelial cell adhesion molecule (EpCAM). CSCs tend to be refractory to chemo- and radiation-therapy and thereby, play an important role in tumor recurrences. Indeed, it has been demonstrated that a single, viable CSC can be responsible for tumor development and/or recurrence

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[4]. Whether CSCs derived from HCC are more, less, or equally susceptible to hyperthermic ablation relative to non-CSC, mature cancer cells (MCCs) has yet to be determined.

Heat shock proteins (HSP) are a family of proteins produced by normal and cancer cells in response to stressful conditions including excess heat [5]. They are classified according to their molecular weight (HSP70, HSP90, etc.). By stabilizing new proteins and refolding proteins damaged by cell stress, HSPs protect cells from injury and death [5]. HSP70 and HSP90 are two of the more extensively studied HSPs. Both play important roles in maintaining hepatocyte viability under stressful conditions and resistance to HCC treatment [6–8].

In this study, we hypothesized that HCC recurrences following hyperthermic ablations occur as a result of CSC thermal-resistance relative to MCCs. Thus, the principal objective was to document and compare CSC and MCC susceptibilities to hyperthermia over a therapeutic time interval. A secondary objective was to determine whether HSP70 and/or HSP90 expression differ in CSCs and MCCs following heat exposure.

2. Materials and methods

2.1. Cell culture

HepG2 cells were grown and cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (1248-028, Gibco) supplemented with 10 U/ml Penstrep (15140-122, Gibco) and 2 mM Glutamine (25030-081, Gibco). Cells were passaged every 3–4 days and allowed to proliferate to no more than 75% confluency in 75^2 cm flasks (90076, FroggaBio Inc.) until sufficient for a given experiment.

2.2. Cell sorting

Cells were washed twice with PBS-CMF (21600-10, Gibco) and 1 ml trypsin-EDTA (25300-054, Gibco) and added to 75^2 cm culture flasks. After a digestion period of 1–2 min, cells were removed and 9 ml of ice cold DMEM containing 10% HI-FBS was added. Cells were then gently washed off the flask with pipetting. Media was collected in 50 ml conical tubes and centrifuged at 4 °C for a period of 5 min at 100 × G. Supernatants were removed and cells resuspended in cell sorting buffer (PBS-CMF containing 2 mM EDTA (ED2SC, Sigma), 1% FBS and 25 mM HEPES (H4034, Sigma) pH 7.0, PBS-CMF). Cells were then exposed to anti human EpCAM (130-113-263, Miltenyi Biotech) tagged with FITC as well as 7-AAD (A07704, Beckman Coulter) as a marker of cell death, as per the manufacturer's recommended concentrations. Cells were sorted by FACS into two groups (EpCAM positive enriched or negative groups) in tubes containing 25% HI FBS in DMEM to a maximum dilution of 10% FBS. After sorting, cells were centrifuged at 100xG for 5 min and resuspended to a concentration of 60K cells per ml and plated on 96 well plates (92696, FroggaBio Inc.) at a density of 6000 cells per well (i.e. 100 μl per well). The first row received 10% of the total number of cells and last row no cells in order to serve as references. Cells were then cultured for 24 h in a 5% CO2 incubator maintained at 37 °C. We have previously reported that EpCAM positive enriched cells isolated by this technique possess the CSC features of self-renewal, diverse lineage differentiation and spheroid/colony formation in anchor free media [9].

2.3. Heat treatment

Heating blocks were heated to the desired temperature of 65 °C for at least 1 h prior to use. Plates were then placed overhanging on the heating block such that lanes 1 and 2 were not in contact with the block and the timer started. Plates were shifted on the block one row at a time after 1, 2, 4, 5, 6, 8, 10, 12, 14 and 16 min and then placed in an incubator and after 24 h, assayed for WST-1 activity as an indicator of cell survival.

2.4. HSP70 and HSP90 determinations

In a separate series of experiments employing the same apparatus and technique, cells were harvested after eight minutes heat exposure and HSP mRNA expression documented by real-time quantitative RT-PCR with the HSP70 primer set 5'-AGGCCGACAAGAAGAAGGTGCT-3' (forward) and 5'-TGGTACAGTCCGCTGATGATGG-3' (reverse) and HSP90: 5'-TGGTCATTGTGCGGTTCTCT (reverse) and 5'-AGAGAACCGCACAATGACCA-3' (forward). All PCR reactions were performed in a ViiA™ 7 Real Time PCR System (Applied Bio-Systems, USA), with the SYBR Green PCR Core Reagent under the following conditions: 1 cycle at 95 °C for 10 min, 35 cycles at 95 °C for 15 s, annealing temperature at 58 °C for 1 min. Data were analyzed by QuantStudio™ Real Time PCR Software (Applied Bio-Systems, USA). Real-time detection of the emission gene cycle threshold (CT) values of the targets was standardized to the beta-actin housekeeping gene: TCCTCTCCCAAGTCCACACAGG (forward) GGGCACGAAGGCTCATCATTC (reverse). Gene expressions were calculated by using the relative quantification method ($2^{-\Delta CT}$) (PMID: 11846609). Experiments were performed in triplicate on a minimum of three occasions.

3. Results

The effects of heat exposure on $EpCAM^+$ CSC and $EpCAM^-$ MCCs are provided in Fig. 1. There were no differences in survival throughout the 16 min heat exposure period in the two cell populations and maximum cell killing was obtained after 12–14 min of heat exposure. In separate experiments where culture conditions were restored after the 16 min of heat exposure for an additional 72 h, no growth of cells was observed.

The results of HSP70 and HSP90 mRNA expression after eight minutes of heat exposure in CSCs versus MCCs are provided in Fig. 2. HSP-70 expression increased in both cell populations (2.14 ± 1.20 and 3.22 ± 2.57 fold respectively) but were not disproportionately upregulated in CSCs. HSP-90 expression actually decreased (0.61 ± 0.25 and 0.60 ± 0.15 fold respectively) in both cell populations following heat exposure.

4. Discussion

The results of this study do not support the hypothesis that HCC site recurrences following hyperthermic ablation reflect relative CSC thermal-resistance. Specifically, the survival curve in CSCs was similar to that obtained with MCCs throughout the 16 min of exposure to 65 °C. That HSP70 and HSP90 mRNA expression were not disproportionately increased in CSCs further argues against the hypothesis.

An important question that emerges from this study is whether the recommended duration of heat exposure with thermal ablation is optimal to obtain complete cell killing. Unfortunately, there are no consistent guidelines as to how long hyperthermic ablations should be applied to HCC tumor tissues. Published literature and device manufacturers suggest 10–15 min for small electrode RFA, shorter if the device "impedes out" at an earlier time point or in the setting of severe patient discomfort [2,10]. For microwave ablations, the most common recommended application time is 5–10 min and for laser ablation; 5 min [11–14]. Thus, depending on the modality employed, many patients will have not received heat treatment for the 12–14 min which may be required to obtain

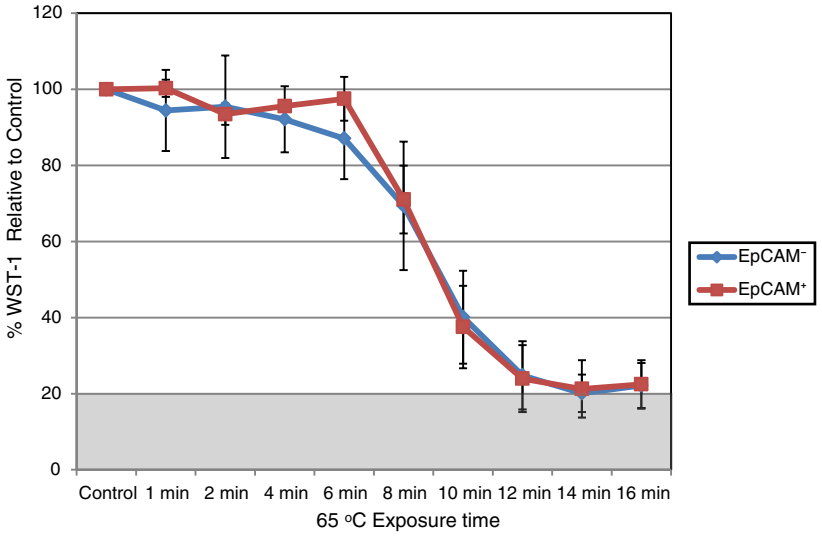

Fig. 1. EpCAM⁺ and EpCAM⁻ cell survival at various times following exposure to 65 °C external heat. The shaded gray area represents background luminescence resulting from cell debris.

maximal cell killing. However, the impact of excess heat on tumor vessel integrity and tumor ischemia must also be taken into consideration as does the dissipation of heat from the point of electrode placement to the periphery of the tumor and progressive thermal injury that may occur hours or days following the application of heat.

Regardless of the cell type (CSC or MCC), the results of the HSP-70 analyses (2–3 fold increases) raise interesting therapeutic considerations in that specific and non-specific HSP inhibitors have been identified and are commercially available [15]. Whether pre-treatment with these agents will enhance the effects of thermal ablative therapies for HCC as described for breast cancer remains to be determined [16].

There are a number of limitations to this study that warrant emphasis. First, as mentioned above, because thermal ablation also interferes with tumor blood flow and denatures potentially important extracellular proteins, our in vitro findings should be interpreted with caution. Indeed, additional studies employing animal models of HCC are required to identify the optimal duration of heat application. Second, although a well-defined human HCC cell line rather than fresh HCC tissue served as the source of the cell populations (in order to maximize CSC enrichment), the possibility that a small percent of MCCs were EpCAM⁺ cannot be excluded. Third, there were too few surviving cells toward the end of heat exposure to determine whether those that survived longest were CSCs or MCCs. Fourth, although previous studies do not suggest a "dose-response" effect for temperatures beyond 60 °C, the absolute intratumor temperatures achieved with the various hyperthermic techniques may influence optimal application times and this could not be addressed within the experimental design employed for this study. Finally, thermal treatment was limited to hyperthermia and cryo-ablation was not studied.

In conclusion, while the results of this study argue against relative CSC thermal resistance being responsible for HCC recurrences following hyperthermic treatments, preventing increases in HSP expression may represent a therapeutic strategy worthy of consideration.

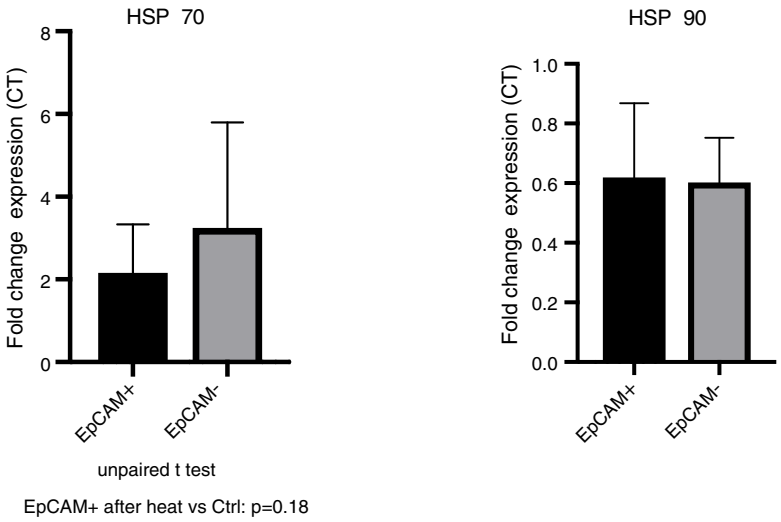

Fig. 2. Heat shock protein (HSP)-70 and -90 mRNA expression following eight minutes of exposure to 65 °C external heat. The differences between EpCAM$^+$ and EpCAM$^-$ cells were not significant.

Conflict of interest

The authors have no conflicts of interest to declare.

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