Impact of Temperature on Cell Death in a Cell-culture Model of Hepatocellular Carcinoma

BETTINA LEBER[1*], URSULA MAYRHAUSER[2*], BARBARA LEOPOLD[2], SONJA KOESTENBAUER[2], KARLHEINZ TSCHELIESSNIGG[2], VANESSA STADLBAUER[1] and PHILIPP STIEGLER[2]

[1]*Division of Gastroenterology and Hepatology and*
[2]*Division of Transplantation Surgery, Medical University of Graz, Austria*

Abstract. *Background: Radiofrequency ablation (RFA) is one treatment option for hepatocellular carcinoma (HCC) where tumour cells are destroyed by heat. However, there is lack of knowledge about cellular reactions after heating. Therefore, we studied cell death after heat application in a cell-culture setting mimicking HCC. Materials and Methods: Intracellularly stained hepatic stellate cells (LX-1) and HCC cells (HepG2) were cultivated in co-culture or alone. Apoptosis was determined by flow cytometry using AnnexinV-PE and eFluor®450. Results: Heating resulted in early apoptosis for 20-30% of HepG2 cells and 10-15% of LX-1 cells. Late apoptosis was observed in a large percentage of cells 24 h after heating at 65˚C for 15 min or 75˚C for 5 min; 65˚C for 10 min resulted in a moderate increase and 55˚C for 15 min resulted in a minor percentage of late apoptotic cells. Conclusion: Heat-treated LX-1 and HepG2 cells die by apoptosis. This finding is important for future planning tools to ameliorate RFA outcome in clinic.*

Hepatocellular carcinoma (HCC) accounts for 85% to 90% of all primary liver cancer cases and is the fifth most common malignant tumour in humans (1). In the majority of cases, HCC is caused by viral hepatitis infection (hepatitis B or C) or liver cirrhosis (*e.g.* alcohol abuse, metabolic disorders) (2). When a tumour develops in a cirrhotic liver, therapy modalities depend on tumour size, localization as well as staging and grading. In many cases, resection or transplantation is not feasible due to the advanced stage of the liver tumour at the time of diagnosis. Alternative treatment options are thermal cancer therapy techniques such as radiofrequency ablation (RFA), percutaneous microwave coagulation therapy (PMCT), and laser interstitial thermal therapy (LITT) (3-7).

Temperatures between 46˚C and 60˚C are associated with irreversible cellular damage, proportional to the exposure time (8, 9). Between 60˚C and 100˚C, protein coagulation occurs instantly with irreversible damage of key cytosolic and mitochondrial enzymes and nucleic acid-histone complexes (9). Temperatures above 105˚C result in tissue boiling, vaporization and carbonization. Therefore the key aim for thermal therapy is achieving and maintaining a temperature range of 60-100˚C throughout the entire target volume (7, 9) in order to solely destroy the affected and not the healthy liver. However, to our knowledge there is no detailed description on a cellular level about the type of cell death cells undergo during thermal treatment. Assessment of cell viability immediately after heat application by histological techniques is generally inaccurate as morphological changes are a poor indicator of cell viability at this stage (10). Heat activation of apoptosis may be involved in the progression of the affected RFA zone and is possibly an important determinant of the completeness of tumour eradication (11-13). However, only few studies define the role of apoptosis in the progression of injury following the application of local thermal therapy in the treatment of primary cancer or metastasis in the liver (11). The destruction of the surrounding tissue after local thermal treatment seems to be crucial in order to destroy the whole tumour (14). Furthermore, respecting the mandatory safety margin required to avoid local tumour recurrence is crucial (15-17). Moreover, heat-activated live tumour cells are known to be transformed into even more aggressive malignancies which may even reduce the patient's survival opportunities (10, 11, 17-31).

This study aims to investigate the effect of hyperthermia on a HCC cell line (HepG2) and a hepatic stellate cell line (LX-1) and to increase the knowledge on the type of cell

*These Authors contributed equally to this work.

Correspondence to: Vanessa Stadlbauer-Köllner, MD, PhD, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. Tel: +43 031638582282, e-mail: vanessa.stadlbauer@medunigraz.at

Key Words: Radiofrequency ablation, HepG2 cells, LX-1 cells, thermal therapy, apoptosis, HCC.

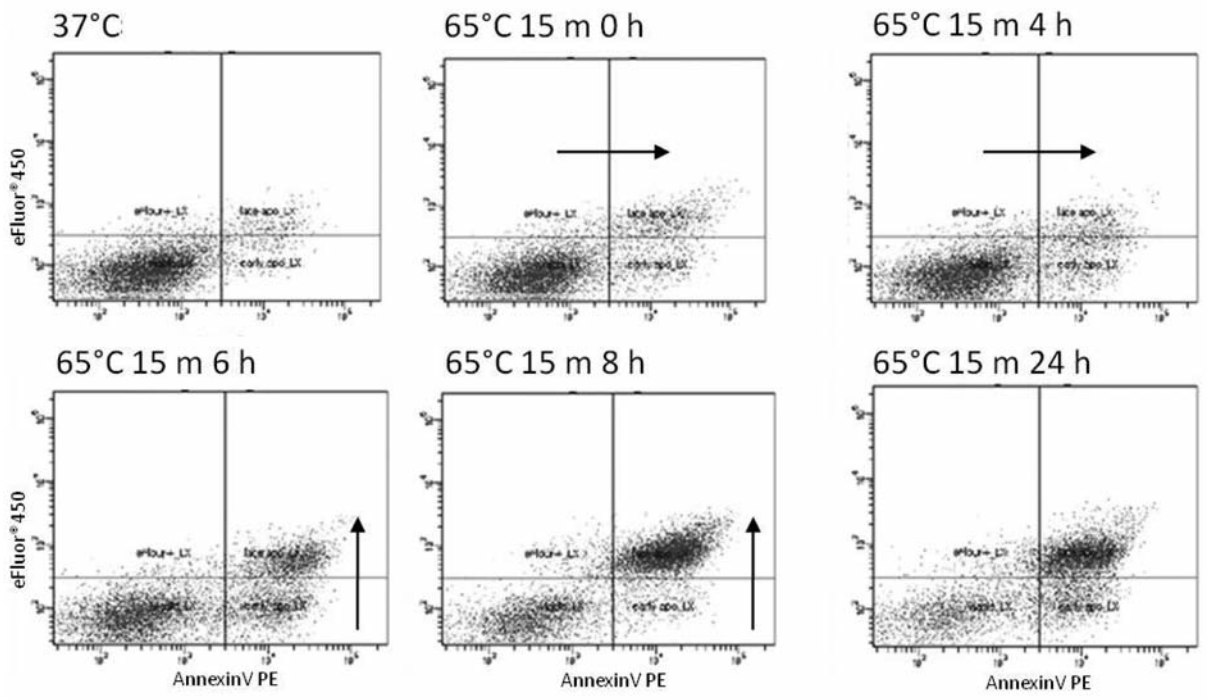

Figure 1. *Representative time course of typical apoptotic death of LX-1 cells after heating at 65˚C for 15 min. Cells shift from viable (annexinV/eFluor®450-negative) to early apoptotic (annexinV-positive/eFluor®450-negative) and finally to late apoptotic (annexinV/eFluor®450-positive).*

death that cells undergo during thermal treatment of HCC. Furthermore, the impact of heat exposure on different grades of simulated fibrosis is documented to gain more information about thermal therapy in livers with different tissue compositions. These experiments are intended to provide helpful findings for planning RFA, PMCT or LITT for patients suffering from HCC in order to achieve complete tumour destruction and to respect the safety margin needed to avoid local tumour recurrence.

Materials and Methods

Cell culture. The human hepatocellular carcinoma cell line HepG2 (ATCC# HB-8062; Rockville, MD, USA) was cultivated at 37˚C in minimum essential medium (MEM, Invitrogen GmbH, Lofer, Austria) containing additional 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin/streptomycin (P/S; PAA Laboratories GmbH) in a humified atmosphere (5% CO_2). The hepatic stellate cell line LX-1 (gratefully provided by Professor Friedman, Mount Sinai School of Medicine, NY, USA (32)) was cultivated in Dulbecco's modified Eagle's medium (DMEM containing 4.5 g/l D-glucose; Invitrogen GmbH) supplemented with 10% FBS, 1% P/S and 1% L-glutamine (PAA Laboratories GmbH) in a humified atmosphere (5% CO_2). For all co-culture experiments, MEM with 10% FBS and 1% P/S was used.

Prior to heating (18 h) 5×10^5 intracellularly stained cells (see below) were seeded in co-cultures (BD Biocoat™ Collagen I 6-well Multiwell Plates; BD Biosciences Europe, Heidelberg, Germany; see Figure 1) and induced with 2.5 ng/ml recombinant human transforming growth factor-beta 1 (TGF-β1; Biovision, Mountain View, CA, USA) in MEM supplemented with 10% FBS and 1% P/S. To simulate different grades of fibrosis, the cells were mixed at 25% LX-1/75% HepG2, 50% LX-1/50% HepG2, 75% LX-1/25%HepG2, 100% LX-1 and 100% HepG2.

Intracellular staining of LX-1 and HepG2. To distinguish cells *via* flow cytometry, cells were stained intracellularly before the experiments. LX-1 cells were stained with DiO (Detected in the FITC channel; VybrantTM Cell-labelling solutions, Molecular Probes Inc, Leiden, the Netherlands) and HepG2 cells with DiD (detected in the APC channel; Vybrant™ Cell-labelling solutions, Molecular Probes Inc, Leiden, Netherlands) in culture flasks 48h before heating.

After removal of the culture medium, LX-1 cells were incubated for 15 min at 37˚C in 4 ml (in a 150 cm^2 flask) staining medium (0.5% DiO in MEM supplemented with 10% FBS and 1% P/S). Thereafter, the cells were washed three times by adding 12 ml PBS and incubated for 10 min at 37˚C.

After removal of culture medium, HepG2 cells were incubated for 5 min at 37˚C in 2 ml (in a 75 cm^2 flask) staining medium (0.1% DiD in MEM supplemented with 10% FBS and 1% P/S). Thereafter, the cells were washed three times by adding 6 ml PBS and incubated for 10 min at 37˚C.

Stained cells were then kept in MEM supplemented with 10% FBS and 1% P/S for further use.

Heating experiments. Immediately before heating, the culture supernatant was removed, 1 ml preheated medium (55˚C, 65˚C,

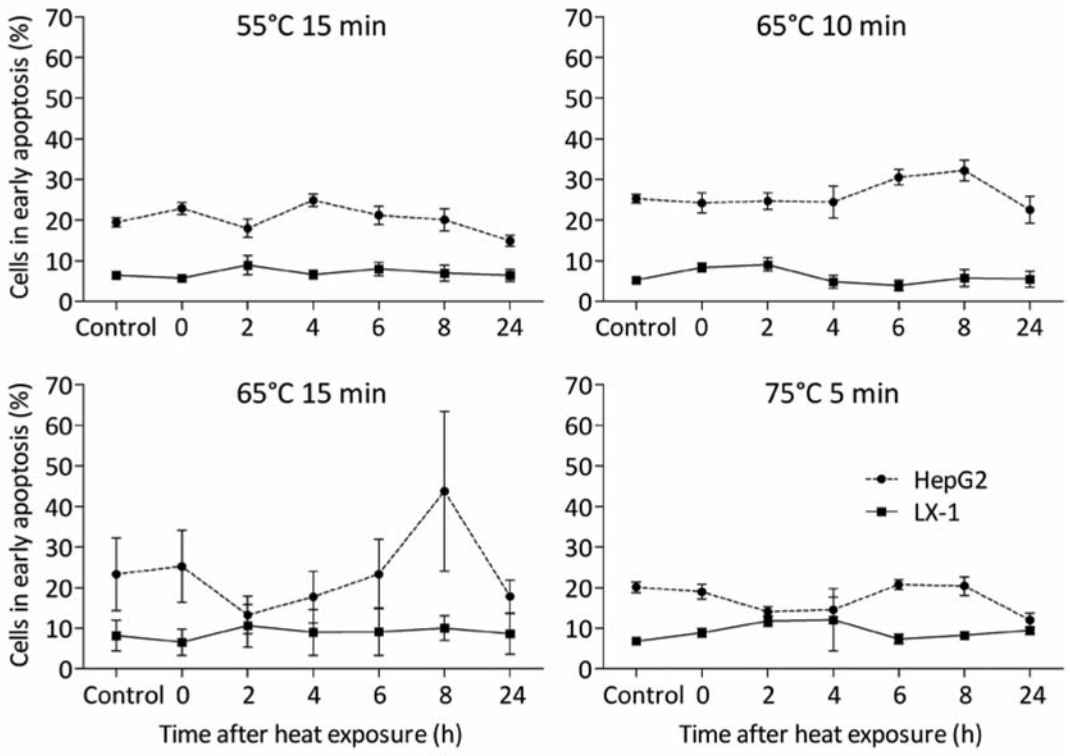

Figure 2. *Percentage of cells in early apoptosis (annexinV-positive/eFluor®450-negative) after heating determined by flow cytometry. For each time point data of all different co-culture compositions were taken together for each cell line generating one data point per time point. Results are expressed as the mean±SEM of data of three independent experiments.*

75˚C) was added and the plates were incubated at this temperature in a preheated heating cabinet for 5, 10 or 15 min. Thereafter the plates were kept at 37˚C until further use or directly used for determination of apoptosis *via* flow cytometry.

To avoid losing dying or dead cells, all supernatants of heated and control cells were collected in one tube (collection tube). At each time point for heated cells and controls, the medium was transferred to the collection tube and cells were washed once with 1 ml PBS (also transferred to the collection tube). Thereafter, the cells were detached by addition of 200 µl 0.025% Trypsin/EDTA (Sigma-Aldrich Handels GmbH, Vienna, Austria) solution and incubated at 37˚C for 5-7 min (depending on the detaching progress observed). Then the cells were resuspended in 1 ml medium, added to the collection tube and used for flow cytometric analysis immediately.

Staining for flow cytometric analysis of apoptosis. For apoptosis staining, annexinV PE (PE AnnexinV Apoptosis Detection Kit I, BD Biosciences Europe, Heidelberg, Germany) in combination with eFluor®450 (detected in the PacificBlue channel; Fixable Viability Dye, eBiosciences, Vienna, Austria) was used. The cell suspension was washed twice with 1 ml cold PBS, resuspended in 1 ml cold PBS and kept on ice. After discarding the supernatant, one half of the cells were resuspended in 500 µl of cold eFluor®450 working solution (diluted 1:15 in PBS; apoptosis staining), the other half was kept in 500 µl cold PBS (unstained control) and incubated for 10 min on ice in the dark.

Thereafter, the cells were washed once with 1 ml cold PBS and the supernatant was carefully removed . Cells were then resuspended in 100 µl 1× annexinV dilution buffer (diluted 1:10 in PBS). The eFluor®450-stained cells were incubated with 5 µl annexinV PE, whereas the unstained control remained in 1× annexinV dilution buffer without antibody. After incubation at room temperature for 15 min in the dark, 400 µl cold 1× annexinV dilution buffer were added. Then the samples were kept on ice and were immediately analysed by an LSRII flow cytometer in combination with FACSDiva 6.1.2 software (BD Biosciences Europe, Germany).

To differentiate between apoptosis and necrosis we observed the shift of the cell populations over 24 h after heat treatment was recorded. We assume cells shifting from annexinV/eFluor®450-negative to annexinV-positive/eFluor®450-negative (phosphatidylserine externalization) and then to annexinV/ eFluor®450-positive (permeabilization of membrane after phosphatidylserine externalization) as being apoptotic, whereas cells shifting from annexinV/ eFluor®450-negative immediately to AnnexinV/ eFluor®450-positive were assumed to be necrotic.

Statistical analyses. Data of three independent experiments were used and the mean±SEM was built from all the results. Graphs were created with GraphPad Prism version 5.0 software for Windows and statistics were calculated by means of PASW18.0 (SPSS GmbH Software, an IBM Company, Munich, Germany). Furthermore, the controls at 37˚C of each time point were set as control data per experiment.

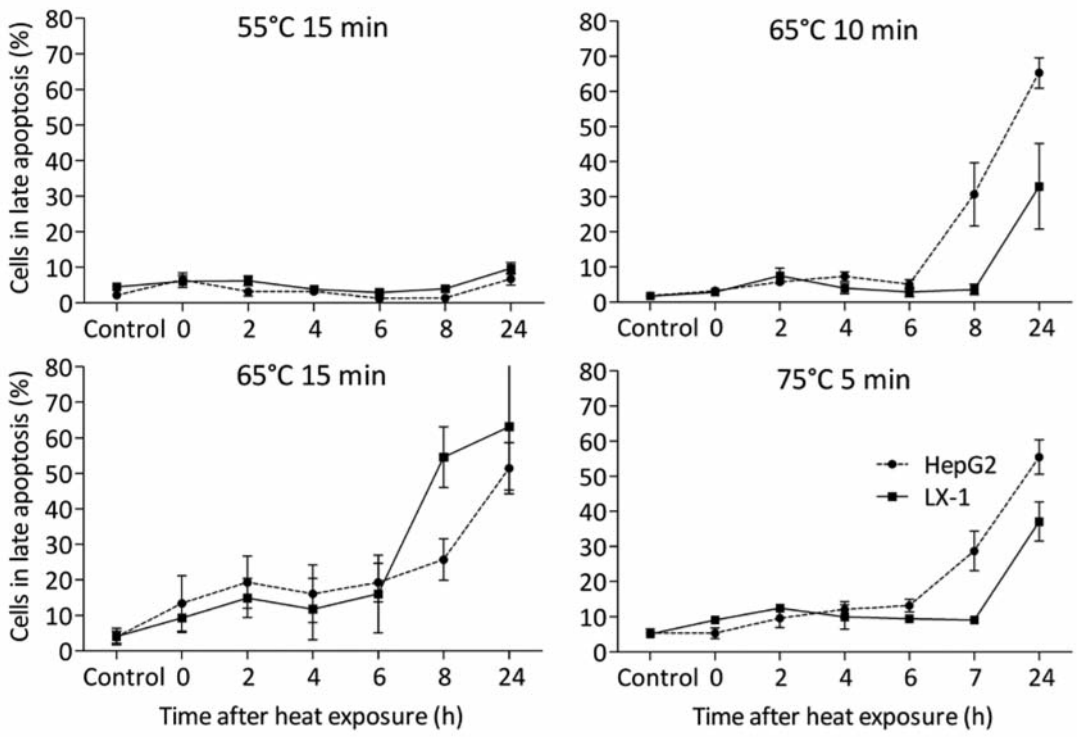

Figure 3. *Percentage of cells in late apoptosis (annexinV/eFluor®450-positive) after heating, determined by flow cytometry. For each time point, data of all different co-culture compositions were collected together for each cell line generating one data point per time point. Results are expressed as the mean±SEM of data of three independent experiments.*

Results

Flow cytometry was used to determine whether LX-1 and HepG2 cells were dying *via* apoptosis or necrosis after heating. Intracellular staining was performed prior to heating to enable identification of the two cell types in co-cultures. All investigated heating conditions (55˚C for 15 min, 65˚C for 10 min, 65˚C for 15 min and 75˚C for 5 min) showed similar patterns as seen in Figure 1.

We did not observe any differences in the percentage cell survival between the different cell compositions (100%, 75%, 50% and 25%) therefore the following results represent the mean±SEM of all results for each cell line at each time point.

About 20-30% of HepG2 cells were identified as being early apoptotic (annexinV positive/eFluor®450-negative) over the 24 h time period of measurement for all heating conditions. One exception was the unexpectedly high percentage (about 60%) of early apoptotic HepG2 cells 8 h after heating at 65˚C for 15 min. The proportion of early apoptotic LX-1 cells was constantly between 5% and 15% under all heating conditions (Figure 2).

After heat exposure at 55˚C for 15 min only a minor proportion of LX-1 and HepG2 cells were in the late apoptotic state (about 5% AnnexinV/eFluor®450 positive cells; Figure 3). This percentage did not change over the whole 24 h time span of observation. In contrast, 8 h after heating at 65˚C for 10 min, 30% of HepG2 cells were in the late apoptotic state and this percentage rose to about 65% 24h after heat treatment. Only a low percentage of LX-1 cells heated to 65˚C for 10 min were in late apoptosis 8 h after heating (4%) and this number increased to 30% 24 h after heat treatment. Immediately after heating at 65˚C for 15 min, a marginal increase of late apoptotic LX-1 (~10%) and HepG2 cells (~15%) was found. This rose to 55% late apoptotic LX-1 cells 8 h after heating and 60% late apoptotic LX-1 cells 24 h after heat treatment, while late apoptotic HepG2 levels rose to 25% 8 h and to 50% 24 h after heating, respectively. Heating to 75˚C for 5 min revealed 5%-10% late apoptotic LX-1 cells within 8 h after heat treatment and increased to 35% 24 h after heat treatment. In contrast, the proportion of late apoptotic HepG2 cells constantly increased from 5% to 15% over the first 6 h after heat treatment and rose drastically to 30% 8 h and to 55% 24 h after heat treatment (Figure 3).

Discussion

In contrast to resection and transplantation, RFA is a minimal invasive thermal treatment method for HCC that can be performed percutaneously, even for patients with

decreased general health conditions (7). However, there is still not enough knowledge on the response of different cell types to hyperthermia. Focal hyperthermia creates a temperature gradient that progressively decreases away from the site of the probe insertion that is known to negatively impact on the ablation outcome (10). Several studies showed that prolonged hyperthermia above as little as 40˚C leads to inactivation of vital enzymes and may initiate apoptosis (12, 13); differing results have been published focusing on the occurrence of apoptosis in the periphery after focal thermal treatment (22, 33, 34).

The aim of our study was to investigate the effect of hyperthermia on a HCC cell line (HepG2) and a hepatic stellate cell line (LX-1) to better understand the impact of thermal treatment on liver cell lines. We investigated temperatures ranging from 55˚C to 75˚C, which are commonly used during thermal therapy. Temperature exposure time was chosen based on the general RFA, LITT and PMCT conditions, which currently last about 10 to 20 min (35-38) and are adapted for single layer cell-culture models based on previous findings (39). We chose the HepG2 cell line because it is a common HCC cell line, which contains no hepatitis virus and is not resistant to apoptosis (40). This cell line is often used for *in vitro* hyperthermia studies (25, 40, 41). Differentiation between necrosis and apoptosis might be of great interest in terms of tumour recurrence, as well as tumour progression, due to the fact that damaging of cells in the boundary area of the tumour, which do not undergo apoptosis due to lower temperatures (21), is known to develop even more aggressive neoplastic lesions (17-23).

Previous experiments of our group revealed that a temperature exposure of 85˚C for 5 min, 75 for 10 min and 65 for 15 min is necessary to completely down-regulate metabolic activity in all HepG2 and LX-1 cells within 48 h (39). Comparing those data with data of the present study, we found similarities in cell survival after heating at 55˚C for 15 min and at 65˚C for 10 min in both settings. However, immediately after heating at 65˚C for 15 min a clearly higher viability was measured in this study (60%-90%) compared to the one found by Mayrhauser *et al*. [10%-60%; (39)]. This indicates that cells lose their metabolic activity before undergoing apoptosis, whereas 24 h after heating, survival in both experiments revealed similar results again. In the previous study, heating at 75˚C for 5 min led to similar cell survival and no change during 24 h, whereas in this study, a decrease of viability was detected within 24 h after treatment (39).

In clinical practice, a tumour should be ablated totally and *in sano* so that no tumour cells survive and the damage to the surrounding tissue should be as low as possible. However, the heat-sink effect caused by large vessels (>3 mm in diameter) can lead to a deflection of the ablation zone and some viable cells can remain (42, 43). It is essential to destroy all tumour cells because heating without destroying tumour cells has been shown to induce further transformation of tumour cells in culture (25), as well as in clinical and experimental studies (10, 11, 24-31). The results of this study were partly implemented in an image-based multi-scale modelling for validation and prediction of treatment results after RFA (44, 45) in order to develop an improved simulation and validation tool for RFA treatments (46).

In conclusion, our results showed that heat-treated HepG2 and LX-1 cells die by apoptosis, activated very early after heating. In order to avoid any tumour progression or even transformation of tumour cells, exact planning tools for local thermal treatments might be crucial to enable the radiologist to completely destroy the tumour with respect to the mandatory safety margin, therefore avoiding tumour recurrence and improving patient survival.

Conflicts of interest

The Authors declare no conflicts of interest.

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