Immunohistochemical and radiological characterization of wound healing in porcine liver after radiofrequency ablation

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Summary. Background: Radiofrequency ablation (RFA) is a minimal invasive therapeutic option for patients with hepatocellular carcinoma or liver metastases. We investigated RFA-induced cellular changes in the liver of pigs.

Material and Methods: Healthy pigs (n=18) were sacrificed between day 0 and 3 months after RFA. The wound healing process was evaluated by computed tomography (CT), chromotrope anilinblue (CAB) staining of large-scale and standard tissue sections. Immunohistochemistry (IHC) for heat shock protein 70, Caspase-3, Ki67, Reelin, Vinculin, Vimentin and α-SMA was performed.

Results: One day after RFA, CAB staining showed cell damage and massive hyperaemia. All IHC markers were predominantly expressed at the outer borders of the lesion, except Reelin, which was mainly detected in untreated liver regions. By staining for Hsp70, the heat stress during RFA was monitored, which was most distinct 1-2 days after RFA. CT revealed decreased lesion size after one week. Development of a Vimentin and α-SMA positive fibrotic capsule was observed.

Conclusion: In the early phase signs of cell damage, apoptosis and proliferation are dominant. Reduced expression of Reelin suggests a minor role of hepatic stellate cells in the RFA zone. After one week myofibroblasts become prominent and contribute to the development of the fibrotic capsule. This elucidates the pathophysiology of RFA and could contribute to the future optimization of RFA procedures.

Key words: Radiofrequency ablation, Healthy pig, Liver, Immunohistochemistry, Wound healing

Introduction

Radiofrequency ablation (RFA) is the standard of care for patients with early stage hepatocellular carcinoma and liver metastases, who are not suitable for surgery (Khatri et al., 2007, Easl-eortc clinical practice guidelines, 2012). For RFA, electrode probes are placed into a tumour percutaneously and the tissue around this electrode is coagulated using a radiofrequency generator (Lau and Lai, 2009). The goal of this thermal therapy is achieving and maintaining a temperature range about 60-100°C throughout the entire target volume which induces coagulation necrosis (Gaiani et al., 2003; Vanagas et al., 2010b) in order to destroy the affected tissue with a safety margin in the peritumoural tissue (Easl-eortc clinical practice guidelines: Management of hepatocellular carcinoma).

During, as well as after the RFA-procedures, control-imaging studies like computer tomography (CT) or magnetic resonance imaging (MRI) are performed. It is known that a few days after RFA a hyperattenuating
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rim around the site of destruction is visible radiologically, reflecting post interventional hyperaemia or tissue regeneration (Stippel et al., 2004; Antoch et al., 2005; Vanagas et al., 2010a). On a cellular level the RFA lesion can be classified in four different zones: The application zone (A-zone) where the tissue is directly exposed to the thermal electrode is characterised by irreversible carbonisation. The central zone (C-zone) is adjacent to the A-zone and consists of necrotic tissue. The transition zone (T-zone) is a haemorrhagic area surrounding the C-zone, containing apparently undamaged liver cells with signs of tissue infiltration with blood cells. The reference zone (R-zone) contains the normal, undamaged tissue surrounding the R-zone (Germer et al., 1998; Ohno et al., 2001; Godwin and Coad, 2009).

Information about the time related changes of the RFA lesion is limited and controversial. In imaging studies it is reported on the one hand that the lesion size is stable over one year or longer (Feliberti and Wagman, 2006) and on the other hand it seems to shrink (Tsuda et al., 2001; Morimoto et al., 2002; Filippone et al., 2007; Fukuda et al., 2011).

As it is crucial to understand the physiological changes after RFA in the affected tissue in order to be able to accurately apply RFA for the individual patient and predict treatment success we aimed to investigate the cellular changes after RFA in a healthy pig model over time. Therefore we compared radiological findings with large scale sections and immunohistochemistry.

Material and methods

Ablation procedure

All animal experiments (application number: 66.010/69-II/10b/2008), including medication, anaesthesia and euthanasia, were carried out according to the Austrian animal law and were performed by I.W. (veterinarian) and RH.P (interventional radiologist).

All pigs (n=18) were anaesthetised using Remifentanil (Ultiva®, GlaxoSmithKline Pharma GmbH, Vienna, Austria) 1 mg ad 50 ml NaCl 0.9% (20 g/ml) 0.08-0.1 g/kg/KG/h and Sevourane (SEVOfluran®, Abbott Laboratories, Illinois, US) 2% enitdial. After intubation a catheter was placed in the vena cava for collection of blood samples and injection of the contrast agent for CT monitoring.

After transfer to a 320-row CT unit (Aquilion One, Toshiba Medical Sytems, Austria) two large area reference electrodes (Rita Dispersive Electrode, RITA Medical System, CA, US) were fixed on the pigs’ femoral muscle. After a topographic scan, a volume CT of the upper abdomen was acquired and reconstructed to 3 mm axial slices in order to analyse the liver anatomy for adequate RFA-probe placement. By the use of a laser visor, the probe (Rita StarBurst XL, Electrosurgical Device, RITA Medical System, CA, US) was inserted through the skin into the liver during advised respiratory arrest. Three images were taken in a distance of 5 mm around the chosen position (-5 mm, 0 mm und +5 mm) during respiratory arrest. Then the prongs of the RFA electrode were extended to 3 cm. The liver was scanned in 3 phases (arterial, portal venous and hepatic venous) using 40 ml/kg contrast agent under respiratory arrest. The RFA using the ModelX RF Generator (AngioDynamics, NY, US) lasted 7 minutes after reaching the target temperature of 100°C. This procedure was done a second time to produce a second lesion. In the end a third phase scan was performed. In total two RFAs were performed in each pig (one near the large vessels = “central”; one near the liver surface = “peripheral”). Follow up CT scans after RFA treatment were performed at different time points (one day, two days, one week, two weeks (n=2 per time point), one months (n=3), two, three months (n=2). Animals were sacrificed and the whole liver was explanted at the given time points for further histological analysis.

Statistical analysis of the lesion volume

The volume of RFA lesions was analysed on CT scans directly after treatment and on the day of scarification of the pigs. On a commercially available imaging workstation (Sienet Magic View; Siemens Erlangen, Germany), the CT-studies were reviewed and analysed with interactive multi-planar viewing by an independent radiologist. An electronic caliper was used to assess the diameters of the lesions in three dimensions (mm*mm*mm). According to the formula of ellipsoid of rotation (V=4/3*π*a*b*c) the theoretical volume was calculated. The volume after the surviving time was compared to the volume directly after treatment. For each pig both lesions (“central” and “peripheral”) were measured individually. Data from pigs surviving different time spans were collected. The arithmetic mean of all datasets at each time point was calculated. ANOVA with Tukey’s post hoc test was used. A p<0.05 was considered as statistically significant.

Large scale paraffin blocks

Large scale paraffin blocks were prepared by in situ fixation-perfusion as described in (Koestenbauer et al., 2011). Then sections large scale sections were stained with chromotrope anilinblue (CAB), dried and scanned using a digital desk scanner (ScanMaker i800, Microtek, Everst GmbH, Willich, Germany) with a resolution of 4800dpi. ScanWizard 5 vs.7.22 for Mac (Microtek, Everst GmbH, Willich, Germany) software was used for scanning.

Preparation and immunohistochemical staining of standard paraffin sections

Tissue was fixed in 4% formalin, dehydrated by Tissue-Tek® Vacuum Infiltration Tissue Processor (Tissue-Tek® VIP®, Sakura, USA) and paraffin
embedded using Tissue-Tek® Tissue Embedding Console (Tissue-Tek® TEC™, Sakura, USA). Sections (1.5µm-3µm) were mounted on StarFrost slides (ca. 76x26mm, cut edges; Knittel, Germany), air-dried and used for CAB staining or immunohistochemistry.

Tissue sections were stained for Heat shock protein 70 (Hsp70; Abcam, UK; 1:100), Caspase-3 (Caspase-3; R&D Systems, Germany; 1:25), Ki67 (Dako, Austria; 1:200), Glial fibrillary acidic protein (GFAP; Thermo Scientific, Belgium; 1:500), Vinculin (Sigma Aldrich, Germany; 1:20000), Reelin (Chemicon, USA; 1:400), Vimentin (Dako, 1:400) and alpha-smooth muscle Actin (α-SMA; Sigma Aldrich; 1:5000). Sections were pretreated with sodium citrate buffer (0.1M pH 6.0) for 15 min (Hsp70), 30 min (GFAP) or 40 min (Vinculin, Caspase-3, Ki67) in a microwave at 150 watt, with Target Retrieval Solution pH 9.0 (Dako) for 40 min in a microwave at 150 watt (Reelin, Vimentin), or without antigen demasking (α-SMA). Subsequently sections were incubated with the primary antibody for 1 h at room temperature. For staining the UltraVision LP Detection System (Thermo Scientific) and chromogen DAB (Ki67, GFAP, Reelin and Vinculin; Dako) or AEC (for Caspase-3, α-SMA, and Vimentin; Dako) were used. The antibodies were diluted in Dako REAL™ Antibody Diluent (Dako). As positive controls porcine tonsils, spleen and fibrotic liver tissue were used. Counterstaining was performed with hemalaun. Standard isotype controls were used for each antibody.

Results

Characterisation of RFA lesions by CT scan

There was no morphological difference between central and peripheral lesions. Therefore data of both lesions per animal were combined. One and two days after RFA the lesions tended to increase in size and significantly shrink starting one week after RFA (p<0.05, Fig. 1). In one animal, after three months, no lesions were detectable on CT scan anymore. Table 1 shows the volume of lesions at the day of sacrifice and the size changes in percent compared to the day of intervention (0d).

Characterisation of wound healing after RFA by histology

Immediately after RFA the area on large scale sections showed a well defined lesion with carbonisation in the middle and hyperaemia at the border to the healthy tissue (Fig. 2).

CAB staining of small scale sections and subsequent light microscopical evaluation enabled a more precise differentiation between healthy and damaged liver tissue (Figs. 3-5). Morphologically intact liver cells showed a violet cytoplasm and large nuclei. Damaged cells had a pink cytoplasm and pyknotic nuclei. Erythrocytes were stained in red, collagen fibres in blue. Immediately after RFA (0d), hepatocytes in the T zone were morphologically intact, but loosened their arrangement within the liver plates. Although the liver lobules themselves stayed intact, an increased amount of erythrocytes was observed in the damaged area. At day one, hepatocytes appeared damaged. In addition, a massive hyperaemia, visible as red rim, could be observed in the T zone. This red rim persisted until week one and was enclosed by a blue rim as sign for enhanced collagen deposition. After two weeks the red rim in the T zone had disappeared and a marked blue collagen deposition was visible. During the next 3 months a thick fibrotic capsule developed with a clear blue rim adjacent to the healthy tissue. Liver lobules inside the capsule appeared massively damaged with enhanced blood supply and dilatations in the region of the central veins.

Characterisation of wound healing after RFA by immunohistochemistry

Hsp70 expression is documented in Figs. 3-5. Hsp70 occasionally showed diffuse cytoplasmic and nuclear staining of healthy liver cells. Immediately after RFA, Hsp70 expression did not differ between T-zone and R-zone. After one day however, a distinct expression of Hsp70 could be detected in morphologically intact liver cells outside the hemorrhagic area at the border to the T-zone. In the T-zone, HSC-like cells with strong Hsp70 expression were observed in damaged regions of the liver lobules. In addition, Hsp70 was dominantly expressed in the periportal fields including the interlobular vessels. After two days, a marked Hsp70 expression was still present at the border to the T-zone. Moderate Hsp70 expression, with occasional faint nuclear and cytoplasmatic staining was observed in the R-zone after one week. Between two weeks and three months after RFA, Hsp70 expression was observed at the border zones of damaged liver lobules contacting the fibrotic capsule. Damaged hepatocytes did not express Hsp70 themselves, whereas some cells of the fibrotic capsule showed Hsp70 expression.

Vimentin serves as a marker for cells of...
Fig. 1. Computer tomography associated RFA in healthy pig. Arrows indicate the RFA lesion. d: days after RFA, w: weeks after RFA, m: months after RFA.
mesenchymal origin including endothelial cells, smooth muscle cells, fibroblasts and macrophages (Kupffer cells), α-SMA as a marker for smooth muscle cells, myofibroblasts and partly endothelial cells. The expression of Vimentin and α-SMA in the liver is shown in Figs. 5-7. Immediately after RFA, no prominent changes in the expression of Vimentin and α-SMA expression were found. After 1 and 2 days, a striking accumulation of round-shaped Vimentin positive cells was demonstrated at the outer margin of the corresponding hyperaemic rim adjacent to the T-zone. α-SMA positive cells were distributed in similar regions, but at a lower extent compared to Vimentin. After one week, the number of Vimentin positive cells in the T-zone was highly increased compared to the R-zone and positive cells were also found in the newly formed fibrotic capsule. α-SMA positive cells were predominantly located at the inner and outer margins of the fibrotic capsule and in adjacent liver lobules of the T-zone. After two weeks, both Vimentin and α-SMA were strongly expressed in the fibrotic capsule. In addition, numerous Vimentin positive cells and fewer α-SMA positive cells were observed in lobules of the T-zone. After one month, most prominent Vimentin expression was observed at the margins of the fibrotic capsule protruding towards the C-zone. Interestingly, these

Fig. 2. CAB staining of liver large scale sections of RFA treated pigs. CAB staining shows development of the ablation zone surrounding tissue over 2 months (d: days, w: weeks, m: months). Immediately after RFA a hyperattenuating rim occurs (red rim) and persisted until day 2. At day 2 an additional faint blue rim (fibrotic cells) was already visible surrounding the red rim. After one week the lesion was encapsulated by a fibrotic border (blue rim). This blue rim was visible until month 2. One month after RFA the capsule started to shrink compared to date of RFA. Relative changes in RFA lesion volume are compiled in Table 1. Scale bar: 1 cm.
Fig. 3. Effects of RFA in liver tissue 0 days – 1 week after RFA treatment evaluated by CAB staining and Hsp70 expression. Immediately after RFA treatment, CAB staining revealed a loosened arrangement of liver cells and an increased amount of erythrocytes in the transition (T) zone. Arrows mark the border to the untreated reference (R) zone. Hsp70 expression did not differ between T and R zone. At day 1, a massive hyperaemia, visible as red rim could be observed in the T zone after CAB staining. A distinct expression of Hsp70 could be detected in morphologically intact liver cells outside the haemorrhagic area at the border to the T zone. In damaged regions of the liver lobule, Hsp70 was predominantly expressed in the perportal fields including the interlobular vessels (arrow). After 2 days, the red rim at the T zone persists and a marked Hsp70 expression was still present at the border to the T zone. After 1 week, a fibrotic capsule (F) visualised by CAB staining as blue rim, enclosed the haemorrhagic region. Moderate Hsp70 expression, with occasional faint nuclear and cytoplasmic staining was observed in the R zone. C: central zone of RFA; T: transition zone; F: fibrotic capsule; R: reference zone. Scale bar: 500 μm.
Fig. 4. Effects of RFA in liver tissue 2 weeks – 3 months after treatment evaluated by CAB staining and Hsp70 expression. CAB staining revealed that after two weeks the red rim (hemorrhage) in the transition zone had disappeared. During the next 3 months a thick fibrotic capsule developed with a clear blue rim to the adjacent healthy tissue. Liver lobules inside the capsule appeared massively damaged with enhanced blood supply (arrows) and dilatations in the region of the central veins (asterisks). Hsp70 expression was observed at the border zones of damaged liver lobules contacting the fibrotic capsule and in some cells of the fibrotic capsule (asterisks). C: central zone of RFA; F: fibrotic capsule; R: reference zone. Scale bar: 500 µm.
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Fig. 5. Effects of RFA in liver tissue 0 days – 1 week after treatment evaluated by Vimentin and α-SMA expression. Immediately after RFA, no prominent changes in the expression of Vimentin and α-SMA expression were found. After 1 and 2 days, a striking accumulation of Vimentin positive cells was demonstrated at the outer margin of the corresponding hyperaemic rim adjacent to the R zone. α-SMA positive cells were distributed in similar regions, but compared to Vimentin at a lower extent. After 1 week, the number of Vimentin positive cells in the T zone was highly increased and positive cells were also found in the newly formed fibrotic capsule. α-SMA positive cells were predominantly located at the inner and outer margins of the fibrotic capsule and in adjacent liver lobules of the T zone. C: central zone of RFA; T: transition zone; F: fibrotic capsule; R: reference zone. Scale bar: 500 µm.
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Fig. 6. Effects of RFA in liver tissue 2 weeks – 3 months after treatment evaluated by Vimentin and α-SMA expression. Both Vimentin and α-SMA were strongly expressed in the fibrotic capsule. In addition, numerous Vimentin positive cells and fewer α-SMA positive cells were observed in lobules of the T zone after 2 weeks. During the next months, most prominent Vimentin expression was observed at the margins (arrows) of the fibrotic capsule protruding towards the C zone. Interestingly, these protruding “tip cells” (arrows) remained α-SMA negative. In the C zone, expression of Vimentin and α-SMA was mainly restricted to the periportal field and blood vessels (asterisks). C: central zone of RFA; F: fibrotic capsule; R: reference zone. Scale bar: 500 µm.
Fig. 7. Expression of Hsp70, Vimentin and α-SMA in a liver lobule located in the transition and reference zone, 1 day after RFA. CAB staining showed damaged liver cells with faint cytoplasm and pycnotic nuclei, as well as enhanced amounts of erythrocytes in the T zone. Hepatocytes in the R zone had darker cytoplasm and morphologically intact nuclei. Hsp70 was strongly expressed in morphologically intact hepatocytes in close vicinity to the T zone and in periportal fields (arrow) surrounding damaged lobules. Few Hsp70 positive stellate-like cells were observed in the damaged liver lobule of the T zone. Compared to the R zone, the expression of Vimentin and α-SMA was increased in the T zone. T: transition zone; R: reference zone. Scale bar: 100 µm.
Fig. 8. Expression of Caspase 3, Ki67, Reelin and Vinculin in a liver lobule located in the transition and reference zone, 1 day after RFA. Compared to the R zone, increased numbers of Caspase3 and Ki67 positive nuclei were observed in liver lobules of the T zone. Also Vinculin expression was higher in the T zone, whereas the expression of Reelin was lower. T: transition zone; R: reference zone. Scale bar: 100 µm.
protruding “tip cells” remained α-SMA negative. In the C-zone, expression of Vimentin and α-SMA was mainly restricted to the periportal fields and blood vessels.

Compared to the R-zone, increased numbers of Caspase-3 and Ki67 positive nuclei were observed in liver lobules of the T-zone (Fig. 8). In addition, Ki67 expression was observed in cells of the fibrotic capsule (data not shown).

Vinculin stained endothelial cells including liver sinusoids, smooth muscle cells, bile ducts, connective tissue and HSC. Damaged liver lobules exhibit a higher expression of Vinculin than untreated reference tissue (Fig. 8). Reelin was described to be expressed in quiescent and activated hepatic stellate cells (Kobold et al., 2002). In addition, we found that nerves, bile ducts and partly endothelial cells were stained by Reelin (data not shown). Compared to the R-zone, there are only sparse HSC in damaged liver lobules. Staining for HSC in pig tissue was difficult. GFAP did not stain HSC, but showed a specific binding to nerve fibers (data not shown).

Discussion

This study investigated the effects of RFA in liver tissue over time in a porcine model to better understand pathophysiological changes after thermal injury. We monitored changes in and around the affected area in healthy liver tissue over three months using CT images, large scale histological sections and conventional immunohistochemistry.

Two lesions, one in an inner position bordering the large liver vessels and the other in a peripheral position nearby the liver surface, were investigated. The volume of the lesion was calculated on the basis of CT scans performed immediately after RFA treatment and compared to the volume calculated on the basis of the follow up CT scans at sacrfication date (0 days to 3 months after RFA). We found no differences between central and peripheral lesions regarding their size indicating that in healthy liver tissue the heat sink effect seems to be neglectable.

In the first days after treatment the lesions tended to increase in size and after one week lesions started to shrink. This is in accordance with a human study where a decrease in size of the ablation zone compared to the pre-treated HCC tumours after 24 weeks was described (Morimoto et al., 2002). Another study found a significant increase in the size of the necrosis zone 48 h after laser-induced thermotherapy in patients with liver metastases and a subsequent decrease after three months. Several studies found a reduction in size after different ablation techniques during long-term follow up (Tsuda et al., 2001; Filippone et al., 2007; Fukuda et al., 2011). In contrast, the group of Feliberti reported a nearly equal size of the ablation zone after RFA in liver metastases over time (Feliberti and Wagman, 2006).

This controversy might be caused by the different biological behaviour of different tumours, the exact localisation of the tumour and patient specific factors, such as age. Furthermore the surrounding tissue (healthy or cirrhotic) could have an influence on the progressing development of the lesion.

The observed initial increase in size of the ablated area is most likely due to cell swelling prior to cell death. In a previous in vitro study, we could demonstrate that cells damaged by heat need up to two days to undergo apoptosis (Mayrhofer et al., 2011a,b; O’Neill et al., 2011; Leber et al., 2012). Here we could show apoptosis already after one day. These data are in accordance with a study of Nikfarjam et al. They induced local hyperthermia via laser in CBA strain mice with liver metastases and sacrificed them after different time points. Their study showed apoptosis in liver tissue the following 72 h, peaking after 12 h (Nikfarjam et al., 2005). These findings are therefore important to correctly interpret imaging studies after thermal tumour destruction.

By staining for Hsp70, the heat stress during RFA was monitored. Heat shock proteins are involved in denaturation, folding, activation and transport of other proteins and are synthesized under stressful conditions. Released from the cell, they act as messengers communicating the cells’ interior protein composition to the immune system for initiation of immune responses against intracellular proteins (Jolesch et al., 2011). Moreover, these proteins play a major role in tumour immunogenicity by activating resting antigen-presenting cells to take up and process the tumour antigens and up-regulate the expression of co-stimulatory molecules necessary for T-cell activation (Janetzki et al., 1998; Przepiorka and Srivastava, 1998; Srivastava et al., 1998). Our data supplement the current knowledge that Hsp70 is not found immediately after RFA (Rai et al., 2005). We found the most distinct Hsp70 expression 1-2 days after RFA mainly at the border of T-zone and R-zone, indicating that in this region sub lethal heat has been applied. This leads to cellular stress and enhanced immunogenicity and in turn can lead to cytotoxic cell injury.

Inside the RFA lesion most cells probably die from necrosis but up to two weeks after RFA we also detected apoptosis by Caspase-3 staining, whereas later on Caspase-3 positive cells were scarce and only found inside the fibrotic capsule. Apoptotic cell death was previously shown early after RFA in the T-zone (Vanagas et al., 2009), but this seems to be a tissue specific or species specific effect, since it was not detected after RFA of rat myocardium (Dos Santos et al., 2012).

To complete the picture, we also studied proliferation by Ki67 staining. Ki67 positive cells were detected inside the RFA lesion in the early period (up to one week) thereafter proliferation was mainly found in the fibrotic capsule. This finding most likely reflects tissue repair after RFA in healthy tissue. Since Ki67 has been shown to be a reliable marker for disease progression in hepatocellular carcinoma (Sofocleous et al., 2012), the induction of Ki67 expression in
previously healthy liver tissue has to be kept in mind with caution. Ideally no proliferation should be found inside the RFA lesion because this would indicate potentially viable tumour tissue.

Furthermore, we intended to clarify if the generation of the fibrotic capsule is triggered by HSC or by migrating myofibroblasts. HSC are a highly versatile type of liver cells known to play an important role in liver regeneration. HSC become activated by injured hepatocytes, differentiate to myofibroblasts and produce large amounts of extracellular matrix. Upon healing activated HSC can undergo apoptosis or revert to quiescent HSC (Atzori et al., 2009). The role of HSC after thermal injury of the liver remains unclear. To differentiate between HSC and other myofibroblasts we intended to use GFAP, Vinculin and Reelin. However, only Vinculin and Reelin seemed to detect HSCs on porcine liver tissue.

Contrary to our hypothesis that HSC are responsible for fibrogenesis (Pinzani, 1995), we found decreased numbers of Reelin positive but increased numbers of Vimentin positive HSCs in heat damaged tissue. Reelin is an extracellular matrix protein that has been proposed as a marker to distinguish HSC from myofibroblasts. Although Reelin expression was up-regulated during liver injury and in cirrhosis, Reelin deficient mice recovered completely from injury suggesting either a more distinct role in tissue repair reactions or a case of redundancy through the action of related proteins (Koboldt et al., 2002; Magness et al., 2004; Botella-Lopez et al., 2008). Vimentin is a focal adhesion protein and is expressed in quiescent and activated HSCs but might also be expressed in myofibroblasts (Kawai et al., 2003; Van Rosen et al., 2009). We could not find GFAP positive HSCs in porcine liver tissue. According to literature, in healthy human liver tissue, only few GFAP positive HSCs are detectable, whereas the number increases in chronic diseases such as chronic viral hepatitis (Morini et al., 2005; Carotti et al., 2008). So the reason for not finding any GFAP positive HSCs in our study might lie in the model (porcine tissue) or in the technique (different detection systems).

Not much is known about the role of HSC in thermal injury yet. In a rat model of focal thermal injury and common bile duct ligation HSC seem to play an important role in collagen deposition around the injury (Okayama et al., 2008). From our data we are not able to conclude that HSCs play a major role in repair from thermal injury.

We found both Vimentin and α-SMA positive cells within and outside the fibrotic capsule surrounding RFA lesions. We used both markers to distinguish between subpopulations of myofibroblasts of different origin (Gabbiani, 1996; Knittel et al., 1999). Myofibroblasts were found in the area of the fibrotic capsule. Most prominent Vimentin positive cells were found at the protruding margins of the fibrotic capsule towards the C-zone, whereas α-SMA was found closer towards the inner part of the capsule. Different types of myofibroblasts seem to originate from different cell types (Hinz et al., 2007). In the liver, the most accepted myofibroblast progenitor cells are HSCs, located along each sinusoid, between the centrolobular vein and the portal tract. Expression of α-SMA is a sign of differentiation and results in stronger contractile activity than in α-SMA negative cells (Hinz et al., 2007). Therefore these myofibroblasts might be responsible for the shrinkage of the fibrotic capsule.

Previously performed in vitro studies by our group revealed that reaction of liver cells to heat exposure is dependent on the grade of fibrosis. We were able to show that with increasing grade of fibrosis, liver tissue gets less sensitive to heat exposure. The finding that higher grades of fibrosis require higher temperatures to affect cell viability was visualized by Life/Dead® staining and confirmed by metabolic activity testing in a cell culture model mimicking various grades of fibrosis (Mayrhoouser et al., 2011a,b). This could suggest that further studies in pig models would be of great interest to characterize the role of fibrosis and cirrhosis in RFA outcome.

In conclusion, we could confirm size changes of the RFA lesion over time and found that CAB staining is a reliable method to discriminate between damaged and healthy tissue. By immunohistochemistry we could show heat stress and apoptosis shortly after RFA. Regeneration and increased numbers of myofibroblasts lead to the development of a fibrotic capsule. We could not confirm the role of HSC in this setting. Further experiments are mandatory to better define the role of involved cell types in order to find potential diagnostic and therapeutic targets.

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