Pathological Proof of Cellular Death in Radiofrequency Ablation Therapy and Correlation with Flash Echo Imaging — An Experiment Study —

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Summary: The aims of this study were to clarify the geographic distribution of complete cell death in the radiofrequency ablated area in a porcine liver experiment, and to evaluate the efficacy of ultrasonography using contrast media in detecting the area of Radiofrequency-induced cell death. Radiofrequency ablation was performed at 3 sites in each liver in seven swine with a RF2000TM radiofrequency generator using an expandable type needle electrode. The ablation area was investigated histologically by Hematoxylin-Eosin staining and NADH staining. The area of radiofrequency-induced cell death was correlated to the ultrasonographic findings using contrast media, by means of contrast harmonic imaging, flash echo imaging-subtraction and flash echo imaging-power Doppler. The ablation area showed three distinct regions. Although the HE staining did not indicate necrosis, the NADH staining showed a complete loss of cellular activity in the inner and middle layers of the ablation area. However, in the outer layer cells displaying cellular integrity were intermingled with the necrotic cells, indicating that some of the cells in this layer had a chance to survive. Further, in some cases the outer layer of the ablated area had irregular margins. The flash-echo power-doppler images were accurately correlated in size and shape to the pathologically proved region of complete cell death in the radiofrequency-induced lesions. In the marginal part of the radiofrequency ablation area, cell death was incomplete. Flash echo imaging-power doppler was a useful and sensitive real time imaging technique for accurate evaluation of the region of complete cell death.

Key words: hepatic malignancy, RFA, levovist, flash echo imaging, NADH

INTRODUCTION

Surgical resection is the only proven curative therapy for malignant hepatic tumors. Unfortunately, many patients are not surgical candidates due to multifocal intrahepatic disease or deteriorated liver function with cirrhosis. A number of novel methods for destroying liver tumors without resection have been evaluated; these include percutaneous ethanol injection [1] cryotherapy [2], microwave ablation [3] and radiofrequency ablation (RFA) [4,5]. While each technique continues to have its advocates, RFA has become increasingly popular in recent years due to its ability to ablate large tumors with relative ease and low complication rates. RFA preserves liver function and offers repeatability, and is now expected to achieve satisfactory long-term local control [6,7] as a possible alternative to surgical resection.

However, it is important to be aware that complete destruction of the tumor was not necessarily achieved by RFA even in small Hepatocellular car-
cinoma (HCC) [8], and that there have been some cases of rapid growth of local recurrence after RFA [9,10]. In RFA, a high frequency alternating current (350 to 500 kHz) flows from the uninsulated tip of the electrode into the tissue, producing ionic agitation in the tissue. This agitation results in frictional heating in the tissue around the electrode. The temperature is increased to 80-100°C, and cell death is induced. Recently, the development of multiple electrode probes [11] or probes with electrode cooling systems [12-14] has made possible the destruction of large lesions [15]. However, whether all the tissue in these lesions reaches the same temperature, and whether homogeneous tissue destruction is achieved has not been clarified. Many previous studies tried to elucidate cellular death in the RF ablated area by HE staining, however, cellular necrosis could not be proven histologically because of the tissue fixation effect of high frequency waves [16-19]. Clinicians have therefore not been able to obtain a full understanding of the true extent of tissue destruction in the ablated area. Therefore, investigations of the geometrical distribution of the induced cell death in the RF ablated area are indispensable.

Further, it is also very important to determine the best real-time imaging modality to evaluate the extent of the region of cell death induced by RFA. Although ultrasonography is the dominant image guidance technique for RFA, the accuracy of ultrasonography in depicting the extent of ablated tissue has been controversial. Most clinicians rely on contrast-enhanced CT or MRI to evaluate the size of the ablated area. However, newly introduced ultrasound techniques such as contrast harmonic imaging (CHI), flush echo imaging-subtraction (FEI-S) and flush echo imaging-power Doppler (FEI-PD), which utilize sonographic contrast media, may have the potential to depict tissue and tumor perfusion image sensitively [20-24].

In this study, RF ablated areas were examined histologically by NADH vital staining to geometrically clarify the region of complete induced cell death. Then the ultrasonographic perfusion images from CHI, FEI-S and FEI-PD were compared and correlated to the pathological findings.

SUBJECTS AND METHODS

Seven farm pigs weighing 15-20 kg were used for this study. The animals were handled according to the guidelines of the National Institutes of Health. (Guide for the Care and Use of Laboratory Animals, NIH publication #85-23 revised 1985).

RFA technique

For all procedures, the pigs were sedated with general anesthesia. Introduction was achieved using an intramuscular injection of 5 mg/kg of ketamine hydrochloride, Nomopain (Daichi Pharmaceutical, Tokyo.) and 4.8 mg/kg xylazine, Quinorazin (Daichi Pharmaceutical, Tokyo). The animals were then intubated and given 5 L/min of 0.5%-1.5% halothane. After adequate anesthesia was achieved, the pigs were placed in a supine position.

All animals were laparotomized by abdominal median incision. Under ultrasound guidance (Power Vision 6000, Toshiba, Tokyo), LeVeen Needle Electrodes with a diameter of 2 cm (15G, eight umbrella-shaped electrodes, Boston Scientific. Boston) were inserted into the liver parenchyma away from the liver surface in the right-middle lobe, left-middle lobe, and left-lateral lobe of each animal. Ablation was started after verifying the position of the needle using ultrasound. Current was passed after confirming that the tips of the eight umbrella-shaped electrodes were not protruding from the liver. An RF frequency of 460 kHz and maximum power output of 60 watts was applied using a monopolar RF generator, RF2000 (Boston Scientific, Boston). Each ablation was performed following the manufacturer’s recommended algorithm, which calls for a single 2-phase application. In the first phase, the RF energy was applied beginning at a power level of 30 watts and was increased by 10 watts each minute until a level of 60 watts was reached. The power setting of the generator was maintained at 60 watts until either 15 min. total application time had elapsed or until impedance had risen to over 200 ohms, at which time the power passively decreased to less than 10 watts. After waiting 30 sec, a second phase was started and the application of RF energy continued until the impedance again rose to over 200 ohms or until a total of 10 min had elapsed.

Imaging examination

Contrast media consisting of galactose-based microbubbles, Levovist (Schering AG, Berlin) was administrated by bolus intravenous injection at 300 mg/ml × 0.1 ml/kg of body weight. Before ultrasonographic examination, a 10-minute interval was implemented to allow for the disappearance of gas generated by ablation. In order to improve the enhancement of Levovist, the anesthesia was changed to nitrous oxide off and 0.3 of FiO2 more than 10 min.
before the start of the ultrasonographic examination, because the Levovist microbubbles collapsed under high partial pressures of oxygen and nitrogen in the blood [25]. Using a TOSHIBA Power Vision 6000 (SSA-370A), the RF-ablated area was first examined by fundamental B-mode, and consecutively by harmonic imaging as CHI [22], FEI-S [23], FEI-PD [24]. The transmission frequency was 4.2 MHz in B-mode imaging. In harmonic mode imaging, the transmission and received frequencies were 2.5 MHz and 5.0 MHz, respectively. Pulse rate frequency was basically 4.5 KHz. The low cut filter was set to about 1.3 KHz. The mechanical index value was 0.8-1.2, depending on the focus and transmission frequency. The focus was generally set at one point at the lower margin of the target. CHI was performed for 30 sec in the arterial phase and 90 sec in the portal phase after contrast agent injection. FEI-S and FEI-PD were performed using commercially available hardware for intermittent harmonic mode, digital subtraction function and power Doppler function. Thirty seconds after contrast agent injection, 3-second intervals were used between rapid sequences of one to 5 acquisitions, and 90 sec after injection, 5-second intervals were used between rapid sequences of one to five acquisitions. The order of each harmonic imaging technique was determined according to a table of random numbers in each ablated area, and contrast media was injected for each harmonic imaging examination.

Pathological examination

After the imaging evaluation, the pig was sacrificed and the liver was excised. The ablated area was carefully excised from the liver, making sure to leave the track marking aid in place so as to identify the orientation of the RF probe entry. The liver was cut at the maximum diameter of the ablated region and along the identical scan line of the echo imaging under RFA procedure. Sections for Hematoxylin-Eosin (HE) staining were prepared from the region containing the ablated area and the surrounding tissue, and sections for NADH staining [26,27] were prepared from the opposite region. NADH staining denotes the intracellular mitochondrial viability of cells based on the presence of cell respiration, the concept being that a cell that is not respiring is not viable.

Immediately after preparation, the sections for NADH staining were frozen in optimal cutting temperature (OCT) compound, Tissue-Tek (Sakura Finetek, Tokyo.) using liquid nitrogen, and stored at −80°C. Eight-μm tissue slices were prepared using a cryotome and placed on slide glasses. The incubation medium was consisted of 1ml of 2.5 mg/ml reduced a-NADH (Sigma Aldrich, Tokyo) adjusted with distilled water, 2.5 ml of 2.0 mg/ml nitroblue tetrazolium chloride adjusted with distilled water, 1 ml of physiological saline, and 0.5 ml of Ringer solution. One hundred μl of the incubation medium was dripped on the slide glasses which were then kept at room temperature for 15 min. The slides were washed with distilled water for two min. Cover glasses were placed on the slides after being dipped in water. The slides were subjected to evaluation of cell viability 24 hrs after the operation. Normal liver tissue sections were used as the positive control, and normal liver tissue sections warmed to 100°C in physiological saline were used as the negative control.

Statistical analysis

Statistical analysis of the size differences between each sonographic image and the pathological measurement was performed by Student’s t-test, and p<0.05 was defined as significant.

RESULTS

Twenty-one RF ablated areas from 7 pigs were investigated.

Pathological findings

At gross pathology, the ablated area in all pigs

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Fig. 1. Gross pathology of radiofrequency ablated area immediately after ablation. A sharply demarcated three-layered structure is observed, consisting of an inner dark-brownish region, middle ash-colored region and outer reddish region.
was sharply demarcated and showed three distinct regions (Fig. 1). The outer layer of the ablated area was 3-5 mm wide and reddish, and the middle layer was ash-colored. The inner layer was a dark brownish region, and contained microcysts that were supposed to be formed by the electrodes and bubbles generated during ablation. The mean major and minor axes of the macroscopic ablated area including all three layers were 27.5±2.3 mm and 21.7±4.8 mm, respectively.

The histological examination of HE staining revealed few changes in the hepatocyte nuclei in the inner layer and middle layer (Fig. 2A). The cytoplasm appeared eosinophilic, and the cell membranes were unclear. These findings showed degeneration, but did not elucidate necrosis. In the outer layer, severe congestion of the sinus filled with red blood cells and thinned hepatocytes were observed (Fig. 2B). As in the inner and middle layers, few changes were observed in the hepatocyte nuclei and cytoplasm. In NADH staining (Fig. 3), no cells were stained in the inner and middle layers, proving the absence of cellular activity. The outer layer was stained pale-blue at low magnification, and stained and non-stained cells were intermingled at high magnification, indicating that some of the cells had cellular integrity (Figs 4A and 4B).

Therefore, only the region including the inner and middle layers was recognized as the zone of complete RF destruction. The mean major and minor axes of the lesions including only the inner and middle layers were 26.3±2.1 mm and 20.2±3.8 mm, respectively.

**Imaging findings**

B-mode, CHI, FEI-S and FEI-PD ultrasonographic imagings were examined for all of the ablated areas.

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*Fig. 2.* Microscopic findings of radiofrequency ablated area in HE staining. A, The inner layer. Few changes of hepatocyte nuclei are observed, and the hepatic cord is preserved well. The cytoplasm appears eosinophilic. B, The outer layer. Severe congestion of the sinus is observed. The hepatocyte nuclei and hepatic cord are preserved.

*Fig. 3.* Magnified image of the radiofrequency ablated area in NADH vital staining. The surrounding non-ablated area is stained blue by NADH staining. The central part of the ablated area corresponding to the macroscopic inner and middle layers is not stained. The marginal part corresponding to the macroscopic outer layer is stained pale-blue.
Fig. 4. Microscopic findings of the radiofrequency ablated area in NADH staining. A, Low magnification. B, High magnification. Stained cells and non-stained cells are intermingled in the layer stained pale-blue in the magnified image.

Fig. 5. Ultrasonographic imagings of the radiofrequency ablated area. A, B-mode. Hyperechoic lesion in the center and surrounding hypoechoic lesion are observed. The boundary is indistinct. B, Contrast harmonic imaging. The boundary is distinct compared with B-mode imaging. C, Flash echo-subtraction imaging. The ablated area is depicted as a distinctly homogeneous low echoic area. D, Flash echo-power Doppler imaging. The ablated area is depicted as a non-colored area distinctly demarcated from the surrounding tissue.
In the B mode ultrasonographic examination, a hyperechoic area in the center and a surrounding hypoechoic area were observed, but the boundary between the ablated area and surrounding tissue was unclear (Fig. 5A). The mean maximum diameter of the B-mode-determined ablated area was 25.0±2.7 mm along the major axis. In the CHI ultrasonographic examination, as in the B-mode image, a hyperechoic area in the center and a surrounding hypoechoic area were observed (Fig. 5B). The

Fig. 6. Tomographic scanning of the radiofrequency ablated area by FEI-PD. Sequential multiple plane imaging of the whole ablated area is obtained.

Fig. 7. Size discrepancy between ultrasound-determined ablation area and macroscopic complete-destruction area. The size discrepancy between the ablation area determined by flash echo-subtraction or flash echo-power Doppler and the actual macroscopic area is significantly smaller than that of B-mode.

Fig. 8. Case of radiofrequency ablation adjacent to major vasculature. A, Gross pathology. The outer layer has a wide and irregular form and extends into the central layer. B, Magnified image in NADH staining. The marginal irregular layer stained pale-blue corresponds to the macroscopic outer layer. C, Flash echo-power Doppler. FEI-PD findings showed a narrow and irregularly shaped non-perfusion area, reflecting the irregular shape of the inner and middle layers.
boundary between the area and the surrounding tissue was generally clearer than that in the B-mode image, however, in 9 ablated areas the boundary was indistinct. The mean maximum diameter of the ablated area determined by CHI was 25.5±2.0 mm. In FEI-S findings, the non-perfusion area, corresponding to the ablation area, was imaged as a homogeneously hypoechoic area that was clearly distinguished from the surrounding tissue (Fig. 5C). However, due to the cardiac pulse the subtraction image was not always stable. The mean maximum diameter of the non-perfusion area measured by FEI-S was 26.1±2.0 mm. In FEI-PD findings, the non-perfusion area corresponding to the ablation area was clearly distinguished from the surrounding tissue in all cases (Fig. 5D). The mean maximum diameter of the non-perfusion area measured by FEI-PD was 26.2±1.9 mm. In FEI-PD, unlike FEI-S, sequential multiple plane imaging of the entire ablated area were obtained as shown in Fig. 6, because the enhanced effect for FEI-PD examination lasted more than 5 min.

Differences in the maximum diameter of the major axis between ultrasonographic imaging values and macroscopic values including measurements of only the inner and middle layers, which represent the extent of complete RF destruction, are shown in Fig. 7. Differences from the macroscopic measured value varied in B-mode and CHI images, but there was little variation in the differences in the FEI-S and FEI-PD images. The values measured in FEI-PD images correlated particularly well with the macroscopic values. The values obtained by FEI-S and FEI-PD correlated significantly with the macroscopic value, compared to the values obtained by B-mode.

**Influence of major vasculature on RF ablation**

In cases where radiofrequency ablation was performed in a location including a large blood vessel, the macroscopic three-layer structure was not regularly in shape. The outer layer was irregularly wide and extended to the center (Fig. 8A). The pale-blue NADH-stained layer, showing incomplete destruction of the cells, corresponded to the irregular outer layer (Fig. 8B). In these cases, FEI-PD showed a narrow and irregularly shaped non-perfusion area, reflecting the irregular shape of the inner and middle layers (Fig. 8C).

**DISCUSSIONS**

In spite of the popular clinical use of RFA for hepatic tumors, histological investigation of cell viability in the ablated area has not been sufficient. Goldberg et al. [27] and Scudamore et al. [28] resected the ablated area of the liver after RFA in patients with liver tumor, and observed tissue damage after RFA by HE staining. They reported that cell death could not be confirmed conclusively. In order to assess the viability of cells, the HE staining technique relies on visual examination of the condition of cell membranes and structures. In RF ablation, because of the tissue fixation action of high frequency waves [15,16,29], the hepatic cord seemed to remain intact and cells containing preserved nuclei were observed from the central zone to the peripheral zone of the ablated area in HE staining. Therefore, cells in the ablated area were not considered nonviable. In many of the previous reports concerning RFA, the term “coagulation necrosis” was optimistically used to denote irreversible thermal damage to cells, despite the fact that the ultimate manifestations of cell death did not fulfill the strict histological criteria of coagulation necrosis. Kuromatsu et al. [17] investigated the changes of RF ablated areas by electron microscopy. They demonstrated that destruction of various organelles, such as mitochondria in the cytoplasm, showed cellular death of the ablated area. However, the geometric distribution of complete cell death in the ablated area could not be clarified accurately by electron microscopic examination. Therefore, it was not clear whether or not homogeneous cell death occurred in the entire ablated area. In the present study NADH staining was used to elucidate cell viability. NADH staining denotes intra-cellular mitochondrial viability based upon the presence of cell respiration (metabolism). Only viable cells show active diaphorase, and hence a blue coloration, and this activity ends almost immediately following RF-induced cell death [25]. Therefore, a very clear, visual sequestering of living and dead cells is possible, providing an accurate delineation of the extent of tissue necrosis.

The RF ablated area showed a three-layer structure macroscopically. The present study demonstrated that complete cell death with a complete loss of cellular integrity occurred in the cells in the central part corresponding to the macroscopic inner and middle layers. On the contrast, the marginal part corresponding to the macroscopic outer layer showed pale-blue in NADH staining, indicating an intermingling of stained and non-stained cells. According to these findings, we concluded that the region of complete cell death consisted only of the inner and
middle layers of the RF ablated area, and that some of the cells in the outer layer had a chance to survive. It was noteworthy that the outer layer, which was stained pale-blue by NADH staining, extended irregularly and widely into the central layer in cases of ablation adjacent to major vascular structures, as shown in Fig. 6. This finding might be due to a heat sink effect caused by blood flow during RF ablation. The hypervascularity reduces the RF-induced thermal effect by mediating tissue cooling, and alters heat conduction within the treatment zone [30]. In a clinical setting, the irregular distribution of cell death in the margins of the ablated area represents a risk of potential therapeutic failure, as early clinical studies suggest that tumor ablation often failed at the margins [31]. Clinicians can misjudge the region of cell death if they assume that the region of complete cell death is regular and can be predicted by the size of electrode. When real time imaging is able to accurately indicate a potentially viable area within the ablated lesion, immediate additional ablation can be performed to achieve successful RFA.

Although ultrasonography represents real time imaging, most studies showed that conventional ultrasonography, such as gray-scale, color doppler, and power doppler sonography, correlated poorly with the extent of RF-induced necrosis [32-34]. However, recently introduced sonographic contrast media is reported to provide better perfusion images for assessment of tissue vascularity than does conventional ultrasonography [35]. In this study, several ultrasonographic techniques were examined. The size of ablation area determined by each imaging technique was compared to the pathological size of the actual destructive lesion, which included only the macroscopic inner and middle layers. In B-mode examination, the border of the ablated area was unclear, and the imaged size differed from the size of the pathological ablated area. This result was similar to those in previous studies. Although obtained using contrast media, CHI images also did not correlate well with the pathological size of the ablated lesion. In CHI, the border between the enhanced surrounding tissue and non-enhanced ablated area was unclear in some cases, as the contrast effect was influenced by echogeneity of the original tissue and residual gas formation. In B-mode and CHI examinations, the size determined as the ablation area was generally larger than the size of the macroscopic inner and middle layers. These imaging methods probably pick up the change of echogeneity of the congestive outer layer and interpret it as an ablated area, in spite of the tissue perfusion. On the other hand, the FEI-PD imaging accurately correlated with the pathologic size of the inner and middle layers, where complete cell death was induced. Also, the irregular shape of the inner and middle layer correlated to that of the non-perfusion area in FEI-PD. Tissue perfusion may be a reliable sign of the existence of viable cells, and FEI-PD sensitively reflected the viable cells in the outer layer. Tissue perfusion in the outer layer should be reduced, however, contrast media accumulated more than in the intact tissues because of congestion. Therefore, ultrasonographic perfusion image was thought to be sensitively depicted tissue perfusion of the outer layer. Cioni et al. [31] reported the efficacy of harmonic power doppler sonography without flush echo mode in evaluating the RF ablated area. However, harmonic power Doppler sonography without flush echo mode detected only enhanced flow of the major vessels, not tissue perfusion. Theoretically, flush echo imaging depicts tissue perfusion more sensitively, by destroying or disrupting the microbubbles of contrast media that accumulated in the perfusion tissues during the interval of the flush echo pulse. Furthermore, in FEI-PD the perfusion images could be carefully observed in sequential multiple planes in the entire ablated region, because the enhanced effect lasted more than 5 min. The FEI-S technique allows the acquisition of several rapid sequences at one trigger with high acoustic power and stores every image of this sequence for later review. Consequently, digital subtraction of the contrast harmonic B-mode image is automatically obtained by subtracting the last-frame image from the first-frame image. This technique was reported to be sensitive for detecting tissue perfusion [22]. In the present study, however, FEI-S sometimes produced a failed image because of the influence of breathing and heart-beat. FEI-PD is not a high-end function nowadays, and it can be utilized even in standard models of sonographic machines. The gas formation generated during ablation showed a diffuse strong echoic lesion, and disturbed the ultrasonographic observation immediately after RFA. However, most of the gas disappeared within 10 minutes after irradiation, and thereafter adequate FEI-PD images were obtained.

Although dynamic CT or MRI can evaluate the ablation area accurately [5,13,18], these technologies are expensive and are inconvenient for real-time monitoring of RFA. Ultrasonography offers some obvious benefits over other imaging modalities such as low cost, easy repetition, and real-time guidance.
for therapy [19]. We concluded that FEI-PD was a simple, reliable and useful imaging modality for evaluation of the complete destructive region of RFA, and might be applied to other local treatments for evaluation of therapeutic effectiveness.

In the present study we used Levovist as the contrast media. However, Levovist, a galactose-based microbubble contrast media, is unstable and breaks up easily in the circulation. It collapses readily in the presence of high concentrations of oxygen and nitrogen in the blood, and it was necessary for us to give the animals only room air for 10 min before our examination. As Levovist is the only commercially available contrast media in Japan, we used this media in the present study. We look forward to the development of next generation contrast media such as Optison and Definity which have the potential to provide much improved tissue perfusion images.

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