In vivo real time imaging of the liver with confocal endomicroscopy permits visualization of the temporospatial patterns of hepatocyte apoptosis

Running head: In vivo imaging of hepatocyte apoptosis

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Abbreviations  FITC, Fluorescein isothiocyanate; FOV, field of view; TUNEL, TdT-mediated dUTP nick end labelling

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ABSTRACT

Background: Apoptosis is a dynamic process of programmed cell death and involved in multiple diseases. However, its mechanisms and sequence of events are still incompletely understood, partly because of the inability to visualize single cells continuously in vivo. The aim of the present study was to monitor hepatocyte apoptosis with confocal endomicroscopy in living rodents.

Methods: In 73 anaesthetized mice, apoptotic liver injury was induced by injection of the CD95-agonistic antibody Jo2. Individual hepatocytes were followed for up to 240 min with a handheld confocal probe (FIVE1, Optiscan, Australia) providing 0.7 µm resolution (1000-fold magnification). Different fluorescence staining protocols were used for cellular staining, vascular and cellular barrier function imaging, and caspase activation visualization.

Results: The time course of apoptosis could be visualized in vivo while liver perfusion and tissue integrity were maintained. In contrast to most ex vivo studies, initial cell swelling was observed that coincided with early defects in barrier function of sinusoids and hepatocytes. Cytoplasmic vesicle formation, nuclear condensation, cellular disintegration and macrophage infiltration were captured sequentially. Labeling of caspases allowed molecular imaging.

Conclusions: Our study allowed for the first time to continuously follow distinct morphological, functional and molecular features of apoptosis in a solid organ in vivo and at high resolution. Intravital confocal microscopy may be a valuable tool to study the effects of therapeutic intervention on apoptosis in animal models and humans.
INTRODUCTION

Apoptosis is a tightly regulated mechanism of organized self-disassembly of cells to maintain tissue homeostasis. Imbalance of this delicate process has been identified as a major contributor to initiation and aggravation of many liver diseases (12, 36) such as viral hepatitis (31, 43), non-alcoholic steatohepatitis (8), acute liver failure (42), and hepatocellular carcinoma (40). Modulation of apoptosis signaling can be beneficial in the treatment of these diseases. To understand the complexity of apoptosis, imaging of the cellular alterations has been subject to intensive research ever since the first description of apoptosis in ischemic rat livers (23). Morphologic hallmarks associated with apoptosis are nuclear condensation, plasma membrane blebbing, and cell shrinkage and fragmentation (24). Apoptotic bodies are subsequently eliminated without provoking an inflammatory response. Apoptosis is a dynamic process. However, much of our knowledge of the morphology of apoptosis has been gained ex vivo on fixed samples, subject to processing artifacts and yielding static information rather than continuous imaging, or from cell culture which can only partially reflect the biologic complexity of apoptosis in the intact, multicellular organism.

Intravital visualization of apoptosis has been limited by the lack of appropriate reporter and imaging systems in animal models and patients (2). Recently, miniaturized confocal microscopy has allowed in vivo imaging in animal models (14) and humans (13, 25) with subcellular resolution. With this novel approach, many of the features of bench top confocal microscopy are available for examination in live animals, including high resolution subsurface imaging with different intravital dyes (14), monitoring of dynamic events such as blood flow (16) and cell shedding (26), bacterial translocation (33) and even molecular imaging (18). A major advantage is the ability to maintain tissue integrity during imaging, thereby being able to visualize tissue in its natural surrounding almost free of artifacts. The aim of the current study was to continuously visualize the sequence of morphologic features of hepatocyte apoptosis with confocal microscopy in live rodents.
MATERIALS AND METHODS

Apoptosis model

Apoptosis was induced by injection of the FAS ligand Jo2 (0.1 μg/g, (BD Pharmingen, Heidelberg, Germany) in C57BL/6 mice (20–30 g, > 6 wks). Animals were bred and kept at the Animal Facility of the University of Mainz in a temperature-controlled environment on a 12 h light-dark cycle and were fed regular pelleted rodent maintenance diet and water ad libitum before experiments started. Of 73 animals, 10 died before imaging was finished. 49 treated and 14 untreated control mice were evaluated. Animals were euthanized at the end of the experiment by a ketamine-xylazine overdose. Examination in the first five animals started 120 min after injection of Jo2 until up to 365 min to establish morphologic criteria of late apoptosis. For the next 58 animals, examination started immediately after injection and was limited to 240 min. The study protocol was approved by the local review board (TVA 23 177-07/G07-1-028).

Confocal laser microscopy and staining protocols

The FIVE1 system (Optiscan, Melbourne, Australia) with a rigid confocal probe delivered an excitation wavelength of 488 nm, light emission was detected at 505-585 nm. Serial en face optical sections of 475x475 μm were obtained with a lateral resolution of 0.7 μm (1024x1024 pixels) at adjustable imaging depth, as previously described in detail (14, 15, 26). Laser power was adapted from 300-600 μW (FLIVO: 1000 μW) to obtain appropriate tissue contrast.

15 μl 0.01% Acriflavine (Fluka, Sigma Aldrich, Steinheim, Germany) were injected i.v. in 18 Jo2 treated animals and 4 controls. Fluorescein isothiocyanate (FITC)-labeled dextran (Fluka, Sigma-Aldrich, Steinheim, Germany) was injected at 10 mg/ml (w/v) in distilled. MW of the dextran was 4 kD (n=5 Jo2, n=2 controls), 70 kD (n=3 Jo2, n=3 controls), and 150 kD (n=5 controls).
Jo2, n=3 controls). 3 animals were administered Jo2, acriflavine and FITC-dextrans simultaneously. FLIVO™ (ImmunoChemistryTechnologies, LLC Bloomington, USA) was injected at 5 µl/g simultaneously with Jo2 in n=5, 105 min after Jo2 in n=5, without Jo2 (controls) in n=3 mice. In case of fading contrast during observation, contrast agents were re-administered.

**Surgical and imaging protocol**

Mice were deeply anesthetized using ketamine-xylazine (120mg/kg and 16mg/kg i.p., resp.). A 26 gauge intravenous line (prefilled with 0.9% saline; BD Biosciences, Heidelberg, Germany) was inserted into the tail vein. Median laparotomy was performed with careful cauterization of bleeding sites. Exposed tissue was kept moist and animals were kept warm throughout the examination. Initially, the liver was briefly screened with confocal microscopy. The probe was then mounted onto a stereotactic frame and put on the liver surface with gentle pressure. To overcome movement artifacts by breathing, liver and probe were stably aligned by a self-constructed wire frame with a perforated dressing to accommodate the confocal window.

To minimize i.v. volume load, Jo2 and contrast agents were injected simultaneously. Imaging was started immediately to capture the early events of apoptosis. Within the first 60 min, 5-10 confocal images were recorded every 5 min, then every 10 min. Imaging plane depth was changed by 4 µm increments within superficial sections during such series. To prevent photobleaching, laser power was reduced to 0 µW, if imaging was paused. A total of 150 – 250 images were digitally recorded per session.

**Ex vivo correlation**

At the end of the examination, liver specimens were obtained, fixed in 4% buffered formalin and embedded in paraffin. Since initial confocal screening did not show marked differences in
the distribution of apoptotic hepatocytes among the liver lobes, specimens were not specifically targeted or oriented. Serial sections of 4µm were stained with Hematoxylin&Eosin (H&E). Additional specimens were snap-frozen in liquid nitrogen and stored at -80°. TdT-mediated dUTP nick end labelling (TUNEL) was performed on 5µm sections with the In Situ Cell Death Detection Kit for fluorescein (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Positive controls were deoxyribonuclease I-treated, negative controls without enzyme solution. After 1 hr incubation, samples were washed in PBS and incubated with converter AP (anti-fluorescein, Roche) for 30 min at 37°C. Fast Red (Roche, Mannheim, Germany) was used as a substrate, counterstaining was performed with haemalum for light microscopy (Olympus BX 41).

Statistics
Statistical analysis was performed using the software package GraphPad Prism (v5.00; La Jolla, CA, USA). All p values were generated using 2-sided tests. For quantitative analysis of frequencies in the acriflavine groups and of vascular permeability in the dextran groups, unpaired Mann-Whitney rank sum tests were used with 95% confidence intervals. P values of \( \leq 0.05 \) were considered statistically significant.

RESULTS
Morphology of apoptosis and its time course
Cellular changes during the course of apoptosis were evaluated with acriflavine. After establishing the morphology of later stages of apoptosis in the first five animals, 19 individual cells were selected from subsequent six animals that were imaged for three to four hours, starting immediately after apoptosis induction. To optimize generalizability, only such cells were chosen that did not show any sign of apoptosis at the beginning of the observation
Goetz M et al., In vivo imaging of hepatocyte apoptosis

period, but definite signs during the course of monitoring. The time where those cells were still hexagonal and did not show changes in their fluorescence was defined as the starting point (t=0). Initial changes were evident five to 55 min after apoptosis induction. Changes of cell shape, fluorescence intensity, cytoplasmatic texture, and the nucleus are shown in Fig. 1, and the time course of these alterations is depicted in Fig. 2. First morphological changes included the formation of vesicles in close proximity to the cell membrane (Fig. 1a). Early contact of such cells with mononuclear cells was also noted early in the course of apoptosis (Fig. 4, see below). Budding of vesicles from the cell membrane, their enlargement and transition to cytoplasmic vesicles was visualized, but loss of fluorescence (an early event in apoptotic cells, yet also observed in some non-apoptotic cells) hampered the further visualization of these events (Fig. 1c). First morphological changes of the cell bodies were swelling combined with rounding and loss of hexagonal shape (16 of 19 cells, Fig. 1-3). Swelling prevailed for approx. 15 min before the onset of cell shrinkage. First changes in nuclear morphology were the loss of clear visibility of the nuclear membrane after 25 min. Nuclear blebbing was found after 55 min (Fig. 1g), and pyknosis after 135 min (Fig. 1i). Chromatin condensation resulted in brightly stained nuclei (Fig. 1 g,i,k). After reaching the state of pyknosis, cellular morphology remained stable. Individual hepatocytes undergoing apoptosis are shown in Fig. 3 a-l and Supplementary Video 1.

**Macrophages in apoptosis**

Contact between macrophages and hepatocytes was found already in the early phases of apoptosis. In some instances, the high resolution of confocal microscopy even permitted the visualization of macrophage pseudopods contacting apoptotic material of hepatocytes (Fig. 4). Macrophage movement was serially recorded every 10 min, and video sequences can be found as Supplementary Video 2. Engulfment of apoptotic bodies by adjacent hepatocytes
was only rarely observed (Fig. 1g, left upper corner). These hepatocytes showed bright cytoplasm and intracytoplasmic vesicles but no other signs of apoptosis. Engulfment in macrophages was not observed.

**Quantification of apoptotic changes**

Loss of fluorescence, blebbing, vesicle formation and loss of the nuclear membrane were quantified in 10 animals stained with acriflavine from 20 min to 140 min within a field of view (FOV) of 240x240µm, corresponding to 170 to 200 cells per FOV. Medians were calculated for the time points 30 min and 140 min. Since not all animals showed all signs of apoptosis at the same time, data from 4 to 10 animals are included per criterion. At 20 min, only 5 apoptotic cells were identified per FOV. Every 20 to 30 min, this number increased by approximately 5 additional apoptotic cells. Thus, apoptotic cells doubled after 50 min, quadrupled after 90 min and were six-fold elevated after 140 min.

**Specificity of observations**

To rule out that the above described morphological changes occurred as a result of anesthesia, surgical procedure, acriflavine staining protocol or confocal microscopy, n=4 mice were examined without Jo2 injection. The number of dark cells with low fluorescence intensity was not significantly different in the controls. While all apoptotic cells were dark initially, not all dark cells eventually underwent apoptosis. To rule out tissue acidification (a low pH can quench fluorescence) intact liver slices were incubated ex vivo in phosphate buffered saline with pH of 7.0, 7.4, and 8.0. After topical staining with acriflavine, the number of dark hepatocytes was unchanged.

When relative frequencies of signs of apoptosis in dark cells after apoptosis induction were calculated at 80 min, 83% [±16 SD] of these cells showed blebbing and 73±27% showed loss
of nuclear membrane integrity, whereas in untreated controls only 12±9% and 6±4% of dark hepatocytes displayed these signs of apoptosis (p<0.006 and p<0.002, resp.). Furthermore, these changes were stable and did not show signs of progression. Hepatocyte swelling, shrinkage, loss of integrity of cellular and nuclear membranes or nuclear alterations were not observed in the control group.

**Imaging of vascular and cellular barrier function**

To quantify vascular and cellular permeability during apoptosis, FITC-labeled dextrans of different size were injected together with Jo2 antibody. Labeled cells were counted per 475x475 µm FOV and numbers compared to controls. While no difference was found for 4 kD with staining of multiple hepatocytes in controls and induced animals, 70 kD and 150 kD dextrans labeled significantly more hepatocytes in the mice with apoptosis induction than in the controls (15.3 ±5.2 vs. 0.7 ±0.7 for 70 kD; 19.4 ±10.9 vs. 1.7 ±1.5 for 150 kD, Fig. 5). Stained cells were often grouped together in the periphery of the liver lobule, and nuclei visible as darker spots in the brightly stained cytoplasm. Cell counts remained relatively stable over the observation period.

To examine whether enhanced leakage coincided with morphological signs of apoptosis, double staining with acriflavine and FITC dextrans (150 kD) was performed in three animals. A complete evaluation was not possible due to the overlay of low fluorescence for apoptotic cells stained with acriflavine and simultaneous bright staining with FITC dextrans. Although the lobular distribution was different (diffuse with acriflavine, peripheral with FITC dextrans), some cells showed both clear morphological signs of apoptosis and functional compromise of barrier function (Fig. 5d).
**Labeling of caspases**

Activated caspases were targeted with FLIVO. While simultaneous injection of FLIVO and Jo2 did not result in staining of apoptotic cells, since binding of FLIVO inactivates caspases and thus inhibits apoptosis. When FLIVO was injected 105 min after Jo2, groups of cells showed nuclear and weak cytoplasmic staining that was not found in controls or the simultaneously injected group. In apoptotic cells molecular staining of activated caspases co-localized with typical morphological signs of apoptosis (Fig. 6 a,b). Both controls and induced cells showed cytoplasmic inclusions of strong fluorescence that are likely due to particulate material in the solution and/or unspecific pinocytosis by hepatocytes. FLIVO labeled cells appeared to be more frequent than hepatocytes with morphological signs of apoptosis, most probably due to the fact that activation of caspases is an earlier event than subsequent morphological alterations.

**Ex vivo microscopy**

The presence of apoptotic hepatocytes was confirmed in all animals. Using H&E staining, apoptotic hepatocytes were identified in high numbers throughout the liver tissue Similar to in vivo findings, TUNEL assay identified multiple apoptotic cells with homogenous distribution within the parenchyma, in contrast to non-Jo2-induced animals (Fig. 6 c-f).

**DISCUSSION**

In the present study, first evidence is provided that in vivo confocal microscopy can be used to continuously follow apoptosis with subcellular resolution in intact liver tissue of live animals for up to four hours. Our current novel approach differs in several ways from previous reports (4, 7, 10, 30, 35, 37): Rather than combining multiple snap shots of different cells from culture or biopsies we now continuously followed individual cells within their multicellular context. Associated mediator profiles were preserved in our study reflecting the natural environment for
apoptosis. Clinical observation during the examination, lack of necrosis and examination in control animals suggest that liver perfusion and tissue integrity were maintained throughout the examination. Different staining protocols allowed evaluation of morphological, functional and molecular events in vivo at high resolution and enabled us to set a time frame for apoptosis based on continuous observation of individual hepatocytes. To our knowledge, such an intravital follow-up has not been reported so far. Apoptosis has been observed in cell culture with computerized video time-lapse microscopy (10) or real-time fluorescence microscopy (30), or in vivo by ophthalmoscopy after annexin V-labeling (37), but not in conjunction with high resolution microscopy.

Intravital imaging of apoptosis offers significant advantages over ex vivo visualization. It is less prone to artifacts by tissue processing which are known to potentially interfere with morphological hallmarks of apoptosis, such as invasive sampling, dehydration, sectioning and staining. TUNEL staining - although apparently simple - may incur similar limitations (11, 19, 29, 34, 41). In our study cell membrane blebbing started as early as five to 10 minutes after induction of apoptosis, and first changes in nuclear morphology were recorded after 25 minutes. Pelling et al. found changes in cell culture within a similar time frame when using simultaneous confocal and atomic force microscopy, with depolymerization of the cytoskeleton beginning 10 min and nuclear compression 25 min after apoptosis induction (35). In our model, most cells eventually undergoing apoptosis showed early transitional swelling after approximately 15 min which was only discussed in a minority of ex vivo studies (4, 7). This was followed by a cell volume decrease after approximately 40 min. Similarly, an initial burst of membrane blebbing was followed by cell swelling for up to four hours observed by computerized video time-lapse microscopy after radiation of lymphoid cells (7). Swelling of cells may be particularly delicate to capture in processed dehydrated tissues and dependent on the complex tissue context as mentioned above. Furthermore, hepatocyte swelling was only transient and thus may have escaped detection in ex vivo studies.

Our FITC-dextran-studies suggest that larger molecules can leak from sinusoids into the hepatocyte cytoplasm early in the course of apoptosis. This is of specific interest in light of
recent discussions on the intimate connection of hepatocellular apoptosis and an endothelial barrier defect (22). Together with our previous findings that specific ablation of hepatocellular apoptosis protects from endothelial leakage and parenchymal bleeding this supports the notion that hepatocyte apoptosis drives sinusoidal leakage (39). This finding coincided with initial swelling of hepatocytes.

The darkening of cells that eventually undergo apoptosis is not entirely clear. We observed a darkened cytoplasm also in healthy hepatocytes. A photobleaching effect cannot explain this phenomenon, since it would not be limited to single cells. Condensation of ribosomes has been found in apoptotic cells (32) and may explain for altered fluorescence intensity, however such details could not be resolved in vivo. An altered pH has been described in cells undergoing apoptosis (6), and fluorescent agents such as fluorescein are known to show a pH-dependent fluorescence intensity (27). However, variation of pH from 7 to 8 in intact liver slices did not result in altered fluorescence, and not all cells becoming dark underwent apoptosis, although apoptosis was always preceded by darkening. Alternatively, mitosis may induce changes in the fluorescence intensity, however can be differentiated morphologically (14).

Interaction of mononuclear cells, likely Kupffer cells, with apoptotic cells could be visualized dynamically early in the course of apoptosis. The starting point of this interaction is difficult to define based on morphology alone, since normal liver tissue contains numerous resident macrophages (Kupffer cells) that can interact with healthy hepatocytes (38). However, our finding of frequent early cellular interactions is in accord with a recent report suggesting that cells starting to undergo apoptosis are rapidly cleared by macrophages, independent of local production of cytokines (28). This would explain our inability to see neutrophil migration to sites of apoptosis, as opposed to previous studies on necrosis (17).

Confocal microscopy also permits molecular imaging in vivo (9, 18, 21). We could confirm its utility for apoptosis by imaging caspase activation with a fluorescein-labeled poly-caspase inhibitor (FLIVO). Since FLIVO may interfere with the apoptotic cascade (5, 20), novel reporter molecules may prove more useful in resolving the spatio-temporal pattern of caspase activation by fluorescent imaging (1). In addition, other molecules of interest can serve as targets for in
vivo confocal molecular imaging, provided that its expression is high enough to allow detection with currently available devices.

Our approach, although allowing dynamic microscopic visualization, has some shortcomings. First, apoptotic cells cannot be identified prospectively, necessitating a large field of view in order to capture early events. Furthermore, the probe has to be positioned on healthy tissue prior to apoptosis induction. Although we initially quickly screened the liver, we cannot exclude that fine details could have been missed at sites distant from the imaging site. This may give some inaccuracy to the suggested time frame. Second, imaging was confined to superficial areas of the liver. Third, other than in bench top laser scanning confocal microscopy our current instrument settings do not allow to examine different wave lengths. At present this poses limitations for multi-staining.

Our findings can contribute to translational research in several ways. Intravital confocal microscopy can be used for sequential imaging in the same animal in survival experiments, such as in acute liver failure and its regeneration. The visualization of the effect of e.g. cancer therapeutics may improve our understanding for the complex cascade involved in cell death after chemotherapy and/or radiation. Imaging of cell death and regeneration has already been performed in animal models of human diseases with confocal endomicroscopy (14, 17). In humans, apoptosis has been visualized in intestinal epithelial homeostasis (26) and in graft-vs.-host-disease after bone marrow transplantation with confocal endomicroscopy (3). However, none of these studies followed cellular events over several hours.

In summary, we demonstrate that apoptosis of individual cells, with its morphological, functional and molecular changes can be visualized in the intact liver of living animals over several hours. Such in vivo microscopy allows identification of distinct features of apoptosis that are not uniformly described in ex vivo studies and may represent a promising tool for future studies of apoptosis and its pharmacological manipulation in experimental animals and humans.
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REFERENCES


FIGURE LEGENDS

**Fig. 1:** Morphologic changes during apoptosis: Alterations of cell shape and fluorescence intensity: a, swelling and peripheral vesicles (arrows); b, shrinkage (arrows); c, reduced fluorescence. Alterations of cytoplasm: d, blebbing at cell membrane; e, formation of intracellular vesicles (arrows); f, loss of identifiable cell boundaries and disassembly of cytoplasmatic architecture (arrows). Nuclear changes: g, blebs; h, karyorrhexis (arrows); i, pyknosis (arrow). Edge length 50 µm.

**Fig. 2:** Time course of apoptotic changes of 19 individual hepatocytes as box plots (median, 25% and 75% quartiles): Whiskers identify maximum and minimum, + means. t=0 min was defined as the first visible changes in cell morphology.

**Fig. 3:** In vivo imaging of apoptosis: Three cells (arrows) show initial signs compatible with early apoptosis. These cells were followed for 180 minutes from apoptosis induction. The two cells on the right show initial swelling and undergo karyorrhexis, whereas the upper left cell still only shows intercellular blebs (animated in Supplementary Figure 1).

**Fig. 4:** Macrophages in apoptosis: a. Three dark apoptotic hepatocytes (with loss of clearly defined cell boundaries, blebbing, and shrinkage) are surrounded by three macrophages. One of these cells (M) extends a pseudopod towards the cytoplasm of a hepatocyte (arrow). b-g. Mononuclear cells in contact with apoptotic hepatocytes are resident (asterisks in b) or migrate within apoptotic areas (arrows in b-g). Images were captured every 10 minutes. An animated version can be found as Supplementary Figure 2.

**Fig. 5:** Barrier function in apoptosis: a,b: In controls, 150kD-FITC dextrans were retained within the vasculature and exclusively stained the sinusoids (a). Black dots correspond to red
blood cells. After apoptosis induction (b), localized extravasation visualized the cytoplasm of single hepatocytes (inset, arrows). Stained cells were more frequent for both 150 kD and 70kD dextran in the apoptosis group vs. controls, whereas no difference was found for 4 kD dextran (c). Double staining with acriflavine and FITC-dextrans: 150 kD dextran co-localized with apoptotic hepatocytes after 105 min (arrows, d). Edge length 475 µm.

Fig. 6: Molecular staining of apoptosis: Labeling of activated caspases in untreated controls (a) and mice after Jo-2 injection; (b) shows unspecific pinocytosis of contrast agent and biliary excretion labeling the canaliculi. In apoptotic cells, loss of integrity of cellular membranes (red arrows) and nuclear fragmentation (white arrows) co-localize with staining of caspases by FLIVO. Many cells show signs of apoptosis 105 min after induction. Scale bars 100 µm. Ex vivo correlation from tissue specimens at the end of the experiment: H&E staining (c) identified multiple apoptotic hepatocytes (arrows) in different stages of apoptosis. TUNEL staining from the same specimen (d) confirmed DNA strand breaks. In a control mouse, no apoptotic changes can be visualized (e, H&E; f, TUNEL). Original magnification 400x.

Suppl. Fig. 1: Hepatocytes undergoing apoptosis, time course from 15 min to 180 min (video rendering).

Suppl. Fig. 2: Macrophage movement in hepatocyte apoptosis from 20 min to 220 min (video rendering).