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

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Goetz M, Ansems JV, Galle PR, Schuchmann M, Kiesslich R. In vivo real-time imaging of the liver with confocal endomicroscopy permits visualization of the temporospatial patterns of hepatocyte apoptosis. Am J Physiol Gastrointest Liver Physiol 301: G764–G772, 2011. First published July 21, 2011; doi:10.1152/ajpgi.00175.2011.— Apoptosis is a dynamic process of programmed cell death and is 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. In 73 anesthetized 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) providing 0.7 μm resolution (1,000-fold magnification). Different fluorescence staining protocols were used for cellular staining, vascular and cellular barrier function imaging, and caspase activation visualization. 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. 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.

apoptosis; confocal endomicroscopy; confocal microscopy; macrophages; hepatocytes

APOTOTIS 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), nonalcoholic 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). Morphological 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 biological 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 benchtop 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 morphological 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 wk)]. Animals were bred and kept at the Animal Facility of the University of Mainz in a temperature-controlled environment on a 12:12-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. Forty-nine treated and 14 untreated control mice were evaluated. Animals were killed 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 morphological 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, and light emission was detected at 505–585 nm. Serial en face optical sections of 475 × 475 μm were obtained with a lateral resolution of 0.7 μm (1024 × 1024 pixels) at adjustable imaging depth, as previously described in detail (14, 15, 26). Laser power was adapted from 300 to 600 μW [fluores-
Fifteen microliters of 0.01% Acriflavine (Fluka, Sigma-Aldrich, Steinheim, Germany) were injected intravenously in 18 Jo2-treated animals and 4 controls. Fluorescein isothiocyanate (FITC)-labeled dextran (Fluka, Sigma-Aldrich) was injected at 10 mg/ml (wt/vol) in distilled water. The molecular weight of the dextran was 4 kDa (n = 5 Jo2, n = 2 controls), 70 kDa (n = 3 Jo2, n = 3 controls), and 150 kDa (n = 5 Jo2, n = 3 controls). Three animals were administered Jo2, acriflavine, and FITC-dextran simultaneously. FLIVO (ImmunoChemistryTechnologies) was injected at 5 μl/g simultaneously with Jo2 in n = 5, 105 min after Jo2 in n = 5, and without Jo2 (controls) in n = 3 mice. In case of fading contrast during observation, contrast agents were readministered.

Surgical and imaging protocol. Mice were deeply anesthetized using ketamine-xylazine (120 and 16 mg/kg ip, respectively). A 26-gauge intravenous line (prefilled with 0.9% saline; BD Biosciences, Heidelberg, Germany) was inserted in 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 on 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 intravenous 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 and 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. Because 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 4 μm were stained with hematoxylin and eosin (H&E). Additional specimens were snap-frozen in liquid nitrogen and stored at −80°C. TdT-mediated dUTP nick end labeling (TUNEL) was performed on 5-μm 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-h incubation, samples were washed in PBS and incubated with converter AP (anti-fluorescein; Roche) for 30 min at 37°C. Fast Red (Roche) was used as a substrate, and counterstaining was performed with haemalum for light microscopy (Olympus BX 41).

Statistics. Statistical analysis was performed using the software package GraphPad Prism (version 5.00; La Jolla, CA). All P values were generated using two-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 ≤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 3–4 h, 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 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 (time 0). Initial changes were evident 5–55 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. The 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 and their enlargement and transition to cytoplasmic vesicles was visualized, but loss of fluorescence (an early event in apoptotic cells, yet also observed in some nonapoptotic cells) hampered the further visualization of these events (Fig. 1C). The first morphological changes of the cell bodies were swelling combined with rounding and loss of hexagonal shape (16 of 19 cells; Figs. 1–3). Swelling prevailed for ∼15 min before the onset of cell shrinkage. The 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, and K). After reaching the state of pyknosis, cellular morphology remained stable. Individual hepatocytes undergoing apoptosis are shown in Fig. 3, A–L, and Supplemental Video 1 (Supplemental data for this article may be found on the American Journal of Physiology: Gastrointestinal and Liver Physiology website).

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 Supplemental Video 2. Engagement of apoptotic bodies by adjacent hepatocytes was only rarely observed (Fig. 1G, top left). These hepatocytes showed bright cytoplasm and intracytoplasmic vesicles but no other signs of apoptosis. Engagement 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 to 140 min within a field of view (FOV) of 240 × 240 μm, corresponding to 170–200 cells/FOV. Medians were calculated for the time points 30 and 140 min. Because 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 five apoptotic cells were identified per FOV. Every 20–30 min, this number increased by approximately five additional apoptotic cells. Thus, apoptotic cells doubled after 50 min, quadrupled after 90 min, and were sixfold 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 PBS 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, respectively). 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 475 × 475 μm FOV, and numbers were compared with controls. Although no
difference was found for 4 kDa with staining of multiple hepatocytes in controls and induced animals, 70 and 150 kDa 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 kDa; 19.4 ± 10.9 vs. 1.7 ± 1.5 for 150 kDa; 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 kDa) was performed in three animals. A complete evaluation was not possible because of 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. 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 week cytoplasmic staining that was not found in controls or the simultaneously injected group. In apoptotic cells, molecular staining of activated caspases colocalized with typical morphological signs of apoptosis (Fig. 6, A and B). Both controls and induced cells showed cytoplasmic inclusions of strong fluorescence that are likely the result of 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 because of 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. With the use of 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, the 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 4 h. 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 5–10 min after induction of apoptosis, and the first changes in nuclear morphology were recorded after 25 min. Pelling et al. (35) 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. In our model, most cells eventually undergoing apoptosis showed early transitional swelling after ~15 min, which was only discussed in a minority of ex vivo studies (4, 7). This was followed by a cell volume decrease after ~40 min. Similarly, an initial burst of membrane blebbing was followed by cell swelling for up to 4 h observed by computerized video

Fig. 4. Macrophages in apoptosis. A: 3 dark apoptotic hepatocytes (with loss of clearly defined cell boundaries, blebbing, and shrinkage) are surrounded by 3 macrophages. One of these cells (M) extends a pseudopod toward 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 min. An animated version can be found as Supplemental Video 2.
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 the 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 seven to eight 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 that 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 FLIVO. Because FLIVO may interfere with the apoptotic cascade (5, 20), novel reporter molecules may prove more useful in resolving the spatiotemporal 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 expres-

![Fig. 5. Barrier function in apoptosis. A and B: in controls, 150 kDa 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 and 70 kDa dextran in the apoptosis group vs. controls, whereas no difference was found for 4 kDa dextran (C). Double staining with acriflavine and FITC-dextrans: 150 kDa dextran colocalized with apoptotic hepatocytes after 105 min (arrows, D). Edge length 475 μm.](http://ajpgi.physiology.org/content/301/5/G769/F5)

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Our approach, although allowing dynamic microscopic visualization, has some shortcomings. First, apoptotic cells cannot be identified prospectively, necessitating a large FOV to capture early events. Furthermore, the probe has to be positioned on healthy tissue before 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 benchtop laser scanning confocal microscopy, our current instrument settings do not allow examination of different wavelengths. At present, this poses limitations for multistaining.

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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This trial includes aspects of the MD thesis of J. V. Ansems.

DISCLOSURES

M. Goetz and R. Kiesslich: The confocal microscopy system (FIVEI) was provided by Optiscan Pty Ltd, Melbourne, Victoria, Australia.

REFERENCES


