Modern Methods for Assessing the Regenerative Potential of the Liver after Partial Hepatectomy (Review)

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The review addresses the main methods for assessing the function and regenerative potential of the liver. They include both the traditional methods, commonly used in clinical practice, and the latest promising techniques suitable for the analysis of cellular and tissue pathology and having a proven diagnostic value.

It is known that the dynamics of liver regeneration is reflected in the metabolic status of liver cells, their morphology, and the molecular rearrangement. Therefore, by looking at these parameters we will be able to assess the regenerative potential of the liver as a whole.

At present, the most promising method is represented by multiphoton microscopy able to generate the second harmonic; there are also such techniques as coherent anti-stokes Raman spectroscopy (CARS), stimulated Raman scattering (SRS) microscopy, and fluorescence lifetime imaging microscopy (FLIM). In addition, a number of options for analyzing metabolic and structural changes are provided by mass spectrometry, in particular time-of-flight secondary ion mass spectrometry (ToF-SIMS). Studies using these methods with *in vivo* models and with human biopsy samples demonstrate their relevance in biomedical research and in clinical practice alike.

Key words: regenerative potential of the liver; liver function; liver regeneration; hepatocyte proliferation; FLIM; ToF-SIMS; CARS.

Introduction

Liver regeneration is an important component of the reparative process after a part of the functioning parenchyma is lost due to damage (necrosis) or surgical resection [1]. The regenerative process in the liver is represented by hyperplastic activation of viable cells (hepatocytes and sinusoidal cells) in the remaining undamaged tissue. Moreover, in the case of extensive loss of hepatic mass (80–90%), the stem/progenitor cells are activated. The regeneration process is compensatory in nature: it includes the restoration of the liver volume, mass, and function so that the liver is able to maintain its role in metabolism of the whole body [2–4].

In this review, we do not discuss mechanisms of the stem reserve activation; instead, we consider the process of restoration driven by the proliferation of functioning mature liver cells.

A comprehensive assessment of the regenerative potential and function of the would-be remnant enables to plan the surgical intervention for liver resection in primary and metastatic tumors, as well as for liver transplantation from a relative donor [1].

It is known [5–9] that 25% is the minimum volume of hepatic remnant for adequate recovery in patients with morphologically intact liver. In the presence of drug-induced damage (chemotherapy) or with background liver diseases (cirrhosis, fibrosis, hepatosis), at least 40% of the initial liver volume is needed to remain. If a liver mass exceeding this limit was removed, the remaining liver would not be able to restore its physiological function. Even if smaller fragments of the liver are removed, fatal acute post-resection liver failure can develop (occurs in 5–8% of patients) leading to death.

Pre-surgery assessment of the regenerative potential of the liver and its ability to recover is important for

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predicting the result of resection or transplant surgery [10]. The standard preoperative tests do not solve this problem. Currently, the decision whether to perform a resection is based on preoperative tests and intraoperative assessments made by the operating surgeon.

We believe that preference should be given to the intraoperative decision for the following reasons. The preoperative assessment of the liver functional reserve may be not fully informative because, in the course of surgery, some part of the intact liver is removed in order to observe the principle of ablasticity (R0) and prevent the formation of ischemic zones or bile leaks.

In addition, at the time of resection, liver tissue can already be pathologically altered below the detection limit of standard markers such as bilirubin, albumin, blood coagulation profile, transaminase, and alkaline phosphatase levels [11, 12]. The decay period of the standard hepatic markers used in routine clinical practice is too long to reflect changes in the liver function in real time. Finally, the functional and restorative activity of the liver may not correlate with its volume. Surgery resection along the anatomical lines can have an unpredictable effect on the residual function of the liver, depending on the individual characteristics of patient's anatomy [1, 13].

In recent years, to estimate organ volume and mass, as well as their hemodynamic, ultrasound, CT and MRI modalities, were commonly used. The cell proliferative activity is assessed by morphological and immunohistochemical methods. Physical methods are being developed to study the structure, function and metabolic activity of cells and tissues: elastography, various types of spectroscopy, multiphoton microscopy, the method of second harmonic generation, and fluorescence lifetime imaging. Using these methods could expand the ability to assess the regenerative potential of the liver, including the intraoperative option.

This review discusses modern tools for assessing cell proliferation activity, as well as methods for analyzing the restoration of liver volume, mass, and function, both in the experiment and in clinical settings.

Assessing the linear dimensions, volume, and mass of the liver in the experiment

In experimental science it is feasible to measure the ratio of liver mass to body mass before and after its partial resection; it allows for a quantitative assessment of liver recovery [14]. Such an assessment is the simplest method of analysis; however, it does not report on the recovery processes and can only be used in animal studies [1].

Thus, in experiments on rats, the average ratio of the liver mass to body mass, as well as the masses of liver lobes (or the ratio "lobe mass/whole liver mass") were determined [15, 16]. Based on this ratio, after removing and weighing a liver fragment (for example, the left lateral lobe), the mass of the whole liver can be estimated and the ratio of its mass to body weight can be calculated. Later, after some recovery period, the animal is sacrificed, its liver extracted and weighed. By comparing the real liver weight after recovery with the estimated weight just after its partial resection, one can assess the processes of liver regeneration.

Traditional methods for assessing the linear dimensions, volume and structure of the liver in clinical practice

An assessment of liver morphology is made possible using modern imaging techniques. In clinical practice, the linear dimensions, volume, and structure of the liver are examined using traditional imaging techniques such as ultrasound, CT, MRI, and ultrasound elastography. In suspected pathology, the liver parenchyma is examined for its regenerative potential and the risk of liver failure after surgery [17, 18].

Ultrasound procedure. Evaluation of the liver size by ultrasound is a common clinical practice for diagnosing a liver disease, detect a response to treatment (chemotherapy, chemoembolization of the hepatic artery, etc.), and for long time post-surgery monitoring. Ultrasound is an affordable method of real-time imaging that is not associated with ionizing radiation [19]. The low cost and non-invasiveness make it possible to repeatedly examine the acoustic properties of the liver parenchyma.

The test is carried out by comparing the echogenicity of the liver and other tissue (often, the kidney). In the presence of fat droplets in hepatocytes, the brightness of the liver parenchyma increases and becomes greater than the brightness of the renal parenchyma. However, this method has several disadvantages, namely: the presence of speckle noise (caused by energy interference due to random re-reflection of the signal from structures with different densities that are too small to detect), poor image quality [20], and limited diagnostic value in differentiating hepatic pathology. Specifically, as described by Zhang et al. [21], it is difficult to reliably discern between fibrosis, fatty liver disease with inflammation and other chronic disease having similar echogenicity.

Doppler ultrasound allows for analysis of blood flow in the liver vessels. This method is also applicable to the intraoperative assessment of perfusion in a liver remnant or a graft [22]. In addition, the Doppler modality is helpful in diagnosing various liver diseases (in particular, cirrhosis) [23]. Blood flow characteristics and resistance index of the main hepatic vessels (hepatic artery, hepatic vein, and portal vein) have been well described. However, in case of liver pathology, its blood flow may change and affect the wave displacements in the three main liver vessels [24].

Computed tomography and magnetic resonance imaging. The gold standard for estimating the liver volume is CT volumetry [25] and MRI [26]. Organ volume

is determined by obtaining multi-layer images of the liver, which then undergo post-processing. The liver size and dimensions are derived from a resulting 3D image.

In the absence of a preoperative biopsy, damaged or diseased parenchyma often remains undetected until surgery [27]. CT technique allows one to measure the liver volume from sliced images of the human body. From these images, the ratio of the liver remnant volume to the liver total volume can be calculated [28]. By now, many formulas and computation algorithms for calculating the liver volume from post-processed CT images have been proposed. These formulas are specified for age and gender of the patient [29]. On average, the liver volume of a man is 1467.0±28.0 cm^3, and that of a woman — 1271.1±28.9 cm^3 [30]. However, by using this method alone, it is impossible to correctly predict the state of the liver tissue after surgery, especially in the presence of background diseases (steatosis, cholestasis) [31].

For a deeper assessment of the regenerative potential of the liver before surgery, single-photon emission computed tomography is used; that allows for simultaneous assessment of the liver function and its volume [32, 33].

MRI with gadolinium-based contrast agents produces more accurate (as compared to CT) visualization of benign or malignant liver lesions. The rate of gadolinium clearance indicates the functional ability of the liver parenchyma. This contrast-enhanced MRI is already part of the standard preoperative procedure for liver resection in various centers around the world [34].

In addition, liver function can be assessed by using the contrast label Gd-EOB-DTPA. Absorption of this gadolinium-based marker in the liver sinusoids occurs with the help of organic anion transport proteins and Na^+-taurocholate cotransport polypeptides; then the label is excreted into the bile without biotransformation. The prospect of using MRI with Gd-EOB-DTPA as a functional test is currently under consideration [6, 27, 31].

It should be borne in mind that liver density may vary depending on deposition of lipids, glycogen, and non-resident inflammatory cells, which may not be associated with the regenerative or hyperplastic activity [14]. Therefore, the accuracy of CT and MRI in assessing the function and restorative potential of the liver may be limited. The disadvantages of CT volumetry include the need for using relatively low doses of radiation and of the contrast agent, which could impact the quality of the CT images. In MRI, there is no problem with radiation but the need to control the contrast agent dose remains [27].

Visualization of the vascular bed. Analysis of the vascular tree (the portal and/or hepatic vein) gives an indication of liver perfusion in the process of liver regeneration. This technology is able to identify the areas where the blood outflow is obstructed. Visualization of the vascular tree is performed using a spiral CT scan with a contrast agent or using non-contrast MR angiography.

This technology is able to assess vascular regeneration and restoration of intrahepatic vascular architecture, as well as to analyze the areas where the proliferative response of hepatocytes is not homogenous. This heterogeneity is often observed in the process of liver repair and depends on an adequate blood supply in this area [35]. However, the use of contrast agents can lead to cardiovascular complications. In addition, the limitations of this technology include the long time of contrast clearance, which interferes with re-visualization if it is planned at time intervals of less than 24 h [36].

Elastography. With the development of nuclear imaging technology, the methods of ultrasound elastography and magnetic resonance elastography have emerged as major diagnostic tests for assessing liver fibrosis. It has been demonstrated that elastography showing the liver tissue stiffness is more indicative of developing liver fibrosis and cirrhosis than morphological changes in MRI images [37, 38]. Srinivasa Babu et al. [39] describe main limitations of this method, in particular, an inaccuracy in measuring the liver stiffness in patients with severe obesity and ascites. However, as shown by Castéra et al. [40], the use of a new probe (XL probe FibroScan; Echosens, France) could solve this problem.

Evaluation of fibrogenesis using elastography is also relevant for testing the regenerative potential of the liver as its function is significantly decreased with collagen deposition and development of portal hypertension associated with hemodynamic changes in the remnant.

Clinical methods for assessing the proliferative activity of hepatocytes

The proliferative activity of hepatocytes is an integral part of the liver repair process; it is studied with various morphological and immunohistochemical methods based on direct calculation of the mitotic index (the ratio of the number of dividing hepatocytes to the total number of hepatocytes).

Counting the number of mitotic cells. Counting cells undergoing mitosis in histological sections stained with hematoxylin and eosin is a traditional method for assessing cell proliferation. However, it is not without some drawbacks. Firstly, it is difficult to implement this technique in a clinical setting due to the small volumes of samples obtained by percutaneous puncture biopsy of the liver. Secondly, mitosis is a relatively fast process taking only 1 h of the 24-hour cell cycle; accordingly, this method does not allow for calculating the absolute number of actively proliferating hepatocytes. Thirdly, it is known that mitotic activity in different parts of the liver is not identical. Its intensity is higher in the periportal region (acinar zone) than in the middle lobe or the pericentral region. Therefore, biopsy samples taken from different parts of the lobule contain different numbers of mitotic cells [41–43].

In addition, the mitotic activity of cells can be quantified by nucleoside incorporation into the DNA molecule or by using other markers [44].

Staining with thymidine. Measuring the number of 3H-thymidine molecules incorporated in DNA as a marker of phase S activity is one of the most common methods used to monitor the liver recovery process [45].

This assay is used *in vitro* to study liver biopsy samples or isolated hepatocytes. To that end, the cells are incubated with the 3H-labeled thymidine for 1 h, after which the radioactive label is quantified by autohistoradiography. This method involves counting the number of nuclei labeled with thymidine per 1000 cells in a wide microscopic field [46]. The data obtained with rat hepatocytes stained with 3H-thymidine show that at rest, only 0.3% of liver cells will incorporate the marker, whereas in the period of maximum regeneration (after 70% hepatectomy), up to 40% of the cells contain the label. Proliferation of sinusoidal cells also varies from about 0.9% in the resting liver to 30% in the regenerating one. It is known [47] that hepatocytes and sinusoidal cells have different peaks of proliferative activity: 24 h after resection, the largest number of dividing hepatocytes is found whereas the maximum proliferative activity of sinusoidal cells is observed 42–50 h after the resection. This method is not free from limitations: e.g., it is not applicable for *in vivo* studies in humans and requires quite a long time for biopsy sample processing. In addition, the incorporation of thymidine occurs not only during DNA synthesis but also during DNA repair and RNA synthesis.

Incorporation of bromodeoxyuridine in the DNA molecule. Bromodeoxyuridine (BrdU), like thymidine, also incorporates into DNA during cell proliferation. One of the advantages of using BrdU in assessing the liver recovery is that it can be administered in the same dose for 4–5 days with water or food; therefore, the label accumulates in all cells going through mitosis over this period. Its incorporation into DNA can be detected by immunohistochemical analysis using specific antibodies or flow cytometry [48, 49].

The number of hepatocytes that absorb BrdU in the resting liver is less than 1%, and it can reach 25–36% of hepatocytes and sinusoidal cells 24 and 48 h after partial hepatectomy [50]. The main advantages of BrdU labeling are a shorter period (relative to thymidine) required to obtain the results, and the absence of artefacts caused by a radioactive label itself. The main disadvantages are similar to those of the thymidine method. In addition, the *in vitro* BrdU detection is more expensive because it requires special equipment and reagents, and the cell incubation requires special conditions [51].

A common serious drawback of methods using DNA labels is the possible incidence of mutations that may affect gene expression [50].

Flow cytometry (combined method). The method is used to measure fluorescence of chemical compounds that make up the cell (autofluorescence), or fluorescence of exogenous markers added to the cells. The method allows measuring fluorescence of cells, microorganisms, cell nuclei, and chromosomal material when the flowing cells cross the light source. The light scattering values directly reflect the structural and morphological properties of cells. The method allows studying the biochemical, biophysical and molecular characteristics of the examined cells. The excitation of the fluorophore is determined by a detector that converts the fluorescence into an electric signal suitable for processing. Using flow cytometry, it is possible (among many other applications) to estimate the amount of thymidine and BrdU incorporated in DNA [52, 53].

An advantage of flow cytometry is that the resulting data is highly accurate and reproducible. In addition, flow cytometry can be used to determine the stages of cell mitosis during liver regeneration. Cells in the G1 phase are diploid, G2 are tetraploid, and in the S phase, they have an intermediate amount of DNA. Disadvantages include the high cost of the equipment and the need for tissue destruction, which leads to the loss of connections between cell subpopulations [53].

Immunohistochemical methods. Methods are based on the use of antibodies to endogenous bio-molecules. The following markers are typically used to analyze tissue regeneration: the proliferating cell nuclear antigen (PCNA), α DNA polymerase, and the Ki-67 nuclear antigen.

PCNA protein. It is an auxiliary protein of Δ-DNA polymerase in eukaryotic cells, which is essential for DNA replication. Its expression depends on the cell cycle: it appears at the end of the G1 phase and reaches its maximum in the S phase. Strongly colored nuclei are believed to reflect the G1/S or G2 phase [46, 54].

The PCNA results correlate with other markers of cell proliferation. The method is also applicable for studying archival bio-materials and stored specimens [48]. However, by using this method it is impossible to assess the intralobular mitotic events, which may lead to misinterpretation of the results from different zones of the same lobule. In addition, the color intensity attenuates with time, because the reagents lose the immunoreactivity upon drying or light exposure [54, 55].

Ki-67. This nuclear antigen is associated with all phases of the cell cycle. It can be detected at the beginning of phase G1 and peaks in phases S and M. Ki-67 is detected by immunoperoxidase staining with the monoclonal antibody MIB-1. The resulting index is expressed as percentage of Ki-67 positive hepatocytes per 1000 cells in a wide microscopic field [56]. The Ki-67 assay is convenient for clinical use and its results well correlate with other indicators of cell proliferation. Along with that, the antigen is sensitive to fixation; therefore, the color intensity may decrease over time. However, high-temperature treatment allows the color to be restored after a long period [7, 14].

Polymerase chain reaction (PCR). Assessment of cell proliferation can be done using the real-time PCR

method. To analyze the liver regenerative process, it is relevant to determine the mRNA cyclin expression associated with activation of the cell cycle. Ccnd1 and Ccne1 are specific cyclins of the G1 and S stages, while Ccna2 and Ccnb1 are specific for the G2 and M phases. In addition, this method allows evaluation of the expression of genes encoding for enzymes involved in the metabolism of amino acids (*Hal*), carbon (*Got1*), and fatty acids (*Gcdh*), which makes it possible to analyze not only proliferative, but also the synthetic activity of the regenerating liver [57–59]. The disadvantages of this method include the requirement for expensive equipment and reagents. In addition, the analysis is associated with cell destruction, which may lead to a loss of cellular material and wrong results.

Serum proteins: thymidine kinase, ornithine carboxylase, and fibronectin can also act as markers for proliferation. Changing concentrations of these proteins in the blood plasma may indicate the activation of the respective synthetic reactions in the liver.

Thymidine kinase. This enzyme regulates the DNA synthesis by catalyzing thymidine phosphorylation and its subsequent incorporation into DNA of proliferating cells. The enzyme is present in the supernatant fraction of hepatocytes after their homogenization and centrifugation [60, 61]. Its presence is detected by incubating the supernatant with radiolabeled thymidine. Radioactivity of the protein-bound fraction is measured with a liquid scintillation counter, and the results are expressed as the amount of the radioactive label per milligram of protein [62]. The main advantages of this technique include an easiness of its implementation and a low cost of the procedure. The results correlate well with the PCNA protein levels and the 3H-thymidine incorporation. The disadvantages include the need for radioactive material and the dependence of the enzyme activity on age and nutritional status of the patient.

Ornithine decarboxylase. This enzyme is involved in the synthesis of polyamines that are essential for the normal course of liver regeneration. The enzymatic activity is assayed in liver homogenates by quantifying the amount of CO_2 released from the C^{14} isotope-labeled ornithine substrate. The peak activity is observed 6 and 24 h after partial hepatectomy. Ornithine decarboxylase levels are measured by ion-exchange chromatography. The disadvantages include the relatively long time needed to obtain the results and the need to use a radioactive label [14, 63]. In addition, the method is not sensitive enough; therefore, large regenerative stimuli (for example, extensive resection of the liver) are required to produce detectable changes in the enzymatic activity [64].

Fibronectin. This protein is a plasma-soluble glycoprotein synthesized by hepatocytes and endothelial cells [65]. Concentration of fibronectin in the blood plasma is determined by a gelatin-coated latex agglutination method. Its main advantage is simplicity of the procedure. However, fibronectin is not a specific marker of liver cell proliferation as it may increase with tumor cell degeneration or during pregnancy [14].

Clinical methods for assessing functional and structural changes in the liver during regeneration

Clearance tests. Currently, there are a number of functional tests used to assess the release (clearance) of exogenous substances by the liver: aminopyrine breath test, galactose elimination test, phenylalanine breath test, sorbitol elimination test, lidocaine metabolism test, and indocyanine green (ICG) clearance test [66, 67].

Of all these tests, the most commonly used in clinical practice are clearance tests with ICG, galactose elimination test, and the lidocaine test.

Indocyanine green is taken from the blood plasma by hepatocyte transporters located on the basolateral membrane; then ICG is excreted into the bile without biotransformation. Therefore, the test allows evaluation of the detoxification function of the liver [11, 68, 69]. A standard method for the quantitative determination of ICG clearance is an *ex vivo* photometric analysis of consecutive blood samples obtained within 15 min after an intravenous ICG injection [70]. However, there are limitations to using this method. As noted by Urade et al. [71], fluorescence tomography with ICG does not allow to identify the landmarks of liver segments having a complex three-dimensional structure, which makes it difficult to conduct anatomical laparoscopy. In addition, ICG fluorescence in the near infra-red range has a limited penetrative ability in biological tissues.

The rate of galactose elimination from the blood correlates with the process of galactose phosphorylation by galactokinase in liver cells. Concentration of galactose in the blood plasma and urine is analyzed spectrophotometrically from the reaction between galactose and nicotinamide adenine dinucleotide (NAD) with the formation of galactolactone and NADH. This test allows for an indirect assessment of function and metabolic activity of hepatocytes. A low rate of galactose elimination may indicate a liver failure or other pathology [72, 73].

In addition, the detoxification function of the liver can be evaluated using a lidocaine test. Lidocaine is metabolized in the liver. About 90% of the administered dose undergoes N-dealkylation with the formation of monoethyl glycinexylidide and glycinexylidide. Due to its rapid metabolism, lidocaine pharmacokinetics may be affected by conditions that impair liver function. In patients with hepatic dysfunction, the half-life of lidocaine may be increased two-fold or more [72].

With some degree of accuracy, the functional tests predict complications and survival in patients after partial hepatectomy. However, these methods evaluate the liver function only at the time of surgery, not allowing to determine the postoperative function of the remnant [70]. It is also worth noting that the result of functional tests,

as established by Sumiyoshi et al. [73], can be distorted by abnormal hepatic blood flow; this factor limits the method application in patients with intrahepatic shunts. The test is also not applicable for patients with excretory defects or jaundice.

Single-photon emission computed tomography. In recent decades, a number of nuclear imaging technologies for non-invasive evaluation of liver function have been developed. Specifically, it is the method of single-photon emission computed tomography (SPECT) with the 99mTc isotope. A number of 99mTc-labeled agents are available, including 99mTc-sulfur colloid, 99mTc-GSA (human galactosylated serum albumin) and 99mTc-IDA (dimethyl-acetanilide-iminodiacetic acid). The latter two radiopharmaceuticals are eliminated by hepatocyte transporters, while 99mTc-sulfur colloidal scintigraphy is based on phagocytosis by liver macrophages [69, 74]. The 99mTc-GSA is designed as a synthetic asialoglycoprotein that binds to the receptor on the sinusoidal side of hepatocytes and thus allows for liver visualization. Due to that, the SPECT method with 99mTc-GSA can be used as a functional liver test [11, 74–76]. The main drawback of the SPECT-based methods, according to Rassam et al. [77], is a relatively low spatial resolution (as compared with MRI), which makes these methods insufficiently sensitive in detecting liver lesions.

Promising methods for assessing the structure, function, and metabolism of a regenerating liver

The methods presented in this section are not currently used in clinical practice. They provide additional information about the cell energy metabolism, molecular composition, and morphology. Evaluation of pathological changes at the cellular level makes it possible to perform early diagnosis of hepatic diseases and predict the development of the regenerative process after surgery. Such capabilities of the below methods make their use highly desirable.

Mass spectrometry. In the past few years, methods based on spectrometry, such as infrared, ultraviolet spectroscopy (IR and UV spectroscopy) and mass spectrometry have been increasingly used.

Biomolecules have specific infrared absorption spectra. By identifying specific resonance frequencies of the functional molecular groups upon irradiating a sample, one can determine the chemical composition of the sample [72]. IR spectroscopy has become a valuable tool for analyzing the molecular composition of the test sample. At the moment, specific IR spectral characteristics of healthy and tumor tissue have already been obtained. This method allows for an additional analysis of changes in the cell structure and metabolism [78, 79].

Spectroscopy in the UV range allows us to study autofluorescence of intra- and extra-cellular components of normal and diseased tissues. Many metabolic markers have characteristic fluorescence spectra with excitation wavelengths in the UV range. In addition to determining the location of metabolites of interest, this method allows detecting changes in the microenvironment of cells including their oxygenation by measuring the local fluorescence emission [80]. Obviously, the level of hepatocyte oxygenation is a significant marker of “well-being” of the postoperative remnant. In addition, changes in the metabolic markers (such as NADH, collagen, tryptophan, lipo-pigments, elastin, and pyridoxins) are important signs of the metabolic status of liver cells.

Methods based on mass spectrometry, in particular the time-of-flight secondary ion mass spectrometry (ToF-SIMS) enable the studies on biomolecules present on the cell surface.

In addition, these methods can analyze not only cell components with their specific chemical structure, but also the distribution of cell metabolites based on the chemical mapping data [81].

In the ToF-SIMS, a focused pulsating ion beam causes desorption and ionization of molecules on the sample surface. The generated secondary ions “fly” from the sample to the detector; their time of flight correlates with their masses. Using this method, it is possible to identify both complex lipids (such as phosphatidylcholines, phosphatidylethanolamines, sulfatides or glycosphingolipids) and low molecular weight lipids such as fatty acids, cholesterol, cholesterol sulfate, bile acids or vitamin E [82, 83].

ToF-SIMS can be used to assess chemical changes typical of liver steatosis. Further studies in this area will help elucidate the mechanisms underlying fatty liver disease and hopefully identify the markers of diagnostic and prognostic values [82].

When using the ToF-SIMS for imaging, the sample does not require additional chemical processing or staining; that is the main advantage of the method. At present, ToF-SIMS is mostly used in medical research to identify lipid or amino acid composition of various cells; in the future, ToF-SIMS is expected to help in diagnosing various diseases. For example, the method was used to identify the distribution of different lipids in atherosclerotic plaques in the aortic wall [84], the lipid spectrum in skeletal muscle samples from patients with Duchenne dystrophy [85], and the accumulation of cholesterol in the cerebral cortex in Alzheimer’s disease [86]. This method is promising for determining the lipid rearrangement and other chemical variations in a regenerating liver [87].

Using various modalities of mass spectroscopy, it has been shown that after partial hepatectomy, the role of lipid metabolism becomes predominant as compared with glucose metabolism. Enhanced lipogenesis leads to rapid accumulation of intracellular triglycerides in the regenerating liver. A transient increase in hepatic triglycerides can be observed already on day 2 after surgery [88, 89]. However, in the case of chronically

abnormal lipid metabolism, liver regeneration may be injured. Thus, in the presence of hepatic steatosis, the regeneration process is significantly reduced [90, 91].

The main drawbacks of the method are its low sensitivity to secondary ions with high masses and its low-resolution capacity. Achieving an XY resolution of less than 1 mm in biological samples remains a difficult task, therefore, the current version of ToF-SIMS cannot be used for the analysis of smaller lipid drops [92]. This limitation can be overcome by combining ToF-SIMS with other imaging modalities, specifically, with fluorescence microscopy, as shown by Saka et al. [93]. Scientists have also demonstrated a correlation of ToF-SIMS results with confocal and STED microscopy.

Fluorescence imaging of liver tissue at the cellular level. Non-invasive biomedical imaging is crucial for detecting liver diseases, but most modalities do not have sufficient sensitivity, spatial resolution, and specificity to identify the stage of the disease and also cannot detect molecular changes specific to the regeneration process.

There is an increasing interest in the methods of tissue imaging capable of studying cellular events *in vivo*. Fluorescence microscopy allows evaluating the presence and distribution of specific molecules and investigating cellular events in real time. Recent data obtained with fluorescence imaging provide some insight into the structure and function of normal and pathological liver [17]. For example, the microstructure of hepatocytes was studied using confocal single-photon [94] and multi-photon microscopy (MPM) [95, 96].

The use of single-photon confocal laser endomicroscopy for histological examination of the liver in animals and humans was described by Goetz et al. [97]. This technology allows for intraoperative microscopic imaging and can potentially be used for monitoring of structural changes in the liver tissue [97–99]. However, in this approach, even with due precautions, the sample undergoes photo-burning and photo-damage, which reduces the image quality and damages the tissue [100].

Multiphoton microscopy has several advantages, which are important for visualizing living objects. Because of the low energy of the exciting long-wavelength radiation the photo-damage decreases and the penetration depth increases [96, 101]. Major cellular components — elastin fibers, NADH, and flavin adenine dinucleotides (FAD) — generate fluorescent signals and can be detected using this method. For NADH and FAD, the signal amplitude reflects the reduced forms of NAD and FAD, and therefore, indicates the intracellular redox balance [95, 102, 103]. A deficiency of NADH in the damaged liver, as reported, is detrimental to energy metabolism and intracellular signaling, which ultimately slows down the regenerative process. In addition, a deficiency of NADH in the liver can affect the oxidation of fatty acids and lead to transient steatosis, a characteristic feature of liver regeneration [104].

Thus, the MPM can be considered a real time analogue of intravital histology that provides dynamic information about the living tissue; in the future, this technology is expected to assess the degree of liver pathology, especially, in fibrosis. Further development of this method will hopefully make it possible to test the regenerative potential of the liver.

Second harmonic generation. A visual assessment of a liver undergoing fibrogenesis cannot fully reflect the degree of pathology without a quantitative assessment of fibrous tissue. Histological methods can distinguish between a fibrous tissue and a normal one, but this analysis takes several days and does not provide information about pathological changes in dynamics. The second harmonic generation (SHG) method makes it possible to qualitatively and quantitatively evaluate the accumulation of collagen in tissue and thus determine the stage of fibrosis [105–107].

Second harmonic generation is the process of formation of secondary electromagnetic waves with a doubled frequency as a result of nonlinear interaction of an electromagnetic wave with matter. SHG allows visualizing highly organized structures, including type I collagen. Available SHG data clearly shows an increase in fibrillary collagen during the development of fibrosis — from the initial stage (portal fibrosis) to the final one (cirrhosis) [108]. This method is applicable for visualization of living tissues, since it does not cause photo damage or photo burnout of the test sample. It can also be used for archival research and analysis of both cryo-preserved and dewaxed tissue samples [109].

At present, a simple method for the qualitative and quantitative assessment of liver fibrosis based on SHG imaging is in use; the results correlate well with the degree of fibrosis according to the METAVIR scale. The SHG system has been tested in the clinic and can become an alternative to the traditional histological analysis, significantly reducing the time needed for clinical diagnosis [110]. Likewise, the SHG data correlate with morphology, structure, and thickness of collagen in Glisson capsules [107].

CARS and SRS. Hyperspectral microscopy based on coherent anti-stokes Raman spectroscopy (CARS) and stimulated Raman scattering (SRS) microscopy is a promising approach that allows for qualitative and quantitative assessment of lipids in cells and tissues. These methods provide information on the chemical structure of lipids and on their distribution, by detecting internal vibrations of chemical bonds. CARS and SRS methods do not require any sample staining. These systems are highly sensitive to molecular vibrations of aliphatic C–H bonds in lipid molecules, and are, therefore, relevant for visualization of lipid-enriched biological structures [108, 111–113]. The combination of CARS and SRS with fluorescence microscopy, in particular with laser scanning microscopy, will increase the resolution of the methods [114]. For example, it will be possible to determine specific vibrational

spectra of chemical bonds between carbon and other elements (usually hydrogen, oxygen, and nitrogen), as well as characteristic vibrations of various functional groups (hydroxyl-OH, amino-NH_2, etc.) [115]. Using hyperspectral SRS microscopy, Fu et al. were able to identify certain types of cholesterol esters and triglycerides in lipid drops [114, 116]. Excess of triglycerides is associated with various (possibly pathological) molecular changes in liver cells, which may indicate a decrease in regenerative potential.

An effective way to simultaneously visualize fibrous and adipose tissue of the liver is to combine MPM, SHG, and hyperspectral microscopy into one imaging platform called multimodal nonlinear optical microscopy [117]. Such an aggregated system has a higher potential for assessing molecular changes than the individual modalities. The advantage of multimodal microscopy is the simultaneous visualization of various histological characteristics of tissues without using staining reagents. In addition, the system allows not only to determine the presence of fat droplets in hepatocytes, but also to conduct a qualitative assessment of their lipid composition [118].

Fluorescence lifetime imaging microscopy. At present, novel methods of fluorescence imaging able to evaluate the metabolic and synthetic activity of cells, are emerging [119]. A change in the metabolic status of hepatocytes is a sensitive parameter for assessing the severity of hepatic pathology and the adequacy of the regeneration process. In addition, the metabolic activity of hepatocytes reflects the function of the liver as a whole.

The use of MPM in combination with fluorescence lifetime imaging microscopy (FLIM) allows determining the fluorescence lifetimes of intracellular fluorophores. Fluorescence lifetime is the time of electron transition to the excited state and its return to the ground state. Changes in the fluorescence lifetimes of NADH and FAD indicate changes in metabolic pathways such as glycolysis, oxidative phosphorylation, the pentose phosphate pathway, and the complex of biosynthetic processes in the cell [120–122]. Several reports showed the applicability of FLIM for assessing metabolic changes in primary sclerosing cholangitis and biliary tract fibrosis, chronic fibrosis, steatosis, hepatocellular carcinoma, and liver ischemia-reperfusion syndrome [95, 96, 100]. In addition, Ranjit et al. [123] showed the potential of FLIM for analyzing the process of lipogenesis in cells.

Thus, the use of MPM in combination with FLIM is promising for the analysis of early pathological changes that cannot be detected by histological analysis, functional tests, and other traditional methods. The results obtained so far require additional analysis and correct interpretation; further accumulation of data on changes in the metabolic status of hepatocytes under various pathological conditions of the liver and in the process of its regeneration is needed.

Conclusion

Despite the progress in drug therapy for liver diseases, partial hepatectomy and liver transplantation remain significant treatment modalities. The regenerative potential of a diseased liver, as well as its functional status, are of paramount importance for planning of surgical interventions or liver transplantation. Comorbidities often affect the liver function and regenerative potential and thus increase the risk of liver failure in the postoperative/posttransplantation period. Standard morphological and immunohistochemical methods that are widely used in clinical practice are not fully applicable for monitoring early signs of pathology or of changes in cell proliferation. Clinical imaging methods and various functional tests provide information on the volume and overall function of the liver, which, however, does not reflect its regeneration potential. In this regard, the search for relevant methods that would allow for assessing the functional ability and regenerative potential of the liver before and during surgery with the possibility of intraoperative monitoring remains an urgent task.

The liver repair process involves metabolic changes, structural rearrangement, and chemical transformation of liver cells. Further studies are expected to determine the criteria for predicting and monitoring cell functional and proliferative potential. The most promising methods to analyze these parameters include multiphoton microscopy combined with SHG, CARS, SRS, or FLIM and mass spectrometry, especially, ToF-SIMS. The combined use of these methods will allow one to predict the dynamics of the regenerative process in the liver remnant. Assessing the regenerative potential of the liver intraoperatively may greatly improve the treatment outcomes.

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