

# The Hepatic Microcirculation: Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair

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**Vollmar B, Menger MD.** The Hepatic Microcirculation: Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair. *Physiol Rev* 89: 1269–1339, 2009; doi:10.1152/physrev.00027.2008.—The complex functions of the

liver in biosynthesis, metabolism, clearance, and host defense are tightly dependent on an adequate microcirculation. To guarantee hepatic homeostasis, this requires not only a sufficient nutritive perfusion and oxygen supply, but also a balanced vasomotor control and an appropriate cell-cell communication. Deteriorations of the hepatic homeostasis, as observed in ischemia/reperfusion, cold preservation and transplantation, septic organ failure, and hepatic resection-induced hyperperfusion, are associated with a high morbidity and mortality. During the last two decades, experimental studies have demonstrated that microcirculatory disorders are determinants for organ failure in these disease states. Disorders include 1) a dysregulation of the vasomotor control with a deterioration of the endothelin-nitric oxide balance, an arterial and sinusoidal constriction, and a shutdown of the microcirculation as well as 2) an overwhelming inflammatory response with microvascular leukocyte accumulation, platelet adherence, and Kupffer cell activation. Within the sequelae of events, proinflammatory mediators, such as reactive oxygen species and tumor necrosis factor- $\alpha$ , are the key players, causing the microvascular dysfunction and perfusion failure. This review covers the morphological and functional characterization of the hepatic microcirculation, the mechanistic contributions in surgical disease states, and the therapeutic targets to attenuate tissue injury and organ dysfunction. It also indicates future directions to translate the knowledge achieved from experimental studies into clinical practice. By this, the use of the recently introduced techniques to monitor the hepatic microcirculation in humans, such as near-infrared spectroscopy or orthogonal polarized spectral imaging, may allow an early initiation of treatment, which should benefit the final outcome of these critically ill patients.

## I. INTRODUCTION

The microcirculation of the liver is of utmost importance for the physiology and function of the whole organism. It guarantees the supply of the parenchymal tissue with oxygen and nutrients, serves as the gate for leukocyte entrance in hepatic inflammation, and is responsible for the clearance of toxicants and foreign bodies from the bloodstream. Historically, the microvasculature could only be studied by histological techniques, which lack information on dynamic processes. With the introduction of *in vivo* microscopy, however, it became possible to analyze flow and resistance of hepatic microvessels under physiological and pathological conditions. Further development in this technology and the *in vivo* application of fluorescent markers and epi-illumination techniques allow nowadays sophisticated analyses of mechanistic contributions of distinct blood and liver cells to the hepatic microcirculation during physiological regulation and to the microcirculatory dysfunctions in disease.

In clinical practice, acute liver dysfunction and failure represent life-threatening conditions that require immediate intervention. Causes for the deterioration of liver function are warm and cold ischemia and reperfusion (I/R) during liver resection and transplantation, generalized inflammation and sepsis in particular after endotoxin exposure, acetaminophen-induced hepatic intoxication, and the small-for-size syndrome, which is a mismatch between size and need, after extended hepatectomy or split liver transplantation. The small-for-size syndrome is additionally aggravated by an impaired process of liver regeneration. These disease states are in focus of interest in clinical practice of surgery, because they are still associated with a high morbidity and mortality.

Hepatic microvascular activation and microcirculatory dysfunctions have been shown to be determinants for the manifestation of these disease states. Accordingly, the

mechanisms of activation and dysfunction are of pivotal interest, not only for the understanding of the disease, but also for the development of novel therapeutic strategies. In the following, this review will describe the morphology and physiology of the hepatic microcirculation. It will further focus on 1) the deteriorations of the microcirculation in disease, 2) the microcirculatory interactions with cellular activation and mediator response, 3) the mechanisms of these interactions, and 4) how this knowledge can be used to develop new treatment regimens. The cellular response includes the accumulation and activation of leukocytes, platelets, and Kupffer cells (KC), while the mediator response consists of proinflammatory cascades with release of cytokines, chemokines, and reactive oxygen species. The microcirculatory deteriorations are characterized by vasoconstriction, white blood cell and platelet endothelial adherence, shutdown of the sinusoidal circulation, and parenchymal tissue hypoxia, resulting in hepatocellular excretory dysfunction and organ failure. These microcirculatory dysfunctions are the major target for treatment strategies, which may be specific according to the mechanisms recognized, or unspecific when using pleiotropic drugs.

With this review we want to assist in improving the understanding of the physiology of the hepatic microcirculation and its pathology in disease. We hope that the delineation of the complex mechanisms of dysregulation may inspire the design and development of novel treatment approaches.

## II. PHYSIOLOGY OF THE HEPATIC MICROCIRCULATION

### A. Dual Blood Perfusion of the Liver

The liver constitutes 2.5% of the body weight and is, thus, the largest organ in the body. It receives ~25% of the

cardiac output via two inflows, the portal vein and the hepatic artery. Both vessels enter the liver at its hilus accompanied by the hepatic bile duct, lymphatics, and nerves. The portal vein is a valveless afferent vessel that drains the blood from the capillary system of the intestine, spleen, pancreas, omentum, and gallbladder and contributes to the liver's blood supply with ~75–80% of its total inflow. The remaining 20–25% is delivered by the hepatic artery (656, 858). Total hepatic perfusion amounts to  $\sim 1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g tissue}^{-1}$ , and oxygen consumption by the liver accounts for 20% of total body oxygen consumption. In contrast to the well-oxygenated hepatic arterial blood, the portal vein carries partly deoxygenated, but nutrient-rich blood. Nevertheless, >50% of the hepatic oxygen requirements are provided by portal venous blood due to its high flow rate. Hence, hepatic oxygenation depends almost equally on both large afferent vessels. In addition to the existence of the two afferent vessels, the liver circulation includes an efferent system by way of the hepatic veins. The hepatic artery is a vessel of resistance, whereas the portal and hepatic veins are vessels of capacitance. Thus the liver is interposed in an arterial high-pressure and a venous low-pressure system.

The dual blood supply of the liver is a unique feature of the hepatic vasculature and distinctly determines the regulation and distribution of blood flow. There is an intimate relationship between the two vascular systems, termed the "hepatic arterial buffer response" (HABR), representing the ability of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow (Fig. 1). While Burton-Opitz observed an increase in hepatic arterial blood flow upon reduced portal venous inflow already in 1911 (76), the term HABR was stamped first in 1981 by Wayne Lautt (465). In addition

to the intrinsic mechanism of classical arterial autoregulation, i.e., the myogenic constrictive response of the hepatic artery if arterial pressure rises, there exists a second intrinsic mechanism in that the hepatic artery dilates, if portal flow decreases, and the hepatic artery constricts, if portal flow increases (459). The increase in hepatic arterial blood flow is capable of buffering ~25–60% of the decreased portal flow (458, 464). Hereby, the hepatic artery is not regulated by the metabolic demand of the liver, because a hemodilution-induced reduction of oxygen content in the inflow and outflow vessels or a massive increase of oxygen demand do not cause arterial vasodilation (66, 464). Instead, hepatic arterial flow subserves the hepatic role as a regulator of blood levels of nutrients and hormones by maintaining blood flow and thereby hepatic clearance, as steady as possible (461, 462). Because the portal vein cannot control its blood flow, which is simply the sum of outflows of the extrahepatic splanchnic organs, there is no reciprocity of the HABR, i.e., alterations of the hepatic arterial perfusion do not induce compensatory changes of the portal vascular flow (465) or resistance (473).

In contrast to potential mechanisms, including neural and myogenic control, which are considered unlikely (464, 465) or even disproved (12, 531), adenosine has been repeatedly advocated as the putative mediator in the space of Mall driving the communication between the hepatic artery and the portal vein. The space of Mall surrounds the hepatic arterial resistance vessels and portal venules and is contained within a limiting plate that separates this space from other fluid compartments. According to the wash-out hypothesis of adenosine, adenosine accumulates in or less adenosine is washed away from the space of Mall, if portal blood flow is reduced. Elevated adenosine concentrations lead to a dilation of the hepatic artery with a subsequent increase of hepatic arterial flow. There are several lines of evidence which suggest that adenosine largely mediates the HABR. First, adenosine produces dilation of the hepatic artery (458, 460). Second, portal venous application of adenosine increases hepatic arterial blood flow. Third, the adenosine receptor antagonists 3-isobutyl-1-methylxanthine (IBMX) (458) and 8-phenyltheophylline (8-PT) (70, 460, 672) reduce the HABR, while dipyridamole, an adenosine uptake antagonist, potentiates the magnitude of the HABR (458). Adenosine also activates hepatic sensory nerves to cause reflex renal fluid retention, thus increasing the circulating blood volume and maintaining the cardiac output and portal flow (463).

In addition to adenosine, other vasoactive substances, namely, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide ( $\text{H}_2\text{S}$ ), might contribute to this regulatory mechanism; however, they have not extensively been studied in this context yet (85, 302). In addition, sensory innervation and sensory neuropeptides

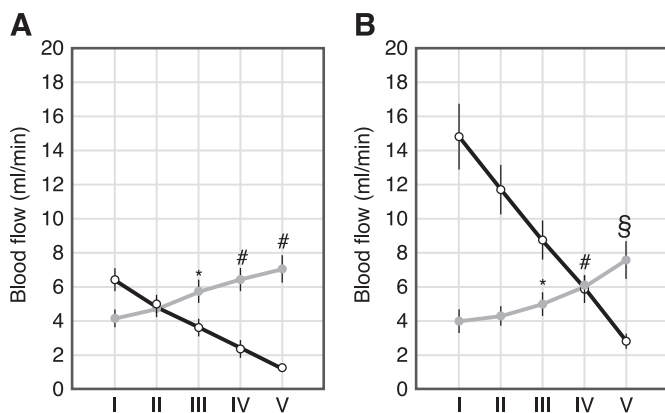


FIG. 1. Hepatic arterial buffer response in cirrhotic (A) and control livers (B). Upon reduction of portal venous blood flow (open circles) to 80% (II), 60% (III), 40% (IV), and 20% (V) of baseline (I), there is a constant increase of hepatic arterial blood flow (solid circles). Note the markedly diminished portal venous blood flow in the cirrhotic livers but the preserved buffer response of the hepatic artery. Values are means  $\pm$  SE of triplicate measurements per animal ( $n = 6$ ).  $\ast P < 0.05$  vs. I, II, III, IV.  $\# P < 0.05$  vs. I, II, III.  $\ast P < 0.05$  vs. I, II. [From Richter et al. (670).]

seem to be involved in the HABR, which is partially mediated by activation of capsaicin-sensitive sensory fibers (47).

The intriguing arterial buffer response of the liver has been shown to operate not only under physiological conditions and prenatally (166), but is also maintained in cirrhotic livers (18, 572, 670) (Fig. 1). Decreased portal venous inflow to the liver leads to a decrease in oxygen supply (261, 670) and thus induces a compensatory increase of hepatic arterial blood flow. It has been shown that both adenosine and NO are main mediators involved in the vasodilation of the splanchnic circulation in cirrhosis (329, 330, 899). Adenosine is released from hypoxic tissues and mediates vasodilation by the adenosine A1 receptor through a NO-dependent pathway (973). The site of resistance of the hepatic arteries is located in the presinusoidal arterioles (782) where NO production is preserved, while in the sinusoidal and postsinusoidal areas deficiency of NO facilitates the effects of vasoconstrictors and thus contributes to the increased intrahepatic resistance in portal hypertension (504).

In liver transplants (144, 286, 749), HABR is also maintained. As increased flow in transplanted livers is predominantly carried through the portal vein, the intact HABR causes a reduction of the hepatic arterial flow, potentially harming organ integrity and function (286). Small-for-size grafts, as seen in split-liver transplantation, are associated with portal hypertension and, as a consequence of an intact buffer response, poor hepatic arterial flow and vasospasm. In severe cases, this leads to functional dearterialization, ischemic cholangitis, and parenchymal infarcts (144). Of note, the HABR-induced decrease of the hepatic arterial blood flow can successfully be counteracted. Splenic artery embolization reduces excessive portal vein flow and, thereby, ameliorates the overactive HABR (651).

## B. Anatomy of the Hepatic Microvascular Bed

The anatomy of the hepatic microvascular bed, in particular the terminal distribution of the afferent vessels and the sinusoids, has been studied in detail by light microscopy, intravital fluorescence microscopy, and transmission and scanning electron microscopy (72, 367, 551, 619, 926).

In the portal tracts, branches of the hepatic artery, the hepatic portal vein, the main bile duct, and the main lymphatic vessels travel parallel to each other through the liver parenchyma. Of the structures present, the lymphatic vessels are often collapsed and inconspicuous, as are the autonomic nerves. Thus three structures are regularly visible with the consequence that portal tracts are often referred to as portal triads. In cirrhotic livers with deterioration of trans-sinusoidal macromolecular ex-

change, it has been shown that lymphatic drainage functions as a compensatory mechanism, guaranteeing the transport of macromolecules that are trapped extravascularly due to diffusion-barrier-induced hindrance of hepatocellular uptake. As a consequence, lymph vessels massively expand and gain visibility due to an increase of density and area within the portal tracts (875).

It is commonly understood that there is some form of communication between the portal venous and the hepatic arterial circulation (55, 72, 398, 541, 543, 601). After repeated branching, the terminal vessels, i.e., terminal hepatic arterioles and terminal portal venules with a diameter of 15–35  $\mu\text{m}$  and a length of 50–70  $\mu\text{m}$  (600), supply the blood to the hepatic sinusoids. At the transition of the terminal portal venule to the sinusoid, inlet sphincters are located and comprise large unfenestrated endothelial cells, covering the vascular lumen and being surrounded by a basement membrane and pericytes, but not smooth muscle cells (72).

In addition to that, the hepatic arterioles wind themselves around the portal venules sending short branches 1) to the portal venules, i.e., arteriolo-portal anastomoses (Fig. 2), and 2) to the capillaries of the peribiliary plexus, which nourish the bile duct and drain into the sinusoids via arteriosinus twigs. Within the periportal tissue at the periphery of the lobule, these twigs have a complete basement membrane and unfenestrated endothelium (72), still resembling capillaries. A short distance downstream into the parenchyma they lose their basement membrane, become fenestrated, and are true sinusoids. The terminal hepatic arteriole-derived capillaries further supply 3) the portal venular wall as vasa vasorum and 4) the connective tissue including the nerves of the portal tract (600, 601). With the use of enzyme histochemistry of alkaline phosphatase activity, the identification of endothelial cells of the arteriolar capillaries within the microvascular system allowed the distinct evaluation of the terminal distribution of the hepatic artery and its involvement in the control of hepatic sinusoidal blood flow (601). The shunting of blood via hepatic arteriolo-portal anastomoses has been shown to guarantee maintenance of nutritional microvascular supply and oxygen delivery in HABR in rats (672) (Fig. 2).

The hepatic sinusoids correspond to the capillary bed of the liver and represent the segment of the microcirculation in which supply of nutrients and removal of metabolic products takes place. Main sinusoids run straight between the liver cell cords over a length of  $\sim 250 \mu\text{m}$  and communicate with each other through shorter interconnecting sinusoids running across the liver cell cords. Sinusoidal diameters increase from  $\sim 7 \mu\text{m}$  in the periportal to  $\sim 15 \mu\text{m}$  in the pericentral area. Sinusoids are invested with a unique type of lining consisting of endothelial cells with flattened processes perforated by small fenestrae. These open fenestrations are arranged in

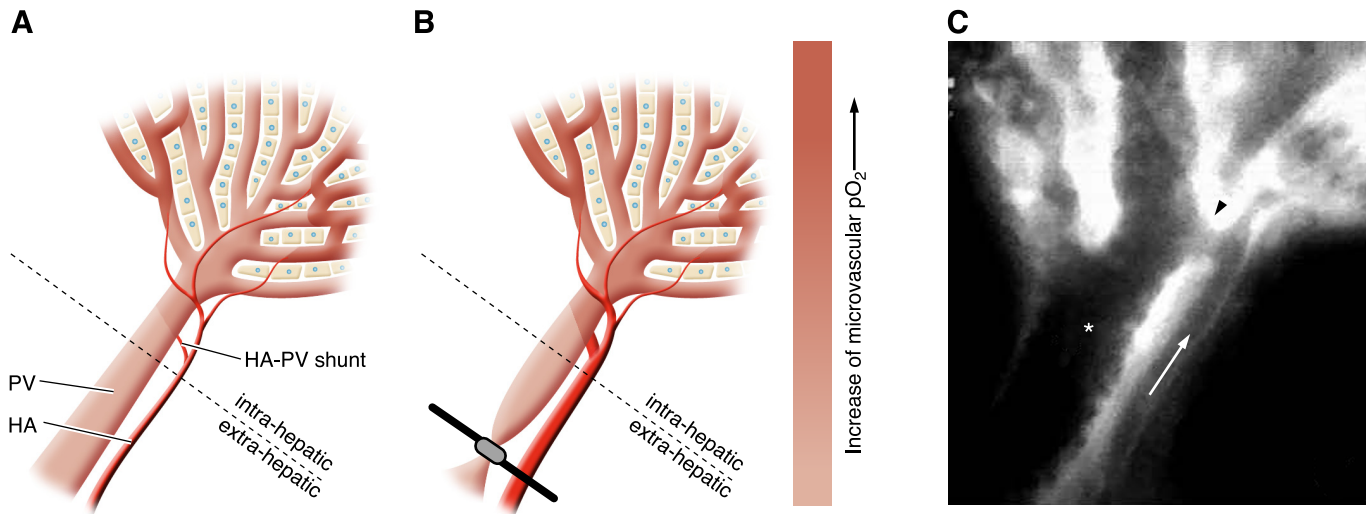


FIG. 2. Schematic representation of hepatic arterial (HA) and portal venous (PV) blood supply to sinusoids with indication of the hepatic arterio-portal venular (HA-PV) shunt. *A*: under normal conditions, all sinusoids are perfused with portal venous blood, while only some of them are additionally supplied directly by terminal hepatic arterioles. *B*: under conditions of reduced portal venous flow with induction of the hepatic arterial buffer response, preferential shunt perfusion leads to a disproportionately increased contribution of perfusion of sinusoids with hepatic artery-derived blood. This guarantees maintenance of oxygen supply despite an overall reduction of nutritive blood flow. *C*: intravital fluorescence microscopic image of the surface of a normal rat liver displaying the most terminal hepatic arteriole (white arrow) and portal venule (asterisk) running in parallel to each other. Note the hepatic arterio-portal venular shunt (arrowhead) located in the distal part of the terminal afferent vessels, but upstream from the sinusoidal network of the lobule. [From Richter et al. (672).]

clusters of 10–50 pores forming so-called “sieve plates” with a diameter of 150–175 nm and represent, apart from the absence of a basement membrane, the structural peculiarity of hepatic sinusoids (903). The sieve plates occupy ~6–8% of the endothelial surface and are not uniform in size or distribution throughout the length of the sinusoids (Fig. 3). There is a decrease in diameter but an increase of frequency from periportal to centrilobular zones, which results in higher centrilobular porosity (64,

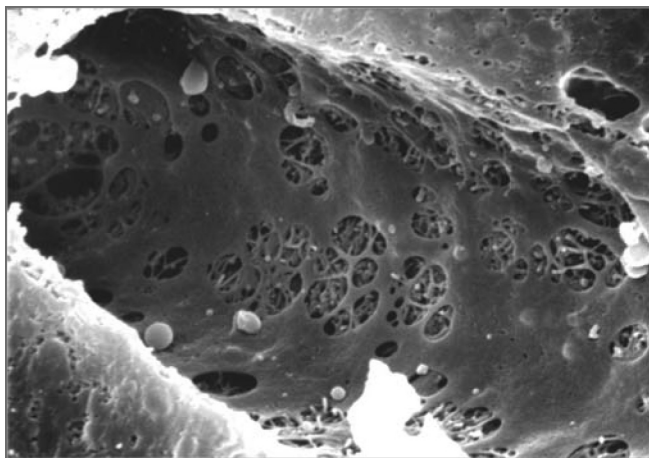


FIG. 3. Lumen of a hepatic sinusoid with the endothelial cell coating, displaying the typical fenestrations arranged as clusters and forming the so-called sieve plates. Freeze-fracture technique and scanning electron microscopy of the rat liver are shown. Magnification  $\times 12,000$ .

902). These membrane-bound pores lack a diaphragm and a basal lamina, thus contrasting the fenestrae described in the kidney, pancreas, and brain (62). The fenestrae are dynamic structures that contract and dilate in response to alterations of sinusoidal blood flow and perfusion pressure. Furthermore, the fenestrae act as a selective sieving barrier to control the extensive exchange of material between the blood and the liver cells, and vice versa, contribute to the homeostatic control of the hepatic microcirculation. In addition, they exert scavenger function by their high receptor-mediated endocytotic capacity and clear the blood from many macromolecular waste products (748). The unique morphology of the liver sinusoidal endothelial cells (SEC) further permits interactions between lymphocytes and hepatocytes, which are accused to allow naive T-cells to interact with hepatocytes and thus to develop immune tolerance as lymphocytes pass through the liver (889). Meanwhile, innovations in correlative imaging techniques by using combined light, probe, laser, and various electron microscope techniques and their application in the hepatic endothelial cell model allow novel insights into the subcellular components and supramolecular structures of the liver sieve (reviewed in Ref. 63). They will help to further understand their biological relevance in various diseases, such as fibrosis, cirrhosis, atherosclerosis, and cancer (64).

A unique cellular component of the hepatic sinusoids is the fat- and vitamin A-storing perisinusoidal cell, known as stellate cell (211). External to the endothelium stellate

cells are located in the space of Disse, which is the space between the basal microvilli-rich surfaces of the hepatocytes and the sinusoidal lining cells. While the cell body of stellate cells is often found in recesses between hepatocytes, their perisinusoidal processes direct along the sinusoids with numerous fingerlike secondary branches, which encircle the sinusoidal tube (56). In addition to their well-known importance in retinol metabolism and as key actors in the hepatic fibrogenic response to injury, stellate cells have been strongly implicated to play a central role in the regulation of blood flow through hepatic sinusoids (681). This is not only based on the close relationship of these cells in a perisinusoidal orientation within the space of Disse, but on the endowment with desmin,  $\alpha$ -smooth muscle actin, and microfilaments and, thus, their remarkable capacity for cellular contraction (681).

In addition to the perisinusoidal stellate cells, the KC constitute a cellular component of the hepatic sinusoids, being anchored to the luminal site of the endothelium and, thus, exposed to the bloodstream. In contrast to stellate cells, which are distributed almost homogeneously throughout the different zones of the liver lobule (766, 873), the majority of KC is found in periportal regions where they are larger and have greater phagocytic activity than those located in the perilobular region (59, 60). This results in a zonal distribution with a specific kinetics of phagocytosis (861). KC are attached to the endothelium by cytoplasmic processes, which sometimes also anchor across the lumen to the opposite sinusoidal

wall. By their large bodies protruding into the sinusoidal lumen, KC represent a flow hindrance and are considered as contractile cells contributing to blood flow regulation through sinusoids (21, 541) (Fig. 4).

After flowing through the sinusoids, blood passes through outlet or efferent sphincters composed of SEC and is collected in small branches of hepatic veins (terminal central veins). Several of these terminal central veins may combine, increasing in diameter and reaching the sublobular vein and hepatic veins, which leave the liver on the dorsal surface and extend to the extrahepatic inferior vena cava (541). Thus the hepatic microcirculatory unit in principle consists of the two terminal afferent vessels, the network of sinusoids running between the liver cords and the efferent terminal hepatic venule. Although the organization of the liver into morphological and functional units is an ongoing matter of debate among anatomists, pathologists, and clinicians, the hexagonal hepatic lobule seems to be the most consistent concept with existing evidence (171, 517) and has displaced the unit termed "simple liver acinus," being first proposed by Rappaport (657, 658). The primary lobule, proposed by Matsumoto and Kawakami in 1982 (533), has gained acceptance as the functional unit of the liver over other conceptual views (657), because it is based on the vessel architecture and includes the classic lobule as a secondary feature (541). The classic lobule, comprising several cone-shaped primary lobules, is a polygonal structure featured by placing the terminal central vein in the center with portal tracts distributed along its periphery. In con-

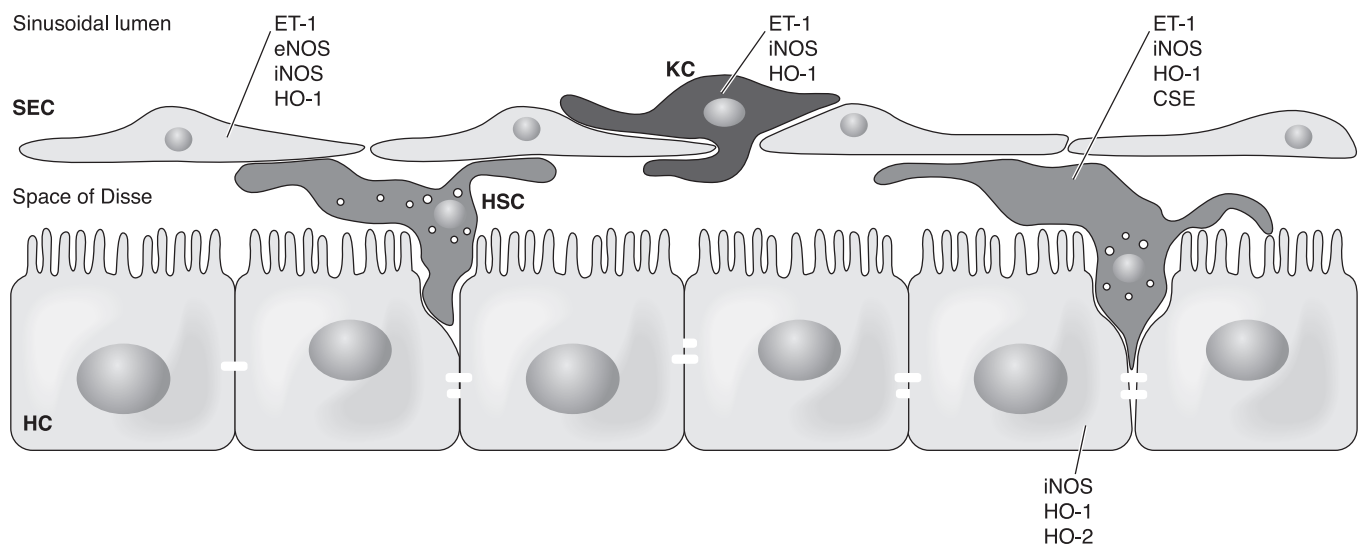


FIG. 4. Local regulators of the hepatic sinusoidal microcirculation. The sinusoid is composed of nonparenchymal contractile cells, such as hepatic stellate cells (HSC), sinusoidal endothelial cells (SEC), and Kupffer cells (KC), which serve as both sources and targets of endothelin (ET), nitric oxide synthase (NOS)-derived NO, heme oxygenase (HO)-derived CO and cystathionine- $\gamma$ -lyase (CSE)-derived H<sub>2</sub>S. In the normal liver, the primary source for NO is most likely the eNOS, which is expressed by the SEC. The cellular source of the vasodilative CO is primarily the hepatocellular HO-2 and to some extent the nonparenchymal cell-confined HO-1. H<sub>2</sub>S is released from the CSE-expressing HSC. In contrast to the K<sub>ATP</sub> channel opener H<sub>2</sub>S, CO and NO exert vasorelaxation by the soluble guanylate cyclase-mediated increase of cGMP within HSC. Counteracting these vasodilative mediators, ET-1 is synthesized and released by SEC, HSC, and KC. While stimulation of ET<sub>B1</sub> receptors on SEC may result in NO-dependent vasorelaxation, ET-1-mediated ligation of ET<sub>A</sub> and ET<sub>B2</sub> receptors on HSC causes vasoconstriction. HC, hepatocyte.

trast to pigs with considerable connective tissue, in tissue of most other species, including humans, the periphery of these lobules is not distinct and easily definable. Primary lobules were renamed as hepatic microvascular subunits, consisting of a group of sinusoids supplied by a single inlet venule and its associated termination of a branch of the hepatic arteriole, finally draining into a central venule. This functional substructure conforms to the basic idea of a microcirculatory unit and is also in line with the proposed gradient of hepatocellular metabolic heterogeneity along the sinusoidal path (806).

### C. Regulation of the Hepatic Microvascular Blood Flow

As outlined above, the liver is morphologically and structurally multifaceted and has been considered second only to brain in its complexity. All vascular segments of the hepatic microvascular subunit, i.e., the afferent portal venules and hepatic arterioles, the sinusoids and the central venules, represent potential sites of regulation of blood flow. In addition to the presence of smooth muscle cells restricted to the afferent and efferent vessels and its branches, the sinusoids contain contractile cells, such as stellate cells, SEC and KC, which all are reported to be involved in the regulation of blood flow through sinusoids.

According to direct blood pressure measurements by micropuncture of hepatic microvessels, the blood pressure in the terminal hepatic arteriole is at least 300–400 mmH<sub>2</sub>O and that in the terminal portal venule is 50 mmH<sub>2</sub>O, while the sinusoidal pressure is estimated as low as 10–20 mmH<sub>2</sub>O (583). More than 50 years ago, Knisely et al. (398) suggested the existence of sphincterlike specialized structures at the entrance to and at the exit from the sinusoids to maintain these steep blood pressure gradients from the portal-venous system, but in particular from the hepatic arteriolar system into the sinusoids (398). The existence of both inlet and outlet sphincters has been confirmed by a number of investigators (55, 927). However, others failed to find any evidence for such structures (657), which might, at least in part, be due to the fact that resistance sites in the portal, sinusoidal, and hepatic venous system are subject to species variations (171). In addition to the vagueness of the existence of hepatic sphincters, there is even more uncertainty about the constituting cellular components. While many studies failed to demonstrate smooth muscle cells or other contractile cells at these locations, electron microscopic studies in the dog have demonstrated branches of the hepatic veins that were equipped with peculiar, periodically arranged sphincter muscles (781). These contained tufts of smooth muscle cells most strongly disposed in the sublobular veins (100–250 μm in diameter), that reduced the luminal

diameters of veins by 34% (7). In addition, double layered smooth muscle cells could be detected in the walls of the terminal hepatic venules of the rat liver by means of electron microscopy (700). Nonetheless, the structure, function, and regulation of intravascular sphincters and their role in controlling sinusoidal blood flow remain obscure.

In contrast, there is a large body of evidence that hepatic microvascular blood flow is regulated and redistributed at the level of the microcirculation (Fig. 4). This view is based on the fact that both stellate cells and endothelial cells can actively control various functions of the microvasculature. Since the description of acetylcholine-induced endothelial cell-dependent relaxation of blood vessels by Furchgott and Zawadzki (216), the discovery of NO as endothelium-derived relaxing factor (614), the identification of the endothelium-constricting factor endothelin (ET) (292, 930) and the demonstration of another two vasodilatory gaseous molecules, i.e., CO (215, 489) and H<sub>2</sub>S (306, 961), these endothelial mediators are known to delicately control vascular tone under both physiological and pathological conditions (Table 1).

Hepatic cellular sources of ETs are SEC, stellate cells, and KC (625, 678) (Fig. 4). Of the three isopeptides ET-1, ET-2 and ET-3, ET-1 is the one of predominant significance in vascular physiology, acting via the ET<sub>A</sub> receptor, which results in a constriction of the effector cell. On the contrary, binding of ET to the ET<sub>B1</sub> receptor on endothelial cells may result in a NO-dependent relaxation of adjacent vascular smooth muscle cells and pericytes, while ET<sub>B2</sub> receptor stimulation induces cell contraction. In addition to the local generation of ET, the ET-dependent action strongly depends on the temporal and spatial distribution of ET receptor expression. This was found greatest on hepatic stellate cells, followed by SEC and KC (307) (Table 1). ET causes reversible and graded contraction of isolated stellate cells in culture (634, 680) and narrows the sinusoidal lumen in isolated perfused livers (679, 954) as well as in livers with intact afferent and efferent nerves upon systemic or intraportal infusion (38). Thereby, the ET-1-induced reduction of sinusoidal diameter was found colocalizing with the body of stellate cells, underlining that these liver-specific pericytes serve as contractile targets for ET (954). In addition to this intrasinusoidal site of action of ET, intraportal infusion of ET causes also an intense contraction of perivascular smooth muscle cells and protrusion of endothelial cells into the lumen of pre-terminal portal venules. This indicates that these microvascular segments can function as presinusoidal quasi-sphincters (365).

Based on the concept of a critical balance between vasoconstrictive and vasodilative agents, ET is counteracted by the vasodilating mediators NO and CO (621) (Table 1). Within the normal liver, the gaseous mole-

TABLE 1. *Vasoactive agents, their pathways, cellular source, and distribution in hepatic (patho)physiology*

| Vasoactive Agent             | Function  | Enzyme System | Cellular Source and Distribution | Target Cell                | Pathways                              | References   |
|------------------------------|---|---------------|----------------------------------|----------------------------|---------------------------------------|--|
| Thromboxane A <sub>2</sub>   | Vasoconstriction, platelet activation and aggregation, leukocyte adhesion | COX-1, COX-2  | (S)EC, KC                        | (S)EC, platelet, leukocyte | TxA <sub>2</sub> R                    | Yokoyama et al., 2005  |
| Prostaglandin I <sub>2</sub> | Vasodilation, inhibition of platelet aggregation                          | COX-1, COX-2  | (S)EC                            | (S)EC, HSC                 | PGL <sub>2</sub> R                    | Fennekohl et al., 1999; Yokoyama et al., 2005                              |
| Angiotensin II               | Vasoconstriction  | ACE           | HSC                              | HSC                        | AT <sub>1</sub> subtype               | Rothe and Maass-Moreno, 2000; Yokoyama et al., 2005; Bataller et al., 2000 |
| Nitric oxide                 | Vasodilation  | eNOS          | (S)EC                            | VSMC, HSC                  | sGC                                   | Suematsu et al., 1996; Shah et al., 1997; Pannen, 2002; Oda et al., 2003   |
|                              | Vasodilation  | iNOS          | (S)EC, KC, VSMC, HSC, HC         | VSMC, HSC                  | sGC                                   | Suematsu et al., 1996; Pannen, 2002; Oda et al., 2003                      |
| Endothelin-1                 | Vasoconstriction  |               | (S)EC, HSC, KC                   | VSMC, HSC, SEC, KC         | ET <sub>A</sub> R, ET <sub>B2</sub> R | Rockey et al., 1998; Pannen, 2002; Oda et al., 2003; Housset et al., 1993  |
|                              | Vasodilation  |               | (S)EC, HSC, KC                   | (S)EC                      | ET <sub>B1</sub> R                    | Pannen, 2002; Oda et al., 2003   |
| Carbon monoxide              | Vasodilation  | HO-1          | (S)EC, VSMC, KC, HSC, HC         | VSMC, HSC                  | sGC                                   | Suematsu et al., 1995; Suematsu et al., 1996; Pannen, 2002                 |
|                              |   | HO-2          | HC                               | VSMC, HSC                  | sGC                                   | Goda et al., 1998  |
| Hydrogen sulfide             | Vasodilation  | CSE (CBS)     | HSC, HC                          | VSMC                       | K <sub>ATP</sub> channels             | Fiorucci et al., 2005  |

(S)EC, (sinusoidal) endothelial cells; VSMC, vascular smooth muscle cells; KC, Kupffer cells; HSC, hepatic stellate cells; HC, hepatocytes; HO-1, inducible heme oxygenase; HO-2, constitutive heme oxygenase; sGC, soluble guanylate cyclase; eNOS, endothelial constitutive nitric oxide synthase (type III); iNOS, inducible nitric oxide synthase (type II); CSE, cystathionine  $\gamma$ -lyase; CBS, cystathionine  $\beta$ -synthase.

cule NO is predominantly synthesized by the constitutive NO synthase of endothelial cells (eNOS) of large resistance vessels but also of SEC (727) (Fig. 4). In arteriolar resistance vessels, abluminal NO release from endothelial cells acts in a paracrine fashion. NO diffuses into smooth muscle cells and reacts with the ferrous iron in the heme prosthetic group of the soluble guanylate cyclase (sGC) that increases the concentration of cGMP and activates a cell-signaling pathway, which results in smooth muscle relaxation (682, 816). Under basal conditions, NO regulates the vascular tone of the hepatic circulation in that either L-arginine or NO donors increase liver blood flow, which can be attenuated by NOS or cGMP inhibitors (563, 727, 951). Hereby, NO seems to serve as a potent vasodilator in the hepatic arterial circulation, but exerts only a minor vasodilatory effect in the portal venous bed. This is most probably due to the higher shear stress and shear stress-dependent NO release in the arterial compared with the venous bed (623). With the use of cultured SEC exposed to flow in a parallel-plate flow chamber, it has been shown that these cells respond to an increase of flow with an increased NO production (727). In line with this, NO is released from isolated perfused livers

in a time- and flow-dependent manner (727). Because the hepatic sinusoids contain no smooth muscle layer, it is tempting to speculate that basal and flow-dependent NO release regulates vessel resistance by modulating stellate cells, whose perisinusoidal location is ideal to regulate sinusoidal diameter through contraction and relaxation. This view is supported by the fact that adenoviral gene vector-based expression of neuronal NOS (nNOS) in SEC, stellate cells, and hepatocytes increases NO production and inhibits ET-1-induced contractility of perisinusoidal stellate cells in normal livers (944).

In addition to biliverdin and iron, the second gaseous molecule CO is released through the physiological catabolism of the heme molecule by the microsomal enzyme heme oxygenase (HO). HO consists of two distinct isoenzymes termed HO-1 and HO-2. Under physiological conditions, HO-1, the inducible form, is only found in KC, while the constitutive form HO-2 is distributed to parenchymal cells (Fig. 4, Table 1). There is convincing evidence for the physiological significance of extrasinusoidal CO-mediated mechanisms for microvascular relaxation and that CO derived from HO-2 in hepatocytes contributes to active relaxation of

the sinusoids (234). By using dual-color digital microfluorography, Suematsu et al. (765) could demonstrate after functional blockade of HO-1 by zinc protoporphyrin IX an inhibition of baseline CO generation, an increase of resistance, a discrete pattern of constriction in sinusoids, and a reduction of the sinusoidal perfusion velocity. Because the major sites of constriction corresponded to local sinusoidal segments colocalized with hepatic stellate cells, CO is proposed to function as an endogenous modulator of hepatic sinusoidal perfusion through a relaxing mechanism involving stellate cells (765). Furthermore, CO maintains portal venous vascular tone in a relaxed state, while there is no intrinsic CO-mediated vasodilation in the hepatic artery (623).

In contrast to CO and NO, much less is reported about the role of H<sub>2</sub>S in the regulation of hepatic microvascular blood flow. H<sub>2</sub>S is a gaseous neuromodulator that exerts potent vasodilatory effects in both the systemic and the splanchnic circulation (201, 202). In normal livers, H<sub>2</sub>S treatment, from either exogenous (NaHS supplementation) or endogenous (L-cysteine supplementation) sources, reverses the norepinephrine-induced increase of portal pressure (201). Unlike NO, H<sub>2</sub>S is not produced by SEC, as they express none of the two H<sub>2</sub>S-generating enzymes [cystathionine- $\gamma$ -lyase (CSE) and cystathionine- $\beta$ -synthetase (CBS)], but is released from the CSE-expressing stellate cells (201) (Fig. 4, Table 1). The fact that H<sub>2</sub>S released by normal rat livers is not modified by an increased shear stress underscores that sites different from that of SEC are involved in the synthesis of H<sub>2</sub>S (201). This is in line with the observation that modulation of intrahepatic resistance by H<sub>2</sub>S is not dependent on NO (201). Although endothelial dysfunction caused by hyperhomocysteinemia comprises defective NO bioavailability, homocysteine-induced impairment of NO release can be reversed by H<sub>2</sub>S in a NO-independent manner, indicating that NO and H<sub>2</sub>S exert additive effects in the liver microcirculation (154).

In aggregate, the major site of blood flow regulation through sinusoids is recognized to reside in the sinusoids themselves, where the most pronounced blood pressure drop occurs in the liver. The interplay of the cellular components and all mediator systems is extraordinary complex. NO and CO may exert synergistic vasodilatory effects due to their common stimulation of sGC. Furthermore, NO may either attenuate or enhance HO-1 gene expression (301, 571). NO and CO can functionally antagonize the vasoconstrictive effects of ET (767). NO limits the ET-1 release from endothelial cells (71) and is capable of terminating ET signaling (235). Finally, NO is a physiological modulator of the endogenous production of H<sub>2</sub>S by increasing the expression and activity of CSE (961).

#### D. Changes of the Microcirculation During Development and Ageing

The anatomy and morphology of the hepatic microvascular bed underlies distinct changes during postnatal development and ageing. Corrosion cast procedures and scanning electron microscopy in neonatal rats revealed the development of a simple capillary network directly pouring into the portal vein towards a peribiliary vascular plexus being composed of arteries and veins in the outer layer and of capillary vessels in the inner layer (272). In parallel, the intrahepatic biliary tree matures within the first 4 wk of newborn rats. The hepatic artery gradually develops. One-day-old animals show small branches of the hepatic artery ending in sinusoids or capillary plexuses around portal vein branches near the hilus. After 4 wk, branches appear up to the peripheral portal networks (272). Survey of the rat liver architecture by *in vivo* fluorescence microscopy demonstrates a progressive growth of the lobular areas with a proportional increase of post-sinusoidal venules from early juvenile to adult (867). In weanling rat livers, lower sinusoidal resistance due to shorter sinusoidal pathways is countered by the smaller caliber of the sinusoids so that overall sinusoidal resistance seems to be not age dependent (160). In addition, age does not affect sinusoidal density and dispersion number, because 24-mo-old rats are reported to present a sinusoidal perfusion rate above 98%, similarly as observed in juvenile rats (867, 888). On the contrary, a 14% reduction in the number of perfused sinusoids with a 35% decrease of sinusoidal blood flow is reported for 27-mo-old senescent mice that might be due to species differences or different age groups (327). In addition to microhemodynamics, age also affects the endocytotic capacity of sinusoidal endothelial cells in mice (327) and rats (471). The age-related thickening and defenestration of the liver sinusoidal endothelial cell, as well as the sporadic deposition of collagen and basal lamina in the extracellular space of Disse, known as "pseudocapillarization" (470) further impair the hepatic clearance capacity in the elderly (471). In addition, loss of fenestrations hampers transfer of lipoproteins from blood to hepatocytes and might explain the impaired hepatic lipoprotein metabolism with old age (469).

### III. TECHNIQUES FOR THE STUDY OF THE HEPATIC MICROCIRCULATION

Measuring blood flow to the liver is fundamental for the understanding of the organ's physiology and pathophysiology. However, it comprises several challenges due to the fact that the liver blood supply is dual, the ratio of portal venous to hepatic arterial flow is not constant, and the direct access to the vessels is difficult. In the follow-

ing, quantitative techniques that are frequently applied in experimental research and clinical settings are addressed.

### A. Radioactive, Colored, and Fluorescent Microspheres Technique

The microspheres technique is relatively simple and extraordinarily accurate, if appropriately applied. Typically, carbonized insoluble plastic microspheres, labeled with radioactive gamma nuclides, such as  $^{141}\text{Ce}$ ,  $^{51}\text{Cr}$ ,  $^{85}\text{Sr}$ ,  $^{95}\text{Nb}$ , and  $^{46}\text{Sc}$ , can be used (126, 857). The method is based on the indicator fractionation principle. A known number of radioactive microspheres is injected into the left ventricle, and a reference sample is simultaneously withdrawn from a peripheral artery using a pump. The injected radioactive microspheres are trapped in the various vascular beds in the body in proportion to the fraction of the cardiac output supplying the individual vascular bed. Upon removal of the organ, radioactivity in the organ samples is assessed by a gamma scintillation counter as radioactive counts per minute (CPM). Organ blood flow ( $Q$ ) is then calculated as follows:  $Q = \text{organ radioactivity} \times \text{reference blood flow} / \text{reference blood radioactivity}$ .

With the use of this technique, only hepatic arterial blood flow can be measured, while portal venous flow must be estimated by adding the separate flows to the splanchnic organs that drain into the portal vein (858).

For the technique to work, the microspheres must be trapped on the first circulation, be evenly distributed, and should have no effect on the liver blood flow and function. In general,  $15\text{-}\mu\text{m}$ -diameter microspheres satisfy both the conditions of distribution similar to red blood cells and the absence of significant nonentrapment (125, 857). Compared with larger microspheres,  $15\text{-}\mu\text{m}$ -diameter microspheres are distributed more like red blood cells, obstruct less the vascular bed, are less variable in size, and can be given in significantly greater numbers, enhancing the statistical precision of the measurements (291). Only a small number of  $15\text{-}\mu\text{m}$  microspheres fail to be trapped in the liver (142, 890). Uniform mixing of the microspheres already at the aortic root is best achieved by injection into the left atrium (dog, sheep, pig) (268) or the left ventricle (rodents) (126, 857). Evenness of microsphere mixing can easily be assessed by comparison of the number of spheres per gram tissue in the left and right cerebral hemisphere or the left and right kidney, which should not differ by  $>10\%$ . Calculation of the number of spheres injected should consider that 384 microspheres must be present in the portion of the organ studied to have a distribution variability within 10% of the mean distribution at the 95% confidence level (291). The more microspheres injected, the higher the precision of measurement is, but also the risk for deterioration of the systemic or regional circulation.

Although the microspheres technique is still regarded as the gold standard to assess nutritive organ perfusion (ml per min and g tissue), the technique requires post-mortem removal of organs and, thus, has no clinical application. The additional limitations of radiation and environmental pollution had meanwhile been overcome by the fabrication of fluorescent or colored microspheres (242, 641) (Fig. 5). By simultaneous blood flow measurement with radionuclide-labeled and colored microspheres, it was shown that the techniques produced comparable values of liver blood flow (39). Currently, the fluorescent microspheres technique seems to be the most promising nonradioactive microspheres technique, because the use of nonradioactive microspheres saves money, facilitates the use of microspheres in chronic animal experiments, and, using histological techniques, allows the assessment of particle distributions in subunits of organs (642). The fluorescent microspheres technique has a high sensitivity and a good spectral separation. Thus the number of microspheres injected can be as small as that used for radioactive microspheres, and at least six labels can be measured simultaneously. In addition, the relatively large volume in which fluorescence is measured

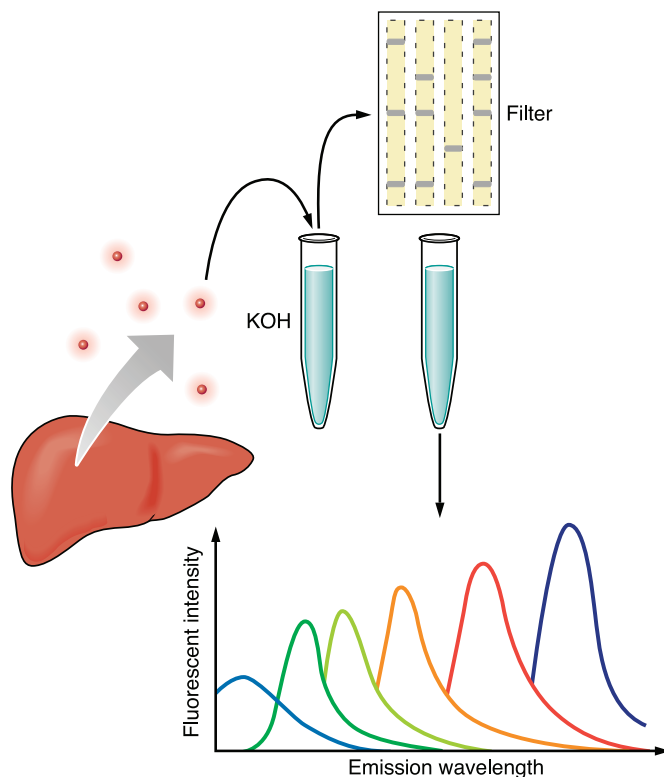


FIG. 5. Microspheres technique for the determination of hepatic arterial blood flow. Upon entrapment of colored or fluorescent microspheres within the liver, the organ is removed and cut into small pieces for subsequent digestion with potassium hydroxide. After microfiltration, the dyes are extracted from the microspheres with a known volume of solvent. Spectrometry allows for separation of the various colors and their concentration.

(~1–3 ml) enables the use of time-saving microsphere isolation techniques and further automation of the quantification process (using either automated spectrometry or FACS analysis) (642) (Fig. 5).

## B. Hydrogen, Xenon, and Indocyanine Green Clearance Technique

Inert gas clearance has been used for more than 30 years to measure hepatic blood flow (507). Injection of a saline solution of  $^{133}\text{Xe}$  is usually made via the portal vein, and the resulting hepatic clearance is monitored with a Geiger-Müller tube, scintillation crystal, or gamma camera (368, 507, 532). Accuracy of flow measurement is critically dependent on the knowledge of the partition coefficient of the gas used. The fact that the gas partition coefficient remains unaffected by liver diseases makes the technique an attractive tool for the measurement of hepatic hemodynamics in humans.

Beside portal venous application, there are reports introducing the intrasplenic (445) and the hepatic intraparenchymal application of  $^{133}\text{Xe}$  for measurement of hepatic blood flow (352, 491). Nowadays, nonradioactive gases, like hydrogen ( $\text{H}_2$ ) or xenon, are commonly administered with the respiration gas. They distribute instantaneously until the tissue reaches saturation. Then the supply of the gases is turned off, and their clearance rates are determined polarographically through platinum electrodes placed on or into the liver to a depth of 3–6 mm (783). The  $\text{H}_2$  clearance technique has been successfully applied in various experimental models for the study of liver blood flow in rats, dogs and pigs (304, 436, 584, 783, 790). In clinical practice, the temporal changes in radiographic enhancement produced by xenon gas inhalation are measured by sequential computer tomography and then the time-dependent xenon concentrations within the various tissue segments are used to achieve local liver blood flow maps (263, 264, 735).

Next to the measurement of the products of liver synthesis, a second approach of assessing liver function is to monitor hepatic clearance function using sulfobromophthalein or indocyanine green (ICG). In addition to serving as an objective test of hepatocellular function (75), ICG clearance has been considered to represent a valid method for determination of hepatic perfusion. In support of this view, simultaneous use of the radioactive microspheres technique, the laser Doppler flowmetry, and the ICG clearance method have revealed similar values for hepatic blood flow in an experimental setting of hemorrhagic shock and resuscitation (884, 885). Of interest, hepatic ICG clearance can be measured directly using near-infrared spectroscopy (see sect. III E). By this, significant positive correlations can be obtained between hepatic ICG rate of uptake and both hepatic blood flow and

microcirculation (172). In addition, ICG uptake measured directly by NIRS is sensitive to assess reduced liver microvascular blood flow in cirrhosis, and its excretion correlates with the degree of liver parenchymal dysfunction (356). Of interest, ICG clearance is also inversely correlated with the collagen content of the liver (846), indicating that the reduction of the microcirculation is part of the manifestation of the disease.

Experimental studies have shown that the blood flow measured by the  $\text{H}_2$  gas method was only 39% of the calculated blood flow by the ICG method. This indicates that the  $\text{H}_2$  gas clearance technique may be a method that assesses hepatic arterial liver perfusion rather than total hepatic blood flow (243). Moreover, ICG clearance is thought to assess only 30% of the blood flow, which was measured electromagnetically by direct placement of flow probes around the hepatic artery and portal vein (138). This may be due to a significant amount of shunting of electromagnetically measured blood flow which is not detected by the ICG method. Overall, these results underline the challenge to accurately assess liver blood flow, which is mainly due to the dual blood supply and the particular interrelation of these two vascular systems.

## C. Multiple Indicator Dilution Technique

The description of the indicator dilution principle to calculate flow and volume goes back to Stewart in 1894 (760) and is discussed with respect to its theoretical foundation by Meier and Zierler (549). In brief, an indicator is injected into an organ with one inflow and one outflow. Observations of the concentration  $C$  as a function of time  $t$  of the indicator in the outflow permits the calculation of flow ( $F$ ), i.e.,  $F = q_0/C(t) \times dt$ , where  $q_0$  is the amount of indicator injected. Typically, the technique comprises the combination of diffusible substances apt to leave the capillaries together with the inclusion of a vascular reference substance (660). Goresky (239) introduced this method to the study of the circulation in the liver and described a linear method to calculate sinusoidal and extravascular volumes (Fig. 6). Due to the specific anatomy of the hepatic sinusoids with lack of a basement membrane and the presence of fenestrae, extravascular substances such as albumin and sucrose are freely diffusible, establishing the pattern of flow-limited multiple dilution curves as opposed to the barrier-limited pattern (238). The technique allows calculating not only volumes of distribution, but also to study transport of hormones, drugs, organic and anorganic ions, bile acids, and metabolic substances (240, 618, 715, 895).

Although over the last three decades there has been major progress made in the field of modeling the liver for investigating the kinetic behavior of substrates, drugs, and metabolites (812), the multiple indicator dilution

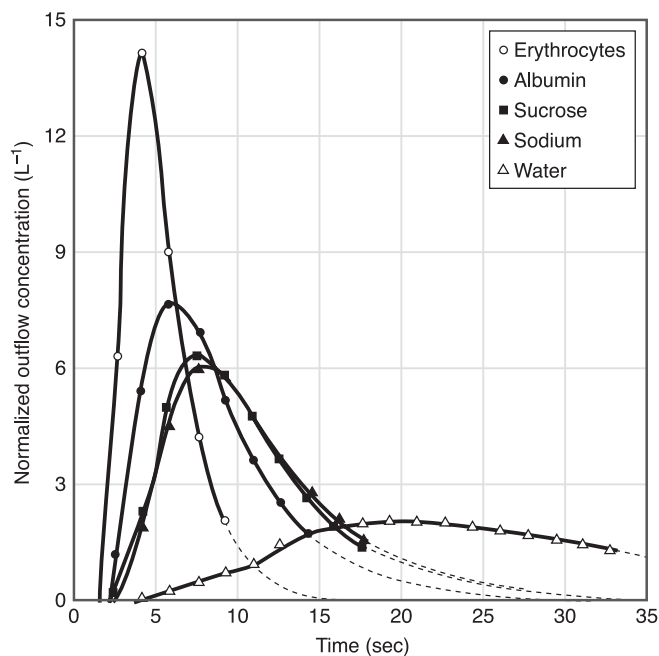


FIG. 6. Multiple indicator dilution technique for assessment of liver blood flow. Displayed are outflow profiles for  $^{51}\text{Cr}$ -labeled erythrocytes (white dot), Evans blue as an indicator for albumin (black dot),  $[^{14}\text{C}]$ sucrose (black square),  $^{22}\text{Na}^+$  (black triangle), and  $^3\text{HOH}$  (white triangle) in the liver of an anesthetized dog. Broken lines represent monoexponential extrapolation of data to correct for recirculation. [From Goresky (239).]

technique is not universalized. Surprisingly, relatively few studies have investigated this method which, for instance, is ideally suited to probe the permeability properties of hepatic sinusoids and to assess receptor-binding kinetics within the hepatic microcirculation (87, 148, 308, 311, 853). By means of multiple indicator dilution technique, Dupuis et al. (164), investigating the hepatic binding of tracer  $^{125}\text{I}$ -labeled ET-1, could demonstrate that there is both substantial clearance and production of ET-1 by the intact liver and that ET-1 clearance is mediated by the  $\text{ET}_B$  receptor with the presence of reversible, nonspecific ET-1 binding at the liver surface. As an extension of the latter observation, it was demonstrated that ET-1 markedly decreases extravascular albumin space in both controls and cirrhosis (659) and that cirrhosis reduces ET-1 clearance probably by capillarization of hepatic sinusoids and reduced access to  $\text{ET}_B$  receptors. This contributes to the increase of circulating ET-1 levels in chronic liver disease (817).

#### D. Laser Doppler Flowmetry

The Doppler effect, named after Christian Doppler, is the change in frequency and wavelength of a wave for an observer moving relative to the source of the waves (669). Laser Doppler flowmetry is an established technique for

the real-time measurement of microvascular red blood cell perfusion in tissue, including the liver (14). The technique relies on the assessment of the Doppler shift in wavelengths of reflections from particles moving with the flow. It works by illuminating the tissue under investigation with low-power laser light from a solid-state diode laser operating at  $\sim 780$  nm, which is guided to the measurement site via an optical fiber. Two identical adjacent fibers receive backscattered light from the tissue which is then transmitted to independent photodetectors (14). This back-scattered portion consists of light scattered from the static tissue matrix which has not been Doppler shifted and a spectrally broadened component resulting from interactions with moving cells. Optical mixing of these components at the photodetector surface produces an electrical signal containing the Doppler shift information. The readout signal is defined as microvascular perfusion (red blood cell flux) = number of cells moving in the tissue sampling volume  $\times$  mean velocity of these cells.

Red blood cell flux is dependent from hematocrit and red blood cell velocity. It has been shown to respond linearly to velocity-mediated flow changes, while increases in tissue red blood cell concentrations are underestimated (14). The ease of operation and the continuous and real-time measurements with a high degree of spatial resolution are considerable advantages of laser Doppler flowmetry over other techniques (797, 897). However, the major limitation of the technique is that laser Doppler devices do not assess absolute blood flow, as different tissues present with different optical properties, but, instead, provide measurement of arbitrary blood perfusion units (BPU). Moreover, the actual sampling volume and the depth at any tissue site cannot be assessed, which definitely limits its quantitative use. Furthermore, baseline values are very variable and, thus, results should be expressed in general as changes relative to baseline (859).

However, comparison between laser Doppler flowmetry and intravital fluorescence microscopy revealed a significant correlation between red blood cell flux and sinusoidal perfusion rate, indicating a reliable assessment of the hepatic microperfusion by this noninvasive technique (405, 637, 798, 864). In line with this, it has been shown that the laser Doppler signal primarily depends on flow in the sinusoids, and not on flow in the efferent portal venules and central veins (798). In addition, a strong correlation between the laser Doppler flowmetry signal and the total liver blood flow (14) and the portal venous and hepatic arterial flow (23) have been demonstrated. In addition to the analysis of the hepatic microcirculation, the laser Doppler technique may also be used to study flow in larger blood vessels, such as the inflow or outflow vasculature of the liver. Accordingly, laser Doppler flowmetry probes were successfully placed onto the hepatoduodenal ligament for measurement of portal ve-

nous inflow changes upon extended hepatectomy (169, 231).

### E. Near-Infrared Spectroscopy

Near-infrared (650–1,100 nm) spectroscopy (NIRS) is a relatively new, noninvasive technique that uses the principles of light transmission and absorption and that allows a continuous monitoring of changes in intravascular (hemoglobin) and mitochondrial (cytochrome  $aa_3$ ) oxygenation in relatively large tissue samples of 2–6 cm<sup>3</sup> (199, 488). The technique depends on the relative transparency of biological tissue to light in the near-infrared region of the spectrum. In contrast to light at visible wavelengths of 450–700 nm, which is strongly reduced in tissue and which can only penetrate a maximum distance of a few millimeters, absorption of light by the tissue chromophores is significantly lower at near-infrared wavelengths of 700–1,000 nm. Sensitive detectors can assess light that has transversed up to 80 mm of tissue (44). There are three main compounds in the liver whose near-infrared absorption characteristics vary with their oxygenation status, i.e., oxyhemoglobin, deoxyhemoglobin, and cytochrome oxidase. The application of NIRS to monitor liver oxygenation has been extensively validated and allows to compute absolute changes in oxyhemoglobin, deoxyhemoglobin, and cytochrome oxidase in micromoles per liter of hepatic tissue (174, 175, 931).

NIRS-assessed cytochrome oxidase (intracellular oxygenation) has been shown to correlate with intracellular  $\beta$  nucleoside triphosphate under graded hypoxia of rat livers (718). It further correlates with the parameters of hepatocellular injury to a higher degree than HbO<sub>2</sub> (extracellular oxygenation) (173). Thus NIRS-assessed cytochrome oxidase reflects cellular ATP metabolism (718). Application of NIRS optodes across the right hepatic lobe of piglets in endotoxemic shock indicates significant correlations between the perfusion parameters and the NIRS readings. This is reflected by a simultaneous decrease of oxyhemoglobin readings and liver oxygen delivery, liver blood flow, and cardiac output. Vice versa, deoxyhemoglobin readings highly correlate with mixed venous lactate and with hepatic vein lactate (577, 578). In humans, intraoperative NIRS is used to determine hemoglobin and cytochrome oxidase content as parameters of perfusion and oxygenation in hepatic tissue (604). NIRS enables quantification of the congestion and impairment of mitochondrial redox state in the anterior segment of a right lobe liver graft caused by deprivation of the middle hepatic vein tributaries (561, 604). In addition, measurement of hepatic ICG uptake by NIRS reveals impairment of sinusoidal perfusion in the veno-occlusive regions of living donor livers (278). Thus NIRS might represent a valuable tool for assessing the indication for venous recon-

struction in living-donor liver transplantation and split donor liver transplantation.

### F. In Vivo Fluorescence Microscopy

The first detailed description of intravital microscopy dates back to the middle of the second last century, when Waller described the passage of leukocytes through microvessels in the frog tongue (878). Later, because of its ease of exposure and its high translucency, the mesentery was acknowledged as a suitable object for the study of microvascular morphology and function (975). Advanced optical imaging in combination with video technology has not only improved imaging quality at up to 1,000-fold magnification using both trans- and epi-illumination technique, but has also considerably widened the spectrum of intravital microscopy, being applied in almost all organs of the body, including the liver (550, 553).

Knisely (399, 400) was the first who reported on a method of illuminating living structures of the liver for microscopic study. He presented the use of properties of fused quartz for conducting light by internal reflection from one end of a rod of this material to the other end (399). During the following 20 years, ~6,000 animals have been analyzed to establish the basic concepts of the living anatomy and certain aspects of the physiology and pathology (for review, see Ref. 55). Of this number, about two-thirds have been frogs, while the other third constituted of rats, mice, bats, guinea pigs, rabbits, dogs, and monkeys. In 1948, Knisely, Bloch, and Warner (397) reported on the regulation of blood flow through the frog liver and presented a schematic drawing of the frog liver lobule, displaying vascular structures, which all could be reidentified and confirmed by subsequent studies. Using also the fused quartz rod transillumination method, Irwin and MacDonald (323) reported on the transillumination of the lower edge of livers of anesthetized guinea pigs, particularly emphasizing the obstacles of this approach. By means of intratracheal insufflation of oxygen and specific surgical exposure of the liver, they overcame the respiration-associated movement of the liver as the factor limiting the resolution of the optical system and provided the first quantitative data on diameters of the various intrahepatic vessels (323). In 1955, Bloch (55) added a few modifications, achieving a twofold increase of magnification. Although the basic concept of the vascular pattern of liver lobules was derived from these transillumination studies, the technique is restricted to the thin edges and boundaries of the liver, because only these tissue portions allow enough light to transmit for visualization of microscopic blood vessels. As a consequence, the number of microvascular segments accessible for intravital microscopic studies is strictly limited. Accordingly, the description of leukocyte adherence (661) and phagocytosis (54)

has been only qualitative in nature. Even in case of quantitative analysis of the transilluminated liver (415, 661, 662), results should be interpreted with caution, because the areas within the liver periphery present with a considerable extent of nonperfused sinusoids already under baseline conditions (167) and cannot be considered representative for the liver microcirculation in general.

With the introduction and use of fluorescent markers, the spectrum of hepatic *in vivo* microscopy could be widened from the transillumination approach to the epi-illumination technique (Fig. 7). Moreover, the availability of an enormous number of different fluorescent markers for *ex vivo* and *in vivo* staining has extended the possibilities of intravital microscopy from purely morphological analysis to the study of complex physiological and pathological events. Apart from all changes in microhemodynamics, i.e., sinusoidal perfusion, sinusoidal diameter, blood cell velocity and flux, as well as volumetric blood flow, various cellular and molecular aspects can be studied (Fig. 7). The quantitative analysis of the hepatic microcirculation includes the perfusion of the terminal afferent vessels, the sinusoids and the postsinusoidal venules, as visualized after intravenous administration of sodium fluorescein (859) or fluorescein isothiocyanate (FITC)-labeled macromolecular substances, such as albumin (112) and dextran (123, 124, 556, 761) (Fig. 7A). Depending on the molecular weights of the individual compounds and on the differences in secretion rates exhibited by hepatocytes in different acinar zones, the kinetics of distribution of the fluorescence across the lobule markedly differ (731). While sodium fluorescein with a

molecular weight of 371 almost freely diffuses from the intravascular sinusoidal towards the extravascular space, there is significant intravascular retention of FITC-dextrans with calculated effective diffusion coefficients, being 2.5 times larger for dextrans with <66,000 molecular weight compared with dextrans of 156,000 molecular weight. In addition to analysis of perfusion, the use of high-molecular-weight dextrans is further suited to study the macromolecular trans-sinusoidal exchange capacity of the liver, which is typically limited upon fibrosis- and cirrhosis-associated deposition of extracellular matrix and thus increased diffusion hindrance (875).

The quality of sinusoidal blood flow can be quantitatively assessed by determining the sinusoidal perfusion rate or the functional density of sinusoids. The sinusoidal perfusion rate reflects the number of perfused sinusoids in percentage of all sinusoids. The functional sinusoidal density represents the length of red blood cell perfused sinusoids per square centimeter area of observation. The recording and assessment of these two parameters, however, are time-consuming. A more easily applicable and time-sparing procedure is the usage of the nuclear marker bisbenzimidazole (H333342; 2  $\mu\text{mol/kg}$  iv) with near ultraviolet epi-illumination to assess hepatic microvascular perfusion failure by planimetric analysis of faint hepatocellular staining in areas of nonperfused sinusoids (870). Application of bisbenzimidazole further allows for the visualization of hepatocyte distribution *in vivo* and can be used for detailed morphological analysis of proliferating hepatocytes undergoing either hyperplasia or hypertrophy (6).

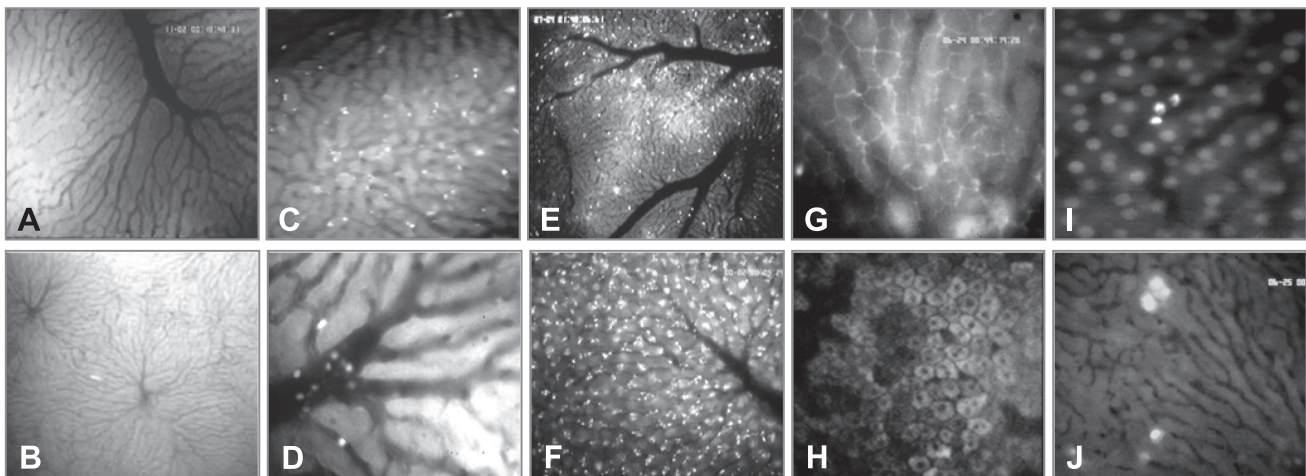


FIG. 7. Intravital fluorescence microscopy of the rat liver in epi-illumination technique. This high-resolution real-time technique allows to visualize the hepatic sinusoidal perfusion (A) and parenchymal NADH autofluorescence (B), platelet (C) and leukocyte (D) adhesive interaction, Kupffer cell phagocytic activity (E) and hepatic stellate cell-associated vitamin A distribution (F), biliary canalicular secretion (G) and hepatocellular transport (H), as well as apoptotic (I) and necrotic cell death (J). A: blue light epi-illumination and sodium fluorescein. B: hepatic autofluorescence upon near ultraviolet epi-illumination. C: blue light epi-illumination and *ex vivo* BCECF-stained platelets. D: green light epi-illumination and *in vivo* acridine orange-stained leukocytes. E: blue light epi-illumination and yellow-labeled latex beads. F: near ultraviolet light epi-illumination and vitamin A autofluorescence. G and H: blue light epi-illumination and sodium fluorescein. I: near ultraviolet light epi-illumination and bisbenzimidazole. J: green light epi-illumination and propidium iodide. Magnification: B and E,  $\times 100$ ; A, C, D, F, G, I,  $\times 200$ ; H, J,  $\times 400$ .

Intravital fluorescence microscopy further allows estimating hepatocellular transport after application of sodium fluorescein or FITC-labeled bile salts. Along with the differences in secretion rates exhibited by hepatocytes in different acinar zones, the sinusoid-to-bile transport times markedly differ among fluorescent compounds and can be used for the study of hepatocyte function and biliary secretion by imaging hepatocellular brightness (Fig. 7H) and the accumulation of the fluorescent dye within the fine canalicular network (731) (Fig. 7G). In addition to that, intra-arterial injection of fluorescently labeled latex particles allows for the assessment of the phagocytic activity of the KC in that calculation of the clearance of beads from the circulating blood (fraction of nonadherent particles over time) nicely reflects KC phagocytosis (508, 636, 707, 747, 861, 871) (Fig. 7E). Most recently, fluorescent-labeled liposomes consisting of phosphatidylcholine and phosphatidylserine have been described as a useful research tool to intravital stain and to clearly delineate KC using both confocal laser scanning microscopy and *in vivo* microscopy (891).

In addition, the intravenous application of acridine orange and rhodamine-6G allows for the *in vivo* staining of leukocytes and platelets and, thus, for the quantitative analysis of the flow behavior along the microvascular path (347, 406) and the interaction of these cells with each other and with the endothelial lining of the microvasculature (380, 474, 551, 865) (Fig. 7D). There is evidence for a zonal gradient of leukocyte velocity in the liver sinusoids, with increasing velocity from zone 1 to zone 3 due to the wider diameters, the lower resistance, and the less tortuous path in zone 3 (406). In addition to *in vivo* staining, circulating blood cells, in particular platelets, can also be isolated and stained *ex vivo* (874). For this purpose, dyes, such as bis-carboxyethylcarboxyfluorescein (BCECF) and calcein, are used, providing an appropriate bright staining of the cells (168) (Fig. 7C). Moreover, *ex vivo* FITC-stained erythrocytes are used for the determination of red cell velocity and can be stored at 4°C after addition of citrate-phosphate dextrose (1.4:10) up to 5 days. Prior to microscopy, cells are diluted 1:1 in saline and are visible as single bright dots (969).

Another potential of *in vivo* fluorescence microscopy is to monitor NADH fluorescence of liver parenchymal tissue (74, 770, 856) (Fig. 7B). Chance and co-workers (95, 96) pioneered the fluorescence property of NADH as an indicator of mitochondrial redox state and, in the presence of sufficient substrate and phosphate, as an indicator of cellular oxygen. NADH is a naturally occurring intracellular fluorophore and one of the main means of transfer energy from the tricarboxylic acid cycle to the respiratory chain in the mitochondria (317). Inhibition of the respiratory chain due to inadequate oxygen supply is reflected by increased intracellular NADH levels. Upon ultraviolet epi-illumination of tissue, NADH, unlike  $\text{NAD}^+$ ,

fluoresces in blue (317). Experimental studies in rats undergoing hemorrhagic shock and resuscitation revealed significant correlations between NADH fluorescence and both hepatic tissue oxygenation and hepatic bile flow (856). A further approach to study the metabolic state of the liver by intravital microscopy is the intravenous administration of the oxygen-sensitive fluorescent dye tris(1,10-phenanthroline)ruthenium(II) chloride hydrate  $[\text{Ru}(\text{phen})_3^{2+}]$  (628). In contrast to NADH, reflecting the mitochondrial redox state and the activity of the mitochondrial electron transport chain,  $\text{Ru}(\text{phen})_3^{2+}$  fluorescence is directly dependent on the tissue  $\text{P}_{\text{O}_2}$ . Thus the simultaneous use of both fluorimetric methods may provide important information about mechanisms of tissue hypoxia with differentiation between oxygen supply and oxygen utilization deficits (628).

In addition to the diffuse NADH autofluorescence of parenchymal tissue, stellate cells are easily visualized by ultraviolet epi-illumination because of the autofluorescence of stored vitamin A (766, 870) (Fig. 7F). Under continuous near-ultraviolet excitation, there are multiple patchy activities along the sinusoids and terminal hepatic venules, which correspond to the fat-storing intracellular droplets of stellate cells. These fluorescent activities show a rapid photobleaching phenomenon and can completely be eliminated by a vitamin A-deficient diet-induced depletion of intrahepatic retinoid contents. Vice versa, repeated administration of vitamin A significantly enhances the patchy fluorescent activities (766, 863). Moreover, it has been shown that hepatic stellate cell-associated area of ultraviolet vitamin A-autofluorescence increases with age and significantly correlates with increasing tissue concentrations of vitamin A metabolites (867). Thus intravital fluorescence microscopy enables the *in vivo* assessment of stellate cells, which are uniformly distributed throughout the liver under physiological conditions. In contrast, there is a striking redistribution and accumulation of stellate cells in fibrous septa upon  $\text{CCl}_4$  exposure of rats, which is associated with a subsequent loss of the vitamin-A storing function due to their transformation into myofibroblast-like cells (873).

More recent developments further allowed extending the microcirculatory analyses with the intravital microscopic assessment of cell viability, differentiating between apoptotic and necrotic cells. Apoptotic cell death can be determined *in vivo* after staining the nuclei of hepatocytes by bisbenzimidazole (H33342; 2  $\mu\text{mol/kg}$  iv) using a near-ultraviolet filter system (330–380/>415 nm) (Fig. 7I). Characteristic signs of cell apoptosis which can be detected by the H33342 staining are condensation, fragmentation, and margination of nuclear chromatin. These can be analyzed quantitatively, providing the number of apoptotic cells per square millimeter observation area (176, 705). To distinguish from apoptotic cell death, propidium iodide can be applied for staining of necrotically

damaged hepatocytes (68, 784, 964) (Fig. 7J). By combining propidium iodide for assessment of necrotic cell death with rhodamine-123 for assessment of mitochondrial depolarization, it could be shown that hepatocytes taking up rhodamine-123 fail to exclude propidium iodide, indicating that depolarization precedes cell death (964).

Fluorescent pH-sensitive probes, such as BCECF, allow for the *in vivo* measurement of liver tissue pH, although spectra may considerably vary depending on the blood content of the illuminated area and the time range in which the measurement is performed (151). With the use of digitized video microscopy and single, cultured rat hepatocytes, quantification of cytosolic pH and vesicular pH is reported by ratio imaging of the pH-sensitive BCECF fluorescence and FITC-dextran fluorescence (713). In addition, digital imaging fluorescence microscopy allows for assessment of changes in cytosolic free calcium by fura 2 loading of hepatocytes (792). In conclusion, due to the high versatility, we feel that intravital fluorescence microscopy is the most ideal tool 1) to analyze complex biological interactions and dynamic hepatic disease mechanisms and 2) to develop and test novel prophylactic and therapeutic approaches aimed at the disruption of microcirculatory and microvascular pathology in liver diseases.

### G. Orthogonal Polarized Spectral Imaging

In orthogonal polarized spectral (OPS) imaging, the tissue is illuminated with linearly polarized light and imaged through a polarizer oriented orthogonally to the plane of the illuminating light (Fig. 8). The OPS technique delivers images of the microcirculation that are comparable to those achieved with intravital fluorescence microscopy (252, 449), but without the use of fluorescent dyes. In rats undergoing hepatic I/R, OPS imaging has been shown to allow accurate quantification of the sinusoidal perfusion rate, vessel diameter, and venular red blood cell velocity. There was a significant correlation of microcirculatory parameters between OPS and *in vivo* fluorescence microscopic imaging (449). For optimal imaging with the OPS technique, a wavelength at which oxy- and deoxyhemoglobin absorb equally (548 nm, isobestic point) is chosen. As a consequence, blood vessels of the microcirculation can be visualized as in intravital transillumination microscopy (252). OPS imaging relies on the absorbance of hemoglobin to create contrast and, thus, is dependent on the presence of red blood cells within the microvessels to be visualized. The power of the system is sufficient to resolve a single red blood cell and individual capillaries with a diameter of  $\sim 5 \mu\text{m}$ .

Apart from the fact that OPS imaging is useful for the quantitative determination of local hematocrit using the

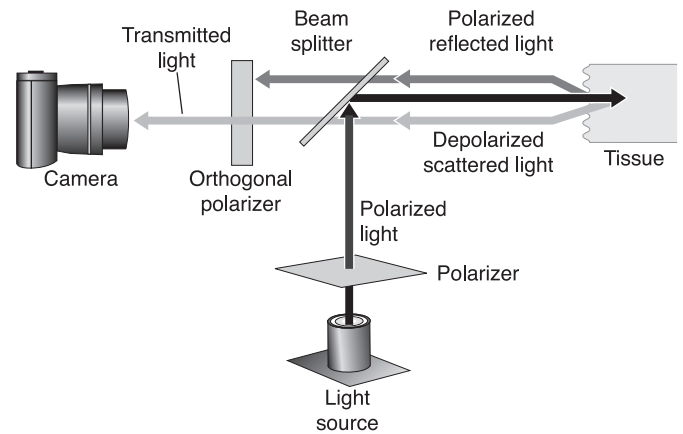


FIG. 8. Schematic representation of the technique of orthogonal polarized spectral (OPS) imaging. Light passes a spectral filter to isolate the wavelength region and is then linearly polarized and reflected toward the target tissue by a beam splitter. By means of a focusing objective lens, a region of  $\sim 1 \text{ mm}$  in diameter of the target tissue is illuminated. The identical lens collects the remitted light, which forms the image of the illuminated region upon the charge-coupled device camera. Hereby, the orthogonal polarizer in front of the camera increases efficacy in that one orthogonal polarization state is reflected while the other is transmitted.

optical density approach in epi-illumination, this noninvasive, small and easily portable, hand-held imaging technology represents a new diagnostic tool for the study of human microvascular physiology and pathology. With the application of OPS imaging in four different regions of interest of the left and right liver lobes in 11 healthy individuals before partial liver resection for living-donor liver transplantation (Fig. 9), OPS imaging could for the first time demonstrate *in vivo* human physiological values of sinusoidal red blood cell velocity of  $0.97 \pm 0.43 \text{ mm/s}$ , mean sinusoidal diameters of  $8.8 \pm 0.9 \mu\text{m}$ , sinusoidal volumetric blood flow of  $58.2 \pm 9.6 \text{ pL/s}$ , intersinusoidal distance of  $22.6 \pm 2.5 \mu\text{m}$ , and functional sinusoidal density of  $391 \pm 30 \text{ cm}^{-1}$  (648). In the setting of hepatic I/R, OPS imaging further proved to be a valuable tool to assess the deterioration of the hepatic microcirculation, which represents a determinant for graft dysfunction (646, 647, 709) (Fig. 9D). Using this technology, liver surgeons will be able to monitor development and progression of microcirculatory disorders as well as effects of treatment on the hepatic microcirculation.

### H. Sidestream Dark-Field Imaging

Sidestream dark-field (SDF) imaging is the successor of OPS imaging. It consists of a light guide, surrounded by diodes, emitting 530 nm light. This is absorbed by the hemoglobin of red blood cells, allowing their observation as dark cells in the microcirculation. The diodes at the tip of the guide are optically isolated from the inner image-conducting core, and pump light deep into the tissue,

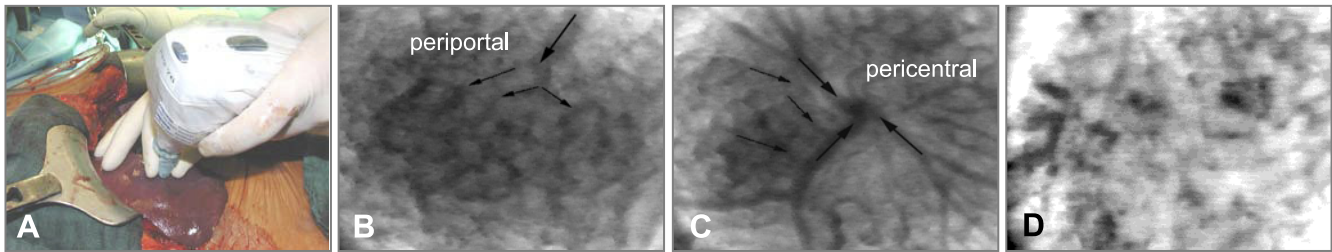


FIG. 9. OPS imaging for assessment of human hepatic microcirculation. *A*: the OPS probe is positioned on the liver surface of a healthy individual before partial liver resection for living-donor liver transplantation. The OPS probe is equipped with a sterile cap and is easily held by hand for imaging. Representative OPS images of the hepatic microcirculation before liver resection (*B* and *C*), displaying the blood flow direction (arrows) from periportal to pericentral sinusoids. *D* illustrates the human hepatic microcirculation upon transplantation, revealing the I/R-induced sinusoidal perfusion failure. *B–D* magnification  $\times 465$ . [From Puhl et al. (648).]

illuminating the microcirculation from within. This dark-field illumination applied from the side avoids tissue surface reflections and thus enables very clear images of the microcirculation with visualization of not only red, but also white blood cells (318). So far, there is one application of SDF imaging to study rat liver microcirculation, revealing a mean functional sinusoidal density of  $402 \pm 15 \text{ cm}^{-1}$ , a sinusoidal diameter of  $10.2 \pm 0.5 \mu\text{m}$ , and a mean postsinusoidal venular diameter of  $34 \pm 13 \mu\text{m}$  (93).

#### IV. MICROVASCULAR INFLAMMATION IN LIVER INJURY

##### A. Kupffer Cell Activation, Oxidative Stress, and Mediator Release

The hepatic macrophage population significantly contributes to the innate immunity, in particular by its initial and rapid response to potentially dangerous stimuli. Together with the SEC they form the reticuloendothelial system of the liver. They constitute the first macrophage population of the body to come in contact with bacteria, bacterial endotoxins, and microbial debris derived from the gastrointestinal tract and transported via the portal vein to the liver. This strategic location with the predominant site in the periportal segment of the hepatic sinusoids suggests a central role of KC in regional and systemic defense (Fig. 10).

KC are found to be distributed over zone 1 (periportal), zone 2 (midzonal), and zone 3 (pericentral) of the rat liver lobule in a ratio of 4:3:2. The periportal KC are larger and show higher lysosomal enzyme activities on a per cell basis with large and heterogeneous lysosomes, and higher endocytic activity than those located in midzonal and pericentral segments of the sinusoids (746). In line with the fact that the functional heterogeneity of KC is related to their position in the liver lobule, periportal KC display low expression levels of MHC class II molecules and show highest production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )

and prostaglandin  $E_2$  (PGE $_2$ ), while pericentral (smaller) KC are more active in Ia antigen expression and elaborate more IL-1, representing important components of the immune response (328).

KC have three complex functions: 1) phagocytosis of particulate matter and uptake of macromolecules, 2) presentation of antigens, and 3) release of soluble mediators (52). For the clearance of blood, KC are endowed with a variety of receptors, facilitating their function as sentinel cells. Scavenger receptors, including the scavenger recep-

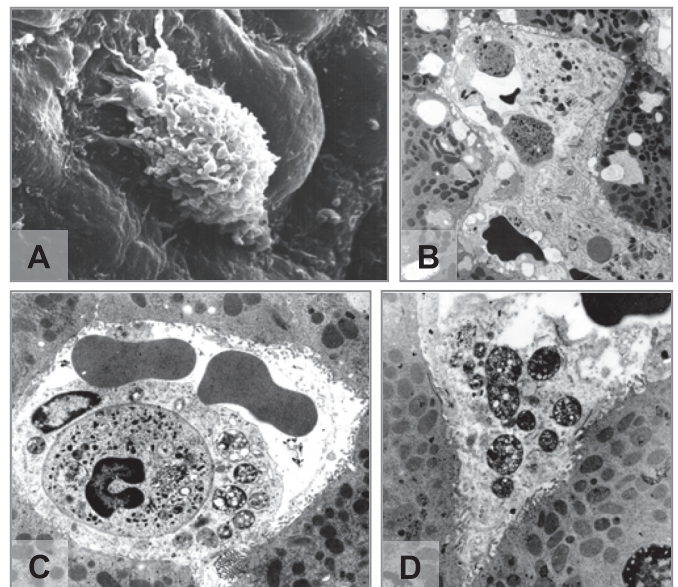


FIG. 10. Scanning (*A*) and transmission (*B*, *C*, *D*) electron micrographs of hepatic Kupffer cells in rat livers upon endotoxin exposure. The activated Kupffer cell, being attached to the luminal surface of the sinusoidal endothelium, presents an irregular surface with numerous microvilli (*A*). Further early signs of activation are an irregular shape, variable-sized vacuoles, and enrichment of lysosomes and phagolysosomes (*B*). Intrasinusoidally accumulated platelets and leukocytes are in direct contact with the Kupffer cell surface (*C*) and might be ingested (*C*). Initial overactivation of Kupffer cells results in severe cell injury with disintegration of cell membranes, focal cytoplasmic lesions, and large phagolysosomes ("autolysis", *D*). Magnification: *B*,  $\times 3,200$ ; *C* and *D*,  $\times 8,000$ . [From Vollmar et al. (871).]

tor cystein-rich (SRCR) superfamily members, are expressed on KC from an early stage of ontogeny and are involved not only in the lipid metabolism (847) but also in the bactericidal action by binding and endocytosis of endotoxin (579, 844, 849). CD163, also well known as ED2 antigen in rats, is a member of the SRCR family class B and functions as a scavenger receptor for hemoglobin-haptoglobin complexes (635), thereby protecting tissue from free hemoglobin-mediated oxidant injury (422). In addition to the CD11/CD18 receptor of KC, being reported to contribute to the clearance of lipopolysaccharide (LPS) from blood by recognition of the lipid A part of LPS (319, 907), the pattern recognition receptors (PRRs) CD14 and Toll-like receptor 4 (TLR4), in combination with the adaptor protein MD2, are essentially involved in the endotoxin-associated KC activation (674, 763, 823). KC are chronically exposed to higher concentrations of endotoxin than circulating peripheral blood monocytes. Therefore, it seems plausible that protective mechanisms have evolved to avoid the inadvertent activation of KC while maintaining scavenger function. For example, KC have relatively low CD14 expression, which however can be upregulated upon various stimuli, including endotoxin (536, 764, 968). Thus changes in CD14 expression can be hypothesized to represent the underlying mechanism that determines the liver and the KC sensitivity to LPS toxicity. Moreover, it has been shown that high hepatic arginase activity, resulting in low arginine concentrations locally in the liver, limits the reactivity of KC towards endotoxin via an arginine-specific and PGE<sub>2</sub>-dependent depression of TNF- $\alpha$  release (81). This may reflect an evolutionary adaptation by KC to their local hepatic environment and strategic anatomic position in the portal circuit, which is optimal for removal of endotoxin and, thus, for protection of the host (81). In line with this, binding of LPS to scavenger receptors does not result in KC activation and production of TNF- $\alpha$ . Even more, studies in scavenger receptor type A knock out mice implied that scavenger receptors can downregulate proinflammatory cytokine production by competing for endotoxin and thus protect the host against lethal endotoxic shock (289). In addition to the scavenger receptors, there is a more recently identified class of PRRs, i.e., the nucleotide-binding oligomerization domain (Nod) molecules, Nod1 and Nod2, which serve as intracellular ligands for LPS (321). So far, the contribution of these molecules in the innate immune response against pathogen infection and in susceptibility to liver injury is not completely understood (633, 764).

Aside from LPS, the endotoxins of Gram-negative intestinal bacteria,  $\beta$ -glycans from bacteria and fungi, and the complement factors C3a and C5a are the most prominent and important activators of KC (52). The transfer of LPS to the LPS receptor CD14 and its TLR coreceptor is facilitated by the acute-phase protein LBP (LPS binding protein), which is produced by hepatocytes and can in-

crease as much as 100-fold during an acute phase response (45). At the same time, LBP participates in neutralization of LPS by transfer to high-density lipoproteins (913), and LBP-deficient mice exert exquisite sensitivity to pathogen challenge, indicating that the critical function of LBP in vivo is to protect animals from bacterial infections (188). In contrast, ethanol administration leads to a significant increase of endotoxin levels in serum and LBP and CD14 mRNA expression in liver tissue, thereby sensitizing the liver to endotoxin-induced tissue injury (974). In line with this, knocking out LBP protects against alcohol-induced liver injury, most likely by blocking the downstream signaling pathway due to reduced availability of LBP (833).

LPS can activate KC directly (763) or indirectly by triggering complement activation, which results in the release of potent anaphylatoxins C3a and C5a and the stimulation of their receptors (219). Subsequently, G protein-enhanced activity of phospholipase C leads to an activation of protein kinase C and the opening of L-type calcium channels with activation of NADPH oxidase and PLA-mediated eicosanoid production (52). Activated complement seems to be responsible for the priming of KC, for the production of reactive oxygen species (ROS) and, thus, for the increased vulnerability of the liver to second hits, such as endotoxin exposure (333, 497).

In addition to neutrophils, KC represent the major source for vascular ROS, in particular upon I/R (333, 335, 336) (Fig. 11). There is a 10-fold increase of plasma oxidized glutathione (GSSG) and a 9-fold higher spontaneous superoxide formation of KC after 60 min of hepatic no-flow ischemia and 90 min of reperfusion (335). In addition, endotoxin has been shown to enhance dose-dependently the release of glutathione (GSH) and its oxidation to GSSG through complement activation (342). Moreover, animals pretreated with KC activating agents, such as retinol, galactosamine (Gal) or propionibacterium acnes, and subjected to I/R reveal enhanced GSSG as an indicator of an increased oxidative stress formation (336). Vice versa, inactivation of KC with gadolinium chloride or methyl palmitate significantly attenuates the postischemic increase of plasma GSSH levels. This strongly indicates that KC are the main source of intravascular oxidative stress. However, it has been shown that lipid peroxidation by these intravascular ROS is not the primary cause of parenchymal cell injury, but may be important as a damaging mechanism for the nonparenchymal cells of the liver, in particular the endothelial cells, which are located close to the sources of the ROS, i.e., the KC (530). Electron microscopic studies confirmed the selective damage of hepatic SEC by ROS, demonstrating a dose-dependent formation of large intracellular gaps and a reduction of the diameter of the remaining endothelial fenestrations. This was shown to have major implications for specific pro-

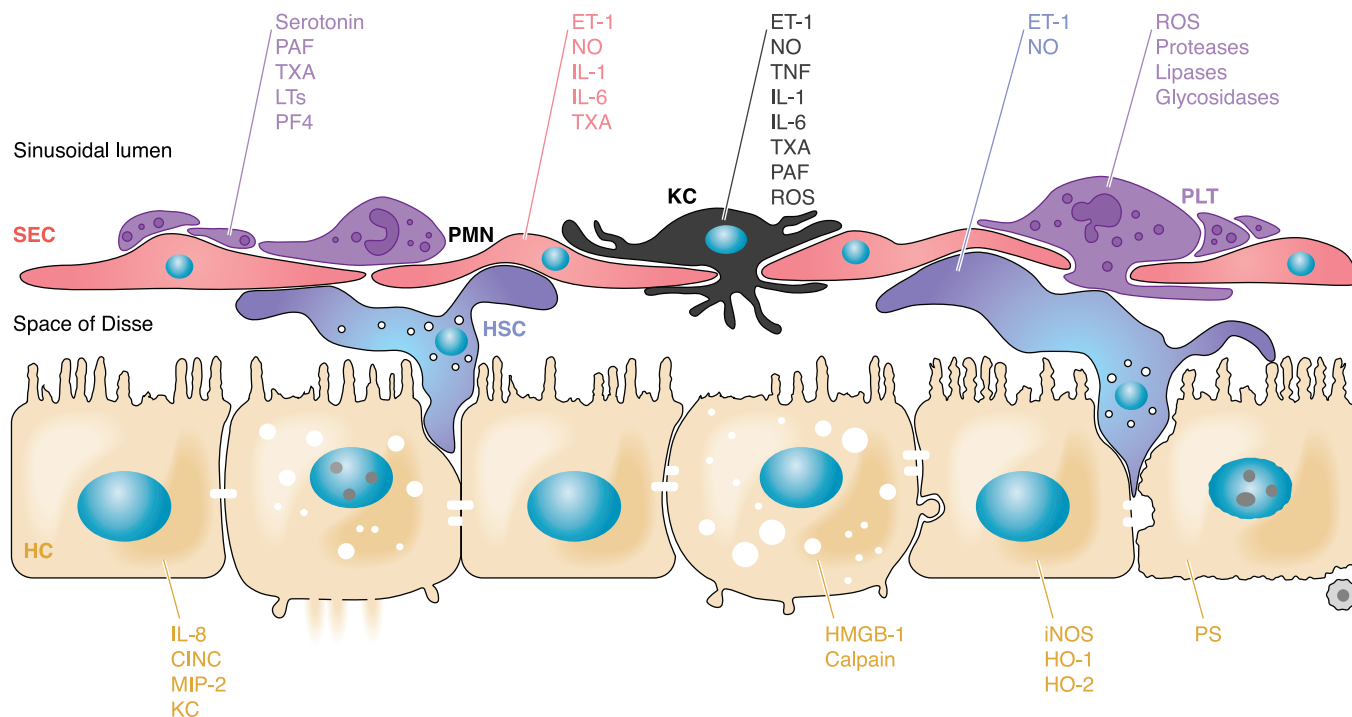


FIG. 11. Cellular mechanisms of microvascular inflammation in liver injury. Injurious stimuli and stress signals cause an activation of sinusoidal endothelial cells (SEC), Kupffer cells (KC), leukocytes (PMN), and platelets (PLT) with release of an armamentarium of aggressive mediators, enhancing the intrahepatic accumulation of inflammatory cells. PMN, PLT, and SEC can interact and bind to each other, leading to further adhesion and accentuation of mediator release. Upregulation of adhesion molecules allows the firmly attaching PMN to migrate towards chemotactic signals (IL-8, CINC, MIP-2, and KC), being released by intact but stress-exposed hepatocytes. Tissue-infiltrating PMN exert direct hepatotoxicity by reactive oxygen species (ROS) and hydrolytic enzyme release. Hepatocytes undergo either necrosis, apoptosis, or mixed aponecrosis, depending on the severity of insult and their intracellular ATP stores. Apoptotic and necrotic hepatocytes can further attract PMN by either surface exposure of phosphatidylserine (PS) or leak of high-mobility group box-1 (HMGB-1).

cesses, such as ageing, cirrhosis and hepatotoxic injury (115).

Although ROS are able to cause cell destruction by lipid peroxidation, in most cases, ROS are more likely to modulate signal transduction pathways by affecting redox-sensitive enzymes, organelles (e.g., mitochondria), and transcription factors (137, 344). Thus ROS can directly induce or regulate apoptotic and necrotic cell death. In addition, ROS can have indirect effects by supporting protease activity through inactivation of antiproteases and modulation of inflammatory mediator formation and adhesion molecule expression (137).

Upon activation, KC release not only ROS, but also an array of inflammatory mediators, such as cytokines, eicosanoids, platelet-activating factor (PAF), nitrogen species, proteases, and chemokines (844) (Fig. 11). The constitutive transcription of TNF- $\alpha$  mRNA in KC allows the rapid and transient release of this so-called "first" cytokine upon LPS-activation of KC (251). This is regularly followed by the secretion of IL-1 and IL-6 (506). In many models of liver injury, TNF- $\alpha$  levels are elevated and correlate with injury (92, 170, 447, 956). Vice versa, inhibition of TNF- $\alpha$  activity by soluble TNF receptors, TNF or TNF-receptor knockout can attenuate liver injury, protect

hepatic morphology, and decrease mortality (212, 764, 828). Though one best studied pathway is the production of TNF- $\alpha$  and the greatest attention has been paid to the role of this cytokine in liver injury, the pathogenic contribution of other proinflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-17, and IL-18 (134, 786, 826, 827, 933), and anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13 (371, 503, 940, 941), is not less important.

It is far beyond the scope of this review to address in detail the complex network of cytokines, as several positively and negatively acting regulatory loops as well as distinct priming functions are possible. Nonetheless, there is a large body of evidence that it is not just the marked elevation of one distinct proinflammatory cytokine or the limited release of a specific anti-inflammatory cytokine that results in global liver injury. Instead, it is the global mismatch and the imbalance of pro- and anti-inflammatory substances that cause a disease (91, 213, 316). Moreover, it has been recognized that KC function as mediator of damage, but protect during processes of regeneration and repair (675). Strategies, such as application of gadolinium chloride or liposome-encapsulated dichloromethylene bisphosphonate (Cl<sub>2</sub>-MBP), which inhibit, deplete, or modulate KC (11, 273, 288, 313), were

effective to protect against liver injury upon toxin exposure (17, 82, 421) and endotoxemia (4, 288, 376, 468, 762, 871) (Fig. 10) as well as warm and cold I/R (429, 594, 823, 956).

On the other hand, there is clear indication that KC can mediate protection in that their depletion increases liver injury after hepatectomy (640). Moreover, KC are demonstrated to be mandatory for sufficient restoration of liver tissue upon hepatectomy (5). Due to the fact that KC-derived hepatoprotective factors, such as IL-10 and cyclooxygenase-derived mediators, such as PGE<sub>2</sub> are also upregulated in response to hepatic damage, KC may also serve important regulatory functions in pathophysiological states of the liver and may help to protect against exacerbation of liver injury. This view is based on the fact that intravenous injection of liposome-encapsulated clodronate almost completely eliminates KC and, by this, significantly increases susceptibility to acetaminophen-induced liver injury when compared with mice pretreated with empty liposomes (357). Along with the observation that 60 min but not 20 min of hepatic warm ischemia followed by reperfusion causes depression of KC phagocytic activity (861), activation of KC by muramyl dipeptide restored KC activity and reduced plasma transaminases and liver tissue necrosis 6 h after a 90-min period of warm hepatic ischemia (255).

Taken together, the currently available data on the role of KC in mediating hepatotoxicity support the idea of an initially protective response which in case of overstimulation develops to turn into damage (675) (Fig. 10, *B* and *D*). This threshold hypothesis of KC activation paved the way for strategies modulating rather than inhibiting the KC-associated innate immune response, including heat shock protein induction (36, 714) and application of glutathione (706), CpG-oligodeoxynucleotides (CpG-ODNs) (747), granulocyte-colony stimulating factor (G-CSF) (866), glycine (916), or statins, to name a few (555).

## B. Leukocyte Recruitment Within the Hepatic Microvasculature

In response to the exposure to KC-dependent inflammatory mediators, both endothelial cells and leukocytes become activated, and the leukocytes accumulate within the hepatic vasculature. The substances known to trigger intrahepatic leukocyte accumulation include TNF- $\alpha$  (118, 395, 904), IL-1 $\alpha$  and - $\beta$  (80, 182), IL-8 (740), PAF (723), activated complement factors (427, 904), cytokine-induced neutrophil chemoattractant (957), and CXC chemokines, such as macrophage inflammatory protein-2 (27, 477) and others (see also Ref. 337). These mediators increase the leukocytic expression of CD11b/CD18, a member of the  $\beta$ -2 integrin family of adhesion molecules, as well as the induction of E-selectin, P-selectin, and

intercellular adhesion molecule-1 (ICAM-1) on endothelium (244). Although these first steps of priming, activation, and recruitment of leukocytes are vital for host defense and removal of cell debris, leukocytes also mediate inflammation-associated tissue injury (244). In line with this, leukocyte recruitment has also been shown to be of paramount importance for the development of liver injury in hepatic I/R (869, 950), endotoxemia (168, 705), acute liver failure (170, 472), alcohol-, toxin-, or drug-induced liver disease (499) and biliary cholestasis (453, 454).

Before transendothelial migration and infiltration into tissue, leukocytes need to interact with the vascular endothelium. The recruitment is dependent on a multistep process, involving the sequential engagement of adhesion molecules. These include selectins with their corresponding ligands for primary leukocyte-endothelial cell interaction and the family of  $\beta$ -2 integrins, which react with endothelial ICAMs to cause a secondary firm leukocyte-endothelial interaction (554). In this multistep sequence, selectin-mediated rolling of leukocytes along the endothelial lining of the microvasculature is a prerequisite for later firm attachment and subsequent transendothelial migration. For this, inflammatory mediators and chemotactic factors play an essential part in initiating, propagating, and directing these dynamic processes (Fig. 11). Although the current knowledge about the molecular and humoral mechanisms, underlying the trafficking of leukocytes to inflammation sites, applies also to leukocyte-dependent liver injury, specific aspects of how leukocytes behave within the individual segments of the hepatic microvasculature still need to be addressed.

Using intravital fluorescence microscopy, a considerable number of animal studies have demonstrated that in most experimental models of hepatic injury (I/R, shock/resuscitation, endotoxemia, acute liver failure, etc.), a particular fraction of leukocytes sequestered in the hepatic vasculature is localized in sinusoids (105, 127, 128, 168, 382, 384, 395, 453, 865, 868, 887). With the assumption that leukocyte-endothelial cell interaction requires selectin-mediated rolling, leukostasis in sinusoids may not involve adhesions molecules, because sinusoidal lining cells express little E-selectin, if any (183), and do not express P-selectin, neither physiologically nor in inflammation, I/R, or rejection (341, 759, 876). In line with this, leukocyte rolling in sinusoids has never been reported (865). This further implies that leukocyte accumulation in sinusoids is mostly independent from the function of the classical adhesion molecules. Accordingly, leukocyte recruitment in sinusoids is not diminished in P-selectin-deficient mice, P-/E-selectin double-deficient mice, and P-/E-selectin double-deficient mice with antibody-induced L-selectin blockade (905). In the absence of selectins, vascular adhesion protein-1 (VAP-1) (443, 444, 545) and liver- or lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN) (31) have

been shown to be expressed by SEC. However, their role in leukocyte sequestration in liver injury has not yet been analyzed. Most recently, spinning disk intravital microscopy revealed that constitutive expression of hyaluronan is restricted to liver sinusoids, and blocking CD44-hyaluronan interactions selectively reduces leukocyte adhesion in sinusoids of endotoxemic mice. This indicates that the CD44-hyaluronan interaction represents a dominant mechanism for leukocyte sequestration in inflamed liver sinusoids (542).

Currently, the prevailing view is that leukocytes become physically trapped in inflamed liver sinusoids. Leukocytes, with a mean diameter of 10–12  $\mu\text{m}$ , need to force themselves in their passage through narrow hepatic sinusoids presenting with a spherical diameter ranging from 5 to 12  $\mu\text{m}$ . Due to this mismatch in size, leukocytes, while passing, compress the endothelial lining cells and the underlying space of Disse. As a result of this endothelial massage, fluid in the space of Disse is pushed downstream, and upon passage of the leukocyte, the space of Disse resumes its original shape with, thus, suction of fresh fluid into the space of Disse, contributing to the physiological process of hepatic transport and exchange (902). Activated leukocytes show a rapid decrease in their deformability or, conversely, an increase of cell stiffness due to polymerization of soluble G-actin to filamentous F-actin at the cell periphery (158, 208, 906). This leukocytic increase in both rigidity and viscosity hampers leukocyte traveling through the liver and supports cell sequestration. In addition to the high tortuosity of sinusoids, promoting lowering of leukocyte flow velocity and leukocyte stasis (406), additional mechanical factors might further hinder sinusoidal leukocyte trafficking and promote intrasinusoidal leukostasis. These include endothelial cell swelling, interstitial edema, but also protrusion of blebs and activated KC (237, 540, 861). Moreover, sinusoidal diameter reduction due to a misbalance of vasodilating and vasoconstricting mediators are known to increase flow hindrance and, thus, aggravating leukocyte sequestration (37, 616, 837). In support of this view, increased leukocyte stasis in sinusoids of posts ischemic livers can be attenuated by an  $\text{ET}_A$  receptor antagonist (835, 836). Conversely, HO-1 inhibition significantly reduces sinusoidal diameters and volumetric blood flow, resulting in a significantly increased number of stationary leukocytes (912). However, it is hard to clearly decipher whether the entrapment of leukocytes is due to changes in resistance conditions or due to classical mediator-stimulated expression of adhesion molecules on the endothelial cells (320), which might endorse leukocyte recruitment.

In contrast to the mechanical trapping of leukocytes in sinusoids, leukocytes accumulate in postsinusoidal venules via interactions, like rolling and firm adhesion, which are mediated by specific adhesion molecules, i.e., L/P-/E-selectin,  $\beta$ -2 integrins, and ICAMs (401, 694, 860).

Rolling interaction of leukocytes in postsinusoidal venules is thought to involve selectins, because postsinusoidal rolling is reduced by anti-P- and anti-L-selectin monoclonal antibodies in mice transfected with TNF- $\alpha$  or IL-1 $\beta$  containing adenoviral vectors (626) and also in P-selectin knockout mice with concanavalin-A-induced hepatitis (519). In adenovirus-activated mice, which exhibit profound liver injury with almost exclusive recruitment of leukocytes in postsinusoidal venules, but not in sinusoids, inhibition of both P-selectin,  $\alpha$ -4 integrin, and E-selectin is necessary to completely block leukocyte rolling and subsequent adhesion (485). In both warm and cold rat liver ischemia models, the role of selectins has been demonstrated by the fact that a soluble P-selectin glycoprotein ligand-1 (PSGL-1) decreases hepatocyte injury, leukocyte adhesion, and subsequent migration, resulting in a significant increase of liver graft survival (163). In addition, CD18 antiserum can also protect against LPS/ranitidine-induced liver injury, suggesting that leukocyte activation is required for tissue injury (147).

ICAM-1 is constitutively expressed on SEC and KC, but expression can markedly be increased by inflammation-associated mediator release, including hepatocytes and stellate cells (285, 605, 696). For example, there is a strong relationship among ICAM-1 expression, TNF- $\alpha$ , plasma endotoxin, and inflammatory changes in alcoholic liver disease (412, 585). The cytokines TNF- $\alpha$  and IL-1 are considered the main mediators responsible for upregulation of ICAM-1 mRNA in the inflamed liver (182) and have been shown to be major inducers of soluble ICAM-1 formation in vivo (332). In addition, IL-2 causes organ-specific TNF- $\alpha$  and RANTES production with increased ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) expression, which may facilitate lymphocytic infiltration and cause liver dysfunction (15). In warm and cold hepatic I/R, anti-ICAM-1 antibody application does not affect sinusoidal leukostasis, but effectively inhibits posts ischemic leukocyte adherence to the venular endothelial lining (117, 587, 666, 860). However, only warm ischemic livers benefit from anti-ICAM-1 blockade with attenuation of tissue injury (117, 581, 587, 860). On the contrary, sequestration of leukocytes in endotoxemic livers has been shown to be independent of  $\beta$ -2 integrins (334) and ICAM-1 (334, 872), suggesting other receptor-ligand interactions which mediate endotoxin-induced leukocyte adhesion to the vascular endothelium.

The inflamed liver develops a vicious circle involving mediators, not only recruiting leukocytes, but also priming them for an enhanced release of ROS. In line with this, inhibition of platelet activating factor, blockade of xanthine oxidase, and induction of leukopenia reduces leukocyte accumulation and hepatocellular injury (925). This vicious circle in turn increases adhesiveness of leukocytes by mobilization of secretory granules, leading to enhanced expression of CD11b/CD18 and shedding of

L-selectin (337). Despite this stimulated inflammatory microenvironment, activated leukocytes logging within the hepatic vasculature are considered not to cause damage, but need an additional chemotactic signal from the parenchymal tissue, which triggers extravasation and attack on target cells, i.e., hepatocytes (Fig. 11).

### C. Leukocyte Transendothelial Migration and Parenchymal Cell Killing

After firm adhesion to the endothelial lining, the leukocytes with their extending pseudopodia migrate between adjacent endothelial cells into the subendothelial tissue and the adjacent interstitial compartment. This complex event, termed diapedesis, is dependent on an array of cellular processes, including adhesion molecule expression and activation, cytoskeletal reorganization, and alteration of membrane fluidity. In contrast to other organs, only a limited number of studies have addressed the importance and contribution of different adhesion molecules and chemotactic stimuli in the emigration of leukocytes from the vascular space of the liver. Nevertheless, it became clear that the liver does not follow the simple paradigm of the inflammatory cell recruitment with 1) selectin-mediated leukocyte rolling, 2) subsequent integrin activation, 3) integrin-mediated firm adhesion, and 4)  $\beta$ -2 integrin and immunoglobulin-dependent diapedesis, as typically established. In mesentery and cremaster muscle leukocyte rolling, adhesion and transendothelial migration occur almost exclusively in postcapillary venules (425, 495). In the liver, the primary site of leukocyte migration into parenchymal tissue, i.e., sinusoids or postsinusoidal venules, is still an unresolved question (105, 865). Extravasation has been shown from all microvascular segments of the liver, including hepatic postsinusoidal venules (384, 865), but the hepatic sinusoids seem to play an undisputable greater role than capillaries in other organs. This is most probably due to the unique type of endothelium, being discontinuous and fenestrated, and lacking basal lamina and tight junctions. Of importance, the sinusoidal endothelium also fails to express E- and P-selectin, and CD34 and VE-cadherin (716, 759). However, it constitutively expresses ICAM-1 (759) and VAP-1 (521, 545), and VCAM-1 upon stimulation, though lower than the endothelium of other vessels (759).

In vitro and in vivo studies provide good evidence that the  $\beta$ -2 integrins CD11a/CD18 and CD11b/CD18 and the counterreceptors ICAM-1 and VCAM-1 on endothelial cells can mediate transendothelial migration (337, 693, 848, 860, 917). In addition, junctional adhesion molecule-A (JAM-A), a receptor expressed on endothelial tight junctions, leukocytes, and platelets, is shown to be up-regulated in hepatic venules and to serve as an endothelial receptor of leukocyte transmigration in warm liver ische-

mia (384). On the other side, there are some reports challenging the clear-cut role of these adhesion molecules for invading leukocytes (401). In particular, when there is extensive endothelial damage, e.g., cold I/R (221, 309, 564) or degradation of membrane constituents by leukocyte-released proteases (78, 192), the access of leukocytes to the parenchyma might become independent from cell adhesion molecules. For example, matrix metalloproteinase (MMP)-9 inhibition attenuates postischemic rolling and adherence of leukocytes in hepatic postsinusoidal venules, CD4+ T cell accumulation in sinusoids, and neutrophil transmigration (383). The relevance of matrix degrading substances for mediating injury is underlined by studies which demonstrate protection against liver damage by a specific neutrophil elastase inhibitor (751) or by MMP deficiency (269). Moreover, MMPs have been shown responsible for sinusoidal endothelial gap formation, allowing initial contact of leukocytes with damaged hepatocytes (326).

Aside from adhesion molecules which serve as tracks for leukocyte movement, chemotactic factors represent the driving force for leukocyte migration. One of the most potent and specific chemoattractants for leukocytes are the CXC chemokines (505), e.g., IL-8, MIP-2, KC, or CINC, being released by hepatocytes in response to TNF- $\alpha$ , IL-1, and endotoxin (155, 369, 482, 687, 737, 811, 957). Excessive CXC chemokine formation in parenchymal cells can recruit leukocytes into the hepatic vasculature (190, 510, 740), induce migration, and cause injury (510) (Fig. 11). The pivotal role of chemokines for leukocyte extravasation and leukocyte cytotoxicity in the liver is convincingly demonstrated by the attenuation of hepatic leukocyte recruitment and leukocyte-mediated injury after neutralizing or inhibiting CXC chemokines in models of hepatic I/R (118, 370, 477), endotoxemia (687, 957), agonistic Fas antibody-induced liver injury (190), and acute Gal/LPS-induced liver failure (830). At the same time, there is evidence that the importance of chemokines may markedly vary between different experimental models used, pharmaceutical approaches applied, and pathophysiological situations present. In fact, immunoneutralization of chemokines is ineffective against endotoxin-induced recruitment of leukocytes in the liver (28), while endotoxin-induced transmigration and extravascular tissue accumulation is dependent of CXC chemokines in Gal/LPS-exposed mice (482). Finally, Dorman et al. (155) have disproved a pathophysiological role of the CXC chemokines MIP-1 and KC in the hepatic injury process after Gal/LPS exposure. This is based on the fact that 1) the time course of CXC chemokine formation did not correlate with leukocyte extravasation; 2) only Gal/LPS caused leukocyte extravasation, although LPS and Gal/LPS induced a similar CXC chemokine response; 3) chemokine neutralization had no effect on leukocyte extravasation and injury; and 4) wild-type and CXCR2 knock-out mice

showed comparable leukocyte extravasation and liver injury (155).

Hepatocytes are recognized as important attractors to control guided transmigration of leukocytes. In mice which display agonistic Fas antibody-induced hepatocellular apoptosis, hepatic induction of CXC chemokines is associated with leukocytic infiltration of the hepatic parenchyma (190). It has further been shown that parenchymal apoptotic cell death coincides with leukocyte transmigration in mice with Gal/endotoxin-induced liver injury (467). Detailed *in vivo* high-resolution multicolor fluorescence microscopy studies have shown that leukocytes frequently colocalize with apoptotic hepatocytes in endotoxemic livers and that these colocalizing leukocytes are the consequence of hepatocyte apoptosis, because intrahepatic leukocyte adherence is markedly inhibited upon blockade of hepatocellular apoptosis by the pancaspase inhibitor zVAD-fmk (168). The fact that in endotoxemic animals inhibition of hepatocellular apoptosis by p53 blockade also reduces intrahepatic leukocyte accumulation further supports the view that apoptotic hepatocytes are capable of recruiting leukocytes (705). Apoptotic hepatocytes might attract leukocytes by specific surface modifications, such as the exposure of phosphatidylserine. Thus diannexin, a 73-kDa homodimer of human annexin V, binds to phosphatidylserine and, by modulating leukocyte and platelet trafficking and EC activation, it suppresses vascular inflammation and decreases apoptosis in rat cold I/R injury (730). Likewise, necrotic hepatocytes can signal by the leak of intracellular messengers, such as high-mobility group box 1 (824) (Fig. 11). Also, calpain is reported to leak out of necrotic hepatocytes into the extracellular milieu and to hydrolyze proteins in the plasma membrane of neighboring cells, leading to progression of injury (547). Experimental intervention with calpain inhibitors substantially mitigates progression of liver injury initiated by toxicants, thereby preventing acute liver failure and toxicant-induced animal death (547).

Although there are a variety of potential mechanisms of leukocyte-dependent hepatotoxicity discussed, two major concepts are under the principal focus of investigation. In cytosolic granules, leukocytes possess a large number of hydrolytic enzymes (192), of which many have been shown responsible for cytotoxicity to isolated hepatocytes (298). These include the classical proteases, lipases, and glycosidases, but also very specialized proteins, such as bactericidal/permeability-increasing protein, defensins, and bactericins. All of these enzymes can induce membrane damage, cell growth arrest, and activation of other hydrolytic enzymes in target cells (298). For example, analysis of conditioned medium from  $\alpha$ -naphthylisothiocyanate-treated leukocytes indicates the presence of both cathepsin G and elastase activities, which causes hepatocellular damage *in vitro* (294). The clear involvement of leukocytic proteases in cell killing has been shown among

others 1) by the protective effect of a neutrophil elastase inhibitor or antileukoproteinase in lethal acute liver failure and in endotoxin-induced liver injury after partial hepatectomy (170, 437, 830), 2) by the attenuation of liver I/R injury through an urinary trypsin inhibitor (484, 924), and 3) by the reduction of liver fibrin deposition, plasma PAI-1 concentration, and parenchymal injury after LPS/ranitidine exposure (147).

In addition, leukocyte-associated oxidant stress has been implied as common mechanism of liver injury. Oxidant stress is considered the condition in which the cellular levels of ROS exceed the neutralizing capacity of nonenzymatic and enzymatic capacities. Most recently, novel approaches to deliver antioxidative enzymes, such as polylipid nanoparticles (PLNP) (282), liposomes (910), or adenoviral-mediated gene transfer (967) have been shown to protect against I/R and endotoxemic liver injury. Despite the ability of antioxidants to block liver damage (908), the relevance of ROS for direct cell killing has likely been overestimated (344). The very high antioxidant capacity of the liver, including catalase and superoxide dismutase (SOD), can neutralize and detoxify extremely high intracellular oxidant stress without adverse effects. The amount of lipid peroxidation, as assessed by highly sensitive and specific gas chromatographic-mass spectrometric analysis of hydroxy-eicosatetraenoic acids (HETES) and F<sub>2</sub>-isoprostanes is one order of magnitude below that which would be necessary to cause direct cell killing (339, 530). Instead, oxidant-induced liver injury is more likely to be mediated by the direct effects of ROS on signal transduction pathway activities through oxidation of kinases and phosphatases (137). There is specific focus directed towards the mitogen-activated protein kinases (MAPK), the extracellular signal-related kinase 1/2 (ERK1/2), the c-Jun NH<sub>2</sub>-terminal kinase (JNK), and the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathways, which play different and complex roles in an often cell-specific manner. For example, ERK1/2 and JNK, but not p38 MAPK, are activated by the liver toxicant toosendanin. Inhibition of ERK1/2 activation sensitizes hepatocytes for death and increases the activity of caspase-9 and -3 in response to toosendanin. In contrast, inhibition of JNK attenuates toosendanin-induced cell death (960). Acetaminophen-induced liver injury involves JNK activation, due to increased ROS generated by GSH-depleted mitochondria, and translocation of activated JNK to mitochondria where JNK induces mitochondrial permeability transition (MPT) and inhibits mitochondrial bioenergetics (270).

Altogether, leukocyte-mediated oxidant stress may induce mitochondrial dysfunction in hepatocytes and, thus, enhance proneness of cells to undergo apoptosis or oncotic necrosis (591). Inhibition of acetaminophen-induced phosphorylation of JNK prevents downstream Bcl-2 and Bcl-xL inactivation and protects from mitochondrial permeabilization and cytochrome *c* release (456). In

addition, JNK inhibition *in vivo* markedly reduces mortality in acetaminophen hepatotoxicity, but not in acute carbon tetrachloride-mediated or anti-Fas antibody-mediated hepatic injury, with a significant reduction in hepatic necrosis and apoptosis (287). Comparable effects for JNK inhibition, i.e., a decrease of hepatic necrosis and apoptosis, have been shown for livers exposed to cold storage and transplantation (832).

#### D. Platelet Adhesive Interactions

In addition to leukocytes, platelets and their intrahepatic adhesion and accumulation have also been implicated in inflammatory liver injury (130, 168, 380, 582). However, their contribution to the injury is far less studied. Decades ago, first studies have reported about histomorphological changes within hepatic sinusoids of endotoxemic livers, such as swelling of KC, accumulation of leukocytes and, in particular, the deposition of platelets and fibrin clumps (296, 539, 540). After a bolus dose of endotoxin, time course analysis of circulation of autologous indium 111-labeled platelets revealed initial hepatic sequestration, resulting in a marked peripheral thrombocytopenia (266, 754).

Although platelets are anuclear, they possess a cellular machinery comparable to that of leukocytes in many aspects. In addition to the cytoskeleton which allows platelets to move, they release an armamentarium of proinflammatory and procoagulant mediators upon activation, such as thromboxane A<sub>2</sub> (TxA<sub>2</sub>), leukotrienes, serotonin, platelet factor 4, and platelet-derived growth factor (PDGF). Due to their potential to modulate leukocyte functional response (689), platelets have been accused of aggravating endothelial damage and leukocyte activation and recruitment to the site of injury. In support of this view, coculture experiments with human leukocytes and platelets revealed a marked elevation of superoxide anion production through a P-selectin-dependent mechanism when thrombin-activated platelets were used (576). P-selectin is found in the  $\alpha$ -granules of resting platelets and in Weibel-Palade bodies of endothelial cells, rapidly occurs on the surface upon cellular activation and recognizes lineage-specific carbohydrate ligands on both monocytes and leukocytes. Thus leukocytes, platelets, and endothelial cells can interact and bind with each other (Fig. 12), leading to further adhesion of leukocytes and platelets with accentuated ROS generation (452, 576).

Leukocytes cultured in the presence of platelets show a dramatic inhibition of apoptosis compared with leukocytes cultured alone (16). Thus adherent platelets might significantly prolong life span of leukocytes and, thus, their capacity for oxidative burst and release of proteolytic substances. Accordingly, interference with platelet sequestration by inhibition of PSGL-1 and GP1b,

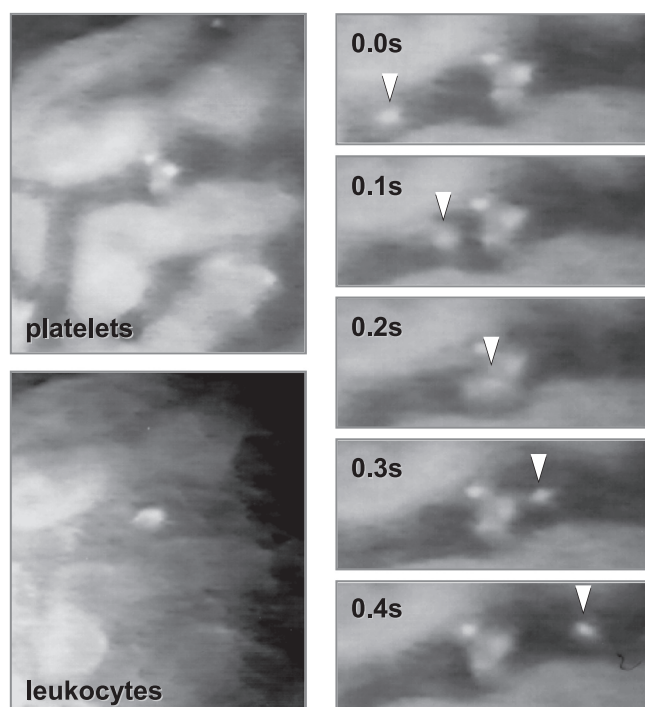


FIG. 12. Intravital fluorescence microscopic images, displaying the cellular passage through hepatic sinusoids. *Left panels* illustrate the identical images studied by different filter sets, demonstrating the colocalization of stagnant platelets and leukocytes. *Right panel* shows a time series of identical images, representing just a higher magnification of the image of the *left panel*. Note that the cluster of adherent cells forms an intrasinusoidal hindrance, which, however, can still be easily passed by an additional inflowing platelet (arrowhead).

P-selectin deficiency, or induction of thrombocytopenia induces protection in normo- and hypothermic postischemic, endotoxemic, and cholestatic liver injury (163, 168, 453, 526, 822, 829, 918). Hereby, endothelial, but not platelet, P-selectin expression seems critical for postischemic platelet-endothelial cell interactions (379). Vice versa, in mice rendered neutropenic with an anti-neutrophil serum, sepsis-induced leukocyte and platelet recruitment is attenuated (743). In an isolated perfused rat liver model, it has been shown that platelets adhere to SEC via sialyl Lewis-X [sLe(x)] oligosaccharide and induce cellular apoptosis (136, 741). The presence of platelets after cold storage and rewarming aggravates liver injury, as assessed by increased release of transaminases (136). After cold I/R of livers with freshly isolated leukocytes and platelets, a synergism of platelets and leukocytes in inducing endothelial cell apoptosis could be found, which was completely abrogated by gadolinium chloride- or pentoxifylline-induced KC blockade. This highlights the need of activated leukocytes, platelets, and KC for full expression of the injury (742). In line with this, animals that were depleted for KC by liposome-encapsulated Cl<sub>2</sub>-MBP and subjected to 20 min of warm ischemia and 120 min of reperfusion showed that platelet adherence in sinusoids

is suppressed, and, as a consequence, sinusoidal perfusion failure, endothelial cell damage, and serum alanine aminotransferase (ALT) levels are significantly attenuated (582). Vice versa, microvascular and hepatocellular injury upon antigen-independent I/R is found aggravated after activation of the endothelium by CD4<sup>+</sup> T cells and recruitment of platelets (382). Activated platelets even contribute to cytotoxic T-lymphocyte-mediated liver immunopathology, such as viral hepatitis (314).

Although this triangular interaction seems to be a major mechanism in reperfusion injury (629), there are also controversial data, showing that neither inhibition of platelet function by clopidogrel nor platelet depletion by repetitive anti-CD41 injections leads to a reduction of postischemic liver injury (596). Instead, studies in platelet-depleted animals indicate that platelets are critically involved in repair after liver injury and in liver regeneration via serotonin-mediated mechanisms (479, 595). Nonetheless, it has to be taken into account that platelet-derived serotonin may at the same time increase oxidative stress and mitochondrial toxicity in nonalcoholic steatohepatitis and aggravate viral hepatitis (446, 595).

### E. Sinusoidal Cell Plugging

The close interrelationship between intraorgan leukocyte accumulation and microvascular injury has given rise to the concept that cells stagnant in capillaries might contribute to the manifestation of perfusion failure and subsequent hypoxic tissue injury. Compelling support for the concept of nutritive perfusion failure induced by leukocytic capillary plugging is provided by studies demonstrating that leukocyte depletion and prevention of leukocyte adherence is associated with a marked reduction of nonperfused capillaries in reperfused myocardium, brain, and skeletal muscle (141, 178, 353).

In postischemic liver tissue, Koo et al. (413) reported an increased incidence of microvascular plugging by leukocytes, causing complete cessation of blood flow in "some" sinusoids. Ferguson et al. (197) demonstrated that at the whole organ level leukocyte accumulation appears to correlate well with microvascular damage, while when the data are analyzed on the basis of 0.05 mm<sup>2</sup> microscopic fields on the surface of the liver, there is no difference in leukocyte accumulation in areas with sinusoidal blood flow compared with areas that were devoid of perfused sinusoids (197). More detailed analysis of leukocyte flow dynamics in individual sinusoids of postischemic rat livers revealed that leukocytes become stagnant in sinusoids and alter sinusoidal blood flow, but do not necessarily occlude the sinusoidal lumen (868) (Fig. 12). The tortuous path of the proximal segments of the sinusoids (406) as well as the lobular distribution of KC (746) and the peculiar junctional site of contractile stellate cells

at the transition of terminal portal venules to the sinusoids (599) are accused of causing the zonal gradient of leukocyte flow behavior with more tethering interactions in periportal and midzonal than pericentral segments (868). This steep gradient of stagnant leukocytes located at bifurcations, with the highest fraction in the periportal and the lowest fraction in the pericentral sinusoids, further indicates a substantial impact of the hepatic angioarchitecture on leukocyte flow behavior. Of utmost interest, however, is the analysis that stagnant leukocytes increase hindrance, as given by the reduction of velocity and number of leukocytes passing sinusoids with stagnant leukocytes (Fig. 12). However, they do not necessarily cause cessation of blood flow. In nonischemic livers, only 3% of the sinusoids with leukostasis simultaneously show perfusion failure, and in postischemic livers, this fraction increases but does not exceed 30%, while >70% of sinusoids with stagnant leukocytes still conduct blood flow (868) (Fig. 12). Thus sinusoidal leukostasis per se does not necessarily determine hepatic perfusion failure. In line with this, it has been shown that large amounts of leukocytes can accumulate in the liver without causing tissue damage, indicating that leukocytes do not contribute to hepatic reperfusion injury passively by plugging of sinusoids, but need an additional stimulus to produce injury (955).

## V. MICROVASCULAR DYSFUNCTION IN LIVER INJURY

### A. Sinusoidal Endothelial Cell Activation

Along with KC, the hepatic sinusoidal endothelium participates in host defense mechanisms and blood flow regulation and represents a major target for injury in the early phase of inflammation. The SEC are secretory and have the capacity to produce immunoregulatory and proinflammatory cytokines, such as IL-1, IL-6, and interferon (193, 748, 901). In addition, they produce eicosanoids, particularly TxA<sub>2</sub> and PGE<sub>2</sub>, as well as important regulators of vascular tone, including NO and ET (901). By forming a thin and continuous layer, the sinusoidal endothelium represents the only structural barrier, separating the hepatic parenchyma from blood constituents passing the liver. However, as SEC are fenestrated and lack tight junctions and an underlying basement membrane, they do not represent a barrier in the same sense that vascular endothelial cells do in other organs. Nonetheless, they still act as a functional barrier. It became appreciated that intact microvascular function requires the finely tuned and integrated activity of the distinct sinusoidal cell populations, i.e., KC, SEC, and stellate cells (757). In line with this, cell morphological changes in the hepatic sinusoid with old age are increasingly recog-

nized as determinants for impaired metabolism of drugs, adverse drug interactions, and susceptibility to toxins (327).

The site of KC activation and leukocyte adhesion is the sinusoid. The release of ROS is the predominant proinflammatory action during the early innate immune response. Although SEC are well-known for their capacity to release ROS, particularly upon *in vitro* exposure to endotoxin, the amount of ROS release *in vivo* is far less (756). Thus SEC are targets rather than significant sources of ROS in the sinusoid. Even more, the SEC have been recognized as antioxidant, being of essential importance for the intercellular oxidant balance with the prooxidant KC (757). As an important adaptive response to oxidative stress during the innate immune response of SEC (757), they regulate the pentose cycle with induction of glucose-6-phosphate (G-6-P) dehydrogenase, the key enzyme of the hexose monophosphate shunt (HMS). It has been shown that the glucose transporter GLUT-1, Mn- and CuZn-dependent SODs (Mn-SOD, CuZn-SOD), and Se-dependent glutathione peroxidase (Se-GPX) are simultaneously upregulated in endothelial cells, allowing accelerated elimination of ROS released from activated sinusoidal phagocytes (758).

Injurious stimuli, such as endotoxin and ethanol, induce similar morphological and functional changes in SEC. Livers from alcohol-fed animals are characterized by massive loss of sieve-plate architecture of the sinusoidal endothelium, which is virtually replaced with a meshwork of enlarged openings with diameters frequently exceeding 1 micron (699). LPS administration further accentuates these alcohol-associated alterations of the sinusoidal lining (699). The fact that reduced fenestration and hyaluronan uptake, being a means for assessing the functional state of SEC, are preventable by elimination of KC, underlines the importance of intercellular communication in the liver (140). Inflammation-induced sinusoidal endothelial gap formation precedes leukocyte adhesion and is shown to be dependent from MMPs, as a MMP-2/-9 inhibitor reduces gap formation by lowering TNF- $\alpha$  production (326). Morphological changes, such as gap formation, are of major pathological implication, because gaps facilitate the contact of leukocytes with hepatocytes (326).

Furthermore, harmful substances, like alcohol and endotoxin, upregulate ICAM-1 expression on SEC, which further primes the sinusoid as site for inflammatory leukocyte adhesion and transmigration (585). Alcohol-associated malondialdehyde-acetaldehyde (MAA)-modified proteins have demonstrated an increase in adhesion molecule expression and the secretion of proinflammatory cytokines and chemokines by SEC, with endotoxin being a cofactor in this scenario (165). SEC are highly responsive to the exposure of endotoxin with TLR4-dependent activation of p38 MAP kinase and the transcription factors AP-1 and NF- $\kappa$ B (99). The expression of ICAM-1 on SEC

correlates with TNF- $\alpha$  mRNA expression and plasma endotoxin concentrations (585). This further demonstrates the complex interplay between cellular, humoral, and molecular events. In line with this, endotoxemia induces a sublobular pattern of leukocyte margination, which is consistent with the concept of cell adhesion regulation by inflammatory mediators released from endotoxin-stimulated KC (37). Vice versa, dexamethasone suppresses TNF- $\alpha$  production by endotoxin-stimulated KC and decreases ICAM-1 expression and leukocyte adhesion on SEC (693).

Along with ICAM-1, VCAM-1 is upregulated by inflammatory stimuli either directly or by mediators released from stimulated KC, resulting in increased adhesion of leukocytes to the endothelial surface (848). In addition, SEC have been shown to recruit leukocytes via VAP-1, which is increasingly induced in inflammatory liver disease and liver allograft rejection (444, 521). Thereby, complex mechanisms regulate VAP-1 to induce adhesion, with 1) direct binding of leukocytes to endothelial VAP-1 protein, 2) indirect enzyme-dependent activation of other endothelial adhesive pathways, and 3) activation of leukocytes by VAP-1 ligand occupancy (444). In addition to leukocyte adhesion, substrate binding to VAP-1 results in SEC activation, which can be abrogated by treatment with the enzyme inhibitor semicarbazide. VAP-1-mediated SEC activation is rapid; dependent on NK- $\kappa$ B, phosphatidylinositol-3 kinase, and MAPK pathways; and leads to upregulation of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 and secretion of the chemokine CXCL8 (444). Moreover, stabilin-2, known as hyaluronic acid (HA) receptor for endocytosis (HARE; also designated FEEL-2) (274), is shown to be involved in lymphocyte adhesion to the hepatic sinusoidal endothelium via the interaction with  $\alpha$ -M beta-2 integrin (358).

In addition to the proadhesive function of activated SEC, they also exert a procoagulant microenvironment. Endotoxin decreases protein S antigen and mRNA levels in SEC via MEK/ERK signaling and NF- $\kappa$ B activation by involving membrane-bound CD14 and TLR4 (281). Thrombomodulin antigen and activity levels are significantly decreased in SEC isolated from endotoxin-treated rats, and thrombomodulin expression in cultured SEC isolated from normal rats is also reduced after treatment with endotoxin and TNF- $\alpha$  *in vitro*. Accordingly, recombinant thrombomodulin is capable of attenuating increased levels of serum fibrin degradation products, fibrin deposition within liver sinusoids, injury of SEC, and liver dysfunction after endotoxin exposure (431). Also, urine thrombomodulin administration prevents intrasinusoidal fibrin depositions and attenuates posthepatectomy liver dysfunction in endotoxin-challenged cirrhotic rats (359). The localization of endotoxin in KC and SEC correlates with the incidence of sinusoidal thrombosis both temporarily and spatially within the septic liver tissue (787), which,

among many other factors, explains the prothrombotic state in sepsis. In addition to endotoxin, also alcohol and alcohol-associated MAA-modified proteins initiate a pro-fibrogenic response by inducing the expression of the fibronectin EIIIA isoform by SEC (808).

Taken together, upon activation SEC exert proinflammatory and proadhesive as well as procoagulant properties, which can substantially contribute to aggravation of injury.

## B. Sinusoidal Vasomotor Dysfunction

Hepatic injury is caused by several mechanisms, including inflammation and microcirculatory dysfunction. The latter involves the action of potent vasoactive mediators. ETs,  $\text{TxA}_2$ , angiotensin II, and catecholamines are proposed as vasoconstrictors, whereas NO, CO, and prostaglandins are indicated as vasodilators that counteract the effect of the vasoconstrictors. Under physiological conditions, the influence of these vasoactive mediators on

intrahepatic blood flow is rather small and their effects are balanced. Any adverse stimulus, however, may lead to an upregulation of constrictor or dilator influences at all microvascular segments of blood flow regulation. These are mostly matched neither for time nor for space and may therefore cause a dysbalance of vasomotor activity and vasotonus. Thus the maintenance of a critical balance seems to be an attractive concept for an adequate regulation of hepatic blood flow, aiming at limiting microcirculatory dysfunction-associated liver injury (625). Although there are innumerable mediators, particular emphasis is given on the intimate relationship of the ET/ET-receptor system and the enzyme systems which liberate the vasoactive gaseous molecules NO and CO, i.e., NOS and HO (625) (Fig. 13).

ROS, endotoxins, cytokines, hypoxia, and vascular shear stress can induce ET gene expression (688). Because all of these mediators play an essential role in I/R, endotoxemia, and sepsis, drug-induced toxicity, and liver resection, increased generation and plasma concentra-

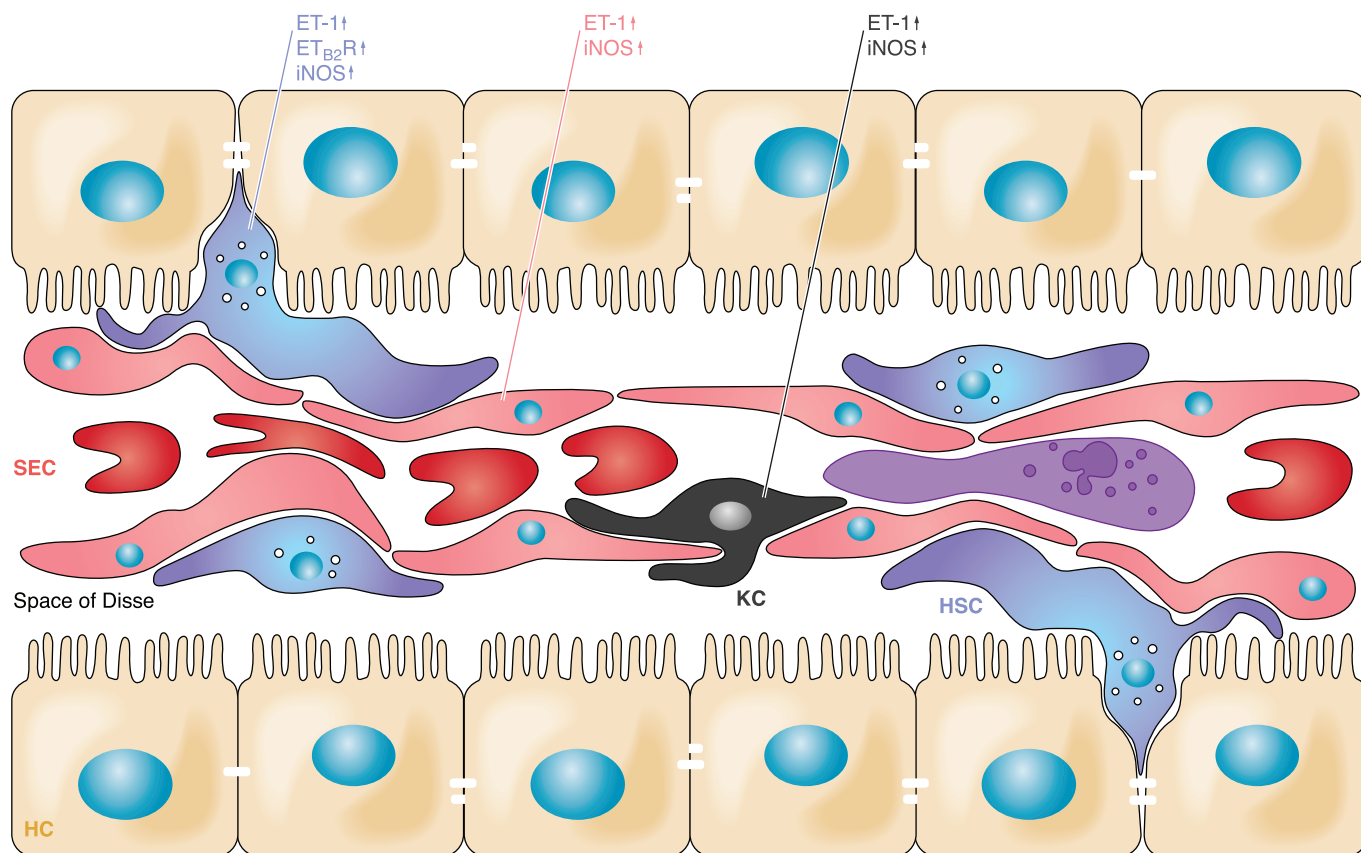


FIG. 13. Consequences of vasomotor control dysfunction in inflammatory liver injury. The dysbalance between the vasoconstrictive ET-1 and the vasodilative gaseous molecules NO and CO with a shift towards the ET system is due to 1) an increased ET-1 production by stellate cells (HSC), Kupffer cells (KC) and endothelial cells (SEC), and 2) an increased expression and sensitivity of ET<sub>B2</sub> receptor on HSC. The increased expression of iNOS further stimulates ET-1 production, most probably through the excessive NO release and, thus, reactive nitrogen species formation. The consequence of this ET-1-dominated loss of balance of vasomotor control is a contraction of HSC, which reduces the diameters of the hepatic sinusoids, resulting in an increase of vascular resistance and a hindrance of blood flow. The narrowing of the sinusoidal lumen does not necessarily result in obstruction by blood cells; however, red blood cells and, in particular, leukocytes have to squeeze to pass the HSC-induced constrictions.

tions of ET are reported in endotoxemia and endotoxin shock (184, 206, 218, 324, 389, 606, 736, 753, 946), post-ischemic reperfusion (389, 753), hemorrhagic shock (496, 664), prehepatic portal hypertension (939), and acute liver failure (931). In addition, substantial changes of the pattern of ET receptor expression can be observed, including an upregulation of ET and a downregulation of the ET<sub>A</sub> receptor expression in hepatic I/R (35, 389, 753, 937), endotoxemia (40, 389, 753), portal vein ligation (939), and other conditions of major inflammatory stimulation. Under these pathological conditions, ET-1 may also induce vasodilation by ET<sub>B1</sub> receptor stimulation on SEC, attenuating its own vasoconstrictive effects mediated via ET<sub>A</sub> and ET<sub>B2</sub> receptors on hepatic stellate cells (625). This implies a self-restricting process, because in the absence of this ET-mediated vasodilatory effect, the extent of I/R-induced liver injury has been found even larger (623). As net result of these molecular and humoral events, the hepatic microcirculation is sensitized to ET-1. The enhanced response of hemorrhagic shock- and resuscitation-primed livers to ET-1 leads to a greater increase of portal driving pressure, total portal resistance, and zero-flow pressures and to a more pronounced decrease of portal flow and sinusoidal diameters (624). Comparably, microvascular hyperresponsiveness to ET-1 has been described in endotoxin-pretreated livers (622) or in livers during polymicrobial sepsis (40). These enhanced ET-1 responses occur at sinusoidal and presinusoidal levels and may contribute to microvascular dysfunction and liver injury.

The vasoconstrictive effects of ET can functionally be antagonized by the vasoactive gaseous monoxides NO and CO (767) (Fig. 13). CO has paracrine effects on endothelial cells, provoking an increase of endothelial cGMP content and a decrease of ET-1 and PDGF expression (569). In parallel, ET-1 secretion from endothelial cells is reduced in the presence of NO donors (71, 562). Although these close interactions might imply that the vasoactive mediators could neutralize each others' effects, there is a large body of evidence that an imbalance of the expression of stress-induced vasoactive mediators is responsible for the alterations of the liver microcirculation and the subsequent tissue injury. Microcirculatory injury in small-for-size liver grafts is related to an upregulation of ET-1 and inducible NOS (iNOS), leading to a deterioration of intracellular homeostasis, as reflected by the downregulation of HO-1 (487). Ischemia and endotoxemia are reported to induce mRNA encoding ET-1 (2.5- and 6-fold), HO-1 (2- and 2.5-fold), and iNOS (6.4- and >24-fold) (753). In addition, HO-1 (16-fold) and ET-1 (9-fold) mRNA, but not iNOS, are found upregulated in hemorrhagic shock-exposed rat livers (663, 664). Increased endogenous production of NO counteracts ET-1-induced increases in sinusoidal resistance, providing protection in models of I/R (620). In support of this, NO donors and

ET-receptor blockers protect the postischemic liver microcirculation by maintaining the ET-NO balance (560, 717, 837). I/R injury might also benefit from blockade of excessive iNOS expression, decreasing NO and hydroxyl radical production (490). In sepsis, iNOS and eNOS seem to be differently regulated. eNOS is thought to protect the liver microcirculation, while the strong upregulation of iNOS might contribute to microvascular dysfunction and hepatic injury (184, 527). This view is further underscored by the fact that the administration of arginine together with a selective iNOS inhibitor during the early phase of sepsis restores plasma arginine, reduces oxidative stress by maintaining NO derived from constitutive NOS, and attenuates neuroendocrine stress responses (915). In contrast, unselective inhibition of NOS activities aggravates liver injury (785).

Comparably to eNOS, HO-1 upregulation confers cytoprotection in many models of organ and tissue injury (691, 912) and CO ameliorates hepatobiliary dysfunction caused by heme overloading in endotoxemic livers (438). Accordingly, HO-1 deficiency exhibits a heightened and dysregulated inflammatory response to endotoxin (814). Of interest, the cytoprotection of the HO-1 metabolite bilirubin is mediated at least in part through the inhibition of iNOS expression, underlining the close interaction of these mediators controlling sinusoidal vasotonus (886). Loss of the delicate equilibrium between NO, CO, and ET induces vasoconstriction and narrowing of the sinusoidal lumen, compromising blood flow, tissue oxygenation, and cell trafficking (Fig. 13).

Furthermore, vasoregulatory imbalances and increased intrahepatic resistance play a central role in the pathophysiology of portal hypertension in cirrhotic livers. The imbalance between the hyperresponsiveness and overproduction of vasoconstrictors (mainly ET-1 and cyclooxygenase-derived prostaglandins) and the hyporesponsiveness and impaired production of vasodilators (mainly NO) is responsible for the increased vascular tone in the sinusoidal and postsinusoidal space (729). Multiple derangements of eNOS-derived NO production are proposed to contribute to impaired sinusoidal relaxation and increased resistance (260, 677, 728). Enhanced expression and interaction of inhibitory protein caveolin binding to eNOS contribute to impaired NO production, reduced NOS activity, and vasoconstriction in the intact cirrhotic liver (728, 936). Higher levels of the NO synthesis inhibitor amino acid asymmetric dimethylarginine (ADMA) (440, 567, 817), reduced phosphorylation of the major eNOS activator Akt (568), and increased expression of the Akt activity inhibiting G protein-coupled receptor kinase-2 (GRK2) by sinusoidal endothelial cells (498) are thought to further impair eNOS function and NO availability in cirrhosis. In addition, hepatic stellate cells as the target cells for NO action are described to exert a blunted NO response due to defects in the GC signaling pathway and cGMP cascade

(161, 631). Moreover, cirrhotic livers show impaired vasodilation to mediators such as CO and H<sub>2</sub>S (201, 260).

Apart from decreased response to vasodilators, vasoconstrictive systems, such as ET receptors and ET-1 levels as well as COX-1-dependent TxA<sub>2</sub> synthesis and 5-lipoxygenase-derived eicosanoids, are upregulated and increased in cirrhotic livers, thereby also contributing to the increase of vascular tone (38, 113, 245, 246). The imbalance in vasoreactivity is primarily mediated by the hepatic stellate cells which, in cirrhosis, undergo activation, thus enhancing their contractility (224, 679). Hereby, smooth muscle like Ca<sup>2+</sup>-dependent contraction patterns as well as calcium-independent mechanisms participate in the contractile function of activated hepatic stellate cells (441). Studies, demonstrating the correction of the hyperresponsiveness to vasoconstrictors by Rho kinase inhibitors, imply a causal role of the RhoA/Rho kinase pathway for the increased vasoconstrictor sensitivity and the elevated intrahepatic resistance of cirrhotic livers (966). Most recently, adenosine has been shown to be a physiological inhibitor of the Rho pathway in hepatic stellate cells. Thus loss of adenosine-induced contraction of hepatic stellate cells might contribute to the substantial functional benefit seen in patients with cirrhosis in the presence of adenosine-induced hepatic arterial dilatation (972). Treatment with nitroflurbiprofen, a NO-releasing cyclooxygenase inhibitor, seems to be a particularly interesting approach to counteract the imbalance in vasoreactivity, because nitroflurbiprofen improves portal hypertension in cirrhotic rats by inhibition of the hyperresponsiveness to vasoconstrictors through the inhibition of COX and by the supply of NO to the intrahepatic circulation (442).

### C. Sinusoidal Perfusion Failure and Hypoxic Cell Injury

Sinusoidal perfusion failure is discussed as a key factor in the pathogenesis of tissue injury in warm I/R (73, 382, 859), cold preservation and transplantation (403, 548, 667), shock and resuscitation (428, 683, 864), endotoxemia (483, 743, 747), acute liver failure (170, 472, 616), bile duct ligation (4, 453), and drug-induced hepatotoxicity (34, 654). Several mechanisms are well established to contribute to sinusoidal perfusion failure, including sinusoidal narrowing caused by SEC edema (98, 864) or stellate cell-mediated vasoconstriction (38, 113, 620). In addition, structural peculiarities of the sinusoid, including diameter, tortuosity of path, branching pattern and number, size and activity of KC with the regional differences between periportal and pericentral areas, predispose to flow heterogeneities within the liver. These cause a gradient of perfusion failure, which is most pronounced in the periportal segment of the sinusoids (68, 864). Upon

entrapment of activated leukocytes, sinusoidal flow velocity decreases due to increased hindrance (868), further inducing perfusion heterogeneity and perfusion deficits. In addition, inflammation- and injury-associated adherence of leukocytes in outflow venules may alter sinusoidal perfusion due to an increase of blood viscosity (104) and, hence, vascular resistance (65). Furthermore, perfusion failure in sinusoids is thought to be caused by sluggish blood flow, intravascular hemoconcentration, and procoagulant conditions (552).

Among others, sinusoidal perfusion failure is particularly dependent on the nature of the injurious stimuli, on the animal and strain of species used, and on the time point of assessment but can amount to 40–60% in models of severe liver injury, such as reperfusion upon 24 h of cold storage (403) or Gal/endotoxin exposure for induction of fulminant liver failure (472). Microcirculatory failure in the liver after stress is characterized by perfusion heterogeneity, resulting in a mismatch between oxygen and nutrient supply and demand. The impaired nutritive blood flow accompanied by reduced oxygen availability decreases cellular levels of high-energy phosphates and contributes to early and late hepatocellular injury and dysfunction. In line with this, regression analyses demonstrated significant correlations between microcirculatory disorders and hepatocellular disintegration or liver dysfunction (859, 862) as well as between reduced blood oxygenation index and increased transaminases (241). Lobular microvascular dysfunction is thought to occur along with parenchymal injury, supporting a functional perfusion deficit-injury relationship, and vice versa. This underlines the determinant role of an intact microcirculation for the adequate organ integrity and function (552). However, in case of direct cytotoxic effects of injurious stimuli, such as TNF- $\alpha$  (883) or endotoxin (170), or in case of remote organ injury (68), hepatocyte death can occur without concomitant sinusoidal perfusion failure. Accordingly, hypoxic hepatitis has been allocated to differing reasons, i.e., decreased hepatic blood flow (venous congestion, heart failure), systemic hypoxemia (respiratory failure), and oxygen utilization failure (sepsis) (289).

### D. Sinusoidal Endothelial Capillarization

In addition to alterations in vasoreactivity, vascular remodeling represents an important component contributing to increased intrahepatic resistance in portal hypertension. Different anatomic lesions have become apparent as important structural changes to the vascular compartment, including fibrosis, sinusoidal collapse, defenestration of sinusoidal cells (capillarization), hepatocyte enlargement, and formation of a basement membrane in the space of Disse, all narrowing the sinusoid (598, 853, 873). In addi-

tion, the result is a reduced access of plasma and plasma-dissolved substances to hepatocytes due to their limited diffusion in the extravascular space. With the use of the multiple indicator dilution curve technique, it was shown that capillarization of SECs plays a lesser role than collagenization of the space of Disse in the reduced exchange between sinusoids and hepatocytes in thioacetamide-induced cirrhotic rat livers (308). Moreover, capillarization of hepatic sinusoids is described to occur only in very limited regions of the cirrhotic parenchyma and seems to be less relevant for functional consequences in cirrhotic livers than the markedly smaller areas occupied by sinusoids per unit of parenchyma and the sinusoid/hepatocyte interfaces disposable for metabolic exchanges (609). Sinusoids of cirrhotic livers further lack features of zonation, thereby contributing to the development and progression of liver failure (609).

## VI. MODES OF CELL DEATH IN LIVER INJURY

### A. Microcirculatory Determinants for Parenchymal and Nonparenchymal Cell Death

As outlined above, liver tissue exposed to various stress stimuli is characterized by deteriorations of the microcirculation. It is generally accepted that microcirculatory disturbances lead to insufficient energy supply, alteration of mitochondrial redox state (232, 838), subsequent decline of hepatic tissue oxygenation (173, 241), and impaired ATP regeneration (214, 522, 523, 655). The strong dependency between an intact microcirculation and the viability of both parenchymal and nonparenchymal cells is indirectly evidenced by numerous studies that demonstrate a cytoprotective effect by implementation of therapeutic strategies improving the liver microcirculation (100, 552, 755). These strategies comprise hemodilution, vasoactive agents, ROS scavenging drugs, immunomodulatory substances, and antiadhesive compounds (373, 466, 803, 963).

The degree of microvascular shutdown during post-ischemic reperfusion can be modulated in that sinusoidal shutdown is largely avoided with flow-controlled reperfusion. In contrast, pressure-controlled reperfusion may result in early and severe microcirculatory shutdown, correlating well with the manifestation of hepatocyte death (109). This underlines again the dependency of tissue injury from microcirculatory disturbances. In line with this, an improvement of the hepatic microcirculation by application of ET-receptor antagonists (35, 152, 560, 834, 835), NO and L-arginine supplementation (8, 152, 734, 785), and PGE<sub>2</sub> or prostacyclin (PGI<sub>2</sub>) (813, 815) results in an attenuation of hepatocellular injury.

In addition to the perfusion failure-associated impairment of oxygen supply to tissue, activated endothelial

cells, leukocytes, and platelets within the injured vasculature release extracellular nucleotides (e.g., ATP, UTP, ADP), which bind to specific cell-surface type 2 purinergic receptors. This process is suggested to further drive vascular inflammation and thrombosis (41). In turn, ectonucleoside triphosphate diphosphohydrolases (NTPDases, i.e., CD39) hydrolyze extracellular nucleotides and, therefore, potentially regulate nucleotide-mediated signaling. This limits the activation of platelet- and leukocyte-expressed P2 receptors and, thus, the generation of adenosine to reverse inflammatory events. The vascular protective activity of NTPDase1, which is expressed in hepatic arteries, portal veins, and hepatic central veins (159), is rapidly inhibited by oxidative reactions, as observed in liver I/R injury (for review, see Ref. 41). Also, endotoxin-induced cholestatic liver injury is associated with a decrease of the NTPDase activity (970), potentially increasing the procoagulatory status of the microvasculature during endotoxemia.

The implication of leukocytes for microvascular injury is unequivocal, but the relative contribution of microvessel obstruction versus adhesion and transendothelial migration versus release of toxic mediators is still a matter of debate. In the isolated perfused liver, nonactivated oxidatively quiescent leukocytes stick in the liver but have no significant effect on either perfused sinusoids or dead hepatocytes, while activated leukocytes cause damage (955). However, detailed spatial analysis disproved a correlation between leukocyte accumulation and microvascular damage on the individual level of sinusoids (197). This supports the observation that stagnant leukocytes increase flow resistance, but do not significantly contribute to perfusion failure of the individual sinusoid (868). On the basis of whole liver upon I/R, linear regression estimates reveal significant correlations between transaminases and bile flow as indicators of hepatocellular integrity and function with the number of adherent leukocytes in postsinusoidal venules, but not of stagnant leukocytes in sinusoids (859). On the other hand, it has been shown that in endotoxemic shock only leukocytes from the sinusoids actually transmigrated at a time when parenchymal cell injury occurs (105). In a remote model of liver injury, i.e., limb I/R, however, the number of stationary leukocytes within both sinusoids and postsinusoidal venules correlated with plasma ALT activities (911).

Substantial differences are described in the vulnerability of the different liver cell types to damage, in particular upon cold I/R. Here, a huge discrepancy could be observed between viability of parenchymal cells and nonparenchymal cells. In addition, there is a qualitative difference between warm and cold preservation injury, with relatively selective damage to hepatocytes or sinusoidal lining cells, respectively (315, 544). For example, after prolonged cold storage in Euro-Collins solution, most

parenchymal cells remained viable (>90%), while severe damage to nonparenchymal cells of up to 40% was observed (79, 525).

## B. Apoptosis, Oncotic Necrosis, Secondary Necrosis, and Aponecrosis

True apoptotic and necrotic cells can be differentiated on the basis of morphological and biochemical characteristics. It is a widely accepted way to define necrotic cell death by demonstrating a lack of apoptotic features. The classical morphology of an apoptotic cell includes cell shrinkage, chromatin condensation, fragmentation and margination, and apoptotic body formation. A swollen cell with evidence for vacuolization, karyolysis, and karyorrhexis is considered to undergo oncotic necrosis. Biochemically, necrotic cell death presents with acute metabolic disruption and ATP depletion, ion dysregulation, and activation of degradative enzymes, finally resulting in plasma membrane rupture and release of cell contents. These features contrast those of apoptosis, representing a cellular differentiation program, leading to resorption of the cell without significant impairment of cellular metabolism (364). In distinction to cell apoptosis, the molecular mechanisms underlying oncotic necrosis are poorly understood, which might be due to the fact that necrosis is still believed to be an uncontrollable and passive form of cell death (364, 387). However, there is meanwhile preliminary evidence that cell necrosis can also be a physiological and regulated, i.e., programmed event (290, 643, 644, 775). In aggregate, it has been recognized that both modes of cell death share common features and pathways, challenging the past view that apoptosis and necrosis are fundamentally different processes. In particular, the MPT, initially described as a reversible  $\text{Ca}^{2+}$ -induced permeabilization of the mitochondrial inner membrane, resulting in simultaneous changes, like increased permeability, induction of ATPase, uncoupling of oxidative phosphorylation, and loss of respiratory control (312), is regarded to play a causative role in necrotic, but also in apoptotic cell death (388, 407, 476, 649) (Fig. 14).

It has been hypothesized that cellular ATP levels might be an important factor in determining the cell death pathway secondary to a harmful insult, an apoptotic pattern when ATP levels are high and a necrotic pattern when ATP levels are low (139, 340, 388, 476, 649). Thus it is thought that the severity of injury is a main determinant for the mode of cell death in that if the insult is severe enough to damage most of the mitochondria, cellular ATP levels will drop and oncotic necrosis occurs. Is the insult less severe or even moderate, enough mitochondria are left intact to maintain cellular ATP levels, allowing apoptosis but preventing necrotic cell death (476, 592) (Fig.

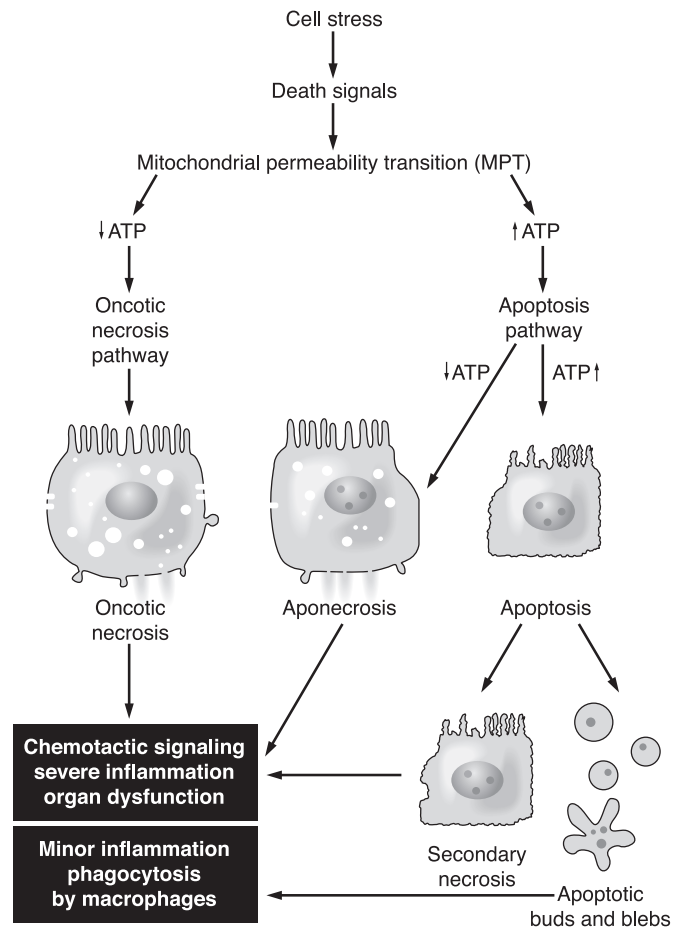


FIG. 14. Apoptotic and necrotic hepatocellular death in response to stress signals. Injurious stress causes mitochondrial permeability transition (MPT) with mitochondrial swelling and lysis, which represents a causative mechanism of acute necrotic cell death. MPT also precedes mitochondrial cytochrome *c* release, which is followed by a cascade of caspase activation, resulting in apoptotic cell death. The important factor in directing the cell death pathway secondary to the insult-induced MPT is the cellular ATP availability. The apoptosis pathway requires ATP, and intracellular ATP prevents the onset of the oncotic necrosis pathway. The characteristics of apoptotic cell death are condensation, fragmentation, and margination of the nuclear chromatin and shrinkage of the cell without damage of the plasma membrane. When MPT depletes ATP, necrotic cell death occurs, which is characterized by cytoplasmic vacuolization and cell swelling as well as plasma membrane blebbing and disruption. Of note, if ATP depletion develops during progression of apoptosis, the cell undergoes aponecrosis. This mixed form of cell death exhibits features of both apoptosis and oncotic necrosis. Apoptotic cells may break up into a cluster of apoptotic bodies, which are removed by neighboring cells or macrophages without a marked inflammatory response. In contrast, oncotic necrotic hepatocytes as well as aponecrotic and secondary necrotic hepatocytes serve as strong chemoattractants for leukocytes, aggravating inflammation and organ dysfunction.

14). The dependency of induction of either necrotic or apoptotic cell death on the oxidative metabolism of the cell was nicely demonstrated by data in a graded model of hemorrhagic shock in rats, suggesting that prolonged low flow/hypoxia induces ATP depletion and pericentral necrosis, while restoration of oxygen supply and ATP levels

after shorter periods of low-flow ischemia propagates pericentral apoptosis (627).

In addition to the severity of the insult, the mode of the insult is also known to influence the nature, kinetics, and extent of the individual cell death. However, there is still constant discussion on which is the predominant pathway in most forms of liver disease. For example, in pig livers subjected to 180 min of warm ischemia, the frequency of apoptotic cells was reported to be only 2.6%, but that of necrotic cells 37% at 24 h after reperfusion (546). This is well in accordance with reports on rat livers exposed to 60 or 120 min of warm ischemia, demonstrating <1% apoptotic hepatocytes and increasing numbers of necrotic hepatocytes up to 57% over 24 h of reperfusion (257). These results challenge data of others, indicating 30 and 50% apoptotic hepatocytes in rat livers after 60 min of warm ischemia and 24 h of reperfusion (404). These controversies on apoptosis and necrosis become even more complex, surveying cold versus warm ischemia and the susceptibility of SEC versus parenchymal cells in normal or steatotic livers (457, 719, 793). In the aggregate, however, warm ischemic-reperfusion injury is thought to preferentially occur through oncotic necrosis (257), while both apoptosis and necrosis contribute to cold preservation injury of the liver (176, 340).

In hepatotoxin-induced injury models, using Gal (90, 391), dimethylnitrosamine (565), acetaminophen (345, 407), carbon tetrachloride (565), alcohol (30), microcystin-LR (153, 894, 896), and endotoxin (22), cytotoxicity has predominantly been related to apoptotic cell death, although necrotic tissue injury is also reported (227, 560, 909). The generation of reactive intermediate metabolites from the metabolism of hepatotoxins, and the generation of ROS during the inflammatory reaction account for the stimulation of a variety of pathways leading to cell death. These include covalent binding, disordered cytosolic calcium homeostasis, depletion of GSH and methionine pools (22), reduced activities of SOD and catalase (30), onset of MPT (153, 896) and, finally, lipid peroxidation (30).

In line with in vitro data, demonstrating that apoptosis and necrosis result from low and high concentrations of the hepatotoxin 7H-dibenzo(c,g)carbazole (DBC) and are dependent upon intracellular ATP levels (597), it became common view that ATP plays a pivotal role in directing apoptotic and necrotic cell killing (476). In proportion of MPT-associated injury of mitochondria and, thus, the extent of ATP depletion, progression of apoptosis is supervised by cell lysis in a pattern of secondary necrosis (27, 771, 772) (Fig. 14). In contrast to primary oncotic necrosis, secondary necrosis is unequivocally preceded by apoptotic hallmarks. For example, carbon tetrachloride and Gal are shown to cause apoptosis in the liver by activating caspase-3, which is released to the plasma by secondary necrosis, indicated by a concomitant rise in glutamate-oxaloacetate transaminase (GOT)

(771, 772). In support of this, secondary necrotic tissue injury, as assessed by the release of transaminases, can be completely prevented by inhibiting the onset of early apoptosis (27, 176).

Given the shared triggers, overlapping pathways, and common signaling events, the terms *necrapoptosis* (475, 476) or *aponecrosis* (205) have been established, describing a syncretic process of cell death sharing characteristics of both apoptosis and necrosis (101, 176, 388) (Figs. 14 and 15). Because necrotic cell death intervenes if ATP depletion occurs during the progression of the cell towards apoptosis, the term *aponecrosis* seems to be superior to the term *necrapoptosis*. Classic apoptosis and necrosis may represent only extremes of a continuum of intermediate forms of aponecrotic cell demise. Aponecrosis has been shown to be responsive to both antiapoptotic and antinecrotic strategies, including MPT inhibition (475, 934, 964), p53 and caspase inhibition (176, 705), application of IL-6 (772) and antioxidants (424, 934), as well as upregulation of Bcl-xL (220). This also underlines the idea of the existence of a mixed form of cell death (Fig. 15).

### C. Cell Death and Secondary Inflammatory Tissue Injury

Cellular oncotic necrosis with rupture of the plasma membrane and release of cell content represents a source of multiple mediators (118, 608), which are best known for their capacity to attract leukocytes. Dying hepatocytes serve as chemoattractants for primed leukocytes, guiding them during their migration. In cholestatic liver injury, more than 50% of all accumulated leukocytes are found extravasated and colocalized with foci of oncotic hepatocytes (259). In postischemic reperfused livers, Colletti et al. (118) described 1) a coincident increase in hepatic neutrophil sequestration, elevated serum ALT levels, and hepatic production of epithelial neutrophil activating protein; 2) a significant suppression of epithelial neutrophil activating protein by immunization against TNF- $\alpha$ ; and 3) an attenuation of intrahepatic neutrophil sequestration and ALT release upon neutralization of epithelial neutrophil activating protein. Moreover, livers benefit from the deficiency of chemoattractants, show reduced leukocyte infiltration and, thus, attenuated leukocyte-dependent secondary tissue injury (947). In addition, inhibition or deficiency of MMPs, which are critically involved in the degradation of extracellular matrix, decreases inflammatory cellular recruitment, because MMPs promote transmigration and are required for the motility of interstitially migrating leukocytes (383, 745). Leukocytes aggravate local injury by their release of ROS, which diffuse into neighboring hepatocytes, cause mitochondrial dysfunction, and trigger MPT-dependent oncotic necrosis (277, 337, 591). This view is further supported by the fact that

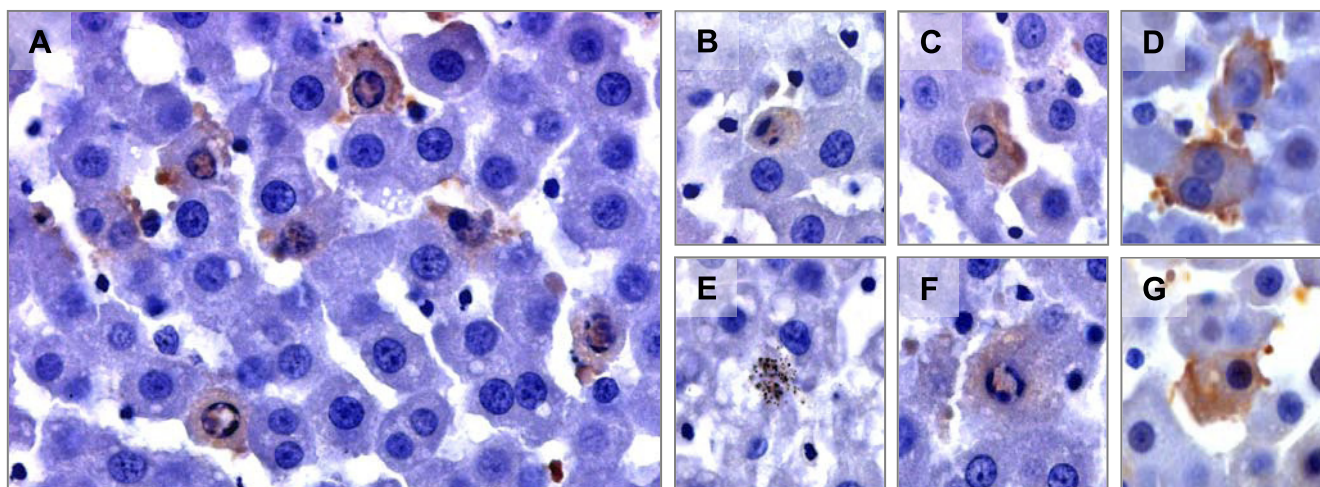


FIG. 15. Cleaved caspase-3 immunohistochemistry of liver specimens, which underwent cold storage and reperfusion. Note the staining of individual contiguous cells rather than groups of cells (A). B and E show caspase-3-positive cells with the classical signs of apoptotic death, i.e., nuclear fragmentation and cell shrinkage. Of interest, however, the caspase-3-positive cell in E displays also marked vacuolization, which is a characteristic of oncotic necrosis. Accordingly, cells in C and F display apoptotic characteristics, such as caspase-3 staining and nuclear condensation and margination; however, these are not associated with cellular shrinkage. Furthermore, cells may express caspase-3, but may lack nuclear changes and shrinkage, and may show, instead, characteristics of oncotic necrosis, such as plasma membrane blebbing (D, G). These cells sharing features of both apoptosis and necrosis are termed aponecrotic. A–G magnification  $\times 500$ . [From El-Gibaly et al. (176).]

glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury, because they cannot adequately encounter intracellular oxidant stress (338). In addition to ROS, proteases released by invading leukocytes (192) are involved in the pathway of leukocyte-dependent aggravation of tissue injury (Fig. 11), as given by the efficacy of protease inhibitors to attenuate liver injury (133, 147, 170, 271, 417, 923).

In addition to the acceleration and aggravation of necrotic tissue injury by infiltrating leukocytes, hepatocellular damage itself may contribute to sustain the detrimental inflammatory response by the release of high mobility group box-1 (HMGB-1) (704). In cytotoxic T-cell-mediated liver inflammation, this nuclear protein is translocated to the cytoplasm of hepatocytes surrounding necroinflammatory foci. It is further supposed to be released into the extracellular space by hepatocytes and responsible for recruitment of antigen nonspecific cells (744). HMGB-1, released from stressed and necrotic cells, has been shown to be a late mediator in systemic inflammation (879), but to act also as an early mediator following I/R injury (825, 892).

In contrast to the paradigm that apoptotic cell death does not induce an inflammatory response (377, 512), apoptotic hepatocytes, like necrotic ones, have been shown to exert chemotactic signals, triggering transmigration of primed leukocytes and sustaining the cell-dependent inflammatory response in severe endotoxemia (467) (Fig. 11). Colocalization of leukocytes with apoptotic hepatocytes in endotoxemic livers can be blocked by pancaspase inhibition, which confirms the causative role of apoptotic cells to provoke recruitment of leukocytes

(168). Infection of hepatocytes in culture with *Listeria monocytogenes* induces cell death by apoptosis with release of neutrophil chemoattractants. Accordingly, *Listeria monocytogenes*-infected mice livers present with apoptotic hepatocytes and recruited leukocytes (684). Similarly as shown for endotoxemia, repression of hepatocyte apoptosis by administration of the caspase inhibitor zVAD-fmk results in an attenuation of neutrophil infiltration and liver injury upon warm I/R (401). Fas ligation on hepatocytes with induction of cell apoptosis has been demonstrated to induce hepatic chemokine expression, which in turn causes leukocyte inflammation of the liver (190). In analogy to antigen-independent unspecific inflammatory liver diseases, cytotoxic T-cell-induced parenchymal apoptotic cell death is aggravated by the intrahepatic recruitment of antigen nonspecific cells. This contributes to the formation of necroinflammatory foci scattered throughout the liver parenchyma (362).

Apoptotic hepatocytes synthesize and release peptide mediators that recruit inflammatory cells and aggravate inflammation-dependent injury. This fact explains the discrepancy between the small number of apoptotic cells and the large protection observed with caspase inhibitors (131, 132, 401). In addition to leukocytes, KC might also promote the inflammatory response upon hepatocellular apoptosis. Activated KC are shown to frequently colocalize with apoptotic hepatocytes (170). They release cytokines and express death ligands upon phagocytosis, which further promotes hepatocyte apoptosis in a feed-forward loop (83) (Fig. 11). In line with data from experimental work, a positive correlation can be observed between hepatocyte apoptosis, hepatic fibrosis,

and inflammatory activity in patients with nonalcoholic steatohepatitis (194). Accordingly, colocalization of apoptotic hepatocytes with neutrophils found in alcoholic hepatitis underlines the relevance of apoptosis-dependent transmigration of leukocytes also in human pathophysiology (971).

## VII. MICROCIRCULATORY AND MICROVASCULAR MECHANISMS OF LIVER REPAIR

### A. Role of Platelets, Leukocytes, and Kupffer Cells

Platelets are recognized for their physiological functions that extend well beyond their role in hemostasis. There is a true body of evidence that platelets which are found activated and sequestered in the hepatic microvasculature are not innocent bystanders in this process. In fact, platelets actively contribute to the pathogenesis of organ damage and organ dysfunction upon I/R as well as in endotoxemia, steatosis, and cholestasis (136, 191, 453, 582, 629, 741, 742). Contrasting their critical involvement in inflammation, platelets may serve as regenerative cells themselves. This view comes from abundant data, connecting platelets to the repair of vascular injury and to the progenitor cell biology (448). For hepatocytes, it has been shown that platelets and platelet-derived serotonin can act as potent comitogens and induce DNA synthesis in primary cell cultures through the serotonin S<sub>2</sub> receptor (29, 189). Also, growth factors, like vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I), and hepatocyte growth factor (HGF), contribute to platelet-induced hepatocyte proliferation (534). Of interest for clinical use, freeze-dried platelets sufficiently maintain their storage of peptide mediators and growth factors and preserve their proliferative action on hepatocytes identical to that of fresh platelets (305). In line with these *in vitro* results, thrombocytopenia (GPIIb- $\alpha$  antibody) or impaired platelet activity (clopidogrel application) have been shown to result in failure of cellular proliferation in a mouse model of liver regeneration (479). In thrombocytopenic animals as well as in mice deficient for tryptophan hydroxylase-1, i.e., the rate-limiting enzyme for the synthesis of peripheral serotonin, it has been demonstrated that a serotonin agonist can reconstitute liver proliferation. This underlines the predominant role of serotonin for hepatocellular regeneration upon both resection and postischemic tissue injury (479, 596).

Depletion or modulation of platelet, leukocyte, and KC function has been convincingly shown to ameliorate inflammation and to protect the liver from subsequent endotoxemic, cholestatic, and postischemic as well as hepatotoxin-induced injury (see sect. IV). However, it is well established that hepatic inflammation is also part of the normal healing process (322). Livers from ICAM-1-

deficient mice exhibit impaired regeneration upon two-third resection. This is associated with a dramatic decrease of leukocyte recruitment and TNF- $\alpha$  and IL-6 tissue levels, which can be reconstituted by IL-6 supplementation (720). As physiological liver regeneration is not associated with markedly enhanced leukocyte-endothelial cell interaction (6), the availability of leukocytes and their cross-talk with KC rather than their activation per se seem to be mandatory for an adequate regeneration.

KC promote the resolution of fibrosis by leukocyte-dependent secretion of MMP-8 (275, 276). Vice versa, it is reported that KC inactivation during liver repair impairs collagen metabolism, inhibits the resolution of fibrosis, and supports the persistence of inflammatory cell infiltrates (685). These repair processes are crucially dependent on the balance of growth factors in aid of HGF (146, 513, 580, 928). In addition, depletion of KC in the regenerating liver alters the pattern of vasoregulatory gene expression, thereby limiting intrahepatic hyperperfusion as an important trigger of proliferation (5). In contrast, dysbalance of vasoactive mediators in postischemic reperfused livers is reported to be KC independent (390). These discrepant data might at least in part be attributed to the fact that marked differences exist between the action of the used KC-targeting substances, inducing either depletion, inhibition, or modulation of KC. Treatment with gadolinium chloride or dextran sulfate, which inhibit KC function, decreases acetaminophen toxicity in mice (455, 557). On the other hand, treatment of mice with Cl<sub>2</sub>-MBP, which completely depletes livers from KC, increases toxicity of acetaminophen (357).

Moreover, the importance of nonparenchymal cells, i.e., KC but also hepatic stellate cells and endothelial cells, in the process of liver regeneration and repair is further supported by data demonstrating activation of KC and hepatic stellate cells upon transplantation of hepatocytes which partly plug the liver microvasculature. This reflects the engagement of KC and hepatic stellate cells in the local host defense and in the initiation of liver repair mechanisms owing to ischemia-related cell damage (900). Furthermore, endothelial cells are mandatory for angiogenesis within the regenerating tissue mass. This is indicated by the fact that acting via induction of endothelial cell apoptosis angiogenesis inhibitors cause a cessation of the regenerative process (247).

### B. Kupffer Cell-Leukocyte Interaction

Although a dysregulated or overwhelming activation of KC and leukocytes is well known to contribute to an exaggeration of tissue inflammation (see sect. IV), physiological functions of these cells facilitate the elimination of invading organisms and represent the first line of defense. Recent reports on the underlying mechanisms in-

dicating that both cell types are complementary in the bacterial elimination process and that KC may play a less significant role in resolving systemic infections (250). While KC initially bind most organisms taken up in the liver on their extracellular surface by the interaction of lectins with the bacterial carbohydrate residues (602), leukocytes internalize and kill these organisms (250). The obligatory requirement of leukocytes in bacterial killing has been established by experiments in leukocyte-depleted animals, demonstrating a sharp decline in the capacity to kill gram-positive and gram-negative organisms trapped in the liver (120, 845, 851). In addition, blockade of the ICAM-1-CD11b/CD18 interaction using specific antibodies, which inhibit the accumulation of leukocytes, decreases the elimination of bacteria (249). These results have established the paradigm that those complementary adhesion molecules, beyond modulating leukocyte traffic and adhesion in the liver, facilitate the accumulation of bactericidal leukocytes at the surface of KC (249, 250). Thereupon, leukocytes eradicate the organisms and are ingested and destroyed by KC (250) (Fig. 10C). Because ICAM-1 is also expressed on other cells than KC, such as SEC and hepatocytes, these cells might effectively contribute in the fight against bacterial load. Accordingly, hepatocytes have been described as nonprofessional phagocytes (248).

The phagocytosis of leukocytes by KC is crucial for limiting inflammation, because by this elimination leukocyte-dependent production of toxic metabolic compounds and degradative enzymes is inhibited. The binding of apoptotic leukocytes or leukocytes expressing surface changes that enable their recognition (732) seems to involve, among other receptors with broad, lipid-binding capacity, a phosphatidylserine specific receptor (186), the  $\alpha$ -4 beta-3 integrin (the vitronectin receptor) and CD36 complex (703). These three molecules probably constitute a single, large receptor complex (185). In addition, carbohydrate-specific receptors are active on KC (492). Upon binding, the cells are ingested by phosphatidylserine receptor-mediated macropinocytosis (303). In consequence, phosphatidylserine blockade by annexin V results in inhibition of phagocytosis by KC (732). There is considerable controversy on the consequence of ingestion of apoptotic leukocytes by KC. There are reports about the release of either anti-inflammatory cytokines (434) or proinflammatory cytokines (435, 831). This might be due to the markedly different *in vitro* settings chosen, but also due to the predominant receptor being involved (187) and to the stage of apoptosis the cells are, as an even silent cleanup of very early apoptotic cells by macrophages is reported (433). If the timing for phagocytosis is retarded, inflammation might occur through unbalanced production of proinflammatory cytokines. This may explain that in mice with bile duct ligation engulfment of apoptotic bodies by KC promotes death ligand and cytokine expres-

sion and, thus, inflammation (83). However, there are also contrasting data demonstrating also in bile duct-ligated animals an abrogation of cholestatic liver injury by KC via IL-6 release (226).

### C. Microvascular Blood Flow and Shear Stress

The maintenance of an adequate hepatic blood flow is crucial for homeostasis (463). One mechanism that acts to maintain hepatic blood flow constant per liver mass is the HABR. In addition to physiological conditions, the HABR has been described to be also maintained in cirrhotic livers of animals and humans (18, 572, 670) and after liver transplantation (286). Of interest, this response is lost upon brain death (236), under increased intra-abdominal pressure (CO<sub>2</sub> pneumoperitoneum) (671), and during endotoxemia (25, 262, 708). The fact that NO (262, 789) but also terlipressin (24) restore the HABR during endotoxemia reflects the temporal and spatial complexity in the misbalance of expression of vasoconstrictive and vasodilative mediators (see sect. v). Restoration of the HABR is shown to prevent subsequent hepatic damage in the course of endotoxemia (789).

In case of supranormal portal blood flow, as it occurs upon extended liver resection or small-for-size split liver transplantation, an intact HABR, though of principal benefit, may cause diminished arterial flow and is accused of significantly contributing to the life-threatening small-for-size syndrome with graft failure (144, 749). In line with this, pharmacological strategies to increase hepatic arterial blood flow (85) and maneuvers reducing the portal hyperperfusion and preventing hepatic artery constriction (57, 231, 325, 420, 501, 796, 819) have been shown to improve the postoperative clinical course after extended hepatectomy. Evaluation of posttransplant biopsies of small-for-size syndrome livers has revealed poor hepatic arterial flow and vasospasm, which resulted in functional dearterialization, ischemic cholangitis, and parenchymal infarcts (144). Beside the arterial hypoperfusion-related hypoxic sequelae (676, 749), portal hyperperfusion in small-for-size livers leads to portal vein and periportal sinusoidal endothelial disintegration and denudation (144). This is associated with the development of fused fenestrae and microvilli protruding into the vascular lumen (61), seriously impairing the postoperative recovery of these livers (325, 518). Liver biopsies from right lobe live donor liver recipients reveal ET-1 overexpression, together with downregulation of HO-1 and heat shock protein 70 (518), underlining the pivotal role of misbalanced vasoactive mediators in the pathogenesis of the small-for-size syndrome (615). In line with this, the hepatic regenerative capacity is found deteriorated by ischemic preconditioning of reduced size livers, most probably due to the preconditioning-associated vasodilation and

thus aggravation of hyperperfusion (169). Vice versa, splenectomy-associated reduction of portal venous blood flow diminishes shear stress-induced liver injury and positively influences postoperative regeneration (231).

Aside from harmful effects of excessive portal hyperperfusion, the hyperperfusion-associated increase of intravascular shear stress is one of the main factors contributing to cell proliferation (593, 701, 702, 810, 880). Physical stimuli are converted into chemical signals with production of endothelium-derived relaxing factors, among those NO and prostaglandins are the best-studied candidates (77, 204). In plasma of partially hepatectomized rats, various growth factors, cytokines, and hormones have been detected, causing proliferation of cultured hepatocytes. This proliferation could be blocked by the NOS inhibitor  $N^G$ -nitro-L-arginine methyl ester (L-NAME) and restored by L-arginine (880). From in vivo models of partial hepatectomy there is convincing evidence for a shear stress-related release of NO and prostaglandins. These molecules most probably trigger the liver regeneration cascade, because proliferation is inhibited by NOS antagonists and because this inhibition is reversed by NO donors or the prostaglandins PGE<sub>2</sub> and PGI<sub>2</sub> (85, 711, 712).

Patients with small, but not with large, liver grafts show an immediate increase of portal pressure, reflecting an increased sinusoidal shear stress, which accelerates liver regeneration through immediate induction of IL-6 and HGF (612). The relevance for appropriate shear stress in liver regeneration is reflected by genome expression analysis. These studies demonstrate differentially expressed genes in livers with a high portal pressure, which have functions related to apoptosis, NO metabolism, and oxidative stress, whereas differentially expressed genes in the livers with low portal pressure potentially regulate the cell cycle (570).

Wall shear stress regulates adaptive vessel growth and angiogenesis, which is of essential importance during the resection-induced restoration of new liver tissue mass. Initial mechanisms, such as the recruitment of all sinusoids and the increase of sinusoidal diameters preserve the functional vessel area after resection, guaranteeing an adequate oxygen supply to tissue (5, 855). Concomitantly, growth factor-driven endothelial cell proliferation with new vessel formation takes place and dictates the regenerative process (247).

#### D. Gaseous Molecules NO, CO, and H<sub>2</sub>S

Beyond the pure vasoactive property of NO, this gaseous molecule is a potentially important paracrine mediator in many organs, including the liver (682). With the advent of mice selectively deficient in the various isoforms of NOS, the role that NO plays in liver injury and

repair can be examined much better than the different and often very unspecific NOS inhibitors allowed. Consistent with the concept that excessive production of NO through inflammation-associated upregulation of the inducible NOS aggravates liver injury and that basal levels of NO deduced from the constitutive NOS mediates rather protection, postischemic liver injury is significantly enhanced in eNOS-deficient but not in iNOS-deficient mice (295, 374, 794). Comparably, eNOS deficiency increases necrapoptosis and fails to improve hepatic microcirculation upon cold storage and reperfusion (807). Accordingly, iNOS-deficient mice have been found less sensitive to the hepatotoxic effects of acetaminophen than wild-type mice (223). The liver-selective NO donor V-PYRRO/NO mediates protection by reduction of oxidative stress, inhibition of TNF- $\alpha$  and TNF-related apoptosis, and possibly by maintenance of hepatic microcirculation to prevent congestion (493, 494). Moreover, TNFR1(-/-) mice have been shown significantly more sensitive to the hepatotoxic effects of acetaminophen, most supposedly due to a more rapid and prolonged induction of iNOS in the liver (222). There is the hypothesis that the use of iNOS inhibitors is only beneficial in the presence of a functioning eNOS system, as some NO is needed to maintain adequate organ function (135). This view is deduced from data demonstrating that liver injury in endotoxemic animals with unspecific NOS blockade profit from simultaneous infusion of a liver-selective NO donor (611).

Generalized vasodilation and hypotension in endotoxemia is attributed to an overproduction of NO and CO by inducible NOS and HO-1. In addition, the most recently recognized gaseous molecule H<sub>2</sub>S is overproduced in vascular tissue of septic animals and is shown to play a proinflammatory role with regulation of severity of sepsis and associated organ injury (310, 480, 953). In endotoxic stress-exposed livers, the expression and activity of both H<sub>2</sub>S-synthesizing enzymes CSE and CBS is found increased (119). Furthermore, H<sub>2</sub>S acts as an important endogenous regulator of leukocyte activation and trafficking by upregulating hepatic expression of the adhesion molecules ICAM-1, P-selectin, and E-selectin (952). However, the role of H<sub>2</sub>S as proinflammatory mediator is not unambiguous. Antiadhesive properties of H<sub>2</sub>S are also reported (520, 948). For example, Zanardo et al. (948) have shown that H<sub>2</sub>S donors inhibit aspirin-induced leukocyte adherence in mesenteric venules. In addition, antiapoptotic properties of H<sub>2</sub>S against leukocytes might cause prolongation of their life span, potentially enhancing the resolution of inflammatory processes (673).

While for each gaseous mediator, i.e., NO, CO, and H<sub>2</sub>S, an immense spectrum of effects is reported, the scenario becomes even more complex, considering the multifaceted interrelation of the molecules and their synthesizing enzyme systems with each other. There is first evidence for the formation of a novel nitrosothiol gener-

ated by the reaction between H<sub>2</sub>S and NO (898). NO can regulate endogenous production of H<sub>2</sub>S by increasing vascular CSE expression (961) and, vice versa, H<sub>2</sub>S enhances the NO-associated vessel relaxation (306). H<sub>2</sub>S upregulates synthesis of CO through induction of HO-1 (650). Furthermore, H<sub>2</sub>S is shown to inhibit NO production and NF- $\kappa$ B activation in LPS-stimulated macrophages through a mechanism that involves the action of HO-1 and CO (603). This most dynamic interplay between H<sub>2</sub>S, the NO pathways, and the CO systems almost precludes a prognosis on the net effect of these mediators in liver disease.

## VIII. MEASURES TARGETING HEPATIC MICROVASCULAR DISORDERS

### A. Antioxidative, Antiadhesive, and Anti-Inflammatory Approaches

One target to prevent inflammatory liver diseases is enhancement of the capacity of endogenous redox defense, because ROS play a pivotal role as cytotoxic and signaling mediators in the pathophysiology of tissue injury. Important sources of ROS formation are KC and invading leukocytes via NADPH oxidase-dependent superoxide generation. In addition, ROS can also be generated intracellularly in every liver cell type after stimulation with cytokines (337, 344). The fact that oxidant-induced liver injury is not only the result of passive interaction of ROS with cellular macromolecules, like the peroxidation of lipid membranes, but in particular of the active modulation of cell signaling cascades by affecting antioxidant enzymes, transcription factors, and intracellular organelles (137), the strategies to augment the antioxidant defense systems are manifold.

The application of radical scavengers (SOD, NAC, tocopherol, and allopurinol) has been proven to be beneficial in experimental and human settings of warm and cold I/R by inhibiting inflammatory leukocyte accumulation (403, 408, 524, 573), microvascular perfusion failure (403, 408, 414, 573), lipid peroxidation (850), and graft dysfunction (809) (Table 2). These salutary effects of radical scavengers have also been shown for endotoxemic livers (914) and steatotic livers subjected to I/R (217) as well as in conjugation with hydroxyethyl starch as antioxidative resuscitation upon hemorrhagic shock (33). Finally, the University of Wisconsin and HTK preservation solution enriched with antioxidative agents are widely established tools in experimental and clinical settings of liver transplantation (195, 639, 769).

Inhibitors of NADPH oxidase, such as diphenylene iodonium, reduce the formation of superoxide anions and liver injury associated with hemorrhagic shock and resuscitation, most probably by the downregulation of the transcription factor AP-1 (3). They also prevent the neu-

trophil-derived oxidant stress in endotoxemia, which includes the formation of hypochlorous acid by myeloperoxidase (258). NADPH oxidase inhibitors further attenuate early alcohol-induced liver injury by inhibiting free radical formation via NADPH oxidase, thereby preventing NF- $\kappa$ B activation and TNF- $\alpha$  mRNA expression in the liver (411). In contrast to that, leukocytic NADPH oxidase-dependent oxidative stress seems to play a minor role in acetaminophen-induced toxicity (129). Next to NADPH oxidase, which is thought to be responsible for ROS production during later time periods of reperfusion (613), Rac1-regulated oxidase has been reported to mediate the production of injurious ROS, which contribute to apoptotic and necrotic cell death after I/R. Targeted inhibition of this oxidase, which is distinct from the phagocyte NADPH oxidase, should provide a new avenue for *in vivo* therapy, aimed at protecting organs at risk from I/R injury (613) (Table 2).

Prevention of inflammatory liver injury can further be achieved by increasing the extracellular antioxidant capacity by infusion of GSH to counteract extracellular and vascular oxidant stress (49, 51, 143). Upon postischemic GSH treatment, intravital fluorescence microscopy has demonstrated an almost complete restoration of sinusoidal blood flow, paralleled by a reduction of inflammatory leukocyte adherence in sinusoids and postsinusoidal venules, resulting in reduced necrotic tissue injury and improved rat survival (706). Micronutrients like selenium are observed to enhance the endogenous antioxidant capacity of the cells by increasing the activity of SOD and the GSH content (177). Accordingly, selenite supplementation significantly improves postischemic hepatic microcirculation by acting as a radical scavenger and by preserving the antioxidative capacity of the liver (949). This is supported by *in vitro* results on the organoselenium ebselen, which has been shown to be a potent inhibitor of NADPH oxidase in KC (882). Of interest, ebselen also protects against ET-1-mediated microcirculatory disturbances and cell damage in alcoholic liver injury (610). Furthermore, glutamine or alanyl-glutamine dipeptide are beneficial in supporting hepatic microcirculation and can protect against postischemic necrotic liver injury by enhancing GSH content (355, 778) (Table 2).

With the idea that inhibition of ROS has not only beneficial but also detrimental effects, because ROS are important in bactericidal defense and may trigger cell signals that are critical for hepatocellular proliferation, therapeutic strategies aim at increasing the resistance of hepatocytes against these reactive species and at targeting of downstream factors specific for the death effects of ROS (137). Heat shock proteins are considered to effectively mediate resistance against subsequent injurious stimuli. Heat shock protein 32, better known as HO-1, is among the most interesting molecule, exerting cytoprotective mechanisms activated during cellular stress (821).

TABLE 2. *Agents targeting the hepatic microcirculation, their site of action, and their mechanisms of inhibiting hepatic injury*

| Agent Family                                      | Family Members                                 | Mechanism of Inhibition of Hepatic Injury            | Liver Injury            | References  |
|---|--|--|-------------------------|---|
| Radical scavengers and antioxidants               | Superoxide dismutase                           | Inhibition of intrahepatic leukocyte accumulation    | I/R                     | Marzi et al., 1992; Müller et al., 1996; Kondo et al., 1999                   |
|   |  | Attenuation of sinusoidal perfusion failure          | I/R                     | Marzi et al., 1992; Müller et al., 1996; Kondo et al., 1999; Koo et al., 1992 |
|   | Deferoxamine-conjugated hydroxyethyl starch    | Inhibition of intrahepatic leukocyte accumulation    | Shock/resuscitation     | Bauer et al. 1999   |
|   |  | Attenuation of sinusoidal perfusion failure          | Shock/resuscitation     | Bauer et al. 1999   |
|   | N-acetyl-cysteine                              | Inhibition of lipid peroxidation                     | Shock/resuscitation     | Bauer et al. 1999   |
|   |  | Inhibition of leukocyte accumulation                 | I/R                     | Koeppel et al., 1996  |
|   |  | Attenuation of sinusoidal perfusion failure          | I/R                     | Koeppel et al., 1996  |
|   | Tocopherol                                     | Increase of portal flow                              | I/R in steatosis        | Fusai et al., 2005  |
|   |  | Inhibition of lipid peroxidation                     | I/R                     | Vardareli et al., 1998  |
|   | Allopurinol                                    | Inhibition of leukocyte accumulation                 | I/R                     | Müller et al., 1996   |
|   |  |  | Endotoxemia             | Xiang et al., 2003  |
|   |  | Attenuation of sinusoidal perfusion failure          | I/R                     | Müller et al., 1996   |
|   |  | Endotoxemia  | Xiang et al., 2003      |   |
|   |  | Reduction of hepatocyte apoptosis                    | Endotoxemia             | Xiang et al., 2003  |
|   | NADPH oxidase inhibitors                       | Reduction of CXC chemokine expression                | Endotoxemia             | Xiang et al., 2003  |
|   |  | Downregulation of AP-1                               | Shock/resuscitation     | Abdelrahman et al., 2005  |
|   | Rac1-oxidase inhibitors                        | Prevention of NF $\kappa$ B activation               | Alcohol                 | Kono et al., 2001a  |
|   |  | Prevention of TNF expression                         | Alcohol                 | Kono et al., 2001a  |
|   |  | Suppression of NF $\kappa$ B activation              | I/R                     | Ozaki et al., 2000  |
|   | Glutathione                                    | Reduction of necrotic cell death                     | I/R                     | Ozaki et al., 2000  |
| Reduction of apoptotic cell death                 |  | I/R  | Ozaki et al., 2000      |   |
| Inhibition of intrahepatic leukocyte accumulation |  | I/R  | Schauer et al., 2004    |   |
| Glutamine   | Attenuation of sinusoidal perfusion failure    | I/R  | Schauer et al., 2004    |   |
|   | Improvement of hepatic circulation             | I/R  | Bilzer et al., 1999b    |   |
|   | Detoxification of KC-derived hydrogen peroxide | I/R  | Bilzer et al., 2002     |   |
|   | Increase of hepatic microvascular blood flow   | I/R  | Szjártó et al., 2007b   |   |
| Micronutrients                                    | Selenium                                       | Regulation of the Bcl-2 to Bax protein ratio         | I/R                     | Jia et al., 2006  |
|   |  | Preservation of hepatic antioxidative capacity       | Pesticide               | El-Khawaga, 2005  |
|   |  | Amelioration of hepatic microcirculatory dysfunction | I/R                     | Zapletal et al., 2008   |
| Heat shock proteins and metabolites               | Heme oxygenase 1                               | Antioxidant function                                 | I/R                     | Zapletal et al., 2008   |
|   |  | Maintenance of hepatic microcirculation              | I/R                     | Maines, 2005; Tsuchihashi et al., 2004  |
|   |  | Anti-inflammatory action                             | I/R of steatotic livers | Yamagami et al., 2003   |
|   |  | Antiapoptotic action                                 | I/R                     | Wunder et al., 2004   |
|   | Carbon monoxide                                | Augmentation of hepatic iNOS expression              | SIRS                    | McCarter et al., 2004b  |
|   |  | Antiapoptotic action                                 | Endotoxemic shock       | Maines, 2005; Tsuchihashi et al., 2004  |
|   | Bilirubin and biliverdin                       | Augmentation of hepatic iNOS expression              | Endotoxemic shock       | Maines, 2005; Tsuchihashi et al., 2004  |
|   |  | Reduction of adhesion molecule expression            | Endotoxemic shock       | Sarady et al., 2004   |
|   |  |  | Endotoxemic shock       | Sarady et al., 2004; Hoetzel et al., 2007                                     |
|   |  |  | I/R                     | Vachharajani et al., 2000   |

TABLE 2—Continued

| Agent Family                           | Family Members                                   | Mechanism of Inhibition of Hepatic Injury         | Liver Injury                                      | References                                   |  |
|--|--|---|---|--|--|
| Anticoagulants                         | Activated protein C                              | Reduction of adhesion molecule expression         | I/R   | Kuriyama et al., 2009                        |  |
|  |  | Reduced production of TNF- $\alpha$               | I/R   | Kuriyama et al., 2009                        |  |
|  |  | Antiapoptotic action                              | I/R   | Kuriyama et al., 2009                        |  |
|  |  | Reduction of CINC mRNA expression                 | I/R   | Yamaguchi et al., 1997a                      |  |
|  | Antithrombin III                                 | Inhibition of intrahepatic fibrin deposition      | Resection of cirrhotic livers                     |  | Yoshikawa et al., 2000                       |
|  |  |   | Release of PGI <sub>2</sub>                       | I/R  | Okano et al., 1996                           |
|  |  | Improvement of ATP recovery                       | I/R   | Okano et al., 1996                           |  |
|  |  | Improvement of hepatic microvascular blood flow   | I/R   | Okano et al., 1996; Maksan et al., 2000      |  |
|  |  | Inhibition of intrahepatic leukocyte accumulation | Cirrhosis   |  | Maksan et al., 2005; Hisama et al., 1996     |
|  |  |   | Reduction of CINC production                      | I/R  | Yamaguchi et al., 1997a; Hisama et al., 1996 |
|  |  | Heparin   | Inhibition of intrahepatic leukocyte accumulation | I/R  | Yamaguchi et al., 1997a; Hisama et al., 1996 |
|  | Reduction of CINC production                     |   |   | I/R  | Yamaguchi et al., 1997a; Hisama et al., 1996 |
|  | Aprotinin  | Inhibition of intrahepatic leukocyte accumulation | I/R   | Maksan et al., 2000                          |  |
|  | Hirudin  | Reduction of intrahepatic leukocyte infiltration  | I/R, endotoxemia                                  | Kubes et al., 2002                           |  |
| Antiapoptotic agents                   | PARP inhibitors                                  | Preservation of hepatic microcirculation          | I/R   | Szijártó et al., 2007a                       |  |
|  |  | Caspase inhibitors                                | Preservation of endothelial cell integrity        | I/R  | Khandoga et al., 2004                        |
|  | Shock/resuscitation                              |   |   | Roesner et al., 2006                         |  |
|  | I/R  |   | Natori et al., 1999                               |  |  |
|  | Liver failure                                    |   | Natori et al., 2003; Wanner et al., 1999          |  |  |
|  | Reduction of intrahepatic leukocyte infiltration | Endotoxemia                                       | Eipel et al., 2004                                |  |  |
|  |  | Liver failure                                     | Wanner et al., 1999                               |  |  |
|  | Attenuation of sinusoidal perfusion failure      | Endotoxemia                                       | Eipel et al., 2004                                |  |  |
|  |  | Liver failure                                     | Wanner et al., 1999                               |  |  |
|  | Caspase siRNA                                    | Reduction of intrahepatic leukocyte infiltration  | I/R   | Contreras et al., 2004                       |  |
| Adhesion molecule antibodies           | sLe(x) oligosaccharide                           | Prevention of platelet adhesion                   | I/R   | Sindram et al., 2000; Khandoga et al., 2002a |  |
|  | Anti-ICAM-1 antibody                             | Prevention of leukocyte adhesion                  | I/R   | Vollmar et al., 1995b                        |  |
|  | Anti-P-selectin antibody                         | Prevention of platelet and leukocyte adhesion     | Endotoxemia                                       | Klintman et al., 2004                        |  |
| Vasoactive drugs<br>Pleiotropic agents | See Table 1                                      | Statins   | Bile duct ligation                                | Demirbilek et al., 2007                      |  |
|  |  |   | Antiapoptosis by induction of HO-1 expression     | I/R  | Lai et al., 2008                             |
|  | Erythropoietin (EPO)                             | Immunomodulation                                  | Endotoxemia                                       | Kruger, 2006                                 |  |
|  |  |   | Acute liver failure                               | Le Minh et al., 2007                         |  |
|  |  | Reduction of hepatocellular apoptosis             | Acute liver failure                               | Le Minh et al., 2007                         |  |
|  |  |   | I/R   | Yilmaz et al., 2004                          |  |
|  |  | Reduction of intrahepatic leukocyte infiltration  | Acute liver failure                               | Le Minh et al., 2007                         |  |
|  |  |   | Acute liver failure                               | Le Minh et al., 2007                         |  |
|  |  | Reduction of sinusoidal perfusion failure         | Acute liver failure                               | Le Minh et al., 2007                         |  |
|  |  | Reduction of oxidative stress                     | I/R   | Sepodes et al., 2006; Yilmaz et al., 2004    |  |
|  | Reduction of caspase activity                    | I/R   | Sepodes et al., 2006; Hochhauser et al., 2008     |  |  |
|  | Decrease of JNK phosphorylation                  | I/R   | Hochhauser et al., 2008                           |  |  |
|  | Decrease of I $\kappa$ B degradation             |   |   |  |  |

TABLE 2—Continued

| Agent Family               | Family Members | Mechanism of Inhibition of Hepatic Injury                         | Liver Injury        | References                                       |
|----------------------------|----------------|---|---------------------|--|
| L-Glycine                  |                | Downregulation of TLR4 signaling                                  |                     | Xu et al., 2008                                  |
|                            |                | Modulation of KC response   |                     | Neyrinck et al., 2005                            |
|                            |                | Inhibition of lipid peroxidation                                  |                     | Deters et al., 1997; Zhong et al., 1996          |
|                            |                | Reduction of intrahepatic leukocyte infiltration                  |                     | Yamanouchi et al., 2007                          |
|                            |                | Upregulation of antiapoptotic genes                               |                     | Zhang et al., 2000                               |
|                            |                | Prevention of sinusoidal endothelial cell apoptosis               |                     |  |
|                            |                | Inhibition of ligand-gated chloride channel                       |                     | Zhong et al., 2003; Frank et al., 2000           |
| Atrial natriuretic peptide |                | Reduction of TNF- $\alpha$ release                                | I/R                 | Duenschede et al., 2006; Yamanouchi et al., 2007 |
|                            |                | cGMP-dependent reduction of caspase-3 activity                    | I/R                 | Gerwig et al., 2003                              |
|                            |                | Activation of p38 MAPK  | I/R                 | Kiemer et al., 2002b; Kobayashi et al., 2007     |
|                            |                | Antiapoptotic signaling by Akt and bad phosphorylation            | I/R                 | Grutzner et al., 2006                            |
| Melatonin                  |                | Restriction of cytosolic hepatocellular Ca <sup>2+</sup> increase | I/R                 | von Ruecker et al., 1989                         |
|                            |                | Improvement of hepatic redox state and hepatic perfusion index    | Shock/resuscitation | Mathes et al., 2008a                             |
|                            |                | Inhibition of lipid peroxidation                                  | I/R                 | Mathes et al., 2008b                             |
|                            |                | Antinitrosative activity  | Steatosis           | Sewerynek et al., 1996                           |
|                            |                | Acetaminophen   |                     | Pan et al., 2006                                 |
|                            |                | Increase of NO bioavailability                                    | I/R                 | Matsura et al., 2006                             |
|                            |                |   |                     | Zhang et al., 2006b                              |

The cytoprotection may result from the elimination of heme and the function of HO-1 downstream mediators that are biliverdin, CO, and free iron (511, 841). The HO system is thought to be protective by four major mechanisms, including 1) the antioxidant action, 2) the anti-inflammatory action, 3) the vasodilation-induced preservation of the microcirculation, and 4) the modulatory function upon the cell cycle. In line with this, HO-1 induction, using heat shock, PDTC, cobalt protoporphyrin, adenoviral-mediated gene transfer, or L-arginine, improves hepatic microcirculation and reduces tissue injury upon I/R (283, 450, 804, 920). It further protects against hepatic parenchymal injury and microvascular dysfunction during experimental rhabdomyolysis (156) and during the systemic inflammatory response syndrome (SIRS) (537). Vice versa, HO inhibition, which is achieved with the use of chromium mesoporphyrin (CrMP), significantly reduces sinusoidal diameters and volumetric flow and causes significantly increased numbers of stationary leukocytes and microvascular perfusion deficits. This results in increased hepatocellular injury and hepatocyte death in models of remote hepatic injury and systemic SIRS (538, 912).

In endotoxemic and septic livers, where iNOS and HO-1 overproduce NO and CO (691, 854), the role of HO-1 is rather ambiguous. A protective role of HO activation has been shown, possibly as a result of an interaction with the LPS-induced increase in NO forma-

tion (584). At the same time, CO, produced by excessively induced HO-1 after cecal ligation and puncture, promotes an immoderate dilation of the sinusoidal space through the upregulation of cGMP, resulting in liver dysfunction. In this case, the administration of HO inhibitors is beneficial for the attenuation of sepsis-induced liver dysfunction (331). In contrast, in perfused livers of endotoxemic rats, HbO<sub>2</sub>, which captures NO and CO, causes marked vasoconstriction and cholestasis (438). Of importance, the reduction of increased cGMP by HO-1 inhibition is associated with an increased mortality (157).

HO-1 is regulated in a tissue-specific manner (774) with an AP-1-dependent induction in liver tissue under oxidative stress conditions, such as hemorrhagic shock and resuscitation. These are assumed to function as a stress-inducible vasodilator system, as does NF- $\kappa$ B-dependent iNOS expression in liver inflammation (665). The particular localization of HO-1 in liver mitochondria has important implications for the protection against stress conditions such as hypoxia or sepsis, in which substantially increased mitochondrial NO and oxidant production occurs (122). In accordance with the tissue-confined regulation of HO-1, CO protection against endotoxic shock involves reciprocal effects on iNOS in the lung and the liver (698). In detail, the HO-1 metabolite CO protects hepatocytes from apoptosis while augmenting iNOS expression and inhibits LPS-

induced cytokine production in lung macrophages, while reducing LPS-induced iNOS expression and nitrite accumulation (698). Taken together, CO, when endogenously synthesized or exogenously applied at low concentrations, can exert anti-inflammatory and antiapoptotic effects in a variety of cellular and rodent models of sepsis and, furthermore, reduces morbidity and mortality in vivo (300) (Table 2).

An intriguing fact is the preconditioning efficacy of volatile anesthetics, which have been demonstrated to increase mRNA HO-1 expression, thereby preventing liver tissue from manifestation of postischemic reperfusion injury (710). In contrast to that, isoflurane fails to show hepatoprotective effects against LPS-induced liver injury. On the other hand, ketamine is capable of attenuating LPS-induced liver injury by upregulation of HO-1 and downregulation of iNOS. This implies that ketamine may be the anesthetic agent of choice for septic patients requiring anesthesia (768).

Current research highlights the complex association between inflammation and coagulation. The molecular links between the two are unquestioned (181). Inflammatory mechanisms upregulate procoagulant factors, in particular intravascular tissue factor expression, elicit the expression of leukocyte adhesion molecules, downregulate natural anticoagulants, and inhibit fibrinolytic activity (181, 739). Of the natural anticoagulants, the protein C pathway appears to be the most negatively influenced by inflammation (180). Activated protein C is a potent anticoagulant serine protease and known to have cell-protective properties by virtue of its anti-inflammatory and antiapoptotic activities. For instance, activated protein C exerts cytoprotective effects after postischemic reperfusion of rat livers. This is evidenced by decreased tissue injury, reduced intrahepatic leukocyte accumulation, probably due to reduced expression of P-selectin, ICAM-1, and CINC, reduced cytokine release, and improved hepatic microcirculation (432, 922). By inhibition of intrasinusoidal fibrin deposition and attenuation of hepatocellular necrosis, activated protein C significantly improves survival of rats in a lethal posthepatectomy acute liver failure model (942). Also, pretreatment with antithrombin III significantly improves the energy status and microcirculation, as well as histological damage in postischemic (607) and cirrhotic livers (515). Potential mechanisms for the action of antithrombin are considered to be the release of PGI<sub>2</sub> and, comparably to activated protein C, the reduction of leukocyte-endothelium interaction by attenuation of CINC (297, 514, 607, 921). Comparably to antithrombin, other anticoagulants, like heparin, aprotinin, and hirudin, are reported to exert hepatoprotection in hepatic injury models (297, 426, 514) (Table 2).

## B. Antiapoptotic Strategies

During inflammatory liver diseases, toxic free radicals and oxidants are produced, causing DNA damage, such as DNA single-strand breaks, which result in the activation of the nuclear enzyme poly(ADP-ribose)-polymerase (PARP). PARP assists in combination with the enzymes of the base-excision and -repair complex of the cell in energy-consuming reparative processes. The consequences of the activation of PARP1, which is the main isoform of the PARP family, are particularly important for the manifestation and propagation of tissue injury: 1) its role in DNA repair; 2) its consumption of oxidized NAD and capacity to deplete cellular energetic pools, which culminates in cell dysfunction and necrosis; and 3) its capacity to promote the transcription of proinflammatory genes (346). ATP depletion by PARP directs cells towards necrotic cell death (267). This is confirmed by in vivo studies in postischemic reperfused livers, where PARP promoted hepatocellular necrosis and PARP inhibition or PARP gene deficiency were capable of reducing transaminase levels (378, 381, 777). In line with this, overactivation of PARP with hepatoprotective effects by PARP inhibitors is also observed in systemic I/R, such as hemorrhagic shock and resuscitation (683, 776, 893) (Table 2).

Intrahepatic apoptotic cell death is a common feature of tissue injury upon various injurious stimuli. Pathways of apoptosis execution include cascadelike proteolytic cleavage and, thus, activation of cysteinelike proteases, such as caspase-2, -3, -6, -7, -8, -9, and -10 (695). In cold I/R, therapeutic strategies with various caspase inhibitors have been shown to exert beneficial effects by counteracting sinusoidal endothelial cell apoptosis (588, 589). Small interfering RNA targeted to caspase-8 and -3 also provide significant protection against I/R injury of the liver (121). Moreover, Fas ligation-induced sinusoidal endothelial cell apoptosis and hepatic microvascular perfusion failure can be positively influenced by pancaspase inhibition using zVAD-fmk (887).

Noteworthy, prevention of P-selectin-mediated platelet adhesion in postischemic livers using sLe(x) oligosaccharides or P-selectin gene-deficient mice (379, 741) decreases SEC and hepatocellular apoptosis, caspase-3 activities, sinusoidal perfusion failure, and liver enzyme release. Comparably, blockade of P-selectin using a P-selectin antibody ameliorates leukocyte recruitment, hepatocellular injury, and apoptosis in endotoxemic mice (396). In line with this, induction of leukocytopenia and thrombocytopenia in endotoxemic rats abrogates hepatocyte apoptosis, similarly as observed when the pancaspase inhibitor zVAD-fmk is applied (168). This indicates that both microvascular leukocyte and platelet adhesion contribute to hepatocellular apoptosis (Table 2).

Therapeutic strategies counteracting hepatocellular apoptosis also include the transfer of genes with anti-

apoptotic effects or the ablation/silencing of proapoptotic genes. For instance, expression of the Bcl-2 gene protects against I/R injury, as shown by a significant decrease in transaminases, necrosis, and apoptosis, and by permanent survival compared with animals treated with the control AdCMVLacZ vector (48). Vice versa, ablation of the proapoptotic bax in postischemic livers reduces postischemic apoptotic liver injury, as indicated by a significantly weaker activation of caspase-3 and reduced numbers of TUNEL-positive cells (43).

It has been demonstrated that the release of cathepsin B, a cysteine protease, from the cytosol in liver injury induces mitochondrial release of cytochrome *c* and the activation of caspase-3 and -9, thereby leading to apoptosis. Thus therapeutic approaches to block cathepsin B prove to be effective, again given by the significantly fewer apoptotic hepatocytes detected immunohistochemically, by a weaker activation of caspase-3, and by less TUNEL positive cells (42). In line, cathepsin B knock-out mice are resistant to TNF- $\alpha$ -mediated hepatocyte apoptosis and liver injury (250) and reveal reduced hepatic inflammation and neutrophil infiltration in cholestatic liver injury (84).

In addition, evidence is emerging that the endoplasmic reticulum participates in the initiation of apoptosis induced by the unfolded protein response and by aberrant Ca<sup>2+</sup> signaling during cellular stress such as I/R injury, nonalcoholic steatohepatitis, and alcoholic liver disease (366, 559). Endoplasmic reticulum-induced apoptosis involves the activation of caspase-12 and C/EBP homologous protein (CHOP), and the shutdown of translation initiated by phosphorylation of eukaryotic translation initiation factor-2 $\alpha$  (eIF2 $\alpha$ ) (366). Physiologically, liver cells cope with endoplasmic reticulum stress by an adaptive protective response termed "unfolded protein response," which includes enhanced protein folding and degradation in the endoplasmic reticulum and downregulated overall protein synthesis (354). The endoplasmic reticulum transmembrane eIF2 $\alpha$  kinase PERK phosphorylates eIF2 $\alpha$  to attenuate protein synthesis, including NF- $\kappa$ B-dependent antiapoptotic proteins. However, insufficient adaptation to endoplasmic reticulum stress, as observed for instance in endotoxin-challenged cirrhotic livers (799), unleashes pathological consequences, like inflammation and cell death, either leading to liver disease or worsening underlying causes of liver injury. There are various agents interfering with the endoplasmic reticulum-induced apoptosis, like the chemical chaperone sodium 4-phenylbutyrate, which reduces the load of mutant or unfolded proteins retained in the endoplasmic reticulum and exerts anti-inflammatory activity (630). In line with this, animals given sodium 4-phenylbutyrate survive with 50% in a lethal model of total liver I/R and reveal a decreased endoplasmic reticulum-mediated apoptosis, characterized by a significant reduction in caspase-12 activation and reduced levels of both phosphorylated eIF2 $\alpha$  and

CHOP (852). Also, temporary hypothermic machine preservation of cold-stored livers results in reduced endoplasmic stress and cleavage of caspase-12, with subsequent preservation of ultrastructural morphology (559). In cholestatic liver injury, which is associated with enhanced CHOP and bax expression, deficiency of mice for CHOP attenuates hepatocellular apoptosis and necrosis as well as subsequent liver fibrosis (788).

### C. Vasoactive Drugs

Oxidative stress and inflammatory reactions may promote hepatic microcirculatory dysfunction, which is known to be a determinant for subsequent hepatic injury. Because an altered liver microcirculation often results from an imbalance in the expression of stress-induced vasoactive mediators, numerous studies have been conducted, confirming the distinct pattern of up- and down-regulation of vasoregulatory enzyme systems (753). The most important candidates are NO, CO, and H<sub>2</sub>S as vasodilative gaseous molecules and ET as vasoconstrictive peptide mediator (725). In addition, derivatives of the arachidonic acid cascade with release of vasoactive prostaglandins (690) and leukotrienes (89) play a role in hepatic microcirculatory deteriorations (Table 1). There are innumerable results from both experimental and clinical studies, supporting the idea of the concept of a critical balance to improve hepatic perfusion and function. Both endogenous and exogenous NO protect hepatocytes and endothelial cells against hepatic I/R injury (97, 114) and endotoxemic liver damage (566). However, also selective iNOS inhibition prevents live bacteria from causing key features of metabolic derangements in porcine hyperdynamic sepsis, most probably due to reduced oxidative stress with improved microcirculatory perfusion and restoration of cellular respiration (527).

Further strategies to improve the hepatic microcirculation comprise the application of ET receptor antagonists in I/R (795, 834), in endotoxemia and endotoxic shock (179, 375) as well as in endotoxin-challenged cirrhotic livers (840). Finally, the direct application of PGs (i.e., PGE<sub>2</sub>) in hypoperfused livers (773) and gaseous molecules, like CO, may protect the nutritive perfusion of the hepatic microvasculature in endotoxemia and endotoxin shock (698).

### D. Immunomodulatory and Pleiotropic Agents

Injurious stimuli evoke tissue damage through a complex pathophysiologic network, involving distinct cell populations and humoral mediators, molecular pathways, and signal transduction cascades. These can barely be counteracted by monocausal therapies. Instead, approaches implementing pleiotropic substances are increasingly considered most appropriate to restore dis-

turbed homeostasis. Among others, promising candidates are statins, erythropoietin (EPO), HO-1, L-glycine, melatonin, atrial natriuretic peptide (ANP), and G-CSF (Table 2).

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, namely, statins, exert pleiotropic actions beyond lipid-lowering effects, such as antioxidative, anti-inflammatory, and immunomodulatory effects in *ex vivo* and *in vitro* studies. Statins ameliorate hepatic inflammation, lipid peroxidation, and tissue injury in rats subjected to common bile duct ligation (145). In addition, statins induce HO-1 expression, which protects livers from apoptotic injury in the setting of I/R (439). The pleiotropic action of statins is also able to protect from unspecific inflammation such as sepsis (423), which might be due to inhibitory actions of statins on leukocyte rolling, adherence, and transmigration, as described for the intestinal microcirculation during exotoxemia (645).

EPO has well been characterized as a renal glycoprotein hormone regulating red blood cell production by inhibiting apoptosis of erythrocytic progenitors in hemopoietic tissues. Recently, EPO has also been recognized as antiapoptotic and tissue-protective pleiotropic viability and growth factor in various nonhemopoietic tissues (20, 230, 350, 351). These salutary effects seem to be independent from the hematopoietic properties, as nonerythropoietic derivatives of EPO have shown an almost comparable action profile (67, 200). EPO reduces serum levels of ALT, TNF- $\alpha$ , and IL-2, indicators of oxidative stress, caspase-3 activation, and the histopathological score, promoting the subsequent reduction of apoptosis in I/R-induced liver injury (299, 721, 935). Comparably, EPO abolishes liver injury in hemorrhagic shock (2). Furthermore, mice with acute septic liver failure show diminished systemic cytokine concentrations, intrahepatic leukocyte accumulation, and hepatic perfusion failure as well as a significantly reduced hepatocellular apoptosis upon pre- but also posttreatment with the long-lasting EPO derivative darbepoetin- $\alpha$  (472).

L-Glycine is a simple, nonessential amino acid, acting as an inhibitory neurotransmitter in the central nervous system and long believed to be biologically neutral. However, recent experimental studies have demonstrated also anti-inflammatory, immunomodulatory, and cytoprotective actions (965). As a consequence, dietary glycine significantly improves the survival rate of endotoxemic mice. This is most probably due to an interference with the endotoxin signaling pathway, downregulating TLR4, NF- $\kappa$ B, and TNF- $\alpha$  and modulating KC response (590, 916). In fact, glycine has been shown to attenuate I/R injury by inhibition of oxidative stress-induced lipid peroxidation, reduction of inflammatory cell activation, and decrease of apoptosis-related cell death (149, 162, 929). In particular, SEC, which are most vulnerable upon cold storage and reperfusion, seem to be protected against apoptosis by glycine-associated maintenance of bcl-2 expression (959).

In addition, cellular protection seems to involve the inhibition of ligand-gated chloride channels, thereby secondarily inhibiting sodium influx, as this has been considered the protective mechanism of glycine for hypoxic hepatocytes (207). In contrast to these positive reports, the nutritional effects of a glycine-rich amino acid solution are found similar to those of a standard amino acid solution for parenteral nutrition in endotoxemic rats (502).

Another potential candidate for hepatoprotection due to its pleiotropic action is atrial natriuretic peptide (ANP). Interestingly, reperfusion injury by activated KC can be attenuated by the hormone ANP (50, 53, 228). The hepatoprotection conveyed by ANP in settings of liver I/R seems to involve the activation of p38 MAPK (385, 402), the restriction of cytosolic calcium increase in hepatocytes (877), the limited release of TNF- $\alpha$  by KC (386), as well as the cGMP-dependent reduction of caspase-3 activity (229) and the antiapoptotic signaling by phosphorylation of Akt and bad (254).

The pineal hormone melatonin has been demonstrated to possess distinct protective potential in models of oxidative stress, i.e., local (486, 726) and systemic (528, 529) I/R injury of the liver as well as liver exposure to acetaminophen (535) and cyclosporine (668). The proposed mechanisms of protection are said to involve direct inactivation of ROS and nitrogen species (791) and indirect effects by inducing antioxidative enzymes (617) and by improving the balance between NO and ET (958). Improved liver mitochondrial function (210) and enhanced bile flow and ATP stores (842) in cold stored livers support the idea of using melatonin as pleiotropic agent for patients, e.g., during liver transplantation.

Thus the use of pleiotropic substances represents an interesting novel approach to attenuate hepatic injury by protecting microvascular and cellular dysfunction. Accordingly, the efficacy of these substances is now required to be proven in controlled clinical trials.

## E. Preconditioning Maneuvers

The idea of preconditioning of tissues to enhance their robustness against subsequent insults originates from experiments in the canine myocardium performed by Murry et al. in 1986 (575). They demonstrated that multiple brief ischemic episodes protect the heart from a subsequent sustained ischemic insult (575). Ischemic preconditioning has been shown to exert protection by an immediate phase and a later phase. The immediate phase involves direct modulation of energy supplies, pH regulation, Na<sup>+</sup> and Ca<sup>2+</sup> homeostasis, and caspase activation. The later phase includes the synthesis of multiple stress response proteins (86). The maneuver is considered a promising approach to effectively minimize hepatic I/R injury (88, 343, 418). In experimental studies it has been

shown that ischemic preconditioning reduces neutrophil-specific oxidant stress in hepatocytes (277), ameliorates hepatic microcirculation (232), attenuates hepatic tissue apoptosis and necrosis (111, 652, 800, 918, 943), and improves overall survival (918). Of interest, ischemic preconditioning also increases the tolerance of fatty (198, 692, 720, 722) and cirrhotic livers (102, 348) to hepatic I/R injury. This is shown by an improved preservation and restoration of tissue ATP contents (720), an inhibition of apoptotic cell death (481), and an attenuation of hepatic perfusion failure, neutrophil accumulation (102, 419, 722), neutrophil-dependent oxidant stress, and lipid peroxidation (277).

With the increasing knowledge on the mechanisms of ischemic preconditioning (88), like the release of adenosine with activation of ATP-sensitive potassium channels, the augmentation of NO production and the upregulation of heat shock proteins and antioxidant enzymes (86), many different approaches, which mimic ischemic preconditioning, were successfully applied to reduce I/R injury. These include the application of adenosine (724), adenosine A(1) and A(2) receptor agonists (9, 19, 451), as well as nitrite ( $\text{NO}^{2-}$ ) as an intrinsic signaling molecule that is reduced to NO (738) and bilirubin as metabolite of the HO-1 metabolism (372). However, protection can also be achieved by induction of heat shock (558, 733), infusion of glutathione (706), or exposure to ozone (10, 478) and hyperbaric oxygen (945).

Experimental studies have demonstrated that ischemic preconditioning protects ischemic livers from microcirculatory failure and leukocytic inflammation during reperfusion (232, 419, 843). Analogous to ischemic preconditioning, pharmacological strategies, like administration of phosphodiesterase inhibitor-3 (430, 779), *N*-acetylcysteine (NAC) (233), and pyrrolidine dithiocarbamate (PDTC) (279) or whole body hyperthermia for HO-1 induction (805, 920) are capable of specifically protecting the postischemic hepatic microcirculation. This protection includes an improvement of microvascular perfusion and tissue oxygenation, a reduction of leukocyte adherence, and the maintenance of KC phagocytic activity.

It was further shown that the hepatoprotective effects of preconditioning can be simulated by  $\text{TNF-}\alpha$ .  $\text{TNF-}\alpha$  initiates the priming phase of liver regeneration via  $\text{NF-}\kappa\text{B}$  DNA binding and IL-6 production and exerts identical downstream effects on cell cycle entry, such as STAT3, cyclin D1, and cyclin-dependent kinase 4 (cdk4) expression (802). Hereby, IL-6, rather than  $\text{TNF-}\alpha$  itself, seems to be the mediator of the hepatoprotective and proliferative effects of ischemic preconditioning (801). Moreover, not only  $\text{TNF-}\alpha$  as death ligand, but also FasL is reported to mimic ischemic preconditioning (349).

Many human trials have confirmed the beneficial effects of ischemic preconditioning on the postoperative course and liver regeneration after hepatectomy (106,

108, 110, 111, 284, 481). These studies reported about improved intraoperative hemodynamic stability (108), reduced blood loss (284), increased formation of adenosine (107), downregulation of potentially cytotoxic functions of leukocytes (106), and decreased liver injury, as assessed by aspartate aminotransferase (AST) and ALT release (110). However, there are also reports, either disproving efficacy of ischemic preconditioning for hepatectomy in humans (26), or demonstrating less advantage over or comparability with intermittent vascular clamping during liver resection (632, 750). Also, preconditioning of liver grafts from brain dead donors did not improve graft injury (409) and clinical outcome (94), or even increased reperfusion injury, termed as ischemic preconditioning paradox (410). In summary, most recent meta-analyses, systematic reviews, and Cochrane reports provide no evidence to support or refute the use of ischemic preconditioning in donor liver retrievals (265) and to recommend it as a standard procedure for hepatic resection (653).

## F. Surgical Procedures

Several surgical approaches have been proposed to reduce portal blood inflow and portal pressure in the situation of portal hyperperfusion after extended hepatectomy and segmental liver transplantation. These approaches are applied to prevent the small-for-size syndrome, which is known to predominate graft dysfunction and poor survival in case of extreme size mismatch. The safety limit is thought to be 40% of the ideal liver weight (393) or 1% of the recipient body weight (500). As portal flow linearly correlates with spleen size, the parameter liver graft-to-recipient spleen size ratio has been suggested as a novel predictor of portal hyperperfusion syndrome in living donor liver transplantation (103). With the aim to counteract the small-for-size syndrome, splenic artery ligation or embolization, splenectomy as well as portocaval and hemiportocaval shunts are favored to relieve the remnant tissue from the deleterious effect of portal overflow (253, 818–820, 780, 919). Experimental studies applying these approaches could prove the efficacy of these techniques, i.e., the reduction of portal hyperperfusion (58, 231, 881).

To overcome the high risk of parenchymal dysfunction and liver failure in case of extended hepatectomy and insufficient remnant liver mass (574), portal vein occlusion has been introduced (516). Several weeks before hepatectomy, either ligation (69) or embolization (1, 509) of portal vein branches induce atrophy of the deprived lobe, while the anticipated lobe undergoes compensatory hypertrophy (1). This should guarantee an adequate mass of the liver remnant after the later hepatectomy. However, recent reports demonstrate that the loss of portal blood flow after portal vein ligation is compensated by the

HABR, which allows normalization of overall blood flow and oxygenation also in the ligated lobe (405, 676, 839). This arterIALIZATION even enhances cell proliferation in the ligated lobe, which may potentially bear the risk of tumor progression (405).

To minimize the anhepatic period and the period of partial rewarming of the ischemic graft in liver transplantation, it is generally accepted to reperfuse the liver with portal blood before arterial reconstruction, although this practice is discussed controversially (363). Detailed analysis of the hepatic microcirculation in a rat liver transplantation model has convincingly proven the superiority of simultaneous compared with delayed arterIALIZATION (638). In line with this, noninvasive OPS imaging performed in human living-donor liver transplantation confirmed the importance of timing of arterIALIZATION to the reperfusion of the liver. In this study, microvascular impairment has been shown significantly enhanced by the interval between portal venous and hepatic arterial reperfusion (646). This supports the view that simultaneous portal venous and hepatic arterial reperfusion should be achieved to prevent microvascular I/R injury in liver transplantation.

### G. Clinical Application of Measures Targeting Hepatic Microvascular Disorders

Although there is a large body of experimental studies demonstrating that microcirculatory disorders are determinants for liver injury and that injury can be attenuated by preventing or reducing microvascular dysfunction, little of this knowledge has been successfully transferred to clinical practice. There is in fact great need of appropriate tools for diagnosis of hepatic microcirculatory disorders in patients with shock and sepsis and those undergoing extended hepatic resection or liver transplantation. While noninvasive techniques in conditions of shock and sepsis are difficult to apply, NIRS and OPS imaging have been successfully introduced to analyze the hepatic microcirculation as well as hemoglobin and cytochrome oxidase content as parameters of hepatic perfusion and oxygenation in hepatic resection and liver transplantation (604, 648). These techniques allowed the detection of microcirculatory dysfunctions (647, 709) and enabled the monitoring and control of microcirculatory improvement after surgical procedures such as hepatic or controlled portal venous arterIALIZATION (646, 709). Nonetheless, microcirculatory diagnostic tools such as NIRS and OPS imaging are still not used routinely in clinical practice.

The fact that experimental studies have demonstrated that portal hyperperfusion and hepatic arterial buffer response-mediated hepatic arterial constriction induce hepatic microcirculatory dysfunctions, and that

downregulation of portal flow is capable of improving the microcirculation, has resulted in translation of this knowledge into clinical practice. Single cases or small series of patients have been successfully treated by splenic artery ligation of transient portocaval shunt to attenuate portal hyperperfusion and to improve hepatic arterial flow (57, 325, 796, 819). These surgical procedures, however, will always represent exceptions with major hepatic injury after extended hepatectomy and small-for-size transplantation.

The application of vasoactive substances, aiming at improving the hepatic microcirculation after cold I/R, has also been successfully transferred into the clinical setting. Therapeutic applications of prostacyclin (361) or prostacyclin donor preconditioning (394), but also PGE<sub>1</sub> (225, 416), have been shown to decrease arterial vascular resistance, improve arterial blood perfusion, increase oxygen supply, and ameliorate postischemic reperfusion injury.

Because many experimental studies have shown that oxygen radicals markedly contribute to ischemic and toxic liver injury, the use of antioxidants is long discussed to be used in critically endangered patients with liver ischemia, sepsis, and shock. In patients with hepatic dysfunction who were mechanically ventilated, either after liver transplantation or during an acute or decompensated chronic liver disorder, the antioxidant *N*-acetylcysteine has been shown to improve both indocyanine green extraction and oxygen transport (150). In addition, the University of Wisconsin (UW) and HTK preservation solutions have been, in addition to other ingredients, enriched with antioxidative agents. These solutions have been proven superior to others not containing antioxidative agents, although the distinct contribution of the individual antioxidants has not been clarified yet. In the clinical setting, both UW and HTK have become the gold standard for liver preservation worldwide (195).

Finally, a considerable number of clinical trials have shown a beneficial effect of ischemic preconditioning on the postoperative course after liver resection (106, 108, 110, 111, 284, 481). However, because there are also some studies in patients which disprove an efficacy after hepatectomy (26) or which could not show an improvement of graft injury in transplantation (94, 409, 410), it has not yet become a routine procedure in major liver surgery.

## IX. SUMMARY AND CONCLUSIONS

The circulation of the liver has to be considered unique in that it consists of a dual blood supply. By this, the overall inflow of blood into the liver is regulated through the adenosine-mediated HABR, increasing hepatic arterial blood flow in the case of reduced portal vein perfusion and decreasing hepatic arterial flow in the case

of portal hyperperfusion. In contrast, intrahepatic blood flow is controlled by the balance between the vasoconstrictive ET and the vasodilative gaseous molecules NO, CO, and H<sub>2</sub>S, which act on smooth muscle cells in feeding hepatic arteriolar and portal venular segments, but also through pericytes in hepatic sinusoids.

A considerable body of experimental work has now demonstrated that malperfusion of nutritive sinusoids in hepatic I/R, transplantation, and endotoxemia is due to a deterioration of the ET-NO balance with a shift towards the ET system, resulting in tissue hypoxia and apoptotic or necrotic cell death. According to this pathway, therapeutic interventions by inhibiting vasoconstrictive ET with appropriate receptor blockers or stimulating vasodilative NO or CO production are effective to attenuate microcirculatory deteriorations and to improve hepatocellular function and survival. These results confirm others achieved in cardiopulmonary research. Of interest, in cardiopulmonary medicine, this knowledge was introduced into clinical practice some years ago, and, today, critical patients with acute respiratory distress syndrome or pulmonary hypertension are treated successfully with NO and ET receptor antagonists (116, 360, 392). Thus future concepts in hepatology should also include the introduction of these treatment modalities for critical patients with liver failure, which is caused by microcirculatory disorders in sepsis, reperfusion injury, and hyperperfusion syndrome.

In addition to the dysfunction of the vasomotor control, the initiation of an inflammatory response contributes to the hepatic microcirculatory deteriorations in I/R, transplantation, and endotoxemia. The early mediators of inflammation are ROS and TNF- $\alpha$ , which induce a massive recruitment of leukocytes and platelets within the hepatic microvasculature. After activation of these cells, their adhesion to the microvascular endothelium is controlled by a sequence of adhesion molecules, expressed on the surface of leukocytes, platelets, and endothelial cells. The function of these adhesion molecules represents a prerequisite for successful transendothelial migration. According to mechanistic analyses with scavengers, antibodies, and gene-targeted animals, experimental studies have demonstrated that the inhibition of oxygen radicals and TNF- $\alpha$  is effective to reduce hepatic microcirculatory dysfunctions, microvascular injury, and organ failure. Nonetheless, these results could not be successfully translated into clinical practice. In septic patients, controlled randomized trials could not demonstrate substantial organ protection and improvement of survival after inhibition of TNF- $\alpha$  (203, 209, 586), while antioxidative selenium may improve clinical outcome, infections, and organ failure (46). Future therapeutic approaches to inhibit inflammation in I/R and sepsis may give more focus on those molecules that also maintain the vasomotor control. This view is based on the fact that ET may act proinflamma-

tory, while NO and CO have been shown to exert anti-inflammatory actions (293, 697). Thus the use of ET receptor antagonists or NO and CO donors may not only attenuate microcirculatory disorders by inducing vasodilation, but may also reduce the deleterious leukocyte- and platelet-mediated inflammation in these critically ill patients. The efficacy of this treatment strategy, however, still needs careful evaluation, because, for example, NO may reduce cytokine-induced leukocyte-endothelial cell interaction by inhibiting endothelial adhesion molecule expression (320); however, it may aggravate oxygen radical-induced hepatic injury by formation of peroxynitrite (13). In addition, future studies should further clarify whether the sum of effects of H<sub>2</sub>S on leukocytic inflammation is indeed anti-inflammatory (948), because recent reports have demonstrated also distinct proinflammatory H<sub>2</sub>S actions (962).

The nature of cell death induced by microcirculatory disorders after hepatic I/R and endotoxemia may involve both apoptosis and necrosis. While endotoxemia induces primarily apoptotic cell death, there is still a major controversy of whether I/R primarily induces apoptosis (404) or necrosis (257). However, recent studies brought evidence that apoptosis and necrosis share common pathways and that individual cells undergoing death after I/R show characteristics of both apoptosis and necrosis (176). Accordingly, caspase inhibitors or p53 inhibitors, which are thought to interfere specifically with apoptotic cell death, do not only attenuate apoptosis but also necrosis, as indicated by reduced levels of liver enzymes (176, 705). A major drawback of these inhibitors, which have been proven in experimental studies successful to attenuate organ injury and survival, is that they exert considerable side effects. Thus they cannot be tested for patient treatment in clinical trials.

Taken together, the dysfunction of the hepatic microcirculation is a determinant for organ failure in ischemic and inflammatory disease and contributes significantly to the high mortality in these patients. This knowledge, however, is still not considered for diagnostics and treatment in daily clinical practice. With the introduction of techniques for hepatic microcirculatory analyses in patients, such as LDF, OPS imaging, and NIRS, this drawback may be overcome, allowing an estimate of the quality of nutritive perfusion. The techniques may be applied most easily during open surgery, which gives direct access to the liver. In these cases, they may help to predict deleterious microcirculatory dysfunctions after prolonged cold preservation and transplantation-associated reperfusion or after extended hepatectomy and split liver transplantation, which develop a small-for-size syndrome. This diagnostic information achieved during surgery should be of great importance, because it would allow the early initiation of treatment to prevent manifestation of liver injury and failure. In line with this, the knowledge deduced from the

experimental work on the mechanisms and treatment of hepatic microcirculatory dysfunctions should also be translated into clinical practice. Including diagnostics and monitoring with OPS or NIRS, the novel therapeutic strategies have to be proven in randomized clinical trials. This may improve future outcome of patients with liver failure caused by microcirculatory dysfunctions.

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