Mechanisms of Liver Injury.

II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions

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Polymorphonuclear leukocytes (neutrophils) are part of the innate immune response to infection and tissue trauma. Because of the high mobility of these leukocytes and the capability to either release or generate potent cytotoxic mediators, the main function of neutrophil recruitment to sites of inflammation is to rapidly eliminate invading microorganisms and/or remove dead or dying cells. Unfortunately, in the liver, as in other organs, an excessive inflammatory response bears the risk of additional tissue damage as demonstrated during ischemia-reperfusion, endotoxemia, or obstructive cholestasis (5, 12, 15). Because the tissue-damaging mechanisms of neutrophils are closely related to their vital host-defense function, it remains a challenge to selectively prevent the detrimental effects. This themes article will attempt to summarize recent progress in the understanding of the mechanisms of neutrophil-mediated tissue injury and discuss relevant therapeutic options against neutrophil cytotoxicity.

Basic mechanisms of neutrophil activation and accumulation in the liver vasculature

Neutrophils accumulate in the liver vasculature in response to the exposure to inflammatory mediators such as TNF-α, IL-1α or IL-1β, CXC chemokines [e.g., IL-8, keratinocyte-derived cytokine (KC), macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant (CINC-1)], activated complement factors (e.g., C5a), platelet-activating factor (PAF), and others (12, 15, 17). These mediators increase the expression of CD11b/CD18, a member of the β2-integrin family of adhesion molecules, and other receptors on the surface of neutrophils by causing the fusion of secretory vesicles with the cell membrane. In addition, components of NADPH oxidase are recruited to the cell surface leading to the priming for enhanced reactive oxygen formation (3). These partially activated and primed neutrophils are recruited into sinusoids and postsinusoidal or portal venules without causing tissue damage (12, 15). In contrast to venular adhesion, neutrophil accumulation in sinusoids does not require adhesion molecules (12, 15). In general, neutrophils responsible for parenchymal cell damage are extravasating from sinusoids (15). If the sinusoidal endothelial lining is intact, the extravasation process involves β2-integrin family members and their counterreceptor on endothelial cells ICAM-1 and β1-integrins and VCAM-1 (12). Most importantly, extravasation into the parenchyma is the prerequisite for neutrophil cytotoxicity (15). Given these critical aspects of the mechanism of neutrophil cytotoxicity, potential therapeutic intervention strategies include eliminating proinflammatory mediator formation or activities to attenuate neutrophil activation and accumulation in the liver. In addition, blocking various adhesion molecules on neutrophils and endothelial cells will inhibit toxicity by preventing neutrophil extravasation. Lastly, interventions that prevent the release of cytotoxic mediators are highly beneficial. Each of these principal therapeutic strategies and numerous variations of them have been successfully used in many models of acute inflammatory liver injury including ischemia-reperfusion (12, 15, 17). However, because all these interventions also affect the host-defense function of neutrophils, these strategies may be of limited value in patients. Therefore, the following discussion will focus on two aspects of the mechanism: extravasation and cell killing, which may hold the promise to selectively prevent the detrimental effects.
Signals for Neutrophil Extravasation

**CXC chemokines.** Extravasation of neutrophils, which is a prerequisite for cytotoxicity, requires a signal from the parenchyma. Glutamyl acid-leucine-arginine (ELR)-containing CXC chemokines, e.g., IL-8, MIP-2, KC or CINC-1, are potent chemoattractants for neutrophils (17). Overproduction of CXC chemokines selectively in hepatocytes caused hepatic neutrophil infiltration, extravasation, and neutrophil-mediated liver injury (12, 17). Neutralizing antibodies against CXC chemokines or CXC receptor antagonists attenuated hepatic neutrophil accumulation and liver injury during ischemia-reperfusion (17). Most recently, evidence was provided that neutrophil-derived matrix metalloproteinase-8 (collagenase-2) may be responsible for neutrophil extravasation after galactosamine/TNF treatment by proteolytically cleaving lipopolysaccharide-induced CXC chemokine (LIX, CXCL5) bound to extracellular matrix proteins (21). These findings indicate that CXC chemokine formation in hepatocytes can be a signal for neutrophil extravasation under certain circumstances. Alternatively, proforms of CXC chemokines bound to extracellular matrix can be cleaved and provide a chemotactic gradient for neutrophils. However, these mediators do not always function as chemoattractant. In the galactosamine/endotoxin (Gal/ET) shock model, MIP-2 and KC are formed in substantial amounts in the liver but are not responsible for neutrophil extravasation (2). Chronic overexpression of IL-8 in transgenic mice increases the number of neutrophils in sinusoids without transmigration. In fact, IL-8 overexpression actually attenuates TNF-induced hepatocellular apoptosis, which correlated with activation of phosphatidylinositol (PI)3-kinase/AKT survival pathways (7). On the other hand, high doses of MIP-2 and other ELR-containing CXC chemokines can also protect against drug-induced liver cell injury by triggering mitosis and liver regeneration (10). Thus CXC chemokines are only relevant for neutrophil extravasation if generated at the appropriate time, released at the right location to form a gradient, and formed in sufficient quantities relative to other potential mediators.

**Apoptosis.** In contrast to the generally accepted dogma that apoptotic cell death does not trigger an inflammatory response, excessive apoptosis can trigger extravasation of neutrophils in the liver in the Gal/ET model (13). Pancaspase inhibitors effectively prevented hepatocellular apoptosis and eliminated neutrophil extravasation into the parenchyma (6, 13). How neutrophils recognize cells undergoing apoptosis remains unclear. Because apoptotic cells can generate CXC chemokines (7), the effect may be triggered by soluble chemotactic factors. However, caspase inhibitors prevented neutrophil extravasation but had no effect on CXC chemokine formation (2). Alternatively, gaps in the sinusoidal endothelial cells may facilitate the direct contact of neutrophils through pseudopods with the underlying apoptotic hepatocytes. The altered membrane composition of apoptotic cells (expression of phosphatidylserine on the cell surface) can trigger migration, phagocytosis, and formation of inflammatory mediators by phagocytes (1).

**Necrosis.** It is well known that necrotic cell death causes inflammation with hepatic neutrophil infiltration. Until recently, it remained unclear what actually initiated this nonmicrobial inflammatory response. It was shown that early activation of the complement cascade, presumably through cell content release of cells damaged by ischemia, can trigger Kupffer cell activation (12, 15). Complement factors stimulate reactive oxygen formation by Kupffer cells and directly activate and recruit neutrophils into the hepatic sinusoids (12, 15). A mediator specifically released by necrotic but not apoptotic cells is high mobility group box 1 (HMGB1), which is a nuclear factor bound to chromatin (20). Neutralizing antibodies against HMGB1 attenuated the formation of proinflammatory cytokines, reduced hepatic neutrophil infiltration, and protected against ischemia-reperfusion injury (20). HMGB1 can bind to the Toll-like receptor 4 (TLR4) on Kupffer cells and can induce the generation of proinflammatory cytokines (19). TLR4- but not TLR2-deficient mice showed reduced injury compared with wild-type animals after hepatic ischemia-reperfusion (25). Because HMGB1 antibodies did not protect in TLR4-deficient mice (20) and functional inactivation of Kupffer cells by gadolinium chloride only protected wild-type animals but had no effect on the reduced injury in TLR4-deficient mice (19), the data support the conclusion that HMGB1 promotes inflammation through cytokine formation after TLR4 binding (19). Thus HMGB1 released from necrotic cells may be an important soluble mediator for inducing and maintaining the nonmicrobial inflammatory response after ischemia-reperfusion.

Because reactive oxygen formation is an important consequence of Kupffer cell activation by complement factors and neutrophil recruitment, a variety of lipid peroxidation (LPO) products is formed during inflammation. Some of these LPO products, e.g., lipid aldehydes, are potent chemotactic factors for neutrophils (11). Because LPO is rarely a quantitatively relevant direct mechanism of cell injury in vivo, the protective effect of iron chelators and lipid-soluble antioxidants such as vitamin E may be explained by the reduced formation of chemotactic LPO products, which aggravate the neutrophil response (11). In addition to mediators originating from injured cells, the activated neutrophil can generate potent chemotactic factors, e.g., leukotriene B4 (LTB4). This eicosanoid is generated in high quantities during the neutrophil injury phase during reperfusion (12). Thus activated neutrophils can recruit more neutrophils and aggravate the inflammatory injury.

**Oxidant Stress and Neutrophil-induced Cell Killing**

Although there is extensive evidence for the importance of reactive oxygen species (ROS) in neutrophil-induced liver injury in vivo (11, 12), coculture experiments between activated neutrophils and hepatocytes identified proteases as the cytotoxic mediators without the involvement of ROS (9, 15). This discrepancy between the mechanisms in vivo vs. in vitro suggested a support role of ROS in protease-mediated cell killing (15). A reasonable explanation for these findings was the hypothesis that in vivo ROS are required to inactivate plasma antiproteases, which may interfere with the activity of neutrophil-derived proteases (15). However, more recent findings shed a different light on this process (Fig. 1). In general, neutrophils phagocytose small particles or adhere to larger targets using β2-integrins (CD18) and their counterreceptor ICAM-1 on hepatocytes (15). The engagement of CD11b/CD18 (Mac-1) on the neutrophil triggers a long-lasting oxidant stress through NADPH oxidase in close proximity to the target (11, 12). Superoxide generated by NADPH oxidase dismutates...
to oxygen and hydrogen peroxide, which is a highly diffusible oxidant. In addition, myeloperoxidase released from the neutrophil’s azurophilic granules can generate hypochlorous acid, another diffusible oxidant and chlorinating agent, which gives rise to other toxic species such as chloramines (3). Consistent with this hypothesis, there is evidence for an increase in tissue GSSG content as indicator for increased hydrogen peroxide levels during the neutrophil-induced injury phase during ischemia-reperfusion (8) and endotoxemia (14). In addition, the increase in intracellular chlorotyrosine residues (5, 6) and hypochlorous acid-modified proteins (8) demonstrated a specific neutrophil-derived oxidant stress in hepatocytes after neutrophil extravasation in these models. Because mice deficient in glutathione peroxidase 1 are more susceptible to neutrophil-induced injury than wild-type animals, these findings support the hypothesis that neutrophil-derived oxidants are critical for cell killing (14). This conclusion is further supported by experiments with inhibitors of NADPH oxidase, which showed reduced oxidant stress and injury (6, 8). These data suggest that neutrophils did not kill healthy cells but accelerated the killing of cells in the very early stages of apoptosis. Consistent with these observations is the well-recognized fact that healthy hepatocytes are generally very resistant to killing by intracellular oxidant stress (11). If neutrophils mainly attack dying cells, does this suggest that neutrophil-induced cell killing is merely an acceleration of cell death and therefore of limited overall pathophysiological consequence? The experience with interventions against neutrophils in models of hepatic ischemia-reperfusion injury, endotoxemia, and obstructive cholestasis clearly indicates that inhibition of neutrophil function causes long-term protection in these models (5, 12, 15). To reconcile the fact that neutrophils do not attack healthy hepatocytes, which have a high capacity to detoxify ROS, with the fact that antineutrophil therapeutic strategies can be highly beneficial, the following concept is proposed (Fig. 2): a cell, which is exposed to a significant level of stress, can either survive or die through apoptosis or oncotic necrosis. If neutrophils sense the stressed cell through chemoattractant mediators or direct cell contact, they will attack and kill this cell. Thus the recruitment of neutrophils into the liver in response to cell damage may severely limit any potential recovery of nonlethally stressed cells and therefore expand the area of necrosis. On the other hand, if the insult is very severe and causes...
Fig. 2. Impact of neutrophil activation on liver injury. Cells have 3 options to respond to stress or injury. 1) The cell survives the insult, and the stress triggers cell cycle activation and regeneration. 2) The insult is more severe and induces apoptosis. 3) The insult is very severe and causes oncosis. Oncotic necrosis and extensive apoptosis trigger an inflammatory response with neutrophil (PMN) recruitment into the hepatic vasculature. If neutrophils receive the appropriate distress signals from the injured parenchymal cells, they will extravasate and attack these target cells, which then die by oncosis. The fundamental difference for the tissue is that the neutrophil attack limits the number of surviving cells and thereby aggravates the overall tissue damage. In addition, increased onotic necrosis will recruit more neutrophils into the liver and further promote the inflammatory tissue injury.

progressive cell death, the recruited neutrophils may never be in a position to have a negative impact.

Relevance of Coculture Experiments?

Why are the mechanisms of neutrophil killing of target cells in vivo fundamentally different from results obtained in neutrophil-hepatocyte coculture experiments? The results are different because the conditions used in the coculture experiments do not accurately reflect the in vivo situation. Investigators used activated neutrophils but control hepatocytes for these experiments (11, 15). In most cases, neutrophils and hepatocytes were obtained from different species (11, 15). As we know now, this is unphysiological because there is no reason for a neutrophil to attack a healthy hepatocyte. In addition, a human neutrophil may not even recognize ICAM-1 expression or chemokines released from a rat or mouse hepatocyte exposed to cytokines. Thus, instead of the adherence-dependent oxidant stress and degranulation observed in vivo (Fig. 1), a neutrophil activated by inflammatory mediators in culture may release proteases and a limited amount of ROS into the medium. Because only proteases are stable for any length of time, they slowly digest hepatocytes eventually causing cell death after 15–20 h. This conclusion is supported by the finding that conditioned media of activated neutrophils are equally as effective in killing hepatocytes as the neutrophils themselves

(9). Elastase and cathepsin G were identified as the key cytotoxic mediators under these conditions (9). However, this selective protease-dependent mechanism of cell killing in culture is fundamentally different from the reactive oxygen-dominated process in vivo. In general, ROS kill by inducing mitochondrial dysfunction and opening of mitochondrial membrane permeability pores, which leads to the breakdown of the membrane potential and necrotic cell death (11, 12).

Proteases and Neutrophil-Induced Cell Killing

The beneficial effects of various protease inhibitors during ischemia-reperfusion injury suggest that proteases play an important role during a neutrophilic hepatitis in vivo (15). Neutrophil-derived proteases are able to directly cause cell death of hepatocytes (9). However, this may not be the most relevant contribution of proteases to the inflammatory injury mechanism. Neutrophils contain a large number of different proteins, which are critical for the migration of neutrophils to foci of inflammation and neutrophil cytotoxicity. The proteins are located in the membrane or matrix of several different vesicles and granules and can be released at different times (4). Secretory vesicles contain membrane-associated receptors including the β2-integrin CD11b/CD18, the complement receptor 1, formylmethionyl-leucyl-phenylalanine receptors, the lipopolysaccharide-binding receptor CD14, and components of the NADPH oxidase (cytochrome b558) (4). Secretory vesicles are mobilized on exposure of neutrophils to membrane-bound or systemically circulating inflammatory mediators. The fusion of the vesicles with the plasma membrane rapidly enhances the expression of the receptors on the cell surface during the early stages of inflammation. The main purpose of the increased expression of these proteins is to enhance the adhesiveness of the neutrophil to the activated vascular endothelial cells and to increase its responsiveness (priming) to inflammatory mediators or bacterial products. Because the matrix of the secretory vesicles contains mainly plasma proteins, fusion of these vesicles does not release cytotoxic proteins (4). During the transmigration process, β2-integrin-ICAM-1 interactions trigger the exocytosis of gelatinase granules with the release of collagenolytic metalloproteinases, which degrade vascular basement membrane components and facilitate the extravasation of neutrophils. Any subsequent migration through interstitial tissue is facilitated by partial exocytosis of specific granules, which contain receptors for extracellular matrix proteins, e.g., fibronectin, vitronectin, laminin, and additional matrix-degrading enzymes, e.g., collagenase (4). Once the neutrophil engulfs bacteria or adheres to a larger target, e.g., a hepatocyte, full degranulation of azurophilic and specific granules occurs. This effect releases a large number of bactericidal proteins, e.g., defensins, bactericidal/permeability-increasing protein, and various serine proteases including elastase, cathepsin G, and proteinase-3 into the phagosome or into the area around the cell, respectively (Fig. 1) (4). In addition, mobilization of azurophilic granules leads to the liberation of MPO, which, together with NADPH oxidase, generates the full range of ROS. In addition to the direct cytotoxic effects, these ROS inactivate plasma-derived inhibitors of the neutrophil serine proteases including α1-antitrypsin, α2-macroglobulin, α1-antichymotrypsin, and the secretory leukoprotease inhibitor. Because the serine proteases are less susceptible to the oxidant

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stress, this generates an area around the neutrophil where these proteases can be active. Thus, during a prolonged neutrophilic hepatitis, the combination of the neutrophil-derived oxidant stress and the activity of neutrophil serine proteases may result in the effective killing of target cells including hepatocytes (Fig. 1).

In addition to facilitating neutrophil migration and killing of target cells, neutrophil-derived serine proteases can also be involved in regulation of the inflammatory process (22). TNF-α is generated as a membrane-bound proform, which needs to be cleaved by TNF-α-converting enzyme to release the biologically active mediator. Proteinase-3, but not elastase, can process the proforms of TNF-α, IL-1β, and IL-18 in vitro (22). Consistent with this finding, the serine protease inhibitor α1-antitrypsin eliminated Gal/ET-induced liver injury by preventing TNF-α release (16). On the other hand, elastase can process the membrane-bound protransforming growth factor-α into the soluble transforming growth factor-α, which can activate the epidermal growth factor receptor (22). Furthermore, elastase can induce the formation of neutrophil- and monocyte chemoattractant chemokines in Kupffer cells (24). Urinary trypsin inhibitor-1 and the elastase inhibitor ONO-5046 prevented this effect (23, 24). Both inhibitors reduced chemokine formation, hepatic neutrophil accumulation, and liver injury during hepatic ischemia-reperfusion (23, 24). In addition to these observations, many more effects of neutrophil serine proteases on the processing of cytokines, chemokines, growth factors, and their respective receptors, of complement receptors, CD14, ICAM-1, and many other cell surface receptors have been reported (22). Most of the findings are from in vitro studies. Although most of these effects of serine proteases need to be confirmed in vivo and their pathophysiological relevance needs to be investigated in detail, the existing data suggest that the importance of these neutrophil-derived proteases extends substantially beyond their function in neutrophil migration and cytotoxicity. The local control of inflammatory mediators and their receptors by serine proteases may prove to be a critical effect in the regulation of neutrophil hepatitis.

In summary, microbial infections and tissue trauma trigger an inflammatory response in the liver with recruitment of primed and activated neutrophils into the liver vasculature; in particular, hepatic sinusoids. If parenchymal cells generate a chemotactic signal, neutrophils will extravasate and adhere to the target cell. Alternatively, gaps in the sinusoidal endothelial cell lining may allow direct contact between the neutrophil in the sinusoid and dying hepatocytes, thereby triggering extravasation and attack. The adherence to the target induces full degranulation with release of many proteases and formation of ROS. Some of the ROS can diffuse into hepatocytes and generate an intracellular oxidant stress resulting in mitochondrial dysfunction and eventually necrotic cell death. Some of the proteases, e.g., cathepsin G and elastase, support the cell killing process, whereas others may generate more proinflammatory mediators. In addition, the release of mediators (e.g., HMGB1, lipid aldehydes) from necrotic cells and the formation of chemotactic factors by the activated neutrophils themselves (e.g., LTB4) will activate and recruit more neutrophils to the site of inflammation and further aggravate liver injury. Although these mechanisms reveal a large number of therapeutic intervention points, due to the potential of interfering with the host-defense function of the neutrophils, most of these strategies are not realistic possibilities for human pathophysiology. However, more selective interventions may be possible. On the basis of the mechanistic understanding, one might predict that strategies, which strengthen the intracellular antioxidant defense mechanisms of target cells, neutralize signals released from necrotic cells (HMGB1), activate cell survival pathways, or initiate cell cycle activation and regeneration in hepatocytes, may represent viable therapeutic options to selectively break the cycle of a self-aggravating inflammatory injury. Ischemic preconditioning, which is a therapeutic strategy that triggers some of these mechanisms, is being successfully applied in experimental and clinical studies (18). Exploiting these cellular defense mechanisms against nonlethal stresses will have the highest probability of success to control excessive inflammatory responses and neutrophil-induced cell damage in the liver and in other organs during ischemia-reperfusion and other acute inflammatory conditions.

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