Role of the Kupffer Cell in Mediating Hepatic Toxicity and Carcinogenesis

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Kupffer cells are resident macrophages of the liver and play an important role in its normal physiology and homeostasis as well as participating in the acute and chronic responses of the liver to toxic compounds. Activation of Kupffer cells directly or indirectly by toxic agents results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species. This activation appears to modulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer. Understanding the role Kupffer cells play in these diverse responses is key to understanding mechanisms of liver injury. Idiosyncratic drug-induced liver disease results in morbidity and mortality, impacting severely on the development of new pharmacological agents. Modulation of the response of Kupffer cells by drugs has been suggested as a cause for the idiosyncratic response. Similarly, liver damage seen in chronic ethanol consumption appears to be modulated by Kupffer cell activation. More recent evidence has noted a contributory role of Kupffer cell activation in the process of hepatic carcinogenesis. Several nongenotoxic carcinogens, for example, activate Kupffer cells resulting in the release of cytokines and/or reactive oxygen species that induce hepatocyte cell proliferation and may enhance clonal expansion of preneoplastic cells leading to neoplasia. Kupffer cells therefore appear to play a central role in the hepatic response to toxic and carcinogenic agents. Taken together, the data presented in this symposium illustrate to the toxicologist the central role played by Kupffer cells in mediating hepatotoxicity.

Key Words: Kupffer cell; hepatocarcinogenesis; liver; hepatotoxicity; mode of action; adverse drug reactions.

A symposium entitled “Role Of The Kupffer Cell In Mediating Hepatic Toxicity And Carcinogenesis” was held at the 45th Annual Meeting of the Society of Toxicology (SOT) in San Diego, CA, in 2006. This symposium, sponsored by the Carcinogenesis Speciality Section of the SOT, addressed the role of the Kupffer cell, the resident macrophage of the liver, in the induction and/or facilitation of acute and chronic liver injury. Kupffer cells play an important role in the normal physiology and homeostasis of the liver as well as participating in the acute and chronic responses of the liver to toxic compounds. Activation of Kupffer cells, either directly or indirectly, results in the release of an array of inflammatory and growth control mediators as well as reactive oxygen species. This activation appears to modulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer. Understanding the role of the Kupffer cell plays in the induction of hepatocyte injury is essential in understanding the mechanisms of the liver injury. Idiosyncratic drug-induced liver disease results in morbidity and mortality and has been a major detriment to new therapeutic pharmacological development. Modulation of the Kupffer cell response by drugs has been suggested as a cause for the idiosyncratic response. Similarly, liver damage seen in chronic ethanol consumption appears to be modulated by Kupffer cell activation. More recent evidence has noted a contributory role of Kupffer cell activation in the process of hepatic carcinogenesis. Several nongenotoxic carcinogens, for example, activate Kupffer cells resulting in the release of cytokines and/or reactive oxygen species that induce hepatocyte cell proliferation and may enhance clonal expansion of preneoplastic cells leading to neoplasia. Kupffer cells therefore appear to play a central role in the hepatic response to toxic and carcinogenic agents.

The liver consists of the hepatic parenchyma and a large proportion of nonparenchymal cells (NPCs) including sinusoidal endothelial cells, icto cells, and the dedicated hepatic macrophage known as the Kupffer cell. The majority of the phases...
I and II metabolism functions associated with the liver’s primary role in detoxification take place within the hepatocytes, whereas the NPC population provide physical and biochemical structure to the liver (Fig. 1). The sinusoidal endothelia line the sinusoidal space and form a barrier through which the Kupffer cells are able to pass in and out of the hepatic space. Collectively, the sinusoidal endothelia, epithelia, Kupffer cells, and stellate cells are known as NPCs or to distinguish them from the hepatocytes, also known as the hepatic parenchyma.

An examination of the data on the role of Kupffer cells in acute hepatotoxicity illustrates very well the idea of the Kupffer cell as both protector but also as a mediator of damage. For example, inhibition of Kupffer cell function or depletion of Kupffer cells appears to protect against liver injury from the alkylating agent melphalan (Kresse et al., 2005), the mycotoxin fumonisin B1 (He et al., 2005), the industrial chemical thioacetamide (Andres et al., 2003), and the immunostimulants concanavalin A and Pseudomonas exotoxin (Schumann et al., 2000). Kupffer cells also contribute to liver injury during ischemia followed by reperfusion (Tsung et al., 2005). On the other hand, depletion of Kupffer cells increases liver injury from partial hepatectomy (Prins et al., 2004), suggesting that these cells play a protective role in this circumstance. One possible interpretation of these data is that the Kupffer cell is the primary target for the toxicants but not for partial hepatectomy where its role is purely protective. However, in the case of...
acetaminophen-induced hepatotoxicity, Kupffer cells have been reported to contribute to injury (Ito et al., 2003; Laskin et al., 1986; Michael et al., 1999) as well as to protect against hepatocellular damage (Ju et al., 2002). In this instance, the Kupffer cell may be the primary site of an initially protective response that develops to cause damage with further stimulation.

As with the other roles of Kupffer cells in chronic injury and carcinogenesis, the mechanisms by which Kupffer cells contribute to acute liver injury are varied. In general, the mechanisms involve release by Kupffer cells of mediators including cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins. Reactive oxygen, nitrogen species, proteases, and lipid metabolites such as prostaglandins and thromboxane are also released. These mediators can act directly on hepatocytes to cause cell death or indirectly through activation of other cells. For example, activation of hepatic stellate cells by some of these mediators leads to contraction of sinusoids (Kharbanda et al., 2004), resulting in arrest of neutrophils. This is a prelude to transmigration of neutrophils into the parenchyma, which is promoted by expression of adhesion molecules on sinusoidal endothelial cells activated by Kupffer cell–derived mediators. These mediators, as well as activated sinusoidal endothelial cells, contribute to activation of neutrophils, which in turn can damage hepatocytes through release of proteases and other factors (Ganey et al., 1994; Harbrecht et al., 1993; Ho et al., 1996; Mavier et al., 1988). In addition, activation of sinusoidal endothelial cells leads to a procoagulant state in liver and activation of platelets. A consequence of increased coagulation is deposition of fibrin and hepatic hypoxia, which can also have deleterious effects on hepatocytes. Thus, the release of mediators from Kupffer cells can initiate a variety of downstream events that may initially stimulate survival and protection but with continued or higher dose exposure, ultimately contribute to hepatic injury during chemical exposure.

This release of mediators by Kupffer cells and the ensuing events described above are critical, early features of inflammatory responses. Indeed, Kupffer cells are activated rapidly in response to the inflammatory agent, bacterial lipopolysaccharide (LPS) (Bellezzo et al., 1996; Portoles et al., 1994; Yao et al., 2004). At relatively large doses, LPS damages liver, and this injury is dependent on Kupffer cells (Brown et al., 1997; Fukuda et al., 2004; Iimuro et al., 1994; Vollmar et al., 1996). Treatment with small doses of LPS activates Kupffer cells in the absence of liver injury perhaps because levels or duration of the release of cytokines and other molecules are below a threshold for injury and may even be protective. This hypothesis in supported by the observation that cotreatment with non-injurious doses of LPS increases sensitivity of liver to a variety of other chemicals including monocrotaline (MCT), cocaine, allyl alcohol, aflatoxin B1, chlorpromazine, halothane, and ranitidine (RAN) (reviewed in Ganey and Roth, 2001). For many of these chemicals, Kupffer cells and Kupffer cell–derived mediators contribute to liver damage from cotreatment with LPS (Labib et al., 2003; Sneed et al., 1997; Yee et al., 2003a).

The data presented above clearly support the idea of a threshold of Kupffer activation and release above which liver damage is induced. This mechanism if illustrated by studies of MCT and RAN, where small doses of LPS enhance hepatotoxicity in a Kupffer cell–dependent manner. MCT is a pyrrolizidine alkaloid known to induce hepatotoxicity in people and animals when they are exposed through consumption of contaminated foods or pastures, respectively (Mattocks, 1986). In rats, relatively large doses of MCT cause acute liver damage by a mechanism that appears to be independent of inflammation (Copple et al., 2003). However, smaller doses of MCT that alone are not injurious are rendered hepatotoxic by coadministration of a small, nontoxic dose of LPS (Yee et al., 2000). This combination damages not only hepatocytes but also sinusoidal endothelial cells. Pretreatment with gadolinium chloride (GdCl), which inhibits Kupffer cell function, reduces both hepatocyte and sinusoidal epithelial cell injury, demonstrating the Kupffer cell dependence of hepatotoxicity in this model of chemical-inflammation interaction.

One of the key components of inflammation important to hepatotoxicity from coadministration of small doses of MCT and LPS is the Kupffer cell–derived cytokine TNF-α. The concentration of TNF-α in plasma peaks about 90 min after exposure to LPS and rapidly returns to baseline (Mastronardi et al., 2001; Noel et al., 1990; Xuan et al., 2001). Cotreatment with MCT prolonged the increase in plasma TNF-α in LPS-treated rats (Yee et al., 2003a). Neutralization of TNF-α reduced damage and also reduced the accumulation of neutrophils in the liver (Yee et al., 2003b). Taken together, these results suggest that Kupffer cells participate in MCT/LPS-induced liver injury through release of TNF-α, promotes accumulation of neutrophils in liver leading to hepatic damage.

RAN is a histamine 2 receptor antagonist that causes idiosyncratic liver injury in a very small percentage of people taking the drug (Ribiero et al., 2000; Vial et al., 1991). Similarly to MCT, RAN is not hepatotoxic in laboratory animals when given alone; however, pretreatment with LPS renders RAN hepatotoxic in rats (Luyendyk et al., 2003). In this model, RAN was administered 2 h after LPS, and at this time, the concentration of TNF-α in plasma was increased compared to animals not treated with LPS (Tukov et al., unpublished). The concentration of TNF-α decreased rapidly in animals that were not cotreated with RAN, whereas in animals that received the drug, TNF-α concentration remained elevated for several hours. Similarly, RAN increased the LPS-stimulated release of TNF-α in cocultures of Kupffer cells and hepatocytes, suggesting that Kupffer cells contribute to circulating TNF-α in LPS/RAN-treated rats. The potential contribution of TNF-α to liver injury in this model was evaluated by pretreating LPS/RAN-cotreated animals either with pentoxifylline to reduce synthesis of TNF-α or with etanercept to interfere with the ability of TNF-α to activate its cellular receptors. Prior administration of
either agent reduced TNF-α activity in plasma as well as liver injury assessed biochemically and histologically. Interference with etanercept decreased the LPS/RAN-induced increase in injury from LPS/RAN. Finally, pretreatment with either pentoxifylline or etanercept interferes with TNF-α to increase the plasma concentration of plasminogen activator inhibitor-1 (PAI-1), a major inhibitor of coagulation and to increase the fibrinolysis. Moreover, interference with TNF-α contributes to increased fibrin depositions, including MIP2, which facilitates accumulation of neutrophils and their activation in the liver. Activated neutrophils play a role in hepatotoxicity from LPS/RAN. Dotted lines represent those effects for which there is not direct experimental evidence.

FIG. 2. Working hypothesis of the role of Kupffer cells in liver injury from LPS/RAN interaction. RAN increases LPS-stimulated release of TNF-α from Kupffer cells. TNF-α promotes activation of sinusoidal endothelial cells, leading to activation of coagulation and deposition of fibrin in livers. TNF also contributes to increased circulating concentrations of PAI-1 that inhibit fibrinolysis. The consequence is enhanced fibrin deposition, ischemia, and hypoxia. The hypoxia probably leads to hepatocellular death. Additionally, TNF-α promotes activation of sinusoidal endothelial cells, leading to increased chemokine concentrations, including MIP2, which facilitates accumulation of neutrophils and their activation in the liver. Activated neutrophils play a role in hepatotoxicity from LPS/RAN. Dotted lines represent those effects for which there is not direct experimental evidence.

THE ROLE OF KUPFFER CELLS IN IMMUNE-MEDIATED ADVERSE DRUG REACTIONS

These data derived from investigation of known hepatotoxicants may help in understanding unpredictable and severe immune-mediated adverse drug reactions (IADR). These IADRs pose a significant problem for clinical practice and the pharmaceutical industry and are the subject of extensive research. One possible explanation for the relatively low incidence of IADR is that most individuals develop tolerance to immunogenic drug-protein adducts. Previous studies examining this hypothesis, using a model hapten, demonstrated that immune tolerance was induced by a protein adduct of the hapten, and that Kupffer cells play an important role in such tolerance. The mechanism of this tolerance is unclear but could rely on Kupffer cells, acting as APCs downregulating antigen-specific T-cell responses.

With < 10% of all adverse drug reactions reported, the magnitude of the problem is significant, with estimates of costs > $US30 billion annually in the United States (1995 value). In addition, the costs of not determining the potential of a drug to produce hypersensitivity in the preclinical phase of drug development can be substantial. It has been estimated that the preclinical phase and clinical phase I, phase II, and phase III costs are approximately $US6 million, $US12 million, $US12 million, and $US100 million per drug, respectively (1999 values). It is important that investigational drugs with the potential to produce hypersensitivity reactions be identified as early in the development process as possible. Some adverse reactions to drugs can be avoided if drug-drug interactions are known or if there is a structure-activity relationship established. However, these methods are inadequate. Appropriate animal models of drug-induced hypersensitivity are needed, especially because hypersensitivity has been cited as the leading reason for taking drugs off the market.

IADRs, including allergic hepatitis, lupus, cutaneous reactions, and blood dyscrasias, account for approximately 6–10% of all adverse drug reactions. IADR caused 137,000–230,000 hospital admissions per year in the United States (1998 values), with approximate annual costs of $275 to $600 million (Adkinson et al., 2002; Lazarou et al., 1998; Ratajczak, 2004). One puzzling aspect of IADR is that among all patients who take a certain drug, only a small percentage will develop IADR. One possible explanation for the low occurrence of IADR is that most individuals develop immunological tolerance, rather than an adverse immune response, to drug-protein adducts. Thus, understanding the molecular and cellular mechanisms of immune tolerance to these adducts will lead to the identification of risk factors that determine patients’ susceptibility to IADR.

Previous studies using a model hapten, 2,4-dinitrochlorobenzene (DNCB), have demonstrated that pretreatment of mice with a bovine serum albumin (BSA) conjugate of DNCB (DNP-BSA) induced tolerance against a T-cell–mediated delayed-type
hypersensitivity reaction caused by DNCB sensitization (Ju et al., 2003). The data revealed that the tolerance could be inhibited when Kupffer cells were depleted with liposome-entrapped clodronate prior to DNP-BSA pretreatment. In addition, the tolerance could be induced in naive mice by adoptive transfer with a Kupffer cell-enriched fraction of liver NPCs obtained from mice tolerized by DNP-BSA pretreatment. In contrast, a Kupffer cell–depleted fraction of liver NPC could not transfer tolerance. These findings suggest that Kupffer cells play an important role in downregulating T-cell–mediated reactions against drug-protein adducts. As suggested above, this could occur by Kupffer cells acting as APCs to down regulate antigen-specific T-cell responses.

Kupffer cells, as the largest population of tissue resident macrophages, not only play an important role in first-line defense against invading pathogens, but may also act as APCs to activate and regulate T-cell responses. It is known that T-cell activation requires two signals. The recognition of a specific major histocompatibility complex (MHC)/peptide complex by a T-cell receptor (TCR) gives signal 1 (Paul, 1999). Signal 2 is provided by the binding of costimulatory molecules expressed on APCs to their ligands on T cells (Germain, 1981). For example, CD40 and B7 molecules (B7-1 and B7-2) on APCs interact with CD40 ligand and CD28 on T cells, respectively (Greenfield et al., 1998; Schoenberger et al., 1998). It has been demonstrated that T cells are anergized rather than activated if they receive only signal 1 but not signal 2, or insufficient amount of signal 2, during antigen stimulation (Lafferty and Cunningham, 1975; Matzinger, 1994). Therefore, one possible mechanism by which Kupffer cells may induce T-cell tolerance is that although they can act as APCs, they express inadequate levels of costimulatory molecules and are thus only partially competent leading to T-cell anergy rather than activation.

This hypothesis was examined by the evaluation of Kupffer cell expression of various APC-function related molecules, such as MHC II, B7-1, B7-2, and CD40. The data demonstrated that, compared with potent APCs such as dendritic cells, Kupffer cells express inadequate levels of costimulatory molecules. Furthermore, the abilities of Kupffer cells and dendritic cells to activate naïve T cells were determined and compared using an in vitro system, in which ovalbumin (OVA)-TCR transgenic T cells were cocultured with Kupffer cells or dendritic cells in the presence of the antigen (an OVA peptide). The data revealed that dendritic cells significantly stimulated T-cell proliferation in response to antigen exposure, whereas Kupffer cells could not induce T-cell activation. Interestingly, when Kupffer cells were included in the cocultures of dendritic cells and T cells, T-cell proliferation was inhibited. This inhibitory effect of Kupffer cells was independent of cell–cell contact, suggesting that Kupffer cells could produce and release soluble immuno-suppressive mediators. These findings suggest that Kupffer cells have some ability to act as APCs but are only partially competent due to their insufficient expression of costimulatory molecules. If this hypothesis is correct, Kupffer cells could actively suppress T-cell activation induced by other APCs. Taken to its logical conclusion, this hypothesis suggests that individuals with a higher propensity to develop IADRs may have more costimulatory molecules in their Kupffer cells.

In summary, the data indicate that Kupffer cells may play a role as primary inducers of immune tolerance against a hapten-induced delayed-type hypersensitivity reaction. Because drug-protein adducts are predominantly formed in the liver, or they may circulate to the liver through blood, these cells may have a similar regulatory effect on immune responses to drug-protein adducts. The mechanism by which Kupffer cells cause T-cell tolerance could involve inadequate expression of costimulatory molecules and the production of immunosuppressive mediators. These findings suggest that genetic and/or environmental factors that cause impairment of the tolerogenic functions of Kupffer cells may lead to increased risk of developing IADR in certain individuals.

**ROLE OF THE KUPFFER CELL IN HEPATIC PRENEOPLASTIC LESION GROWTH**

So far, this review has explored the role that Kupffer cells play in acute liver responses to damage. However, it is clear that activated Kupffer cells are also key to chronic liver responses, including neoplasia. Chemically induced neoplasia is a multistep process involving two key mechanisms: DNA damage and alteration in cell growth control that allow this damage to persist and be promoted to foci and tumors. Genotoxic agents induce tumors by increasing rates of DNA damage, whereas nongenotoxic carcinogens act to promote the spontaneous or accumulated DNA damage present in all tissues. While much less is known about the exact mode of action of nongenotoxic carcinogens they are known to modulate cell growth and cell death, with changes in gene expression and cell growth being paramount in their mechanism(s) of action. These agents frequently function at the promotion stage of the cancer process. The stage of hepatic tumor promotion involves the selective clonal expansion of preneoplastic focal cells. Chemicals that function at this stage of the cancer process (tumor promoters) encompass a structurally diverse group of pharmaceutical and environmental agents, all of which result in increased DNA synthesis during the first few weeks of exposure that typically returns to baseline levels within 2–4 weeks of treatment despite continual chemical exposure in normal liver (Marsman et al., 1988; Schulte-Hermann et al., 1990). In contrast, some tumor promoters produce a sustained increase in the growth of chemically- and spontaneously induced lesions in the liver (Cattley et al., 1991; Isenberg et al., 1997; Kolaja et al., 1995; Marsman et al., 1988). The increased cell replication may enhance the rate of fixation of DNA damage into the genome leading to changes in gene expression, changes that facilitate the clonal expansion of initiated cells, and lead to the formation of hepatic focal lesions (Cattley et al., 1998).
Historically, research has focused on the hepatocyte as the target cell of chemical carcinogens, however, recent studies have emerged that suggest a role for NPCs, specifically Kupffer cells, as important mediators of cell proliferation by tumor promoters (Hasmall et al., 2000a; West et al., 1995, 1996). As described already, activated Kupffer cells release a wide array of biologically active products including reactive oxygen species, interleukins, and cytokines (Decker, 1990; Winwood and Arthur, 1993), all of which may be capable of modulating hepatocellular growth. In particular TNF-α has been linked to the stimulation of hepatocellular growth by tumor promoting compounds (Hasmall et al., 2000b; West et al., 1999). Studies in our group are directed at further defining the role of the Kupffer cell in hepatocarcinogenesis. Our working hypothesis is that activation of Kupffer cells results in the release of cellular growth regulatory signaling molecules that result in an increase in the proliferation of hepatocytes; this is expected to be transient in normal hepatocytes but sustained in preneoplastic, initiated hepatocytes, ultimately resulting in selective clonal expansion of the preneoplastic hepatocytes (hepatic tumor promotion).

Experimental models in which preneoplastic focal lesions are induced in rodents using the DNA-damaging compound, diethylnitrosamine as a surrogate for liver tumor promotion (Isenberg et al., 1997; Klaunig and Kamendulis, 1999; Kolaja et al., 1995, 1996a,b; Schulte-Hermann et al., 1981, 1990) have been used to dissect, in a relatively defined experimental system, agents that influence lesion growth and death. The effects of several tumor promoting agents on growth of preneoplastic lesions in B6C3F1 mice have been evaluated using stereologic methodology (Kolaja et al., 1995; Xu et al., 1998) and immunohistochemistry for quantitation of DNA synthesis. Following exposure to phenobarbital (0, 10, 100, and 500 ppm) for 30 or 60 days, an increase in the number, and relative volume of focal lesions was apparent after exposure to carcinogenic doses of phenobarbital (100 and 500 ppm), while the noncarcinogenic dose did not increase the number or volume of hepatic lesions (Kolaja et al., 1995, 1996a,b). In addition, increases in DNA synthesis remained increased within preneoplastic lesions at both 30 and 60 days while DNA synthetic activity in normal surrounding tissue returned to baseline (Kolaja et al., 1995, 1996a). Similar results on the growth of preneoplastic lesions in mouse liver were observed following exposure to Wyeth 14,643 as well as dieldrin (Isenberg et al., 1997; Kolaja et al., 1995, 1996b). These results illustrate that tumor promoter exposure results in a sustained increase in growth but just in preneoplastic cells. To evaluate the potential role of Kupffer cells on modulation of hepatocyte growth, a number of agents are available that are reported to either affect Kupffer cell activation or result in depletion of Kupffer cells. Although these agents have a marked impact on acute responses to liver injury as already described in the previous section, these protocols have yielded mixed results in experiments on hepatocyte growth. For example, while glycine and methyl palmitate (nonselective agents that inactivate but do not eliminate Kupffer cells) have been shown to decrease hepatocellular growth (Rose et al., 1997a; Watanabe et al., 2000), depletion of Kupffer cells with GdCl has been shown to increase liver cell proliferation in rodent liver (Rai et al., 1997; Rose et al., 2001). These apparently conflicting data could be resolved by the proposal that GdCl inactivation of Kupffer cells initially provokes a burst of growth mediators from the Kupffer cells that in turn cause an increase in DNA synthesis. Recognizing the potential limitations of these approaches, we have adopted a protocol that utilizes clodronate-encapsulated liposomes to deplete Kupffer cells in vivo (van Roojen et al., 1996). Depletion of Kupffer cells by clodronate liposomes occurs through a mechanism involving the phagocytosis of liposomes by Kupffer cells, resulting in the release of clodronate, which leads to adenosine 5'-triphosphate depletion and triggers apoptotic cell death (Lehenkari et al., 2002; Van Rooijen and van Kesteren-Hendrix, 2003).

To assess the effect of Kupffer cell activation on induction of DNA synthesis in hepatocytes as well as to assess the efficacy of inhibition of Kupffer cells (via clodronate liposomes) on hepatocellular growth, male B6C3F1 mice were given LPS (0.25 mg/kg, i.p.) in the presence or absence of clodronate liposomes (by tail vein injection of clodronate liposomes; 200 μl of 2.0 mg/ml clodronate in PBS, 3 times/week). After 7 days of treatment, the number of Kupffer cells was determined using the F4/80 mouse macrophage antibody. The number of Kupffer cells identifiable immunohistologically was significantly decreased by clodronate liposome treatment in control as well as in the LPS treatment groups (data not shown). DNA synthesis was assessed in the liver of these same animals using BrdU immunohistochemistry. LPS produced a significant increase in DNA synthesis (approximately 80%) compared with controls, whereas the induction of DNA synthesis by LPS was decreased by approximately 80% by Kupffer cell depletion (data not shown). In addition, Kupffer cell depletion resulted in a 50% reduction in the basal level of DNA synthesis seen in control liver. These results demonstrate that the clodronate liposome protocol is effective for depleting Kupffer cells and provides evidence in support of the growth permissive role of the Kupffer cell in the induction of cell proliferation.

In a separate study, the role of Kupffer cells in the modulation of preneoplastic lesion growth was evaluated in hepatic focal lesions produced in B6C3F1 mice using diethylnitrosamine. Following lesion development, LPS was given (0.25 mg/kg i.p.) for 7 or 30 days. LPS increased the relative volume of hepatic focal lesions after 30 days (~4-fold increase over control) while Kupffer cell depletion with clodronate liposomes significantly reduced the LPS-induced increase in focal lesion volume (Fig. 3). In addition, LPS increased DNA synthesis within focal lesions (~3-fold increase over control) after 30 days but this was inhibited by depletion of Kupffer cells (Fig. 3). In nonfocal liver, LPS produced an early increase in DNA...
synthesis (after 7 days) which was also prevented by Kupffer cell depletion (Fig. 3). Similarly, an inhibition of phenobarbital-induced preneoplastic lesion growth was also seen following Kupffer cell depletion. These data collectively provide support for the involvement of the Kupffer cell in hepatic carcinogenesis, and suggest that activation of this cell type may function at the promotion stage of the cancer process. The mechanisms of nongenotoxic carcinogenesis and the role of Kupffer cells will be explored further in the next two sections.

**KUPFFER CELL ACTIVATION BY NONGENOTOXIC CHEMICALS: DOES IT PLAY A ROLE IN HEPATOCARCINOGENESIS?**

The previous two sections have discussed the role of Kupffer cells in acute and chronic liver responses to hepatotoxins such as MCP, RAN, and LPS. In these examples, evidence suggests that the Kupffer cell is the primary target of chemical damage. However, the effects of many nongenotoxic liver toxicants are thought to be mediated through activation of nuclear hormone receptors. Peroxisome proliferators, a class of rodent liver carcinogens that are also relevant for human health, are one classic example. The current body of knowledge on these compounds shows an important role for peroxisome proliferator activated receptor alpha (PPARα)–dependent molecular events (Peters et al., 2005). Importantly, PPARα is required for liver carcinogenesis in animals fed peroxisome proliferators chronically (Peters et al., 1997). At the same time, several groups reported that PPARα-independent events, that include an increase in liver oxidants and mitogenic cytokines, also take place. A large body of evidence exists in the scientific literature to demonstrate that a number of acute pleiotropic effects of peroxisome proliferators in rodent liver are mediated by Kupffer cells (Rose et al., 1999b). At the same time, it is not presently known whether Kupffer cell–specific events play a role in long-term effects of peroxisome proliferators, and if these mechanisms are operational in species other than rats and mice. Based on evidence presented already in this review, it seems probable that the pleiotropic effects of peroxisome proliferators are dependent on PPARα but in parallel peroxisome proliferators activate Kupffer cells as a direct target as already described for MCP, RAN, and LPS. Thus, deletion of PPARα leaves the Kupffer cell–mediated component of the response which by itself is unable to drive proliferation and carcinogenesis for this class of compounds.

Peroxisome proliferators increase proliferation of rodent liver parenchymal cells both in vivo and in vitro; however, the effect on isolated hepatocytes from rats and mice is much less robust and persistent. For example, 8- to 10-fold increases in cell proliferation were reported in vivo (Marsman et al., 1988), while only up to twofold increases were demonstrated in vitro, regardless of the dose of the compound used (Goll et al., 1999; Hasmall et al., 1999). Most interestingly, in highly purified rodent hepatocytes, peroxisome proliferators fail to initiate an increase in DNA synthesis (Hasmall et al., 2000a, 2001; Parzefall et al., 2001). It has been hypothesized that one possible explanation for these differences is the involvement of NPCs in whole liver that are lost during purification and culture of liver parenchymal cells. Several laboratories in the past decade have demonstrated that Kupffer cell activation by peroxisome proliferators (1) is independent of PPARα, (2) involves generation of reactive oxygen species, and (3) leads to production of mitogenic cytokines (reviewed in Rusyn et al., 2000a). This hypothesis fits with the suggestion of a two-part response of the liver to peroxisome proliferators: one mediated by PPARα and a second mediated by Kupffer cells.

One of the first experimental facts that suggested activation of Kupffer cells by peroxisome proliferators came from an

![FIG. 3. Effect of Kupffer cell activation and Kupffer cell depletion on preneoplastic hepatic focal lesion growth in B6C3F1 mouse liver. *Statistically different from control (p < 0.05), #statistically different from LPS treatment group (p < 0.05).](image-url)
observation that nafenopin and WY-14,643 doubled uptake of particulate colloidal carbon in isolated perfused rat liver, reflecting activation of Kupffer cells (Bojes and Thurman, 1996). Kupffer cells are the predominant source of mitogens and comitogens in liver such as TNF-α, a cytokine that is one of the master regulators of liver growth and regeneration and is produced by Kupffer cells in liver (Diehl, 2000). Thus, several follow-up studies focused on the potential involvement of TNF-α in the acute effects of these agents in rodent liver. It was reported that neutralizing antibody to TNF-α blocked WY-14,643-induced cell proliferation in the rat liver in vivo, and that TNF-α was produced by Kupffer cells following the treatment (Bojes et al., 1997). It was also shown that TNF-α can induce proliferation and decreases apoptosis in cultured rat hepatocytes, mimicking the effects of peroxisome proliferators with the exception of induction of peroxisomes (Rolfe et al., 1997). Increases in whole-liver TNF-α messenger RNA (mRNA) (Rose et al., 1997b), and serum TNF-α protein (Adinehzadeh and Reo, 1998) levels were also reported following treatment with peroxisome proliferators. Finally, inactivation of Kupffer cells in rat liver in vivo by methyl palmitate or glycine prevented the increases in both TNF-α mRNA and protein, and cell proliferation due to the peroxisome proliferator WY-14,643 (Rose et al., 1997a,b).

Studies with cultured rodent hepatocytes, Kupffer cells, or mixed cell populations provide additional evidence for the role of Kupffer cells. Peroxisome proliferators can directly activate rat Kupffer cells in vitro to produce oxidants (Rose et al., 1999a). Furthermore, it was shown that an autofluorescent peroxisome proliferator LY-171883 was taken up by Kupffer cells via phagocytosis leading to their activation (as reported in Rose et al., 1999b). Addition of NPCs, or conditioned medium from NPC cultures, increased DNA synthesis two- to threefold and suppressed transforming growth factor-β1-induced apoptosis by 50–70% in highly purified rat hepatocytes (Hasmall et al., 2000a). Furthermore, in both rats and mice, removal of NPCs from normal hepatocyte cultures prevented both the nafenopin- and TNF-α-induced increases in DNA synthesis and suppression of hepatocyte apoptosis; this response was restored by returning NPCs to the purified hepatocytes (Hasmall et al., 2000a, 2001).

It appears that activation of Kupffer cells in whole liver occurs very rapidly. In rat liver, activation of the transcription factor nuclear factor-kappa B (NF-κB), which is a major regulator of cytokine production by macrophages, occurred first in Kupffer cells as early as 2 h following treatment with WY-14,643 (Rusyn et al., 1998). Furthermore, peroxisome proliferators were shown to activate production of reactive oxygen species in rat and mouse liver via activation of Kupffer cells within hours of treatment and independent of PPARα or peroxisomal enzymes (Rusyn et al., 2001). One recent gene expression profiling study that examined early transcriptional changes induced by peroxisome proliferators in mouse liver (Currie et al., in press) found that a number of known components of the TNF/interleukin 1 (IL-1) signaling pathways, including Irak2, Myd88, Ikbkγ, and others, were induced very early (2 h) and declined at later times (24 h) after acute treatment, consistent with other studies showing a short-lived time-course of Kupffer cell activation.

Activation of Kupffer cells by peroxisome proliferators is PPARα independent. PPARα mRNA or protein was not detected in Kupffer cells that were isolated from naïve or WY-14,643-treated rats (Peters et al., 2000). Moreover, when Kupffer cells from wild-type or PPARα-null mice were treated with WY-14,643 in vitro, superoxide production was similar (Peters et al., 2000). In addition, NPCs isolated from PPARα-null or wild-type mice were capable of facilitating the proliferation of highly purified liver parenchymal cells in response to nafenopin (Hasmall et al., 2001). Finally, one study examined the potentiation of the effect of TNF-α and a peroxisome proliferator WY-14,643 on cell proliferation in purified cultured rat hepatocytes (Parzefall et al., 2001). It was concluded that an increase in mitogenic cytokine production by Kupffer cells is necessary for stimulation of DNA synthesis in purified rat parenchymal cells since the presence of both the PP and TNF-α is required for a maximal proliferative response similar to that in whole liver.

Activation of Kupffer cells results in production of superoxide anion via nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase (Decker, 1990). Peroxisome proliferators are able to increase superoxide production by isolated rat Kupffer cells in a dose-dependent manner, indicating that they can affect Kupffer cells directly (Rose et al., 1999a). It was subsequently demonstrated that this involves activation of NADPH oxidase, since Kupffer cells isolated from p47phox-null mice (Jackson et al., 1995) show no increase in superoxide production when treated with peroxisome proliferators (Rusyn et al., 2000b). In addition, direct evidence for peroxisome proliferators–induced oxidant production was obtained in vivo using a spin-trapping technique and electron spin resonance (ESR) spectroscopy (Rusyn et al., 2001). Specifically, when rats were given di-(2-ethylhexyl) phthalate (DEHP) acutely for 2 h, an ESR-detectable radical adduct signal was detected. No increase in the radical signal due to DEHP was observed when Kupffer cells were inactivated in vivo with glycine pretreatment, or in p47phox-null mice. The rapid DEHP-induced free radical production in vivo occurred long before H2O2-generating enzymes in peroxisomes were induced, and it was not dependent on presence of PPARα.

Kupffer cell–derived oxidants were suggested to play a role in signaling rapid and robust increases in cell proliferation caused by peroxisome proliferators in rodent liver via a mechanism that also involves activation of NF-κB and production of TNF-α (Rose et al., 2000). Indeed, both activation of NF-κB and increases in cell proliferation due to a single dose of WY-14,643 were prevented completely when rats were pretreated with diphenyleneiodonium, an inhibitor of NADPH oxidase (Rusyn et al., 2000b). Furthermore, WY-14,643–induced
activation of NF-κB, increase in TNF-α mRNA, and acute increases in liver weight and cell proliferation did not occur in p47phox-null mice. Combined, these results provide strong evidence that NADPH oxidase in Kupffer cells is a source of oxidants that is activated very early after treatment with peroxisome proliferators. Since PPARα is not involved in activation of Kupffer cells by peroxisome proliferators, it is not yet clear how exactly such activation occurs upstream of the NADPH oxidase.

A causal link between Kupffer cell–mediated production of cytokines and long-term (effects of peroxisome proliferators in liver has not been established yet. However, it has been shown as described in a previous section that Kupffer cells play a role in the sustained proliferation key to the development of early neoplastic foci. One report suggests that Kupffer cell activation may persist for longer than a few days with WY-14,643 but not DEHP (Rose et al., 1999a). Both agents are known to cause robust increase in hepatocellular proliferation in rodent liver during the first few days of treatment; however, only WY-14,643 sustains rates of proliferation with long-term treatment (as reported in Marsman et al., 1988). It was found that Kupffer cells isolated from rats fed WY-14,643 generated superoxide at rates significantly greater than cells from controls for up to 3 weeks of treatment; however, superoxide production was not stimulated by feeding DEHP for the same period of time (Rose et al., 1999a). Several published reports cast doubt on the sustainability of the Kupffer cell–mediated events under conditions of continuous exposure to peroxisome proliferators. No increase in hepatic proliferation was detected in PPARα-null mice fed the Wy-14,643 diet for 1 or 5 weeks which suggests that subchronic effects of peroxisome proliferators on cell proliferation in mouse liver are mediated exclusively by PPARα but not Kupffer cells (Peters et al., 1997). However, this is perhaps explained by more recent data described earlier in this review where Kupffer cells were key to the sustained proliferation seen in foci but not in surrounding normal liver.

Several published reports have questioned the role for TNF-α in peroxisome proliferator–induced cell proliferation in rodent liver. Several groups have attempted to define the role of TNF-α signaling in hepatocellular growth induced by peroxisome proliferators by administering the potent peroxisome proliferator, WY-14,643 (for up to 4 days), to mice nullizygous for TNF-receptor 1 (TNFR1), TNFR2, both receptors, or TNF-α protein (Anderson et al., 2001; Lawrence et al., 2001). Neither study found evidence of abrogated peroxisome proliferator–induced proliferative response in mouse liver as a result of a null genotype. It should be noted, however, that since cytokine-induced signaling is highly redundant, the knockout models used in these studies may have compensated for the lack of a particular signaling molecule. Furthermore, when cytokine expression was examined in nontumorous liver tissue or adenomas in wild-type mice fed with a carcinogenic dose of WY-14,643 for 52 weeks, no difference in expression of TNF-α, IL-6, or TNFR1 &2 was found (Anderson et al., 2001).

However, IL-1β mRNA was significantly elevated and it was postulated that cytokines other than TNF-α may be important liver comitogens in peroxisome proliferator–treated rodents.

The potential for the Kupffer cell–derived oxidants to contribute to oxidative DNA damage following exposure to peroxisome proliferators has also been questioned. The analysis of expression of base excision DNA repair genes was used to assess whether this sensitive in vivo biomarker of oxidative stress to DNA can be used to determine the source of DNA-damaging oxidants following treatment with Peroxisome proliferators. Using PPARα- and p47phox-null mice treated with WY-14,643 for 4 weeks, the report concluded that DNA-damaging oxidants are generated by enzymes that are induced after activation of PPARα, such as those involved in lipid metabolism in peroxisomes, and are not the result of activation of NADPH oxidase in Kupffer cells (Rusyn et al., 2004).

In conclusion, it is apparent that while PPARα is required for the sustained activation of peroxisome proliferator–induced molecular processes that ultimately result in liver tumors and that Kupffer cell–mediated events play a role only in the early stages of the response (Fig. 4). Peroxisome proliferators do activate Kupffer cells to generate oxidants and increase mitogenic cytokines independent of activation of PPARα; however, the Kupffer cell–mediated events likely require activation of PPARα in parenchymal cells to achieve the overall pathophysiological outcome. Peroxisome proliferators are a class of nongenotoxic chemicals that exert both nuclear receptor–dependent and –independent modes of action in rodent liver, and thus offer a unique opportunity to understand the respective role of each pathway in the overall mechanism of their carcinogenic effects that is likely to be important for understanding the carcinogenicity of other nuclear receptor activators. The data presented in this section underline the potential for Kupffer cell–dependent and independent pathways in the response to PPARα ligands: the following section will explore this hypothesis in more detail.

ROLE OF THE KUPFFER CELL IN MEDIATING THE RESPONSE OF THE LIVER TO LIGANDS FOR PPARα

PPs are rodent nongenotoxic hepatocarcinogens that act via PPARα to cause peroxisome proliferation, induce DNA synthesis and suppress apoptosis in rodent hepatocytes (Roberts, 1999). Humans are exposed environmentally and therapeutically to PPs such as plasticizers, pharmaceuticals, and herbicides. Hence, it is key to understand the mechanisms of the rodent tumors in order to evaluate any potential risk to humans.

The in vivo response of the rodent liver to PPs can be modeled in vitro using isolated rat or mouse hepatocytes (Bars et al., 1993; Bayly et al., 1994; James et al., 1992, 1998; Roberts, 1999). As described previously, PPs added to the medium of these cultures cause an induction in DNA synthesis and a suppression of apoptosis (Roberts, 1999). In order to
evaluate the role of hepatic NPCs, particularly Kupffer cells in this response, murine liver cells were isolated and the NPCs were removed prior to plating the hepatocytes (Hasmall et al., 2001). In these cultures, the response to PPs was lost but could be recovered by adding back the removed NPC cell fraction to the hepatocytes (Hasmall et al., 2001). These experiments demonstrate that NPC are required for the hepatic response to peroxisome proliferators. Indeed, there are multiple papers from many different laboratories that confirm this observation both *in vivo* and *in vitro* as described in more detail in the previous section (Bojes and Thurman, 1996; Rose et al., 1997a, 1999a).

Since NPCs and particularly the Kupffer cells are a source of growth modulating cytokines such as TNF-α, it seems reasonable to propose that the Kupffer cells are required for the response since they release cytokines in response to PPARα ligands. However, we were unable to detect TNF-α activity in NPC conditioned medium although others have suggested it can be detected (preceeding section and Lisa Kamendulis, personal communication). Interestingly, contact between NPC and hepatocytes is required since their separation using a diffusion chamber prevented the hepatocyte response.

Experiments with PPARα-null mice plus other supporting data provide extensive evidence of a pivotal role for PPARα in the hepatic growth response to PPs. So, what role does PPARα play in the Kupffer cell response to peroxisome proliferators? To address this, the NPC separation and recombination experiments described for wild-type mouse liver were repeated but with cells isolated from the PPARα-null transgenic mouse (Hasmall et al., 2001). As expected, hepatocytes from the PPARα-null mouse were unable to respond to peroxisome proliferators and this response could not be conferred using wild-type (PPARα+/+) NPCs (Hasmall et al., 2001). However, NPCs from PPARα-null mice were able to confer a response to peroxisome proliferators on PPARα wild-type hepatocytes (Hasmall et al., 2001). These data demonstrate that PPARα is required in the hepatocyte but not in the Kupffer cells for a growth response to peroxisome proliferators and are in accord with papers demonstrating that PPARα is not expressed in the NPCs (Peters et al., 2000).

If PPARα is not involved in the response of Kupffer cells to peroxisome proliferators, what are the mechanisms of this response? Peroxisome proliferators appear to directly activate Kupffer cells through mechanisms involving oxygen radicals, protein kinase C and the transcription factor, NF-κB (Rose et al., 1999b). In this, it would seem that the response of Kupffer cells to peroxisome proliferators is comparable to the response to other liver toxicants such as MCP, acetaminophen, RAN, and LPS as described earlier. NF-κB activation and binding was seen shortly after Wy-14,643 administration but pretreatment with allopurinol, a xanthine oxidase inhibitor and free radical scavenger, suppressed NF-κB activity by Wy-14,643. It is concluded that NF-κB is activated by reactive oxygen species and plays a central role in the mechanism of action of peroxisome proliferators (Rusyn et al., 1998, 2001). Wy-14,643 was also shown to produce a rapid oxidant-dependent activation of NF-κB in Kupffer cells *in vivo* and activated superoxide production by isolated Kupffer cells. Although it is clear that hepatic NPCs are required for the proliferative response to PPs, the mechanisms of this dependency are unclear. Activated NPCs, particularly Kupffer cells, are implicated in producing oxygen radicals and cytokines such as TNF-α and IL-1. In support of this, TNF-α is able to increase hepatocyte proliferation and suppress apoptosis in cultured rodent hepatocytes (Holden et al., 2000; Rolfe et al., 1997). Furthermore, the hepatocyte growth response to PPs can be prevented by antibodies to either TNF-α or TNFR1 (Bojes et al., 1997; West et al., 1999). Treatment with peroxisome proliferators may not mediate necessarily induced *de novo* TNF-α gene expression, suggesting that the response to this class of compounds may be mediated by bioactivation or release of preexisting TNF-α protein from Kupffer cells. Kupffer
cells are thought to produce free radicals via NADPH oxidase leading to activation of NF-κB and production of TNF-α leading to the induction of hepatocyte S-phase. In contrast to these data arguing for a role for TNF-α, the proliferative response of hepatocytes to PPs is intact both in TNF-α and in TNF-α receptor null mice (Givler et al., 2000). These data appear to refute any specific role for this cytokine in the response to peroxisome proliferators but, as described in a previous section, such knockout experiments with cytokine genes can be confounded by redundancy (in other words, the transgenic mouse embryo adapts its signaling pathways to compensate for the absence of the absent gene). Overall, current data suggest that nonparenchymal Kupffer cells are required but not sufficient for the response to PPs (Fig. 5).

In summary, hepatic nonparenchymal Kupffer cells contribute to the cell proliferation response of the liver to PP. However, the activation of NPCs occurs under many circumstances and is not specific to PP-induced hepatocarcinogenesis.

Overall, these data support a role for Kupffer cells in facilitating a response of hepatocytes to PPs via a mechanism that remains to be determined but is ultimately dependent on the presence of PPARα in the hepatocyte but not the nonparenchymal liver cell population. In addition, the role that Kupffer cells play in the response of the liver to PP shares many similarities to the response to other toxicants and is not specific to PP-induced hepatocarcinogenesis.

CONCLUSIONS AND FUTURE PERSPECTIVES

Overall, it is clear that the Kupffer cell plays a pivotal role in liver cell biology. The data on mechanisms of the Kupffer cell-hepatocyte interplay described in this review fall into two broad categories; the Kupffer cell as the primary target of toxic signals and the Kupffer cell as an accessory in the overall response of the liver to a toxic signal received by the hepatocyte. When the Kupffer cell forms the primary target, many of the mechanisms outlined above involve release of inflammatory mediators that impact on hepatocyte survival, proliferation, and function. As discussed in the introduction, the proposed mechanisms of Kupffer cell mediation are at times conflicting regarding the outcome of this release of cytokines. In some cases, Kupffer cell activation is associated with damage (MCP, RAN), whereas in others there may be no impact (acetaminophen) or even protection (surgery). Perhaps these apparently conflicting data sets can be largely reconciled by a threshold hypothesis; excessive or prolonged release of Kupffer cell mediators can switch a protective mechanism to a damaging inflammatory response. One experiment that is very informative in this context is that TNF-α is a survival signal to hepatocytes (West et al., 1999) but becomes a potent death signal if protein synthesis is inhibited.

In contrast to when the Kupffer cell is the primary target for toxicants, when the hepatocyte is the primary target as appears to be the case for peroxisome proliferators, the Kupffer cell seems to play more of a benign supporting role in the overall response to toxicant. This may also be true for surgical damage to the liver. Evidence suggests that low levels of cytokines release from Kupffer cells constitute a survival signal, protecting hepatocytes from cell death and in some cases, stimulating proliferation.

The detailed mechanisms of the Kupffer cell-hepatocyte interaction and its consequences for both normal and toxicant-driven liver responses remain to be determined. New research openings often arise from cross-fertilization and learning between studies on different classes of toxicant. For example, what impact does the hypothesis on the role of Kupffer cells in immune-mediated drug reactions have on our understanding of the mechanisms of preneoplastic focal lesions caused by dieldrin or phenobarbitone? Evidence from investigation of IADRs provide evidence that Kupffer cells may act as partially competent APCs; could this mechanism be operating during the response to peroxisome proliferators or phenobarbitone? Similarly, data on LPS-induced sensitization of the liver to subsequent toxicant exposure may provide new avenues to understanding how repeated administration of nongenotoxic carcinogens can lead to sustained proliferation. This symposium provided an exciting opportunity to review the state of our knowledge and also facilitated such cross-fertilization giving new ideas and strategic direction.

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