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In vitro Investigation of the Molecular Mechanisms of Hepatotoxicity

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Drug-induced Hepatic Injury

Iatrogenic Hepatitis: Intrinsic and Idiosyncratic Toxicity. Substances capable of producing liver damage and, more specifically, hepatocyte damage are known as *hepatotoxins*. They are classified (Zimmerman and Ishak 1995, Castell et al., 1992) according to whether they exert their effects in all individuals, in a dose-dependent and hence predictable manner (*intrinsic hepatotoxins*), or in certain individuals, ocasionally after several contacts, in a non-dose dependent and therefore unpredictable way (*idiosyncratic hepatotoxins*). These substances can act directly on cells (*active* hepatotoxins), or become toxic after biotransformation (*latent* hepatotoxins). Idiosyncratic hepatotoxicity is the consequence, either of an unusual metabolism of the drug by susceptible individuals which produce too large amount of toxic metabolites (*metabolic idiosincrasy*), or is due to an immune-mediated atack to sensitised hepatocytes *(drug hypersensitivity)*.

The damage caused to hepatocytes can be *cytotoxic*, *genotoxic* or *metabolic* (Zimmermann and Ishak, 1995). The first type of injury is a common feature of many intrinsic hepatotoxins. It is evidenced by important morphological changes in the structure of hepatocytes (vacuolization, steatosis, acidophilia, necrosis, etc.), and is accompanied by serum increases in hepatic enzymes. Genotoxins are substances that produce, in first term, DNA damage and show tendency to induce primary hepatocarcinomas. Finally, drugs can also alter the cellular metabolism of hepatocytes without causing cell death. This usually takes the form of alterations of the functional capacity of hepatocytes and, in particular, of the uptake, conjugation and secretion of bile acids, which, in turn, results in an impaired bile flux (*cholestasis*).

The liver is very active in metabolizing foreign compounds. Although a the *biotransformation* reactions generally parallel a detoxification process, there are many cases for which metabolism of the drug is the cause of deleterious effects to cells (Zimmerman and Ishak, 1995). This is the case of reactions catalyzed by cytochrome P450 enzymes (CYP) that can generate more toxic or more reactive metabolites capable of reacting with nucleophiles, covalently bind to macromolecules or initiating radical-chain reactions within cells. Against these

potential hazards, hepatocytes have effective defence mechanisms (specialized enzymes, reducing molecules like GSH, and DNA and protein repair mechanisms). Ultimately, it is the balance between *bioactivation, detoxification* and *defence mechanisms* that determines whether a reactive metabolite will or will not elicit a toxic effect.

Studies on hepatotoxicity most frequently deal with a type of cell death which is commonly defined as *lytic necrosis*, characterized by a rapid collapse of cell's internal homeostasis and evidenced by alterations of cytoplasmic organelles, cell swelling, membrane lysis, release of cellular content and inflammation of the surrounding areas (Columbano, 1995). The other type of cell death, *apoptosis*, is characterized by a progressive condensation of the chromatin on the inner face on the nuclear membrane, cell shrinkage with subsequent loss of membrane contact with neighbouring cells, and fragmentation of the cell with formation of membrane-bound acidophilic globules (apoptotic bodies). There is now accumulating evidences showing that most of the agents capable of inducing death of liver cells can induce both apoptosis and necrosis. Interestingly, there is very little evidence of the occurrence of necrosis in the liver in the total absence of a preceding or concomitant apoptosis (Columbano, 1995).

Molecular Mechanisms of Toxicity

Impairment of Cellular Metabolism. The alteration of the metabolic functions of hepatocytes by a drug or any of its stable metabolites, is frequently in the origin of hepatotoxicity. Certain compounds can directly act as enzyme or ion transport inhibitors, or compete with cellular metabolites in hepatocyte metabolic pathways (*antimetabolites*, e.g., galactosamine, ethionine). Conversely, many hepatotoxins can indirectly alter the energetic balance of cells by dramatically increasing the energy demand (i.e. increased ATP consumption), reducing ATP production, or both (Swartz, 1995). ATP depletion is, in fact, a common event in the course of drug-induced cell damage, frequently preceding the irreversible stages of cell injury. An interesting example of increased energy demand constitutes the *de novo* synthesis of GSH. Intracellular oxidized glutathione concentration can not go beyond a certain level. Glutathione disulfide, if not effectively reduced by glutathione reductase, is eliminated from the cell. This results in a decrease of the GSH pool, requiring *de novo* synthesis of this metabolite and, concomitantly an important ATP demand. A similar situation can occur if a metabolite is eliminated mainly by conjugation with GSH, and the recovery mechanisms (hydrolysis of glutamic acid and glycine from conjugates, and elimination as mercapturic acid conjugates) are overwhelmed (Reed, 1990).

Mitochondria constitute an important target of drug hepatotoxicity. Mitochondrial dysfunction has immediate effects on the energetic balance of cells becoming a crucial event in the onset of hepatocyte necrosis (Rosser and Gores, 1995). Several mechanisms can be involved in mitochondrial injury: a) direct inhibition of mitochondrial metabolism, for instance electron transport and/or oxidative phosphorylation (Nieminen et al., 1995); b) alteration of the

physicochemical properties of the mitochondrial membrane as a result of oxidative damage (i.e. lipid peroxidation after mitochondrial GSH depletion; Nieminen et al., 1995); c) intercalation of compounds in the lipidic membrane that alter its physicochemical properties, the mitochondrial membrane potential and, hence, the driving force of ATP production; and d) damage to mitochondrial DNA.

Drug-induced Lipid Peroxidation. Lipid peroxidation is also frequently involved in the mechanisms of hepatotoxicity (Poli et al., 1987). It is a free radical process leading to the oxidative degradation of the lipids present in cell membranes, and it is easily propagated to other unsaturated molecules when oxygen is present. The oxidation of lipids ends with the formation of a wide range of degradation products, i.e. hydroperoxides, aldehydes, including malondialdehyde, ketones etc. The first consequence of this process is the profound alteration of the physico-chemical properties of the membrane and of the functionality of membrane-allocated enzyme activities (Ross, 1989).

Cells use several strategies to protect themselves against uncontrolled lipid peroxidation: a) inactivation of active oxygen species, b) trapping of eventually formed radicals, c) inhibition of the radical chain propagation, and d) repair of damaged lipids. Superoxide dismutase, catalase and glutathione peroxidase, together with reduced glutathione (GSH) are the most efficient cellular agents against oxygen species and radicals. Lipid hydroperoxides can be efficiently reduced by glutathione peroxidase. Finally, natural antioxidants, like vitamin E present in biological membranes, act by inhibiting the propagation step of lipid peroxidation.

Drug-induced Oxidative Stress. The term *oxidative stress* is defined as a disturbance in the prooxidant-antioxidant balance of a cell (Sies, 1991). In active aerobic cells, a certain oxidative challenge occurs during the oxidation of substrates taking place in the cell to produce ATP. This may also happen as a consequence of a decrease in the cellular antioxidant elements because of nutritional or physiological changes but it does not constitute an oxidative stress. However, when as a result of the metabolism of a xenobiotic there is an increased formation of active oxygen species accompanied by a loss of GSH equivalents, the balance is altered in favour of *oxidative damage* (Sies 1991). The immediate result is an increased lipid peroxidation, alteration of membrane functionality and, indirectly, the inability of the cell to generate its own energy or to maintain the ionic homeostasis.

The most active substances in eliciting oxidative stress are compounds able to undergo repeated oxidation and reduction cycles within the cell. A representative example of this type of toxins are quinones (Figure 1), which cause cell injury by two mechanisms. First, quinones are electrophiles able to react with nucleophiles such as the thiol group of GSH and proteins. Since GSH plays a key protective role within the cell, depletion of GSH renders the cell more sensitive to physiologically-generated oxygen active species. Second, a number of flavoproteins (e.g. cytochrome P450 reductase) catalyze the NAD(P)H-dependent

reduction of quinones to semi-quinones which can be detoxified by reaction with nucleophiles (GSH), or spontaneously react with molecular oxygen in a one-electron oxidation process to regenerate the parent quinone and superoxide anion. The H_2O_2 which is subsequently produced by superoxide dismutases can lead to the formation of oxygen-derived radicals (OH, $O_2^{\cdot-}$, $^1O_2$) by the $Fe^{2+/3+}$ catalyzed Harber-Weiss and Fenton reactions. The quinone can again be reduced, thus becoming trapped in a series of *redox* reactions. The stoichometric balance of the *redox cycling* is the continuous one-electron reduction of molecular oxygen by cellular NAD(P)H. The consequences are a large amount of reactive oxygen species produced and a depletion of reduced thiol and nicotinamide nucleotide pools, what makes cell biomolecules more susceptible to damage by oxygen active species (Boobis et al., 1989). Oxidative stress can also lead to S-thiolation of proteins, a phenomenon that has been shown to occur with several xenobiotics. Ca^{2+}-dependent ATPases are among the most sensitive proteins to these modifications (Bellomo et al., 1983).

Mitochondria have no catalase and they rely totally on GSH peroxidase, and hence, on mitochondrial GSH, to prevent the effects of active oxygen species generated during oxidative phosphorylation. Since no transport system for oxidized glutathione out of the mitochondria exist, GSSG must be reduced inside mitochondria making this organelle particularly susceptible to GSH-depleting xenobiotics.

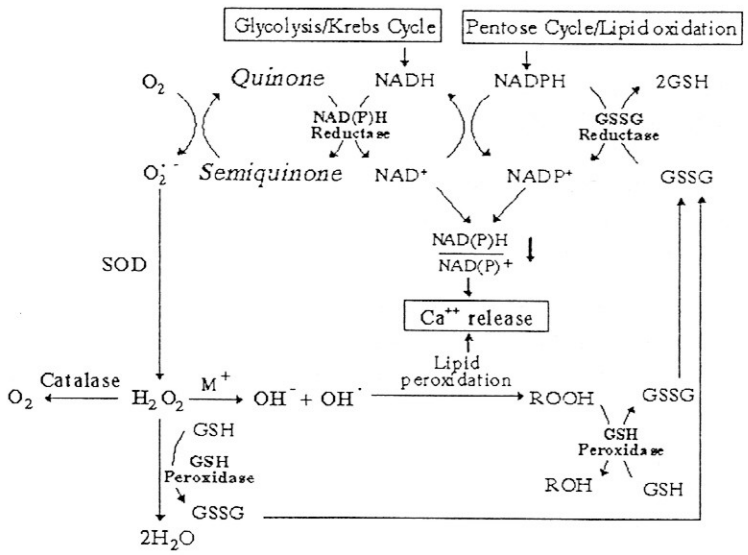

Figure 1. Drug redox-cycling and oxidative stress.

Calcium and Drug-induced Cell Injury. Intracellular calcium plays a fundamental role as a regulator of many enzymes and as an effector for hormones and growth factors controlling a wide variety of physiological processes. The cytosolic free Ca^{2+} concentration in mammalian cells is very low (~0.1 μM) compared with the concentration of extracellular fluids (1-2 mM). The major pathways for Ca^{2+} entry into cells are voltage-dependent channels gated by the electrical potential accross the plasma membrane, and receptor-operated channels (Spedding and Paoletti, 1982). The cytosolic Ca^{2+} concentration is controlled by active sequestration into the mitochondria and the endoplasmic reticulum. The driving force for Ca^{2+} uptake in the mitochondria is the electrochemical gradient across the inner membrane generated during oxidative phosphorylation, while in the endoplasmic reticulum the uptake of Ca^{2+} is done by a Ca^{2+} ATPase pump. Other Ca^{2+} extrusion mechanisms occur in cells associated with the influx of Na^{+} by membrane pumps acting as Na^{+}-driven antiports.

Toxins can alter Ca^{2+} homeostasis either by: a) increasing the external influx or release from intracellular stores; b) impairing the uptake by organelles, or ion extrusion to the external medium. Although xenobiotics able to inactivate Ca^{2+}-ATPases are known, most toxic xenobiotics act indirectly by modifying membrane properties (e.g. lipid peroxidation), reducing the energy status of cells or depleting GSH.

As a consequence of the toxic action of xenobiotics, a sustained rise in cytosolic Ca^{2+} can occur. An immediate consequence of elevated cytosolic Ca^{2+} is plasma membrane blebbing, activation of phospholipases and non-lysosomal proteases which can cause irreparable damage and functional injury to cell components (1989; Shen et al., 1992). The elevated cytosolic Ca^{2+} can also trigger endonucleases activation and a concomitant DNA fragmentation, similar to that observed in apoptosis (Shen et al., 1992).

Covalent Binding. Biotransformation of xenobiotics may result in the formation of chemical intermediates (electrophiles, radicals, conjugates) able to react with cell macromolecules forming stable drug adducts (Hinson and Roberts, 1992). Unsaturated carbonyl compounds (i.e. α, β unsaturated ketones), aldehydes, epoxides and glucuronides are among the most frequently involved reactive groups. Proteins, DNA and RNA are the most frequent targets. Covalent binding is dependent on the proportion of the chemical converted into a reactive metabolite, the half-life of the reactive intermediate, and its ability to react with the target biomolecule. Depending both on the site of generation and on the nature and reactivity of the intermediate, differences in the subcellular localization of adducts occur. The endoplasmic reticulum is where more often protein-drug adducts are found; a fact that probably reflects the proximity of the site of bioactivation (CYP) and of binding (Boelsterli, 1993). Drug-protein adducts have also been identified in plasma membrane (Hinson and Roberts, 1992) a finding that become relevant in relation to the immune-mediated drug hepatotoxicity.

Few examples are known where the drug-protein adduct could be fully identified. CYPs giving rise to the formation of reactive metabolites are among the

most frequently protein targets (Boelsterli 1993; Holtzman, 1995), although other enzymes, (i.e. Ca^{2+}-ATPase, carboxyl esterase, GSH-transferase) have also been reported as targets (Boelsterli 1993; Kenna et al., 1993). Halothane (Van Pelt and Kenna, 1994) and diclofenac (Kretz-Rommel and Boelsterli 1994) have been studied in detail, and the presence of several protein bands bearing the trifluoroacetyl or diclofenac moiety have been identified.

Covalent binding is a phenomenon that although frequently appears in the couse of toxic phenomena, only in a few cases has been possible to associate it with a well defined cell injury. For certain drugs (acetaminophen, bromobenzene), there is a clear correlation between the extent of covalent binding to proteins and the severity of hepatocyte injury (Boelsterli, 1993; Holtzman, 1995). However, with other xenobiotics, there is a lack of correlation with hepatotoxicity. This is the case of bromophenol, and the paracetamol analog N-acetyl-m-aminophenol, which are able to covalently bind to hepatocytes, yet it does not result in liver damage (Boelsterli, 1993). On the opposite, covalent binding is inversely correlated with the extent of hepatotoxicity in the case of diclofenac. Binding of the drug to proteins takes place mainly via trasacylation of diclofenac glucuronide moiety (Hargus et al., 1994). This process does not per se cause cytotoxicity while CYP metabolism of the drug produces toxic metabolites that ultimately cause cell injury (Ponsoda et al., 1995). These two metabolic routes co-exist. Thus, the more diclofenac is metabolized by P450, the less it becomes available for covalent binding through the formation of the glucuronide (Kretz-Rommel and Boelsterli, 1994).

Immunological Mechanisms of Drug Hepatotoxicity

Allergic hepatitis. Among the various forms of drug-induced hepatitis, some of them show clinical features compatible with an hypersensitivity reaction against the drug. *Allergic hepatitis* is a type of idiosyncratic toxicity that occurs in certain individuals in an unpredictable and dose-independent manner and is very difficult to anticipate with animal models.

Drugs are small organic molecules unable to elicit an immune response unless they are bound to a macromolecule (hapten). Covalent binding of the drug (or of a metabolite) to proteins becomes, thus, a necessary stage in the mechanism of toxicity (Figure 2). In addition, the neoantigen has to be accessible to the immune surveillance. This may occur by different ways: a) binding to the CYP that has interacted with the drug and transport to the outer cellular membrane (Robin et al., 1995); b) difussion of the reactive metabolite out of the site of formation and reaction with membrane proteins or proteins that will be transported to the membrane; c) drug-protein adduct formation by membrane allocated CYPs (Loeper et al., 1993).

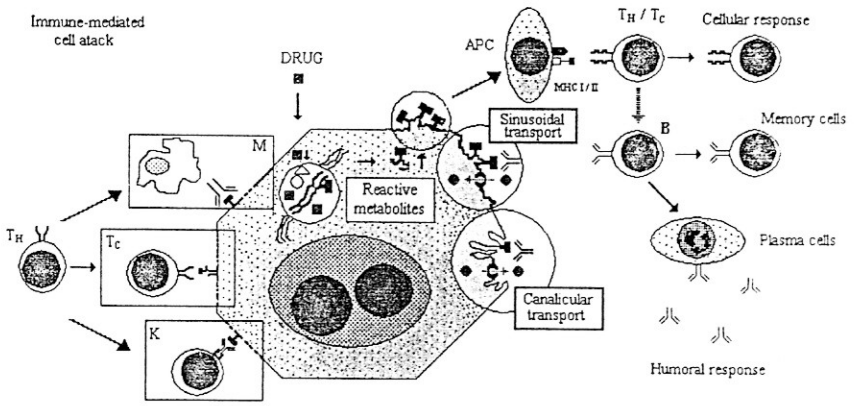

Figure 2. Key events in the course of an allergic hepatitis

Immune-mediated mechanisms of toxicity. Drug-induced allergic hepatitis becomes clinically evident either as *necrosis* (cytolytic hepatitis with increased levels of serum enzyme markers), *cholestasis* (with minor signs of necrosis) or both (mixed hepatitis).

The immune response in allergic hepatitis has an humoral and a cellular component (Homberg et al. 1985; Tsutsui et al., 1992). From the review of a significant number of clinical cases of allergic hepatitis, Homberg et al., (1985) could describe four types of response in patients: a) individuals having drug-directed antibodies in serum; b) those with antibodies directed against CYP isoforms; c) with the presence of auto-antibodies; d) patients with undetectable presence of antibodies against hepatic or drug antigens.

Contrary to what it could be expected, the presence of drug-directed antibodies is not the most frequent finding. Antibodies directed against either CYPs or autoantigens are more frequently reported. One posible explanation to this observation is the fact that the immune response may have arisen against a metabolite rather than to the parent molecule. Recently (Gómez-Lechón et al., 1996), antibodies against erythromycin could be demonstrated in a patient suffering an acute drug-induced hepatic cholestasis. The antibodies were able to recognize only erythromycin that became covalently bound to human hepatocytes after prior incubation and metabolism of the antibiotic with human cells.

Little is known about the linkage between circulating antibodies and the observed liver dysfunction. Antibodies are large macromolecules and can not readily enter the cell. Instead, they could bind to haptenized hepatocyte membranes, thus altering their physicochemical properties and, indirectly, the functionality of membrane-allocated proteins (i.e enzymes, ion pumps, etc.), ion transport and ultimately bile production (Figure 2).

T-lymphocyte sensitization to drugs (or metabolites) is also considered to be a

mechanism involved in allergic hepatitis (Tsutsui et al., 1992). The existence of sensitized T-cell clones can be demonstrated in (sensitized)patients by means of appropriate in vitro tests, they are based on a proliferation assay of peripheral lymphocytes incubated with the suspected drug and/or its metabolites, and a prostaglandin inhibitor. With this combined approach, positive response for a drug could be demonstrated in ca. 80% of patients investigated (Maria and Victorino, 1994).

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