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CHARACTERIZATION OF BIOTRANSFORMATION SYSTEMS IN HUMAN CELLS

FOCUS ON STEM CELLS AND THEIR PROGENY

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Experience is the name everyone gives to their mistakes.

- Oscar Wilde

ABSTRACT

All new chemical entities that are produced have to be carefully evaluated in terms of toxicity. Efforts are continuously being made to improve test procedures so that predictions of toxicity can be made earlier and more accurately. Understanding mechanisms of toxicity is an important part of improving safety assessment, but for this, simplified methods and models to screen for mechanisms associated with toxicity are required.

Glutathione (GSH) is important in protecting cells from oxidants and electrophiles, and the availability of GSH in the nucleus would be highly advantageous in the protection of DNA. A novel immunocytochemical method for visualization of subcellular compartmentalization of GSH was described. Confocal microscopy of stained A549 cells showed that the GSH levels of the nuclear and cytosolic compartments are close to equilibrium, and that the highest levels of cellular GSH are associated with mitochondria. In addition, the method described provided visualization of the compartmentalization of protein-glutathione mixed disulphides formed in A549 cells exposed to diamide. The method described allow for studies of the organization of intracellular GSH in intact cells, and may potentially offer means to monitor the regiospecificity in the GSH levels and the cellular redox state after exposure to xenobiotics.

The liver is a frequent target of xenobiotic-induced toxicity and it is of great importance to assess potential hepatotoxicity. In order to evaluate the use of hepatocyte-like cells derived from human embryonic stem cells (hESC) as a model system for metabolism and xenobiotic-induced hepatotoxicity, the expression of biotransformation enzymes was evaluated. The protein expression of glutathione transferases (GSTs) in hepatocyte-like cells was studied and results show high levels of GSTA1-1, whereas GSTP1-1 is not present, and this was in accordance to the results in primary human hepatocytes. In addition, GST activity was detected in hepatocyte-like cells at levels comparable to human hepatocytes. The mRNA and protein expression of several important cytochrome P450s (CYPs) in hepatocyte-like cells was also investigated. CYP mRNA expression and CYP1A2 and 3A/7 protein expression was detected in hepatocyte-like cells, but at low levels compared to human hepatocytes. The expression of other liver-related genes, such as UDP-glucuronosyltransferases (UGTs), drug transporters and transcription factors were also studied using low density arrays. The mRNAs for a variety of CYPs and liver-related factors as well as CYP1A2 and CYP3A4/7 protein were shown to be inducible. The hepatic phenotype of hepatocyte-like cells was demonstrated by characteristic hepatic morphology, expression of several hepatic markers and transcription factors as well as glycogen storage. Taken together the results indicate that the hepatocyte-like cells have the potential of being used as a model system for studying hepatotoxicity, although further differentiation is needed before they have a fully mature hepatic phenotype.

Just like hESC, human adult stem cells may be a source of differentiated cells for safety assessment, but they may also be an *in vivo* target for xenobiotics and toxicity. To evaluate the biotransformation capacity in human adult stem cells from breast and liver tissue, the expression of GSTs and CYPs was evaluated. The results show a clear difference in the expression pattern of these enzymes between adult stem cells from both tissues, and their differentiated counterparts. This indicates that these stem cells would respond differently to exposure of xenobiotics and that they are not suitable as a model system for safety assessment without differentiation of the cells to more closely resemble the target cell type.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by the Roman numerals as indicated below.

I. **Söderdahl, T**., Enoksson, M., Lundberg, M., Holmgren, A., Ottersen, O.P., Orrenius, S., Bolcsfoldi, G., Cotgreave, I.A. Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells. *Faseb J* 17(1): 124-6, 2003.

II. **Söderdahl, T**., Küppers-Munther, B., Heins, N., Edsbagge, J., Björquist, P., Cotgreave, I., Jernström, B. Glutathione transferases in hepatocyte-like cells derived from human embryonic stem cells. *Toxicol In Vitro*. 21: 929-937, 2007.

III. **Söderdahl, T.** *, Ek, M. *, Küppers-Munther, B., Björquist, P., Edsbagge, J., Cotgreave, I., Jernström, B., Andersson, T.B., Ingelman-Sundberg, M., Johansson, I. Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells. *Biochem Pharmacol,* In press, 2007.
   * These authors contributed equally to this study.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Gustafsson, H., **Söderdahl, T**., Jönsson, G., Bratteng, J.O., Forsby, A. Insulin-like growth factor type 1 prevents hyperglycemia-induced uncoupling protein 3 down-regulation and oxidative stress. *J Neurosci Res*. Jul 15;77(2):285-91, 2004.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AFP	Alpha-fetoprotein
AhR	Aryl hydrocarbon receptor
BMP	Bone morphogenic protein
BSEP	Bile salt export pump
BSO	Buthionine sulfoximine
CAR	Constituitive androstane receptor
CMFDA	5-chloromethylfluorescein diacetate
CYP	Cytochrome P450
dG	Deoxyguanosine
EH	Epoxide hydrolase
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-O-deethylase
FGF	Fibroblast growth factor
FMO	Flavin-dependent monooxygenase
Foxa	Forkhead box A
γ-GCS	γ-Glutamylcysteine synthetase
GPX	Glutathione peroxidase
GS	Glutathione synthetase
GSH	Glutathione
GST	Glutathione transferase
GSSG	Glutathione disulfide
HBEC	Human breast epithelial cell
hESC	Human embryonic stem cell
HNF	Hepatocyte nuclear factor
Hsp	Heat shock protein
IGJC	Intercellular gap junction communication
MDR	Multidrug resistance p-glycoprotein
MRP	Multidrug resistance protein
MT	Metallothionein
NAC	N-acetylcysteine
NAT	N-acetyltransferase
NSAID	Non-steroidal anti-inflammatory drug
NTCP	Na-taurocholate cotransporting peptide
OAT	Organic anion transporter
OATP	Organic anion transporting peptide
OCT	Organic cation transporter
PAH	Polycyclic aromatic hydrocarbon
PAPS	3’-phosphoadenosine 5’-phosphosulfate
PSSG	Protein-GSH mixed disulfide
PXR	Pregnane X receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RXR	Retinoid X Receptor

SCID	Severe combined immunodeficiency
SOD	Superoxide dismutase
SSEA	Stage specific embryonic antigen
SULT	Sulfotransferase
SXR	Steroid and xenobiotic receptor
TAT	Tyrosine aminotransferase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDO	Tryptophan-2,3-dioxygenase
TNFα	Tumor necrosis factor α
UDP	Uridine diphosphate
UGT	UDP-glucuronosyltransferase

GENERAL BACKGROUND

INTRODUCTION

We are continuously exposed to a wide range of foreign compounds, such as drugs and environmental pollutants, and some of these compounds may exert a toxic effect on our cells. Toxicity depends on several different parameters such as exposure, absorption, metabolism and excretion. The substances can have different kinds of toxic effects e.g. oxidative damage, DNA damage, cytotoxicity, and can be targeted to different organs. There are different cellular defense systems evolved to protect against xenobiotic-induced toxicity, such as antioxidant defenses and biotransformation. Biotransformation mainly takes place in the liver which, due to its location between the gastrointestinal tract and the circulatory system, is exposed to a wide range of foreign compounds. Although biotransformation is part of the cellular defense system, it can also contribute to toxicity in a process termed toxication or bioactivation, by converting xenobiotics to more reactive metabolites, such as electrophiles and free radicals. While some xenobiotics are directly toxic, the toxicity of others is due to the formation of reactive metabolites.

When new compounds, e.g. drug candidates, are produced it is of great importance to assess their potential toxic effects in humans. Understanding mechanisms of drug toxicity is an essential step towards improving drug safety testing by providing the basis for mechanism based risk assessment. Assessing the risk from xenobiotic exposure to humans still largely depends on preclinical testing in animals which in many, but not all cases predicts outcomes in humans accurately. Therefore, efforts are being made to develop human/humanized model systems that would improve predictability of human toxicity. The general aim of the work included in this thesis is to improve models and methods to assess xenobiotic toxicity.

ADMET

ADMET is an acronym for Absorption, Distribution, Metabolism, Excretion, and Toxicity and describes the disposition of a xenobiotic within an organism. The first four criteria influence the levels and kinetics of xenobiotic exposure to the tissues. For drugs this may influence the balance between the pharmacological and toxicological activities.

Absorption

Absorption determines a compound's bioavailability to the body. Many compounds enter the body orally and reach the bloodstream via the digestive tract. From the bloodstream, the compound may be taken up into target organs or cells. Some barriers, such as the blood-brain barrier, can substantially prevent this. Factors such as poor compound solubility, chemical instability in the stomach, and inability to permeate the intestinal wall can all reduce the extent to which a drug is absorbed after oral administration.

Distribution

Xenobiotics are distributed into various tissues and organs to differing extents based mainly on its lipophilicity. Biological barriers, such as the blood-brain barrier, and transporter proteins also play a major role in determining the distribution. For drugs, the distribution into target organs is critical in ensuring efficacy.

Metabolism

Drug metabolism, or biotransformation, is discussed in more detail below, in the section Biotransformation.

Excretion

Compounds and their metabolites need to be removed from the body via excretion, usually via urine (kidneys) or feces (bile). This is primarily accomplished by xenobiotic export pumps.

Toxicity

Toxicity is defined as the potential of a substance to exert a harmful effect on the organism, and a description of the effect, and the conditions or concentrations under which the effect takes place.

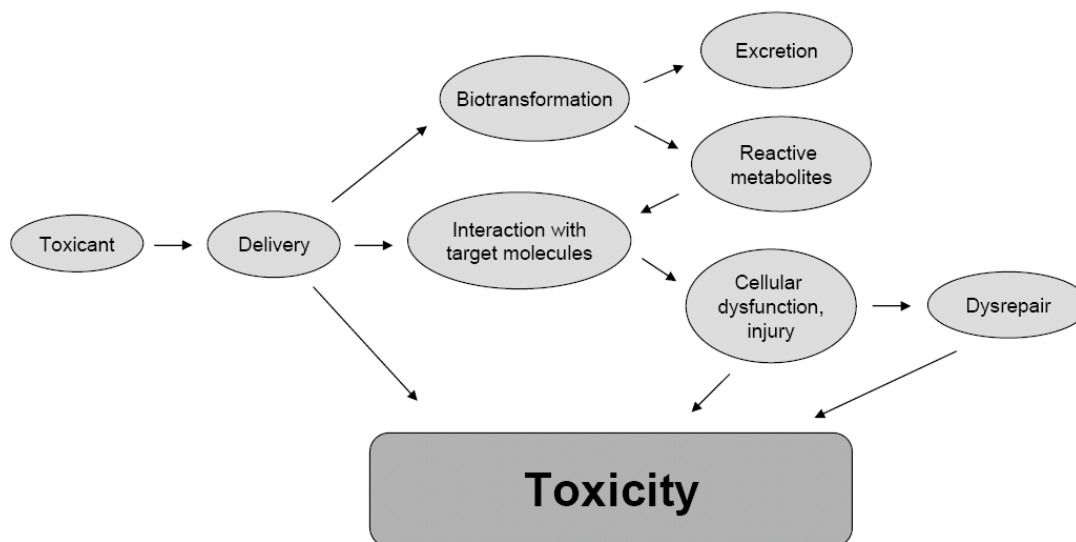


Figure 1. Potential stages in the development of toxicity after xenobiotic exposure.

BIOTRANSFORMATION

While hydrophilic compounds are easily excreted in urine, lipophilic xenobiotics are either stored in lipophilic compartments (e.g. persistent polychlorinated xenobiotics), or

converted by biotransformation to a more hydrophilic compound for subsequent excretion. The consequence of biotransformation is in most cases detoxification, however, metabolism of some xenobiotics generates reactive metabolites that are more toxic than their parent compound. Reactive intermediates can either bind to proteins, potentially causing somatic cytotoxity (apoptosis and/or necrosis), or bind to DNA and other nucleic acids, potentially resulting in genotoxicity and the risk of cancer. In most cases metabolism inactivates the pharmacological response of a drug; however, in some cases metabolites can be pharmacologically active. The liver is the main metabolizing organ in the body, but metabolism can occur in extra-hepatic tissues in the body including intestine, blood and other organs. Biotransformation involves several enzyme systems that are commonly defined into two different phases depending on the reaction they catalyze (Williams, 1959). Transport of conjugates is considered a third phase of biotransformation (Figure 2).

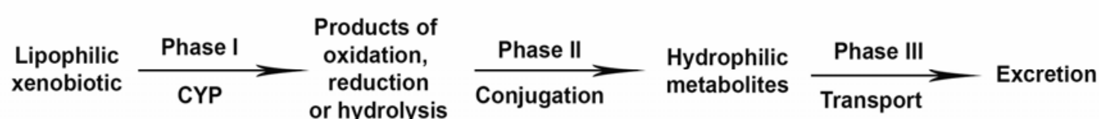


Figure 2. Principal reactions of biotransformation.

Phase I biotransformation

Phase I biotransformation involves oxidation, reduction or hydrolysis of substances and, depending on the nature of the xenobiotic, it can result in inactivation and facilitated elimination, activation of pro-drugs or metabolic activation. Cytochrome P450s (CYPs) are the major enzymes in phase I metabolism of xenobiotics and they carry out oxidation or reduction reactions, including aliphatic or aromatic hydroxylation, epoxidation, N-, O-, S-dealkylation, N-hydroxylation, sulfoxidation, desulfuration, and oxidative dehalogenation. Other phase I enzymes include flavin-dependent monooxygenase (FMO), catalyzing N- and S-oxidation (Krueger et al., 2002, Krueger et al., 2006), peroxidases, catalyzing reduction of hydroperoxidases to alcohols, carboxylesterases and amidases, catalyzing hydrolysis of ester or peptide bonds (Imai, 2006), alcohol dehydrogenases (ADHs), catalyzing oxidation of alcohols to aldehydes (Yin, 1994) and epoxide hydrolases (EHs), catalyzing hydrolysis of epoxides to dihydrodiols (Omiecinski et al., 2000).

Cytochrome P450

Cytochrome P450s are mixed function monooxygenases and their substrates include both xenobiotics and endogenous compounds, such as steroids, bile acids, fatty acids and prostaglandins (Anzenbacher and Anzenbacherova, 2001). In the reaction catalyzed by CYPs molecular oxygen binds to the heme moiety in the enzymes and one oxygen

atom is inserted into the substrate and the other is reduced to water, by electrons supplied by NADPH. Iron can only accept one electron at a time and they are transferred sequentially via NADPH-P450 reductase. There are several classes of CYPs which are classified based on sequence homology. The enzymes of greatest importance for drug metabolism belong to the families 1-3, responsible for 70-80% of all phase I-dependent metabolism of clinically used drugs (Bertz and Granneman, 1997, Ingelman-Sundberg, 2004). The major site of CYP expression is the liver, but other sites include skin, lung, intestine, kidney and testis. Cytochrome P450s are located mostly in smooth endoplasmic reticulum (ER), but also in plasma membrane and inner mitochondrial membrane. Expression and activity of CYPs present large interindividual variations due to polymorphisms. All hepatic drug metabolizing CYPs are polymorphic and this is of major importance for interindividual differences in xenobiotic metabolism and toxicity (Ingelman-Sundberg, 2002). Moreover, CYPs can be induced several fold, or inhibited by specific drugs, resulting in additional, although transient, variability of metabolic activity (Pelkonen et al., 1998).

The CYP1 family of enzymes activates polycyclic aromatic hydrocarbons (PAH) to epoxides which are then converted into highly carcinogenic diol epoxides by epoxide hydrolases. CYP1A1 and 1B1 are mainly extra-hepatic in adults, whereas CYP1A2 is primarily present in adult liver (Shimada and Fujii-Kuriyama, 2004). CYP1 enzymes are less functionally polymorphic than the drug metabolizing CYPs, most likely as a consequence of important endogenous functions (Ingelman-Sundberg, 2002).

CYP2B6, 2C9, 2C19 and 2D6 are the most important CYP2 enzymes for metabolism of therapeutic agents (Anzenbacher and Anzenbacherova, 2001) and, as a consequence, the most clinically important polymorphisms are seen with these enzymes (Ingelman-Sundberg, 2002). CYP2A enzymes are expressed at low levels in adult liver, but are highly expressed in extra-hepatic tissues (Hines and McCarver, 2002). CYP2E1 metabolizes low molecular weight compounds e.g. ethanol, carbon tetrachloride, acetaminophen and caffeine.

CYP3A4 is the most abundant CYP isozyme in human adult liver, accounting for about 25% of the total CYP content and, in addition, it metabolizes the majority of drugs whose biotransformation is known, making it of paramount clinical importance (Bertz and Granneman, 1997). CYP3A5 is highly polymorphic and only expressed in about 20% of humans, whereas CYP3A7 is mainly expressed in fetal liver (Sim et al., 2005). The enzymes of the CYP3A family have overlapping, but not identical, substrate specificities.

In human fetal liver the total CYP content is only about 30-60 % of adult values and there are significant differences in the expression pattern. CYP1A1, 2D6 and 3A7 are present in fetal liver tissue, whereas the presence of e.g. CYP2C8 and 2E1 is controversial (Hakkola et al., 1994, Hines and McCarver, 2002).

Cytochrome P450 induction and inhibition

Many of the biotransformation systems are adaptive, and induction is especially well-studied in the CYP system. Induction is classically defined as the *de novo* synthesis of an enzyme as a result of increased transcription, but in drug metabolism research induction due to translational activation or stabilization is also included (Pelkonen et al., 1998). Induction is a slow process and may take days for full effects to be evident *in vivo* and *in vitro*. Transcriptional activation of CYPs is regulated by nuclear receptors.

The expression of CYP1A and 1B enzymes is regulated by aryl hydrocarbon receptor (AhR) (Nebert et al., 2004). AhR ligands include 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAH). The pregnane X receptor (PXR; also called pregnane-activated receptor; PAR or steroid and xenobiotic receptor; SXR) regulates CYP3A expression and the constituitive androstane (active) receptor (CAR) regulates CYP2B, 2C and 3A expression. CYP inducers interact with CAR or PXR, which translocate into the nucleus, form heterodimers with RXR (retinoid X receptor) and bind to enhancer motifs in drug-related genes (Liddle and Goodwin, 2002). PXR and CAR also regulate expression of several transporter proteins. Some well-known experimental CYP inducers include rifampin (CYP3A, 2C and 2B6), ethanol (CYP2E1), omeprazole (CYP1A2) and phenobarbital (CYP2B6, 2C9, 3A).
In contrast to induction, inhibition of CYPs gives an immediate response and can lead to accumulation of a particular substrate (Pelkonen et al., 1998). The inhibition can be due to either irreversible destruction of the CYP enzyme by covalent binding, or by competitive interaction of two substrates for the same CYP (drug-drug interactions). An example of CYP inhibition is illustrated by furanocoumarin (psoralens) in grape fruit juice, which leads to inhibition of CYP3A by mechanism based inhibition, i.e. the compound is bioactivated by CYP3A to a metabolite that destroys the enzyme.

Phase II biotransformation

Phase II biotransformation involves conjugation, where an endogenous acid is added to the functional group. This can occur without preceding phase I metabolism, if the xenobiotic already has a reactive functional group. The primary enzymes involved in phase II metabolism are UDP-glucuronosyltransferases (UGTs), catalyzing the transfer of glucuronic acid from uridine diphosphate (UDP)-glucuronic acid, sulfotransferases (SULTs), catalyzing the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS), N-acetyltransferases (NATs), catalyzing the transfer of acetate from acetyl-CoA and glutathione transferases (GSTs) catalyzing the transfer of glutathione. In addition, amino acid N-acyl transferases metabolize xenobiotics containing a carboxylic acid group by conjugating to an amino acid, principally glycine in humans (Klaasen, 1995, Knights, 1998).
UGTs are membrane-bound enzymes expressed mainly in the liver. UGTs conjugate a wide variety of xenobiotics, such as drugs and environmental toxicants, as well as endogenous substrates including bilirubin and steroids (Guillemette, 2003). UGT2B7 metabolism of non-steroidal anti-inflammatory drugs (NSAID, e.g. diclofenac) is an example of when biotransformation causes toxication rather than detoxification, and results in formation of reactive acyl glucuronides, which may cause ulcers and damage to the intestine. Induction of UGTs is often coordinated with CYP induction, via e.g. AhR. SULTs are cytosolic enzymes that catalyse the sulfation of xenobiotics, hormones, such as estrogen, and neurotransmitters, such as catecholamine (Coughtrie, 2002). In contrast to most other biotransformation enzymes, SULTs are abundantly expressed in the human fetus, and are believed to be the most important prenatal metabolic system. NAT1 and NAT2 are cytosolic enzymes, but exist also in mitochondria and ER. NAT2 is mainly expressed in the liver and NAT1 is ubiquitous. In contrast to the other phase II reactions, acetylation does not increase hydrophilicity (Hein, 2000, Boelsterli, 2003).

Glutathione transferases

Glutathione transferases (GST) catalyze the conjugation of glutathione with compounds containing an electrophilic center. There are two different families of GSTs; membrane-bound microsomal GSTs, which are important in metabolism of leukotrienes and prostaglandins, and cytosolic GSTs, which will be discussed here. Cytosolic GSTs detoxify electrophilic xenobiotics, such as chemical carcinogens (e.g. benzo(a)pyrene diol epoxide), environmental pollutants, and anti-tumor agents. They also inactivate endogenous α, β-unsaturated aldehydes, quinones and epoxides, as well as hydroperoxides, formed as secondary metabolites during oxidative stress (Hayes et al., 2005). The cytosolic GSTs are homo- or heterodimers, divided into the classes Alpha, Mu, Pi, Theta, Zeta, Sigma and Omega based on amino acid sequence similarities (Mannervik et al., 2005). Subunits can form dimers with members of the same class but not with a member of another class. Cytosolic GSTs have two binding sites, one for GSH (G site), which is common to all GSTs, and one for the substrate (H site), which is specific for each GST. Human GSTs are nearly ubiquitously expressed but the highest levels are found in liver and testis (Rowe et al., 1997). GSTA1-1 is the most abundant subunit in adult liver, contributing about 75% to the total hepatic GST content (van Ommen et al., 1990, Rowe et al., 1997). GSTP is most frequently expressed in lung and brain and is absent from the adult liver.

Expression of GSTs may be modified by genetic polymorphisms, induction, diseases and, presumably, individual differences in efficiency of transcriptional or posttranscriptional events (Coles and Kadlubar, 2005). The expression of hepatic *GSTM1* is highly polymorphic and the frequency of homozygous *GSTM1*0* allele is about 50% (Seidegard et al., 1985). Induction of GSTs has not been extensively studied in humans, however, induction of GSTA1/2 in response to xenobiotics has been shown in primary human hepatocytes (Morel et al., 1993). Anti-cancer drugs that are substrates for GSTs can enhance the expression of GSTs, leading to drug resistance. GSTP is frequently overexpressed in premalignant and malignant cells, and can contribute to anti-cancer drug resistance.

Transport of xenobiotics

Phase III biotransformation involves export of xenobiotics and conjugates out of the cell. There are several ATP-dependent export pumps that belong to the family of ABC transporters. Multidrug resistance p-glycoprotein is encoded by the gene *MDR1*. Its substrates are xenobiotics, and it has no known endogenous substrate. MDR1 is only weakly expressed in liver but abundant in other tissues (Hoffmann and Kroemer, 2004). Multidrug resistance protein 2 (MRP2) is a conjugate export pump and the canalicular form of MDR (Haimeur et al., 2004). It transports bilirubin, glutathione conjugates, xenobiotic glucuronides and sulfate conjugates into bile. Bile salt export pump (BSEP) transports bile acids from hepatocytes to the biliary tree. Phospholipid export pump (MDR3) translocates phospholipids from inner to outer leaflet of membranes. Xenobiotic pumps can concentrate compounds in a certain compartments, eg biliary canaliculi and cause toxicity (Boelsterli, 2003). If the function of the pump is compromised, the damage is usually in hepatocytes. Multidrug resistance in cancer

cells is due to overexpression of MDR and MRP proteins, resulting in efflux of cytotoxic drugs from targeted cancer cells. MDR1 is important in the blood-brain barrier to keep xenobiotics from crossing.
In addition to xenobiotic export pumps, the liver has several transporter proteins e.g. Na+ taurocholate cotransporting peptide (NTCP), organic anion transporting peptide (OATP), organic anion transporter (OAT) and organic cation transporter (OCT). These transporters are also responsible for uptake of xenobiotics that may structurally resemble the endogenous substrate for transport (Boelsterli, 2003).

Reactive metabolites

Reactive metabolites can be produced via oxidation/reduction reactions (Phase I) or by conjugation (Phase II) in the biotransformation process and can react with biological macromolecules such as DNA, RNA, lipids and proteins (Miller et al., 1949, Miller, 1951, Miller, 1970, Klaasen, 1995, Baillie, 2006, Erve, 2006). Such metabolites are important because they can cause genotoxicity, organ toxicity and idiosyncratic adverse drug reactions and they are a frequent cause of delay and attrition of drug development.
Reactive metabolites are generally electrophiles, electron-deficient species with a partial or full positive charge, which can react by sharing electron pair with nucleophiles, or by oxidation. They are often produced when xenobiotics are oxidized by cytochrome P450s or other enzymes to ketones, epoxides, α,β-unsaturated ketones and aldehydes, and quinones. Electrophiles bind covalently to endogenous molecules, such as proteins and nucleic acids. Detoxification of electrophiles generally occurs through conjugation with glutathione, either spontaneously or by the action of glutathione transferases, but can also occur through epoxide hydrolases or alcohol dehydrogenases.
Free radicals are electrophiles which contain unpaired electrons. They are formed by losing or accepting an electron. Radicals, such as that formed from paraquat, can transfer an electron to molecular oxygen, forming superoxide anion radical, thus starting a redox cycle. Detoxification of superoxide anion radical occurs via superoxide dismutase (SOD) (see section Antioxidant defense mechanisms).

OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

Oxidative stress is defined as a disturbance in the normal redox state of the cell and can cause toxic effects through the production of peroxides and free radicals that damage cellular macromolecules such as proteins, lipids and DNA. Molecular oxygen is a biradical and can easily be reduced and lead to production of reactive oxygen species (ROS), such as superoxide radical anion, hydrogen peroxide and hydroxyl radical (Figure 3). Reactive oxygen species can be generated by UV radiation or enzymatically e.g. in the mitochondrial electron transport chain, by cytochrome P450-mediated oxidation, by xanthine oxidase or cyclooxygenase (Boelsterli, 2003, Bayir, 2005, Magder, 2006). Phagocytic cell can generate extracellular ROS due to NAD(P)H oxidase. Xenobiotics can enhance the production of ROS by interacting with an electron transport complex and block electron flow. Superoxide anion radical is formed in many autoxidation reactions and via the electron transport chain. It undergoes dismutation to form H_2O_2 either spontaneously or the action of SOD and is a precursor

for metal-catalyzed hydroxyl radical ($HO^{\bullet}$) formation. Hydrogen peroxide (H_2O_2) is not very reactive itself but, if it is not removed, it can lead to the formation of the highly reactive hydroxyl radical by the Fenton reaction. Reactive nitrogen species (RNS), such as nitric oxide, can also contribute to formation of hydroxyl radicals and oxidative stress.

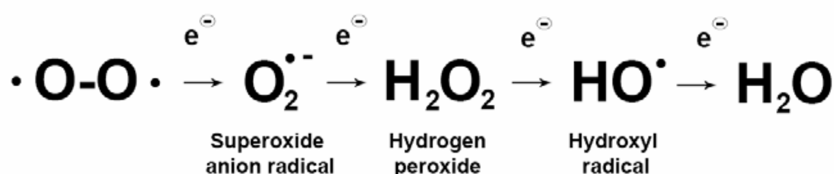


Figure 3. Stepwise reduction of molecular oxygen in biological systems leads to production of ROS (adapted from Boelsterli 2003).

Consequences of oxidative stress

During excessive oxidative stress the antioxidant defense systems may fail to counter the formation of ROS and this may lead to direct accumulation of damage of cellular macromolecules. In addition, a secondary form of stress may be induced, e.g. activation of cytokines that leads to inflammation, production of superoxide anion radical and increased oxidative stress.

Oxidative DNA damage

DNA is continually damaged by free radicals, which may react with DNA causing a reversible and irreversible damage, leading to mutation, carcinogenesis or cell death. 8-oxo-deoxyguanosine (8-oxo-dG) is the most common DNA adduct, formed during oxidative DNA damage and its production is linked to an increased risk of mutation/mutagenesis (Marnett, 2000, Martinez et al., 2003). Mitochondrial DNA usually has a higher rate of damage due to higher exposure of ROS, lack of histones and more inefficient repair. Heavy metals often cause oxidative damage to DNA and some are carcinogenic.

Oxidative protein damage

ROS may lead to oxidation of amino acid side chains, formation of protein-protein cross-links and protein fragmentation, due to oxidation of backbone (Boelsterli, 2003). Cysteine and methionine (sulfur containing amino acids) are especially susceptible to oxidation, which may lead to disulfide bonds and sulfoxide formation, respectively. Aromatic amino acids are also prone to ROS attack.

Oxidative lipid damage

One of the major toxic effects of oxidative stress is damage to cellular membranes by the process of lipid peroxidation (Magder, 2006). Peroxidation of lipids in membranes may be amplified and propagated in a chain reaction (Figure 4). The products of lipid peroxidation, including alkoxyl radicals and toxic aldehydes, may be equally as reactive as ROS.

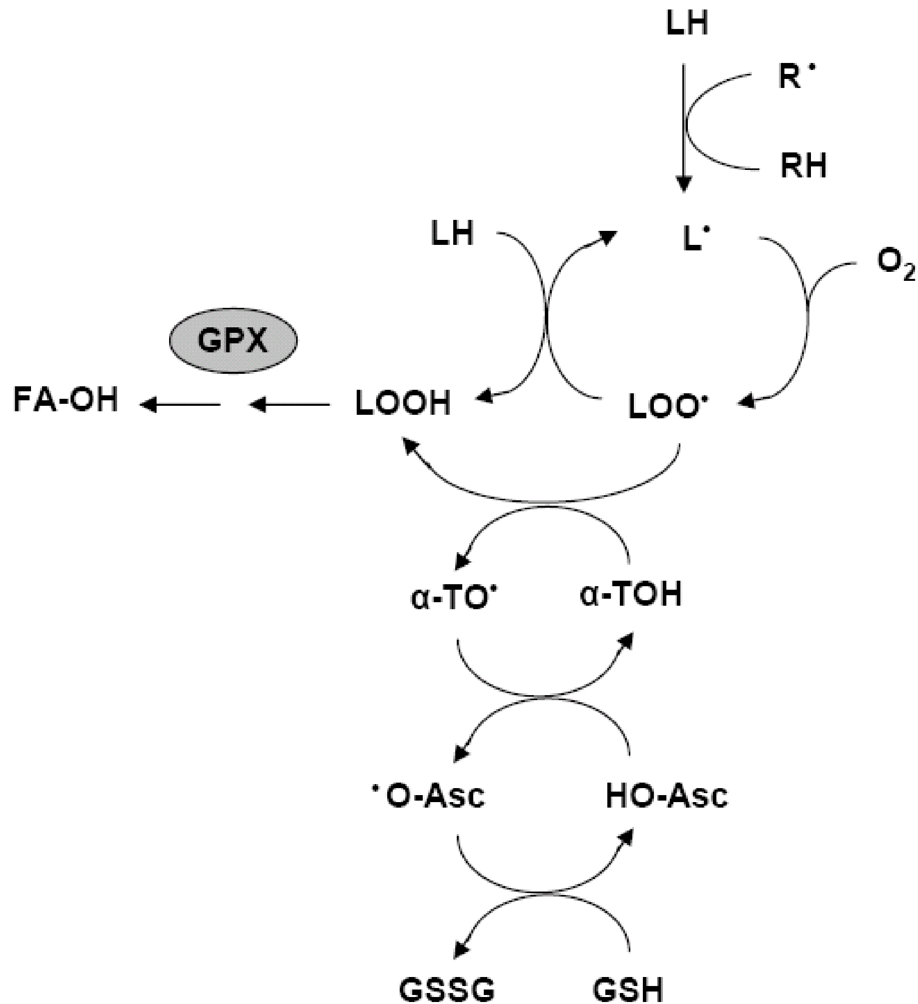
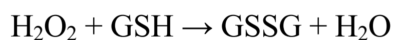


Figure 4. Schematic overview of lipid peroxidation. Abbreviations: LH, lipid substrate; LOOH, lipid hydroperoxide; LOO·, lipid peroxyl radical; α-TOH, α-tocopherol; α-TO·, α-TOH radical; Asc, ascorbic acid; FA-OH, hydroxy-fatty acid.

Antioxidant defense mechanisms

The major functions of the antioxidant defenses are to scavenge ROS and RNS. Additional functions are to keep cellular thiol redox status in a primarily reduced form, to prevent oxidation of lipids, as well as to sequester redox-active metals and thus prevent Fenton reactions. Antioxidants are differentially distributed in the body and this may, in some cases, be the cause for organotropic damage. Antioxidants may be

induced upon prooxidant stimuli. Superoxide radical anion is removed by superoxide dismutase (SOD), catalyzing the reaction where one molecule of superoxide anion is reduced and one is oxidized, forming hydrogen peroxide and oxygen (Noor et al., 2002). Catalase, which is present in peroxisomes, and glutathione peroxidase (GPX), present in the cytosol, are the two major enzymes involved in degradation of hydrogen peroxide (Flohe, 1988, Calabrese and Canada, 1989, Kirkman and Gaetani, 2007). The reaction catalyzed by GPX is:



$$H_2O_2 + GSH \rightarrow GSSG + H_2O$$

Metallothionein (MT) is a protein with 30% cysteine residues that can bind several metal atoms and inhibit their toxicity. α-Tocopherol has a phenolic group that can reduce lipid peroxides and terminate the lipid peroxidation chain reaction. Vitamin E is a mix of tocopherols. Oxidized α-tocopherol is regenerated by ascorbic acid, which is, in turn, reduced by glutathione (Figure 4).

Glutathione

Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH) plays an important part in detoxification of both oxidants and electrophiles. It acts as an antioxidant both chemically, via direct GSH-ROS interaction, and through glutathione peroxidase, and it detoxicates electrophiles via conjugation by glutathione transferases. The redox properties of GSH also predispose its involvement in processes such as protein and DNA synthesis and transport of amino acids (Griffith et al., 1979, Meister and Anderson, 1983). The γ-bond (instead of α-) between the glutamyl moiety and cysteine keeps GSH protected from protease digestion and, as a result, GSH is present intracellurlarly at high (mM) concentration and extracellularly at lower concentrations. Glutathione is synthesized by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), of which the γ-GCS catalyzed reaction is the rate-limiting step (Griffith and Mulcahy, 1999). The reactive thiol group is responsible for the antioxidant capacity that enables GSH to act as a radical scavenger, co-substrate for GPX, as well as to keep the cell in a reduced state and be involved in regeneration of oxidized proteins (see section Repair mechanisms). In these reactions with radicals, GSH is oxidized to glutathione thiyl radical (GS·) and dismutated to glutathione disulfide (GSSG). Glutathione disulfide is reduced to GSH by glutathione reductase and NADPH. For an overview of the actions of glutathione see Figure 5. Oxidative stress is implicated in diseases such as oxidative lung injury, inflammation, AIDS and reperfusion injury and treatment with GSH would be important for restoring the redox balance. However, since cysteine is unstable, N-acetylcysteine (NAC) is commonly used therapeutically as a precursor of GSH.

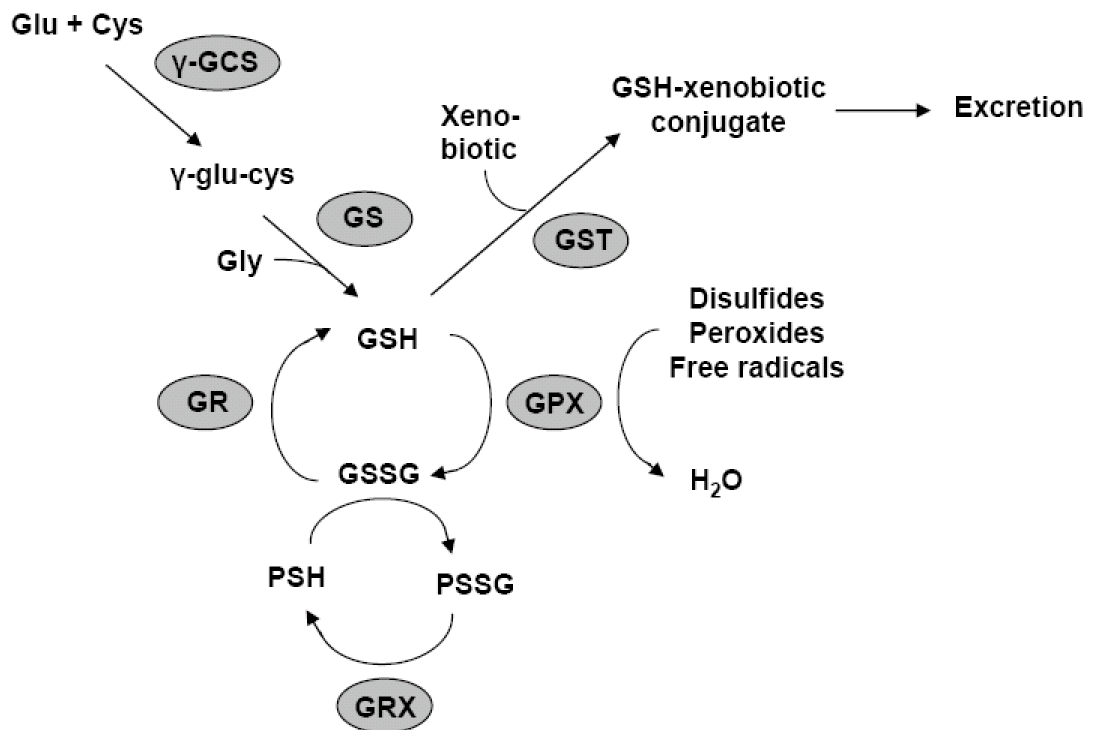
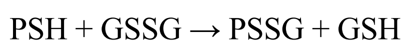


Figure 5. Schematic overview of the mechanisms of glutathione. The enzymes involved in the reactions are γ-glutamylcysteine synthetase (γ-GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione peroxidase (GPX), glutaredoxin (GRX) and glutathione transferase (GST).

Protein-glutathione mixed disulfides

Oxidative stress can lead to formation of mixed disulphides between protein thiol groups and low molecular weight thiols (S-thiolation), primarily with glutathione (S-glutathionylation). Protein glutathione mixed disulphides (PSSG) are formed mainly by the reaction:



PSH + GSSG → PSSG + GSH

Formation of PSSG may act to protect sensitive protein thiol groups from alkylation and/or irreversible oxidation, but also contribute to toxicity, by rendering the protein inactive (Giustarini et al., 2004, Ghezzi, 2005). In addition, reversible S-glutathionylation may be a link between the intracellular redox state and adaptive stress responses (Cotgreave and Gerdes, 1998).

Repair mechanisms

Repair of proteins

Molecular chaperones, such as heat shock proteins (Hsps), are synthesized in response to protein denaturation and are important for refolding damaged soluble proteins. Degradation of damaged proteins is initiated by conjugation with ubiquitin, allowing their recognition by proteasomes, where degradation occurs. Thiol groups are essential for the function of many proteins and oxidation of protein thiols to protein disulfides or protein mixed disulfides may be reversed by thioredoxin and glutaredoxin (Klaasen, 1995, Boelsterli, 2003).

Repair of DNA

Damaged DNA is continually restored by the efficient DNA repair machinery involving several repair mechanisms (Houtgraaf et al., 2006). Direct reversal by e.g. O^6-alkylguanine-DNA-alkyltransferase acts to remove DNA adducts. Excision repair mechanisms removes damaged bases. Minor lesions are repaired by base excision, involving the actions of DNA glycolsylase and AP (apurinic/apyrimidinic) endonuclease, and more bulky lesions are repaired by nucleotide excision. Recombinational (postreplication) repair occurs when excision fails to occur before DNA replication begins. Mismatched bases due to errors by DNA polymerase may be corrected by mismatch repair. Repair of double strand breaks occurs by non-homologous end joining and homologous recombination.

Repair of lipids

Peroxidized lipids may be repaired by a complex mechanism involving glutathione peroxidase (GPX) and glutathione reductase (see Figure 4).

THE LIVER

The liver accepts venous blood from the small intestine, stomach, pancreas and spleen containing high levels of nutrients, xenobiotics and other compounds. Therefore, this organ has a central role in the biotransformation of xenobiotics, and in metabolism of carbohydrates, lipids and proteins (Klaasen, 1995). The plasma glucose level is maintained by storage of glucose as glycogen in the liver (glucogenesis) or depolymerization of glycogen to glucose (glucogenolysis). The liver is also important for oxidation of triglycerides to produce energy, synthesis of lipoproteins, conversion of excess carbohydrates and proteins to fatty acids and triglycerides as well as in the synthesis of cholesterol and phospholipids. Moreover, it is implicated in deamination and transamination of amino acids, synthesis of urea, non-essential amino acids and most plasma proteins e.g. albumin. The liver is the major site of biotransformation of xenobiotics and, due to formation of reactive metabolites as a result of metabolism, it is a frequent target of toxicity.

The liver has a unique architecture and its functions are performed by several types of cells with differing phenotypic characteristics functions. The smallest functional unit of the liver is called the acinus, comprising the liver parenchyma supplied by a terminal

branch of the portal vein and hepatic artery (Figure 6). The lobule is a structural unit of the liver that consists of cords of hepatocytes and sinusoids radiating from the central vein. Sinusoids are vascular channels lined with fenestrated endothelial cells and bounded by hepatocytes in hexagonal arrangements (Figure 7). Hepatocytes are arranged with one side to the sinusoids and one to form canaliculi, the first channel in the biliary system. At the vertices of the lobule are portal triads, with bile duct, terminal branch of hepatic artery and portal vein. Blood enters the liver from the hepatic portal vein and artery, passes through the sinusoids and empties in the central vein. The acinus is divided into three zones, where zone 1 is periportal and will receive blood with highest pressure and oxygen content. These cells are equipped for oxygen-depending functions such as β-oxidation, amino acid catabolism, gluconeogenesis and ureagenesis. Zone 3 cells are perivenular and perform less oxygen-dependent functions such as glycolysis, liponeogenesis and biotransformation. Zone 1 cells have higher levels of antioxidant enzymes, whereas zone 3 cells have higher levels of CYPs. Zone 2 cells are intermediate in function.

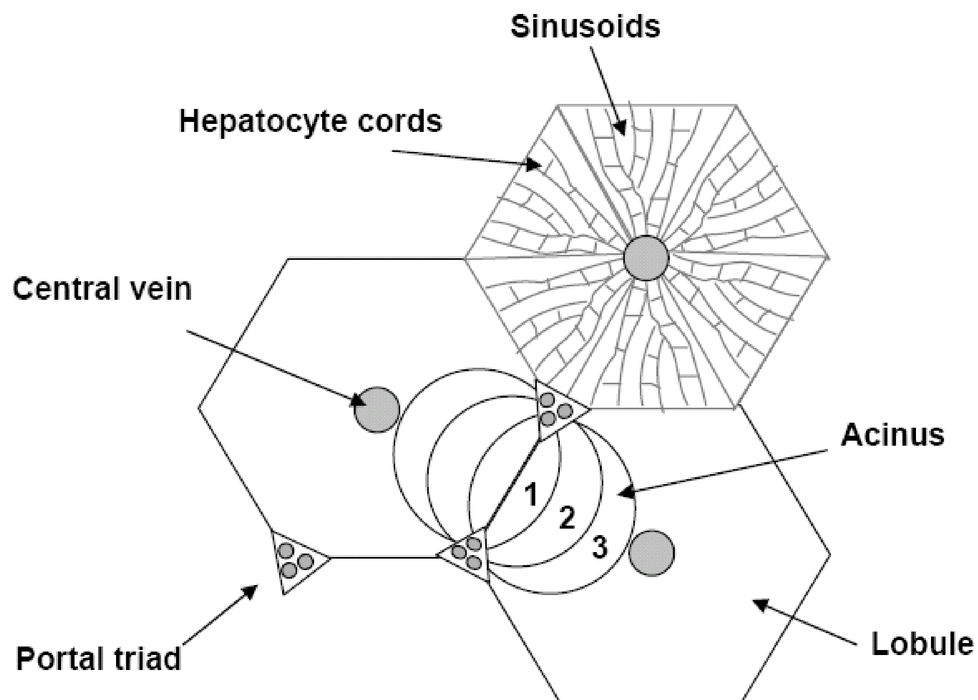


Figure 6: Liver architecture.

Hepatocytes comprise about 60% of the cell population (80% of the volume) (Gebhardt, 1992) in the liver and are the most important cells for most hepatic functions including biotransformation, protein and lipid synthesis and glycogen storage. In addition to hepatocytes, the liver is composed of several non-parenchymal cell types. Stellate cells are located in the space of Disse, between endothelium and hepatocytes (Figure 7), and store fat and vitamin A. Kupffer cells are macrophages in the sinusoids that remove particular material and microbes from the blood that comes from the portal vein. They are developed in bone marrow and reach the liver as monocytes. Activation

of Kupffer cells directly or indirectly by toxic agents results in release of inflammatory mediators e.g. ROS, eicosanoids, nitric oxide, carbon monoxide, TNFα and other cytokines (Kmiec, 2001). This activation appears to modulate some cases of acute hepatocyte injury and chronic liver responses, including cancer.

During mild to moderate liver injury, regeneration occurs by proliferation of normally quiescent hepatocytes (Michalopoulos and DeFrances, 1997). However, after substantial hepatocyte loss liver progenitor cells, known as oval cells (Farber, 1956), may play a role (Fausto, 2000, Lowes et al., 2003, Laurson et al., 2005). Oval cells are small, rare cells found in canals of Hering (in the portal tract margins, adjacent to terminal bile ductules and periportal hepatocytes) (Theise et al., 1999). The terms oval cell and liver stem cell are often used synonymously, however, this is controversial. The suggestion that oval cells may be the activated progeny of liver stem cells has been presented (Alison et al., 1996, Alison, 1998, Alison and Sarraf, 1998, Strain et al., 2003). Hepatocytes can also be regenerated by cells from bone marrow (Theise et al., 2000).

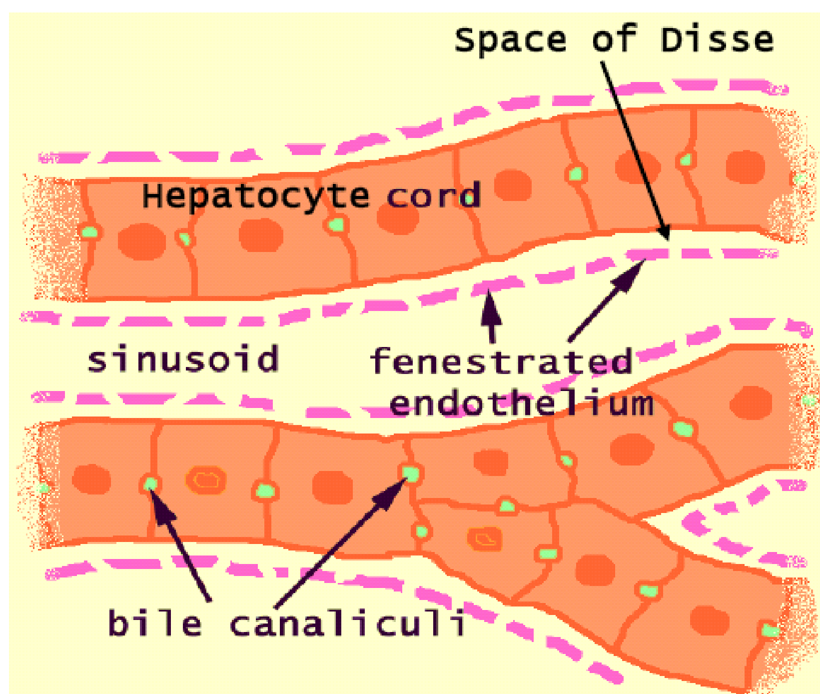


Figure 7: Hepatic sinusoids (adapted from www.siumed.edu).

Liver development

During liver development the definitive endoderm gives rise to the ventral foregut which ultimately gives rise to liver, lung, thyroid and ventral pancreas (see Figure 8). Definitive endoderm differs from visceral endoderm which arises from an extra-embryonic lineage and gives rise to yolk sac, which has many markers in common with hepatic tissue. Hepatic specification requires signaling of fibroblast growth factor (FGF) and bone morphogenic protein (BMP) from mesodermal cells (cardiac mesoderm and septum transversum) (Zaret, 2001). Without this signaling the cells will differentiate along the pancreatic lineage. Hepatocytes arise from bipotential albumin-expressing cells, hepatoblasts, which can differentiate into hepatocytes and bile duct

cells (cholangiocytes), the two types of epithelial cells in the liver. The transcription factors forkhead box A (Foxa) and GATA can act as genetic potentiators for hepatic differentiation and as mediators of competence in foregut endoderm. Expression of both Foxa1 and 2 are essential for hepatic specification (Kaestner, 2005).
There are several commonly used markers for different stages of hepatic differentiation e.g. transcription factors such as Foxa1 and 2, hepatocyte nuclear factor (HNF) 4 and 6, carrier proteins, such as α-fetoprotein (AFP) and albumin and enzymes such as tryptophan-2,3-dioxygenase (TDO), tyrosine aminotransferase (TAT) and CYPs (Lavon and Benvenisty, 2005). There are also some functional assays used to identify hepatic cells such as glycogen storage, urea and albumin synthesis. However, most of these markers and functions are not exclusive for the liver. For instance, AFP and Foxa2 are also expressed in extra-embryonic yolk sac (Meehan et al., 1984). To date, the only markers believed to be specific for hepatocytes are CYP7A1 expression and urea synthesis (Asahina et al., 2004, Lavon and Benvenisty, 2005).

PRECLINICAL SAFETY ASSESSMENT

The pharmaceutical industry aims to develop safer drugs, by identifying and avoiding substances that may cause formation of reactive metabolites. Preclinical safety studies are performed to support clinical trials and characterize toxic effects with respect to target organs, dose-dependence, relationship to exposure and potential reversibility. Recent high profile withdrawals increase the pressure on the pharmaceutical industry to improve safety testing early in the drug discovery process. Today, preclinical safety testing generally includes single and repeated dose toxicity studies, reproduction toxicity studies, genotoxicity studies, local tolerance studies and in some cases carcinogenicity studies. Preclinical studies also include ADME (pharmacokinetics) and safety pharmacology, i.e. assessment of effects on vital functions such as cardiovascular, central nervous and respiratory systems.

Hepatotoxicity testing

Liver toxicity is a frequent cause of drug withdrawal (e.g. various NSAIDs, troglitazone) and the liver is of great interest in safety assessment. Preclinical animal experimentation is often insufficient for predicting hepatic toxicity in humans (Olson et al., 2000) and the use of human model systems, such as cultured hepatocytes, is highly advantageous. However, since human hepatocytes do not maintain their characteristic functions in culture (Wu et al., 1990, Baker et al., 2001, Rodriguez-Antona et al., 2002), several approaches have been developed to mimic the *in vivo* hepatic situation and preserve hepatic function. Various culture systems have been developed for this purpose, such as sandwich culture systems and bioreactors (Table 1) (Dunn et al., 1991, Gerlach et al., 2003). Moreover, hepatic toxicity may be mediated or modified by non-parenchymal cells, such as Kupffer cells (Kmiec, 2001, Edwards, 2004, Roberts et al., 2006) and model systems with co-cultures of parenchymal and non-parenchymal cells would be advantageous. Human stem cells offer a great potential in toxicity testing since they can potentially be differentiated to hepatocytes, and other hepatic cell types, replacing such cells derived from strictly limited patient material, and used in culture systems such as those mentioned above. This would improve predictability of

preclinical safety assessment, both by its use in front loading approaches to screen for, detect and thereby avoid potential hepatotoxic compounds during drug discovery and in problem solving of preclinical or clinical findings.

In addition to primary human hepatocytes (Butterworth et al., 1989, Guillouzo et al., 1993) there are several different human model systems available for *in vitro* hepatotoxicity testing (Brandon et al., 2003, Dambach et al., 2005). They include liver slices (Renwick et al., 2000, Groneberg et al., 2002), hepatocyte-derived cell lines (Gomez-Lechon et al., 2001), cells lines heterologously expressing drug metabolizing enzymes (Guengerich et al., 1997, Crespi and Miller, 1999) and immortalized hepatocytes (Cascio, 2001). These are useful mechanistic research tools, but there are technical difficulties such as availability, variability and standardization and most of them are not used routinely in the pharmaceutical industry.

Culture Model	Concept of cell configuration	Maintenance of specific functions
Hepatocytes in suspension	1D culture	A few hours
Hepatocyte monolayer	2D culture	A few days
Hepatocyte sandwich	3D culture Mimic hepatic sinusoid	A few weeks
4-compartment bioreactor	Mimic hepatic lobule	A few months

Table 1. Liver culture models.

Genotoxicity testing

According to the recommendations of the International Conference on Harmonisation (ICH, 1997) the standard battery for genotoxicity testing is a test for gene mutation in bacteria (typically Ames test), an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma TK assay and an *in vivo* test for chromosomal damage using rodent hematopoetic cells (micronucleus). Normally no mechanistic studies are included, but occasionally DNA binding studies (^{32}P-postlabeling, mass spectrometry) for detection of DNA adducts, and comet assay are performed. Since most procarcinogens require metabolic activation to become genotoxic, preincubations with the 9000 x g supernatant (S9) of rat liver is widely used. The use of human hepatocytes for this application would have advantages, since genotoxicity may be species-dependent and intact cells retain their capacity generate and inactivate reactive metabolites (Maurel, 1996).

HUMAN STEM CELLS

The characteristic properties of stem cells include their ability for long-term self-renewal and ability to give rise to differentiated cells. These characteristics give them

the ability to give rise to unlimited amounts of differentiated human cells, which could be used for many various applications, including regenerative therapies, for treatment of diseases such as diabetes and Parkinson's disease, and toxicity screening. There are two types of human stem cells, those of embryonic origin and those derived from adult tissues.

Human embryonic stem cells

Human embryonic stem cells (hESC) are derived from the inner cell mass of pre-implantation blastocysts. They are pluripotent and can give rise to cells of all three embryonic germ layers; endoderm, ectoderm, and mesoderm, and further on, to all somatic and germ cells (Figure 8) (Geijsen et al., 2004, Heins et al., 2004). Thomson et al. were the first to report the successful derivation of hESC from human embryos (Thomson et al., 1998). In order to establish hESC lines surplus embryos, donated after informed consent from *in vitro* fertilization clinics, are cultured for 4-6 days to blastocysts, consisting of inner cell mass and trophectoderm, which gives rise to extra-embryonic tissue. The inner cell mass is isolated and the cells are grown on mouse embryonic fibroblast (MEF) feeders. Human ESC require the presence of MEFs to maintain their undifferentiated phenotype (Trounson, 2001, Daheron et al., 2004), whereas mouse ESC can be maintained by addition of leukemia inhibiting factor (LIF) (Bradley, 1990). To establish pluripotency of hESC, cells are transplanted to severe combined immunodeficient (SCID) mice to form teratomas, which are examined by immunohistochemistry to identify all three germ layers. Other characteristics used to identify hESC include Oct-4 expression, alkaline phosphatase and telomerase activity, stage-specific embryonic antigens (SSEA) 3 and 4, hESC antigens e.g.TRA-1-60, TRA-1-81 (Stojkovic et al., 2004, Trounson, 2006). HESC lines have been grown for several years with maintained karyotype, high levels of telomerase activity and expression of hESC markers (Heins et al., 2004).

Spontaneous differentiation of hESC can occur either in 2D cultures (Reubinoff et al., 2000) or by formation of embryoid bodies (Itskovitz-Eldor et al., 2000). HESC differentiation can also be directed along a specific cell lineage by e.g. exposure to growth factors, activation of endogenous transcription factors, transfection, or co-culture with cells capable of lineage induction (Trounson, 2006). Differentiation into ectodermal lineage is common in spontaneously differentiating hESC cultures (Reubinoff et al., 2000) and is considered a default developmental pathway. Differentiation of hESC into several different cell types, from all germ layers, has been reported, including neuroectoderm (Pera et al., 2004), cardiomyocytes (Mummery et al., 2002, Mummery et al., 2003, Norstrom et al., 2006) and pancreatic cells (Segev et al., 2004, Brolen et al., 2005).

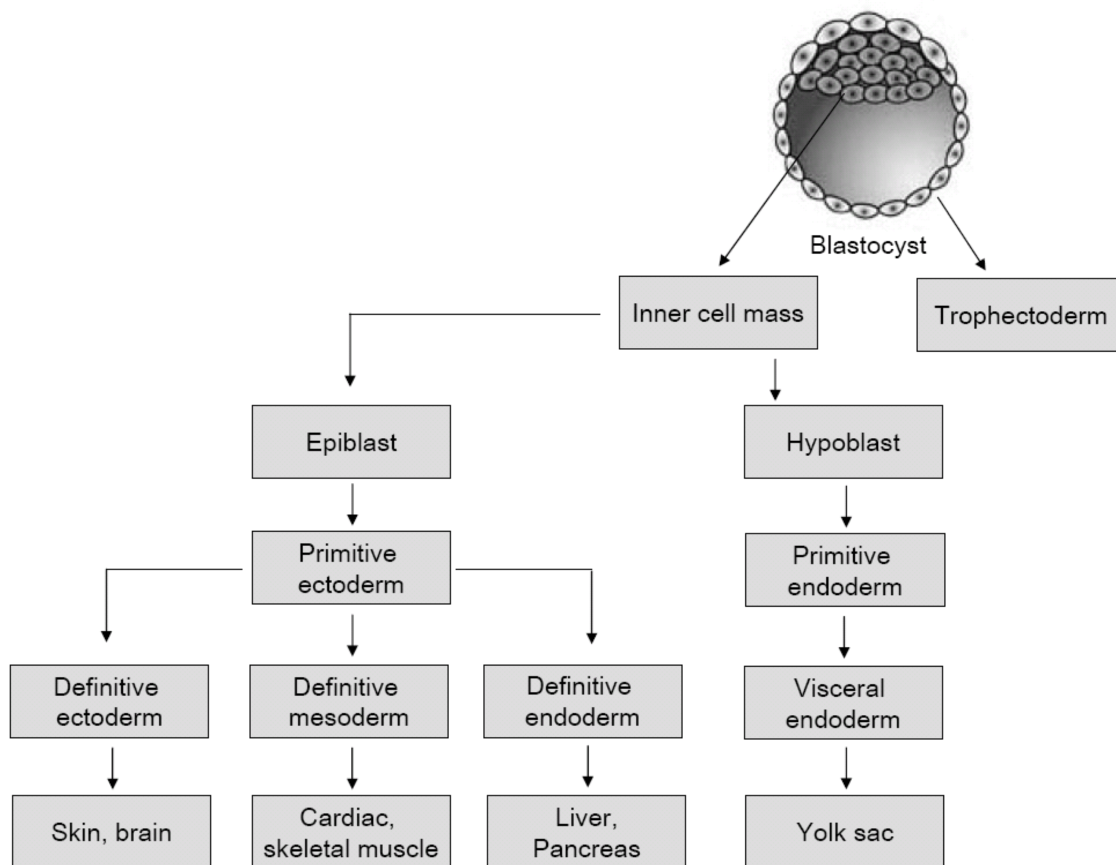


Figure 8. Development of human embryonic tissue.

Human adult stem cells

Adult stem cells share the general characteristics of stem cells i.e. long-term self-renewal and the ability to give rise to differentiated cell types, but are derived from several different adult tissues. The presence of stem cells has been reported from many different tissues, e.g. bone marrow (Bonnet, 2003, Edwards, 2004), nervous system (McKay, 1997, Temple and Alvarez-Buylla, 1999), liver (Alison et al., 1996, Strain et al., 2003), breast (Kao et al., 1995) and skeletal muscle (Seale and Rudnicki, 2000). The primary functions of adult stem cells are to maintain tissue homeostasis and replace cells following injury (Kirschstein and Skirboll, 2001). Adult stem cells are identified using different markers depending on which tissue they are derived from. A feature common to stem cells from different tissues is that they do not seem to express connexins or have intercellular gap junction-communication (IGJC) (Trosko, 2005).

Typically stem cells generate precursor or progenitor cells that in turn give rise to the fully differentiated cell. Dividing stem cells give rise to at least one additional stem cell with the same capabilities as the originating cells (symmetrical or asymmetrical division). Progenitor cells are uni-or multipotential, but in contrast to stem cells, give rise to two differentiated cells when dividing. It is often difficult to distinguish adult stem cells from progenitor cells. Adult stem cells from one tissue has been shown to generate differentiated cell types from another tissue (from the same or another germ layer), in a process termed plasticity or transdifferentiation, but it is unclear if this is of relevance *in vivo* (Bunting and Hawley, 2003, Preston et al., 2003).

PRESENT INVESTIGATION

AIM OF STUDY

General Aim

All new chemical entities that are produced by human activity, e.g. drugs and environmental pollutants, have to be carefully evaluated in terms of ADME and toxicity/safety. Such assays are performed in various test systems, including human and animal cells and experimental animals. There is a continuous effort to improve those test systems used so that predictions of eventual toxicity can be made as early and as accurately as possible. For safety assessment of novel drugs, it is ideal to use human material since that excludes the potential problem of species differences and may reduce the need for animal experimentation. By detailed knowledge on the mechanistic level predictions can be made earlier in the process, but for this, simplified methods and models to screen for mechanisms associated with toxicity are required. Bioactivation is a central issue in xenobiotic toxicity and may lead to cytotoxicity as well as genotoxicity. Therefore, characterizations of the biotransformation capacity of model systems are very important for validation of a predictive tool. The characteristic properties of human stem cells make them highly interesting as a model system for toxicity screening. The long term aim of the studies included in this thesis is to improve test systems and procedures for assessment of toxicity due to xenobiotic exposure.

Specific Aims

Paper I

The aim of Paper I was to describe a method to evaluate the intracellular distribution of glutathione. Potentially, this method could be used to screen effects of xenobiotics on levels and distribution of GSH, particularly in the nuclear compartment, to assess genotoxicity/DNA damage capacity of xenobiotics, as a complement to traditional DNA binding studies.

Preliminary study

The aim of the study was to evaluate the biotransformation capacity of human adult stem cells from breast and liver tissue, with a focus on glutathione transferases and cytochrome P450s. The long term aim of the studies was to couple these basic detoxification data to metabolic events and DNA repair mechanisms in stem cells. These characterizations may also help to develop and validate more relevant human *in vitro* models for screening and predicting reactive metabolite-related toxicity.

Papers II and III

The aim of Paper II was to evaluate the presence of several hepatic markers to establish the phenotype of hepatocyte-like cells derived from hESC, as well as characterize the protein expression of glutathione transferases in these cells using immunocytochemistry, Western blot analysis and a catalytic activity assay.
The aim of Paper III was to similarly investigate the expression of CYPs, UDP-glucuronosyltransferases (UGTs), drug transporters, transcription factors and other liver-related genes as well as protein expression of several important CYPs in hepatocyte-like cells derived from hESC using low density arrays, real time PCR and Western blotting.
The long term aims of the work illustrated in Papers II and III were to characterize drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells in order to evaluate them for use as a models system for hepatic metabolism and drug-induced hepatotoxicity.

RESULTS AND DISCUSSION

Subcellular localization of glutathione in human cells: Relationship to regiospecific bioactivation

Glutathione is important in protecting cells from the effects of oxidants and electrophiles, and the availability of GSH in the nucleus would be highly advantageous in the protection of DNA from attack and damage by adduct formation and/or oxidation. Indeed, depletion of GSH results in increased oxidative stress, e.g. lipid peroxidation, and oxidative DNA modifications, such as 8-oxo-dG (Green et al., 2006). In paper I, we describe a novel method for visualization of intracellular glutathione, based on immunocytochemistry. We used a polyclonal antibody that detects reduced and oxidized glutathione as well as protein mixed disulfides, but does not exhibit crossreactivity with glutamate, cysteine, glycine, γ-glutamyl-cysteine or cysteinyl-glycine (Hjelle et al., 1994). Confocal visualization of stained A549 cells showed that the glutathione levels of the nuclear and cytosolic compartments are close to equilibrium (Paper I, Figure 3) and that the highest levels of cellular GSH are associated with mitochondria. When cells were treated with buthionine sulfoximine (BSO), preventing the *de novo* synthesis of glutathione, the cytosolic and nuclear pools of glutathione were nearly completely depleted within 24 hours, whereas the mitochondrial pool appears more resistant to BSO treatment (Paper I, Figure 2). Figure 2 also present staining using the GSH-labeling agent mercury orange, which only reacts with GSH and not GSSG, as described by Thomas et al. (Thomas et al., 1995). Both methods present comparable results; however, the mercury orange method involves technical difficulties as the reagent reacts readily with protein thiols and the use of the antibody continually produced images of superior intensity and quality. Moreover, the immunocytochemical method presented here offers advantages over other methods aiming to describe the intracellular distribution of glutathione. Our studies are performed on intact cells and the use of antibody labeling excludes the dependence of GSTs that may be a problem with methods using e.g. monochlorobimane or CMFDA

(Bellomo et al., 1992, Voehringer et al., 1998, Stevenson et al., 2002) due to variations (or even absence) in GST activities in particular cell populations. Conjugates may also be unequally distributed in the cell and therefore present a false image of the distribution of GSH (Briviba et al., 1993). In addition, the use of fixed cells allows for multiple stainings and cell sorting using FACS as illustrated in Paper I (Figure 7 and 8), allowing analysis of different cell populations.

The method used in this study could potentially be used to monitor the cellular redox state and the distribution of GSH following treatment with xenobiotics, as well as reflect genotoxicity and cytotoxicity. However, since the results show that mitochondrial GSH is more readily visualized than nuclear GSH in the cells studied here, it could also be used to study events such as mitochondrial redox potential and loss of membrane potential during apoptosis related to oxidative stress.

Visualization of protein-glutathione mixed disulfides in human cells as a marker of oxidative stress

In the first part of paper I the intracellular distribution of the important cellular protectant glutathione was examined. During oxidative stress reduced glutathione (GSH) is oxidized to glutathione disulfide (GSSG) and protein-glutathione mixed disulfides (PSSG). One disadvantage of most methods previously described to monitor GSH in intact cells is that they do not visualize GSH on a cellular basis during oxidative stress. Since the GSH antibody also reacts with GSSG, one option was to visualize PSSG instead. The method described in Paper I provided initial visualization of the compartmentalization of protein-GSH mixed disulphides formed in A549 cells exposed to diamide, a protein thiol oxidant (Paper I, Figure 6). Prior to immunostaining, unbound GSH and GSSG were washed out from the cell, as evidenced by immunocytochemistry and HPLC. The results showed discontinuous staining mainly associated with membrane blebs and the nuclear region. Diamide is known to form PSSG with actin filaments as a result of oxidative stress and bleb formation has been associated with oxidation of thiol groups of actin (Mirabelli et al., 1988, Schuppe-Koistinen et al., 1995).

Overall, the methods described in Paper I allow for an appreciation of the GSH redox status during oxidative stress at single cell level, as well as providing a method to monitor regiospecificity in the GSH levels and redox state within the cell. It provides a potent tool to more carefully study the potential harmful production of reactive electrophilic intermediates in small numbers of test cell, such as those potentially generated from stem cell origin.

Biotransformation systems in human stem cells and their progeny

Biotransformation is an important process in toxicology, since it can lead to both detoxification of xenobiotics and generation of reactive metabolites. The studies presented here, in Papers II and III as well as in a preliminary study, are concerned with the characterization of biotransformation capacity of human adult stem cells from breast and liver tissue as well as hepatocyte-like cells derived from human embryonic stem cells. The focus is on the protein expression of glutathione transferases and

cytochrome P450s, but studies of the mRNA expression of CYPs and other liver-related genes in hepatocyte-like cells are also included.

Characteristics of human adult stem cells used in the present study

Human adult stem cells may represent a vital target for bioactivation and detoxification of xenobiotics, and may be susceptible to somatic toxicity or genotoxicity. Despite this, there is little cohesive data characterizing biochemical parameters in stem cells, such as biotransformation systems, which predispose stem cells to the influences of xenobiotics. In a preliminary study we have characterized human adult stem cells from liver and breast tissue. The stem cells from both tissues have been shown to express Oct-4, a transcription factor considered to be a marker for embryonic stem cells, as detected by immunocytochemistry and RT-PCR (Tai et al., 2005). Two types of human breast epithelial cells (HBEC) were derived from reduction mammoplasty, after informed consent and approval from the local ethics committee (Kao et al., 1995). HBEC Type I cells, with stem cell characteristics, express cytokeratin 18 and 19 and estrogen receptor, can differentiate into Type II HBEC, form organotypical structures in matrigel and have high susceptibility to telomerase activation (Kao et al., 1995). Type II HBEC cells are the differentiated daughter cells of the type I HBEC and show a basal epithelial cell phenotype, expressing cytokeratin 14, α6 integrin, connexins 26 and 43, but lack expression of Oct-4. Liver stem cells (HL1-1) were obtained from the normal part of a liver surgically resected from a male with hemanginoma, after informed consent and approval from the local ethics committee (Tsai et al. unpublished data). These cells express liver stem (oval) cell markers i.e. α-fetoprotein, vimentin and thy-1, and exhibit other stem cell characteristics e.g. high proliferation potential (~ 50 cumulative population doublings), deficiency in gap junction intercellular communication and ability of anchorage independent growth. When these liver cells are grown under differentiating conditions the expression of vimentin and α-fetoprotein is reduced and albumin expression is stimulated (Tai et al., 2005).

Characteristics of hepatocyte-like cells derived from human embryonic stem cells used in the present study

The liver is a frequent target of drug-induced toxicity and it is of great importance to assess potential hepatotoxicity in early drug development. The use of hepatocyte-like cells derived from human embryonic stem cells as a model system could improve the predictability of toxicity tests and reduce the need for animal experimentation. In Papers II and III we studied hepatocyte-like cells derived from hESC by a simple direct differentiation protocol (see Paper II). To establish the hepatocyte-like phenotype we investigated expression of several hepatic markers by immunocytochemistry and glycogen storage by periodic acid-Sciff staining. The hepatocyte-like cells showed expression of albumin, α-1-antitrypsin, liver fatty acid binding protein, Foxa2, cytokeratin 18 and, at low levels, the fetal marker α-fetoprotein. Although, none of the markers studied here is exclusively expressed in the liver, the whole panel of hepatic-related markers, together with glycogen storage and the characteristic hepatocyte morphology (Paper II, Figure 1), strongly suggest the hepatic nature of these cells. A few previous reports have been published on differentiation of hESC into the hepatic

lineage (Rambhatla et al., 2003, Lavon et al., 2004, Schwartz et al., 2005, Baharvand et al., 2006, Soto-Gutierrez et al., 2006) but only Baharvand et al. showed expression of the liver specific gene CYP7A1, whereas Soto-Gutierrez et al. and Schwartz et al. show synthesis of urea.

Glutathione transferases in human adult stem cells and hepatocyte-like cells from human embryonic stem cells

We have investigated the presence of glutathione transferases in human adult stem cells (HBEC type I and HL1-1) and hepatocyte-like cells form hESC by immunocytochemistry and Western blotting. Preliminary results from immunocytochemical analysis reveal few cells in the HBEC type I and HL1-1 cultures expressing GSTA1-1, whereas HBEC type II cells are negative for this isozyme (Figure 9). This indicates an immature phenotype in the majority of the HL1-1 cells, but possibly a more mature phenotype in the GSTA1-1 positive HL1-1 cells, since human adult liver present high levels of GSTA1-1 (van Ommen et al., 1990, Rowe et al., 1997). However, this does not seem to be the case for the HBEC cells since the more differentiated HBEC cells of type II do not show expression of GSTA1-1. The HBEC type I, II and HL1-1 cells show expression of GSTP1-1 (Figure 9), but not GSTM1-1 (data not shown). The Western blot analysis confirms presence of GSTP1-1 in all three cell types, but does not reveal expression of GSTA1-1 or GSTM1-1 (Figure 10). The GSTA1-1 positive cells in the HBEC type I and HL1-1 cultures are most likely too few to be seen using a method such as Western blot, where populations rather than single cells are analyzed.

In contrast to the stem cells from adult tissue, the hepatocyte-like cells derived from hESC show high levels of GSTA1-1, whereas GSTP1-1 is not present (Paper II, Figure 2 and 5). The levels of GSTA1-1 were increased in cells treated with a cocktail of drugs known to induce CYPs. In both hepatocyte-like cells and primary human hepatocytes, GSTM1-1 was weakly detected by immunocytochemistry but not by Western blotting. The weak expression/absence of GSTM1-1 may be due to polymorphism, since the frequency of homozygous *GSTM1*0* is about 50% is Caucasians (Seidegard et al., 1985). In addition, GST activity is detected in hepatocyte-like cells at levels comparable to human hepatocytes. These results indicate that the hepatocyte-like cells have a pattern of GST expression and activity that closely resemble those of human adult hepatocytes, whereas the adult stem cells present an immature phenotype as evidenced by expression of GSTP1-1 and lack of GSTA1-1.

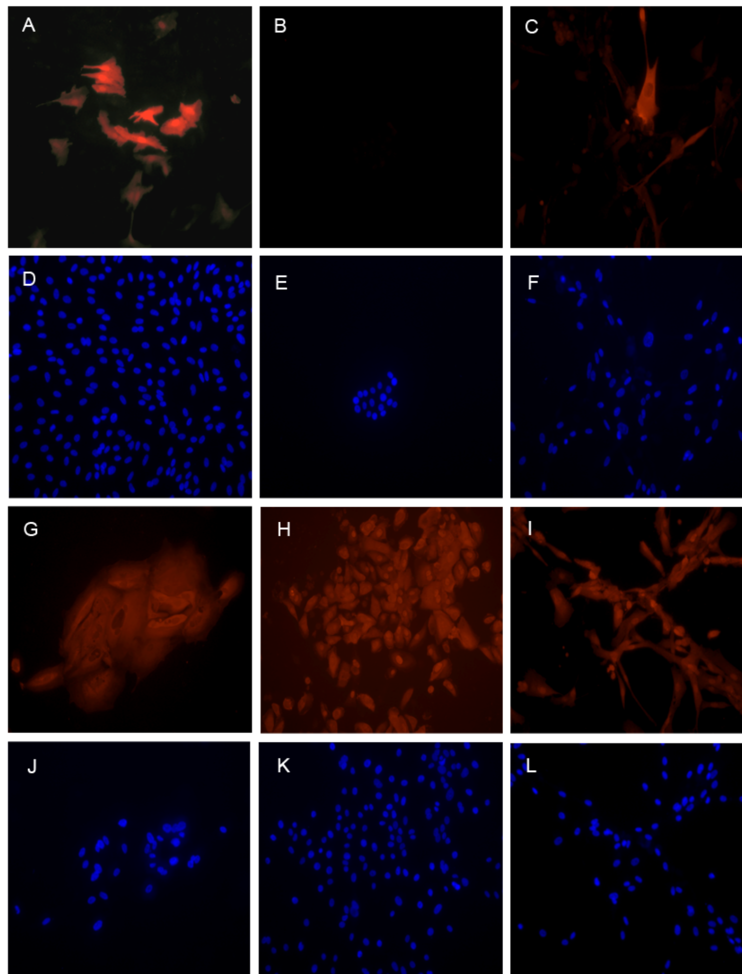


Figure 9: Immunocytochemical analysis of GSTA1-1 and GSTP1-1 in HBEC type I and type II and HL1-1. A-C: GSTA1-1; G- J: GSTP1-1; D-F, J-L: DAPI counterstaining. A, D, G, J: HBEC type I; B, E, H, K: HBEC type II; C, F, I, L: HL1-1.

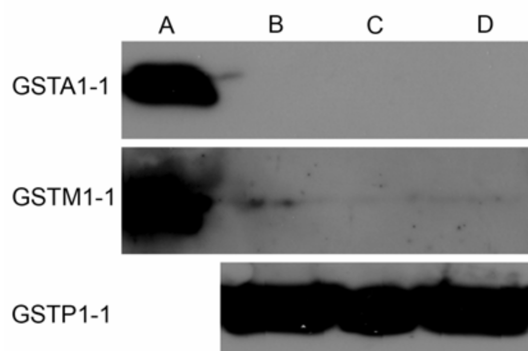


Figure 10: Western blot analysis of GSTA1-1, M1-1 and P1-1 in HBEC type I and type II and HL1-1. A: positive control, B: HBEC type I, C: HBEC type II and D: HL1-1.

Cytochrome P450s in human adult stem cells and hepatocyte-like cells from human embryonic stem cells

Immunocytochemical analysis show weak staining in HL1-1 and HBEC type I and II cells for CYP1A1/2, 2E1 and 3A4/7, but strong staining for CYP1A1/2 in some HBEC type II cells (Figure 11). Western blot analysis confirms presence of CYP1A1, 2E1 and 3A4/7 in type II HBEC cells, but does not reveal any expression of the CYPs tested in HL1-1 and HBEC type I cells, apart from possibly weak expression of 1A1 in type I cells (Figure 12). Using immunocytochemistry, it may, in some cases, be difficult to rule out the possibility of non-specific reactivity. In such cases, Western blot analysis offers the advantage of identification of proteins by their molecular weight and, therefore, increases the specificity of the analysis. The differences in CYP expression patterns between HBEC type I and II emphasizes the differences in maturation. In accordance with the results presented here, it has been reported that mature breast tissue *in vivo* express CYP1A1, 2E1 and 3A4 (El-Rayes et al., 2003) mainly for metabolism of endogenous substrates such as estrogen, but the presence of the CYPs may also have a function in metabolism of xenobiotics that can accumulate in the fatty tissue. The lack of expression of drug metabolizing enzymes in HL1-1 cells indicates an immature phenotype.

In paper III we investigated the mRNA and protein expression of several important cytochrome P450s in hepatocyte-like cells from hESC. Significant CYP expression on the mRNA level was detected in hepatocyte-like cells from one out of two hESC-lines tested (Paper III, Figure 1). CYP1A2, CYP3A4/7 and low levels of CYP1A1 and CYP2C8/9/19 protein were detected in both lines using Western blotting (Paper III, Figure 3). The expression of other liver-related genes, such as UDP-glucuronosyltransferases (UGTs), drug transporters and transcription factors were also studied using low density arrays. The expression of several liver-related transcription factors indicate that the hepatocyte-like cells are committing to a hepatic phenotype. In addition, the mRNAs for a variety of CYPs and liver-related factors were shown to be inducible in both cell lines, and this was reflected in induced levels of CYP1A2 and CYP3A4/7 protein. The results shown in Paper III highlight differences in expression pattern between cells derived from different hESC lines.

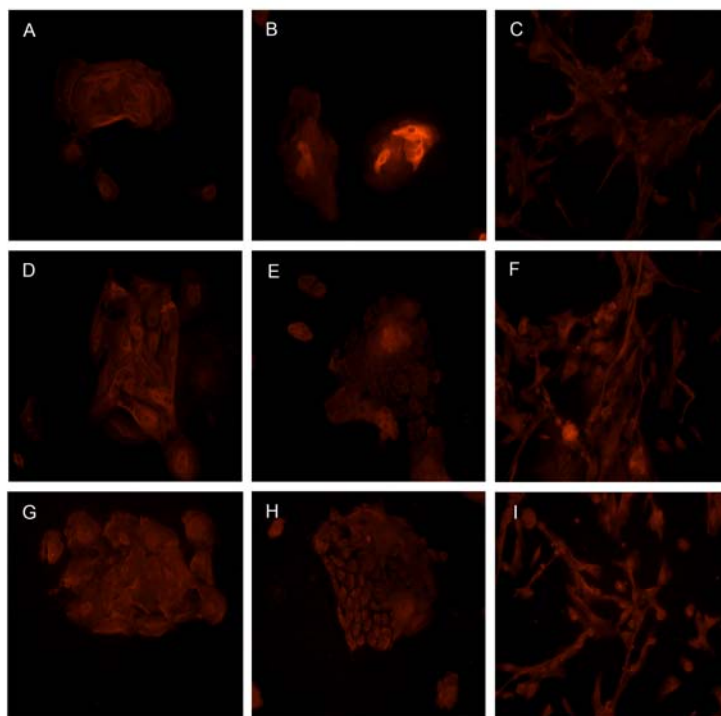


Figure 11: Immunocytochemical analysis of CYP1A1/2, 2E1 and 3A4/7 in HBEC type I and II) and HL1-1. A-C: CYP1A1/2; D-F: CYP2E1, G- J: CYP3A4/7. A, D, G: HBEC type I; B, E, H: HBEC type II; C, F, I: HL1-1.

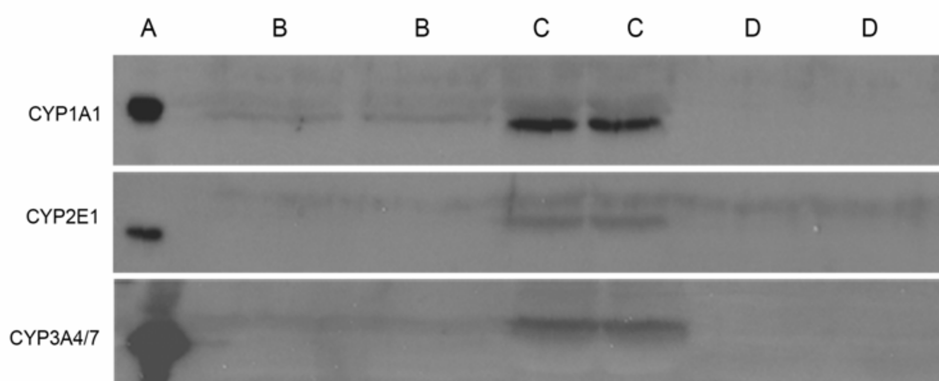


Figure 12: Western blot analysis of CYP1A1, 2E1 and 3A4/7 in HBEC type I and type II and HL1-1. A: positive control, B: HBEC type I, C: HBEC type II and D: HL1-1.

Potential use of human stem cells and their progeny in toxicity screening

Preliminary results show a clear difference in the expression pattern of the biotransformation enzymes studied here between adult stem cells from both breast and liver tissue, and their differentiated counterparts. This indicates that the stem cells

would respond differently to exposure of xenobiotics and since they may in certain cases be the target of toxicity, it could be useful to include such cells in toxicity tests to obtain a more complete prediction of the response in the target tissue.

The differences in biotransformation capacity between adult stem cells and differentiated cells indicated that these stem cells are not suitable as a model system for safety assessment without further development and differentiation of the cells to more closely resemble the target cell type. However, such work would be laborious and time-consuming and therefore beyond the scope of this thesis.

In contrast, the hepatocyte-like cells derived from hESC show several characteristics of human hepatocytes and expression of many important biotansformation enzymes, at levels higher than seen in undifferentiated hESC and adult stem cells from liver tissue. However, the levels are low in comparison to human liver and primary hepatocytes and no CYP activity could be detected in hepatocyte-like cells with the methods used in Paper III. Although these reports on expression of important drug metabolizing enzymes in hepatocyte-like cells derived from hESC represent an important step towards the development of functional hepatocytes, it is concluded that the cells in this study represent a not fully mature stage. Therefore, efforts to further differentiate the cells using optimized protocols are needed before they exhibit similar levels of drug metabolizing enzymes as human primary hepatocytes and liver.

In previous reports, only limited studies of CYP expression and induction in hESC derived hepatocyte-like cells have been included. Schwartz et al. detected phenobarbital-inducible CYP expression, as measured by quantitative RT-PCR and a non-quantitative pentoxyresorufin-O-deethylase (PROD) activity assay in hepatocyte-like cells (Schwartz et al., 2005). However, the PROD assay described, also used by Schwartz et al. in an earlier publication (Schwartz et al., 2002), was questioned by Hengstler et al 2005 (Hengstler et al., 2005). Rambhatla et al. reported inducible CYP1A2 activity as detected by ethoxyresorufin-O-deethylase (EROD) activity in hESC derived cells with hepatocyte-like characteristics (Rambhatla et al., 2003). Nevertheless, the possibility that the activity detected may be due to CYP1A1, which is mainly extra-hepatic, and not 1A2, since EROD is also sensitive toward CYP1A1 (Burke et al., 1994), is not discussed.

At present, primary human hepatocytes or hepatoma cell lines constitute common models for *in vitro* drug metabolism and toxicity testing. However, the availability of human material is limited and activity of drug metabolizing enzymes and many transporter functions is rapidly lost during culturing of hepatocytes (Wu et al., 1990, Baker et al., 2001, Rodriguez-Antona et al., 2002). Many human hepatoma cell lines, such as HepG2 cells, completely lack expression of many important enzymes (Wilkening et al., 2003, Butura et al., 2004). In Papers II and III we show that the hepatocyte-like cells derived from hESC show higher expression levels and activity of GSTs as well as higher expression of CYPs and other liver-related factors, as compared to HepG2 cells.

Hepatocytes derived from mouse embryonic stem cells have successfully been used for studies of hepatotoxicity and this is an important indication of the usefulness of such cells (Kulkarni and Khanna, 2006). The hepatocyte-like cells presented in Papers II and III contain the human metabolizing enzymes, which is highly significant for predictions of toxicity in humans. Although stem cell derived hepatocytes would not detect toxicity mediated or modified by non-parenchymal cells e.g. cytokine release from Kupffer cells (Kmiec, 2001, Roberts et al., 2006) the hepatocyte is established as the major site

of biotransformation in the liver and, therefore, hESC derived hepatocyte-like cells may provide a good model for drug metabolism studies. In addition, hESC have the potential to develop into all cell types of the liver and in the future systems including both parenchymal and non-parenchymal cells derived from hESC may be developed. Since these cells are derived from hESCs, virtually unlimited amounts of hepatocyte-like cells with identical genetic background can be obtained, which is a significant advantage over primary human hepatocytes, which are available only at limited numbers from one single donor. Finally, in contrast to primary human hepatocytes, hESC derived cells have not been affected by diseases, aging, and medications. Thus, hESC derived hepatocyte-like cells can potentially improve predictability of hepatotoxicity tests as well as significantly reduce the need for animal experimentation.

CONCLUSIONS

The methods described in Paper I allow us to more closely illuminate the organization of intracellular GSH in intact cells, the variation in levels of GSH in homogeneous and heterogeneous cells and organelle populations and, importantly, the disposition of GSH in such systems with respect to reversible S-glutathionylation of protein. We observed distinct intracellular pools of glutathione in A549 cells, with the highest levels found in mitochondria, and the nuclear and cytosolic compartments in close to equilibrium. In addition, we observed PSSG mainly associated with membrane blebs and the peri-nuclear region. The application of the techniques may therefore add new dimensions to our understanding of the biochemistry of glutathione and potentially offer means to monitor the regiospecificity in the GSH levels and the cellular redox state following exposure to xenobiotics.

The results of the preliminary study show a clear difference in the expression pattern of glutathione transferases and cytochrome P450s between adult stem cells from both breast and liver tissue, and their differentiated counterparts. This indicates that these adult stem cells would respond differently to exposure of xenobiotics and therefore they are not suitable as a model system for safety assessment without differentiation of the cells to more closely resemble the target cell type.

On the other hand, the hepatocyte-like cells derived from hESC studied in Papers II and III show biotransformation characteristics which indicate a potential of these cells to replace or be used as a complement to primary human hepatocytes in studies of drug metabolism and toxicity. The results presented in Paper II demonstrate that hepatocyte-like cells derived from hESCs show a hepatocyte-like phenotype, as evidenced by characteristic hepatic morphology, expression of several hepatic markers and glycogen storage. Moreover, these cells exhibit a pattern of GST protein levels and activity highly reminiscent of adult human hepatocytes. In Paper III we show that hepatocyte-like cells show mRNA expression of several important CYPs and other liver-related factors, as well as expression of CYP1A2 and CYP3A4/7 protein. In contrast, the undifferentiated hESC, the liver stem cells from adult tissue investigated in the preliminary study and HepG2 cells, showed no or very low expression of the drug metabolizing enzymes studied. However, the expression levels in hepatocyte-like cells are very low compared to human liver and primary hepatocytes and, therefore, the cells need to be further developed by suitable differentiation protocols into hepatocyte like

cells with a mature enough phenotype for future use in studies of drug metabolism and drug-induced hepatotoxicity.

Future perspectives

In order to further differentiate hESC along the hepatic lineage, the culture protocol needs to be optimized. This may be achieved by the use of growth factors, cytokines and surface coatings, including hepatocyte growth factor (HGF), oncostatin M, dexamethasone, sodium butyrate and collagen (Rambhatla et al., 2003, Baharvand et al., 2006, Soto-Gutierrez et al., 2006). One important step on the way is derivation of definitive endoderm, which may be achieved by activin A and low serum as described by D'Amour et al (D'Amour et al., 2005). Following improvement of the hepatic phenotype of the hepatocyte-like cells, extensive characterizations of biotransformation systems are necessary, including investigation of the expression of additional phase II enzymes e.g. UGTs and SULTs as well as transport proteins. Measurements of the activity of the drug metabolizing enzymes are of paramount importance since this is the ultimate functional measurement for cells to be used for studies of metabolism and toxicity. Further, studies of the ability and efficiency of hepatocyte-like cells to recognize/respond and handle genotoxic insults by DNA repair are of great interest. Such studies can be performed by inducing DNA damage by UV radiation and monitoring phosphorylation of histone H2AX, which is an early marker of DNA adduct/damage. At a later stage, validations of hepatocyte-like cells from hESC as a test system are necessary, and this is accomplished by using known toxicants and comparing the results to *in vivo* and *in vitro* systems used today.

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