Regulation of p53 and Susceptibility to Cell Death in Chemically-Induced Preneoplastic Hepatocytes

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Abstract

Induction of preneoplastic lesions, termed enzyme-altered foci (EAF), is considered to be an early step in the development of liver cancer. In view of the relatively homogenous properties of the majority of EAF, these lesions are suggested to arise as an adaptive response to toxic stress. An overall goal of this thesis was to study the role of adaptations to genotoxic stress in EAF development and how resistance to toxicity in EAF was affected.

In association with carcinogenesis much focus has been on the tumor suppressor gene p53. This gene is commonly mutated in cancer cells, thus indicating a critical role in preventing cancer development. Previously, it has been found that EAF lesions from rats treated with the genotoxic agent diethylnitrosamine (DEN) display an attenuated p53 response to genotoxic stress, compared to normal liver tissue. In the first study the underlying mechanisms of the attenuated p53 response was examined in EAF hepatocytes. We found that in contrast to genotoxic agents, treatment of primary hepatocytes with the hypoxia-mimicking agent $CoCl_2$ induced a p53 response in both EAF and normal cells. In addition, we further found decreased levels of serine-15 phosphorylated p53 in EAF tissue upon toxic stress. The kinase ATM is involved in the phosphorylation of p53 after DNA damage, specifically at serine-15, and analysis of EAF tissue, employing immunohistochemistry and western blot analysis, revealed reduced levels of ATM compared to normal tissue.

We hypothesized that an attenuated p53 response in EAF hepatocytes reflects an adaptation to acute cytotoxic and genotoxic stress seen at high doses. In the second study, rats received relatively low doses of DEN for 10 or 20 weeks, and their livers were subsequently analyzed. All doses induced EAF lesions and a majority of these demonstrated a relatively low p53 response after DEN treatment, as compared to surrounding tissue. Compared to rats receiving DEN at high dose rates, rats receiving the same cumulative dose at a low dose rate generally possessed larger preneoplastic lesions that were more "p53-negative". These data argue against our hypothesis and suggest subtler genotoxic effects behind the alteration of the p53 response.

EAF hepatocytes have been found to be relatively resistant to toxicological stress and apoptosis induced by xenobiotics. However, in the third study, *in vitro* treatment with different sphingolipids, such as ceramide and sphingosine, was found to induce cell death, predominantly in EAF hepatocytes. TLC analysis and immunohistochemistry demonstrated altered levels of sphingosine, sphingosine-1-phosphate and glucosylated ceramide in EAF tissue. In *in vivo* experiments, administration of sphingomyelin-supplemented diet to DEN-treated rats was found to reduce the number of preneoplastic lesions. Sphingolipids have been suggested to exert their apoptotic effects by decreasing levels of phosphorylated Akt (pAkt). In the last study, we found that a fraction of EAF displayed higher immunohistochemical staining of ceramide compared to the surrounding tissue, which correlated with lower staining of pAkt. Western blot analysis revealed that sphingosine was able to inhibit insulin-induced Akt phosphorylation *in vitro*. Furthermore, we found that the levels of p27 were increased in EAF and that these cells also were more sensitive to treatment with rapamycin, an inhibitor of mTOR.

In conclusion, our results suggest that the DNA damage signaling pathway, including ATM and p53, is downregulated in EAF hepatocytes. This may result in a resistance to cell death in EAF hepatocytes and confer a growth advantage to EAF in a toxic environment. Furthermore, the sphingolipid levels and the pAkt-mTOR signaling pathway seem to be altered in many EAF cells. This may render these cells more susceptible to cell death induced by sphingolipids.

List of original articles

I. **Silins I**, Finnberg N, Ståhl A, Högberg J and Stenius U.
Reduced ATM kinase activity and an attenuated p53 response to DNA damage in carcinogen-induced preneoplastic hepatic lesions in the rat.
Carcinogenesis, **22**, 2023-2031, 2001.

II. **Silins I**, Nordstrand M, Högberg J and Stenius U.
Sphingolipids suppress preneoplastic rat hepatocytes *in vitro* and *in vivo*.
Carcinogenesis, **24**, 1077-1083, 2003.

III. **Silins I**, Stenius U, Högberg J
Induction of preneoplastic rat liver lesions with an attenuated p53 response by low doses of diethylnitrosamine.
Manuscript, 2003.

IV. **Silins I**, Högberg J, Stenius U.
Susceptibility to cell death in preneoplastic hepatocytes; the role of Akt kinase.
Manuscript, 2003.

Contents

Introduction

The present study

Abbreviations

ATM	Ataxia Telangiectasia Mutated
ATR	ATM-and Rad 3 Related
CAPP	Ceramide Activating Protein Phosphatase
Cdk	Cyclin dependent kinase
CYP450	Cytochrome p450
DEN	Diethylnitrosamine
DMS	Dimethyl sphingosine
EAF	Enzyme-Altered Foci
EGF	Epidermal growth factor
GluCer	Glycosylated ceramide
GSK3	Glycogen synthesis kinase 3
GST	Glutathione-S-Transferase
GST-P	Placental form of Glutathione-S-Transferase
HCC	Hepatocellular Carcinoma
DEN	Diethylnitrosamine
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen activated protein kinase
Mdm2	Mouse double Minute 2
mTOR	mammalian Target of Rapamycin
pAkt	phosphorylated Akt
PDMP	D-threo-1-phenyl-2-decanoylamino-1-propanol
PI3	Phosphoinositide 3
PIP2	Phosphatidylinositol-3,4,5-triphosphate
PIP3	Phosphatidylinositol-3,4-bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PP2A	Protein Phosphatase 2A
PSI	Proteasome Inhibitor
Rb	Retinoblastoma
Sph1P	Sphingosine-1-phosphate
SAPK	Stress activated protein kinase
SM	Sphingomyelin
SMase	Sphingomyelinase
TGF-α	Tumor Growth Factor-alpha
TNF-α	Tumor Necrosis Factor–alpha

Introduction

A large body of evidence supports the possibility that lifetime exposure to chemicals causes parts of the liver tumors developed in humans, either alone or in combination with other factors. Rodent liver models have long been employed for experimental studies of chemically induced cancer. In this regard, exposure to a wide range of different chemicals results in development of putative precursors of cancer in rodent livers (Bannasch *et al.* 2003). Morphological and biochemical characterization of these preneoplastic lesions has improved the understanding of hepatocarcinogenic development and its cellular alterations. Until recently, corresponding studies in humans have been few. However, during recent years, studies have demonstrated that the preneoplastic alterations in human livers share many characteristics of rodent preneoplasia (Su and Bannasch 2003; Su *et al.* 1997; Thorgeirsson and Grisham 2002).

1. Mechanisms Underlying Carcinogenesis

The development of cancer usually proceeds over many years and is considered to be a multistage process involving several distinct steps. It was recently suggested that malignant transformation require six essential alterations. These alterations include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, escape of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. These six capabilities are probably shared by almost all tumors (Hanahan and Weinberg 2000).

It has long been known that tumor suppressor genes often are inactivated during cancer development, whereas proto-oncogenes are activated (Hanahan and Weinberg 2000; Vogelstein and Kinzler 1993). Tumor suppressors normally control cellular growth and cell death. Consequently, mutations in such genes may result in loss of cell growth regulation. Proto-oncogenes are normal genes that can be converted to oncogenes by mutations. This often involves a gain of function. By stimulating mitogenic signal transduction pathways activation of oncogenes often leads to uncontrolled cell growth and proliferation.

Cancer development can also involve modifications of the methylation pattern (Wachsman 1997). This includes silencing of tumor suppressor genes or overexpression of oncogenes. A permanent alteration of the phenotype without any changes in the DNA base sequence is often called an epigenetic change. Epigenetic events include DNA methylation, or more specifically, the 5-methylcytosine content of DNA. Since the methylation of DNA has a critical role in embryonic development and differentiation of cells, the hypermethylation-induced silencing of tumor suppressor genes or hypomethylation-induced overexpression of oncogenes might be possible mechanisms underlying carcinogenesis. The methyl group acts as a chemical signal that can regulate transcription of genes and the resulting methylation pattern can be inherited from cell to cell. Changes in the DNA methylation pattern are consistent findings in cancer cells and

hypomethylation has been found to be an early event in carcinogenesis (Goelz *et al.* 1985; Goodman and Watson 2002).

The paths that cells take on their malignant development are very variable. The specific mutations that occur in tumors may appear early or late in the development (Hanahan and Weinberg 2000; Vineis *et al.* 2003). Importantly, organisms do have a great capacity to repair or eliminate damaged cells and mutations are generally rare events. However, increased mutability may depend on failure of the proteins that defend cells by triggering protective actions, one of those being the tumor suppressor protein p53.

2. Liver and Liver Cancer

The liver controls many important functions in the body. It is responsible for the glucose homeostasis, lipid synthesis and metabolism, storage of vitamins and minerals, regulation of excess amino acids, ammonia, and bile production. The liver is also the main organ for detoxification and metabolism of toxic substances (Arias *et al*. 1988; Parkinson 2001).

Structurally, the liver can be divided into lobules. The lobules are localized around the central veins and can be divided into three regions: the centrilobular (closest to the central vein), midzonal, and periportal. Enzymes involved in the metabolism of toxic compounds have been found along the lobule. High amounts of the metabolizing enzymes cytochrome P450 (CYP450) are predominantly located in the centrilobular region.

Xenobiotics, chemical agents, and endogenously formed hydrophobic substances are metabolized in the liver and excreted from the body. This generally involves two types of reactions. The phase I reaction involves the addition of a reactive, functional group (by oxidation, reduction, or hydrolysis) to the compound, thus yielding an activated, more reactive metabolite than the parent compound. Many pro-carcinogenic compounds need the activation by CYP450s to become carcinogenic. Subsequently, the phase II reaction involves conjugation with sulfate, glucuronic acid, or glutathione. Conjugated substances are relatively harmless and can easily be excreted via the urine and bile (Oinonen and Lindros 1998; Parkinson 2001).

The hepatocytes are the main functional cells of the liver and represent about 60 percent of all liver cells. Depending on the zonal location of the hepatocytes, their function, structure, and shape varies (Arias *et al*. 1988). Additional cells in the liver are endothelial cells, Kupffer cells (liver macrophages), and Stellate (Ito or fat-storing) cells. These cells constitute about 20 percent of the total liver volume and are mainly located in the periportal region (Oinonen and Lindros 1998; Parkinson 2001).

Liver Cancer - Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers worldwide (Anthony 2001; Thorgeirsson and Grisham 2002). It has been estimated that the average time to develop HCC in human is 30-50 years (Bannasch *et al.* 2003). Most of the HCC cases occur in Africa and Southeast Asia. The most common cause of HCC development worldwide is chronic hepatitis B infection. However, the increased incidence of HCC in USA and Europe is most likely due to chronic hepatitis C infection. Other factors proposed to be involved in HCC development are alcohol, smoking, chemicals, and aflatoxin B1 exposure (Anthony 2001; Wogan 2000).

The earliest neoplastic alteration found in the human liver is the dysplastic hepatocyte that corresponds to the rodent liver foci and noduli. The genomic alterations driving the development of HCC are not fully understood, but are considered to involve both genomic (mutations, amplifications, deletions) and epigenetic alterations, such as changes in methylation pattern (Thorgeirsson and Grisham 2002).

Human and rodent hepatocarcinogenesis are likely to follow similar principles. Consequently, analysis of the cellular and molecular mechanisms in both species is of great importance. Some common alterations found in both human and rodent HCC are the early appearance of abnormal DNA hypomethylation, overexpression of genes regulating the mitogenic signaling, changes in tumor suppressor genes and dysregulation of cell cycle genes (Feo *et al.* 2000).

3. Chemically Induced Cancer

Chemical hepatocarcinogenesis is considered to be a multistep process. The first description of various stages in carcinogenesis by chemicals was reported for skin (Boutwell 1964; Slaga *et al*. 1981). Later, the liver became a common target organ for studies on chemical carcinogenesis (Farber 1973; Goldfarb 1973). Experimentally carcinogenesis can be classified into three separate steps termed initiation, promotion and progression (Farber 1984a, 1990). The initiation step is considered to involve irreversible changes in the genome of the cell (mutations or epigenetic alterations). An initiator is a genotoxic agent possessing the capacity to induce genetic and heritable changes in the cells. The genetic change has to be fixed in the genome in order to remain after proliferation (Farber 1984b). Subsequently, the promotion phase involves clonal expansion of the initiated cells and this phase is regarded to be reversible. Promoting agents are usually not genotoxic, but have the ability to select initiated cells to proliferate, whereas normal (not initiated) cell proliferation is inhibited. Initiated cells will not progress further in carcinogenesis unless a promoting stimulation is employed, and vice versa (Schulte-Hermann *et al*. 2000; Pitot and Dragan, 2001). Furthermore, some substances can act as both initiator and promoter. These substances are termed complete carcinogens and one example is diethylnitrosamine, DEN.

Only a few percent of the initiated clonally expanded cell lesions will grow into malignant lesions. During the progression phase, accumulations of mutations may occur. This probably drives the progression further and may eventually result in malignant cells. Many organs show similar progression of stepwise neoplastic alterations as in the liver, e.g. papillomas in skin and colon (Farber 1996; Williams 2001).

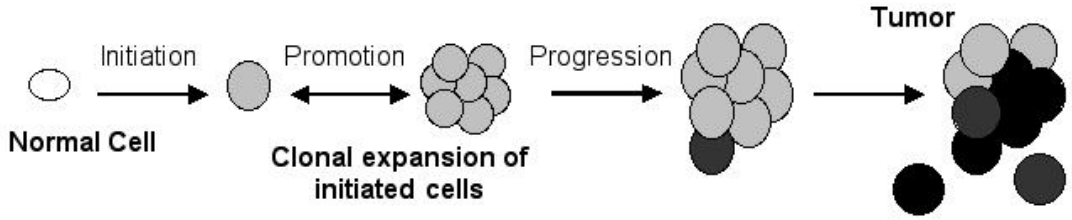

Figure 1. Schematic figure of the different phases in chemically induced cancer.

The above-described phases are well defined in experimental models, however, spontaneously occurring cancers do not necessarily arise through this specific sequence of distinct steps. Their development can be controlled by similar principles, but the complexity of the process is not known. The alterations during tumor progression described by Hanahan and Weinberg illustrate more general aspects of the multistep process. Initiation probably involves one or more changes that confer for another type of change, subsequently resulting in cancer.

Enzyme-Altered Foci

The appearance of preneoplastic enzyme-altered foci (EAF) is considered to be an early step in the development of liver carcinogenesis by chemicals (Pitot 1990). Studies of EAF formation in rodents can be used for different purposes, including evaluation of the general alterations occurring during carcinogenesis, and for chemopreventive strategies. Furthermore, EAF induction in rodents is an important tool in risk assessment studies. In long- and medium-term risk identification assays of chemicals, EAF induction is commonly used as an indicator of carcinogenicity (Bannasch *et al*. 2003; Shirai 1997). Increased understanding of mechanisms underlying EAF initiation may lead to a more reliable evaluation of EAF as an endpoint in risk assessments.

It has been reported that 90 percent of 170 hepatocarcinogens tested induced preneoplastic lesions in rodent livers (Ito *et al*. 1992). In addition, viruses, transgenic oncogenes, certain hormones, and radiation induce EAF lesions in different rat and mouse strains (Farber 1984a). Furthermore, studies have provided evidence that the early lesions found in rodents correspond to similar lesions found in humans (Bannasch *et al.* 2003; Bannasch *et al.* 2001; Su *et al.* 1997).

The physiological aspects of EAF initiation and development have been widely discussed. It has been suggested that formation of EAF in the liver may have a protective role, by delaying the consequences of acute chemical exposure.

However, chronic exposure of chemicals may eventually result in tumor development (Farber and Rubin 1991; Ramel 1992). Thus, the induction of EAF in the liver can be regarded as an adaptation process regulated by the toxic environment, during which EAF hepatocytes develop a resistance to the growth inhibitory effects of many carcinogens (Farber 1996). The acquired resistance to chemicals and radiation will provide EAF hepatocytes with a selective growth advantage (Schwarz 1995).

An indication supporting the role of EAF being liver cancer precursors is the appearance of noduli-in-noduli. Noduli are larger foci, macroscopic in size, as compared to the microscopically small foci. In the center of some noduli, lesions of phenotypically different hepatocytes have been found. Noduli-in-noduli possibly represent a more malignant form of lesion, as compared to the foci and the surrounding noduli, supported by their subsequent metastasizing capacity when transplanted into rodent spleen (Farber 1984b). Other clues supporting the role of EAF as precursors of liver cancer is that the number of EAF correlates to the dose of initiating agent (Scherer and Emmelott 1975), and that appearance of EAF precedes the development of HCC (Kaufmann *et al.* 1985, Bannasch *et al*. 1989). Several studies suggest that EAF lesions are monoclonal in origin (Rabes *et al*. 1982, Scherer and Hoffman 1971).

Due to the relatively homogenic nature of the alterations found in EAF, development of early cancer precursors has been suggested to involve non-mutagenic changes. In fact, recently, epigenetic alterations have been suggested to be responsible for the initiation stage instead of mutations (Bannasch *et al.* 2003; Kopp-Schneider *et al.* 1998; Satoh and Hatayama 2002; Schulte-Hermann *et al.* 2000). Still, this may currently be a subject for debate, owing to the fact that tumors do possess mutations.

In contrast to EAF cells, tumor cells are relatively heterogeneous with respect to their phenotype. Along with tumor progression, the heterogenicity is increased. During liver cancer development, major changes are found in chromosomes and genes, such as translocations, deletions, and mutations (Feo *et al.* 2000). However, no single mutation so far has been found to be critical for the development of preneoplastic lesions in the liver.

Some Phenotypic Alterations of EAF

The alterations of EAF cells are suggested to be part of a genetic program. These include a common cell-to-cell organization, formation of an organized blood supply, and commonly shared biochemical alterations of different lesions (Farber and Rubin 1991). These phenotypic changes are constitutive and are not part of the acute changes induced when the liver is exposed to chemicals (Eriksson and Andersson 1992).

As stated above, early preneoplastic liver lesions are generally rather homogenous, especially regarding changes in their enzyme metabolism. One common alteration

found in preneoplastic liver lesions is lower levels of phase 1 enzymes (activating) and higher levels of phase 2 enzymes (inactivating) (Hendrich and Pitot 1987). Changes of phase 2 enzymes involve increases in epoxide hydrolase, glutathione S-transferases (GST), and gamma-glutamylcysteine synthetase (GGT), which is the rate-limiting enzyme for glutathione synthesis. Lower levels of phase 1 enzymes include decreased CYP450 isozyme activities. Decreased levels of at least three CYP450 isozymes have been found to increase the capacity of proliferation in EAF lesions (Buchmann *et al.* 1987). Reduced CYP450 levels in foci cells may affect the resistance towards chemicals, since the ability to generate reactive metabolites from carcinogens is decreased. Correspondingly, higher levels of phase 2 enzymes give preneoplastic cells an increased capacity to conjugate, and detoxify activated forms of carcinogens (Hendrich and Pitot 1987; Roomi *et al.* 1985). It has previously been shown that after carcinogen feeding, replicating liver cells contained lower amounts of DNA adducts than non-replicating hepatocytes. It was suggested that these replicating cells might be progenitors to EAF cells and that reduced adduct levels could be due to changes in the metabolizing enzyme systems (Huitfeldt *et al*. 1990).

As mentioned above, many forms of GST are increased in EAF lesions and nodules. GSTs are mainly localized in the cytosol where they catalyze the conjugation of glutathione to reactive electrophiles. The placental form of glutathione S-transferase, GST-P or GST 7-7, is expressed in almost all EAF of rats. Small, even single-cell GST-P-positive cells have been identified shortly after exposure to DEN, aflatoxin B1 and other compounds, which suggests that GST-P is a very early marker (Sato *et al.* 1988). This has made GST-P valuable for the detection of initiated cells and EAF lesions (Sato *et al*. 1984; Satoh and Hatayama 2002; Tatematsu *et al*. 1985; Tatematsu *et al*. 1987). The increased GST levels in preneoplastic lesions may be involved in drug resistance (Morrow and Cowan 1990).

4. Hepatocarcinogens

As mentioned previously, the liver is the major organ for metabolism of toxic substances. In spite of a great capacity for metabolizing harmful compounds, there are still a large number of chemicals that have damaging effects on the liver.

Hepatocarcinogens can be classified as genotoxic and non-genotoxic agents. Genotoxic substances react directly or indirectly (after metabolic activation) with DNA. Adducts on DNA increase the probability of mutational events and may eventually result in development of cancer. DNA damage can also be caused by indirect mechanisms, e.g. by reactive oxygen species formed during metabolism of carcinogens (Trush and Kensler 1991). Non-genotoxic agents do not react with DNA. Instead, they increase the proliferation rate of already altered (initiated) cells, whereas proliferation of normal cells is inhibited (Grasl-Kraupp *et al.* 1997; Williams 2001).

Nitrosamines – Diethylnitrosamine

Nitrosamines are among the most potent rodent carcinogenic substances known (Lewis *et al.* 1997). These substances are commonly found in preserved meats, tobacco products, industrial solvents, alcoholic beverages and fishmeal. Furthermore, N-nitroso compounds can be formed endogenously, e.g. via nitrosation of substances ingested through food, water, and air (Brown 1999). The daily human exposure to exogenous nitrosamines is considered to be approximately 10 µg (Lewis *et al*. 1997). Nitrosamines are known to produce tumors in more than 40 different species. However, although nitrosamines are classified as probable human carcinogens (IARC 1978), there is still little evidence that nitrosamines are carcinogenic to humans.

Much focus in experimental models of chemical carcinogenesis in rodents has been on diethylnitrosamine (DEN). It has long been used as an initiating agent for induction of preneoplastic cells and tumors in a large number of investigations concerning dose-response issues, chemopreventive strategies, and investigations of cellular and molecular mechanisms of cancer development.

It has been reported that EAF lesions induced by DEN arise predominantly in the centrilobular region, around the central vein. Since the hepatocytes in this zone express high levels of certain CYP450 isoenzymes, mainly CYP2E1 that metabolize DEN, the formation of reactive DEN-metabolites would be elevated in this zone. Formation of reactive metabolites increases the possibility of causing damage to the affected cells. This may explain the development of EAF lesions in this specific region (Koen *et al.* 1983).

Aflatoxin B1

Food contaminated with *Aspergillus flavus*, which produces aflatoxin B1, has long been suspected to cause human HCC, and aflatoxin B1 is believed to be one of the most potent hepatocarcinogens known. It can be found in nuts, rice, and grains, mostly in the southeast part of Asia and southern Africa.

Aflatoxin B1 is metabolized from its original form to the potent intermediate 7,8-epoxide (Root *et al.* 1997). The epoxide is highly reactive and easily form adducts with DNA, which eventually cause mutations. Aflatoxin B1 exposure is highly associated with mutation of the tumor suppressor gene p53, specifically at codon 249 in human tumors. Results from numerous experiments using rodent models indicate that aflatoxin B1 induce preneoplastic lesions and tumors. However, many investigations report the lack of p53 mutations in preneoplastic rodent lesions, suggesting that this mutation is not an important factor for early cancer development, at least in rodents (Hulla *et al.* 1993; Lee *et al.* 1998; Liu *et al.* 1996).

Dose-Response in Carcinogenesis

It has long been assumed that the carcinogenic effect at low dose exposures of genotoxic compounds is proportional to the effect at high doses (Henderson *et al.* 2000). However, during recent years, this assumption has been widely discussed (Waddell 2003), and effects by genotoxic compounds are now suggested to possibly involve threshold doses. At a cellular level, low dose exposure may elicit different effects, as compared to high dose exposure.

Kinetic Factors

Firstly, depending on the substance, the carcinogenic action may be dependent on metabolic activation by enzymes, such as the CYP450 enzymes. Exposure to high doses may result in saturation of these enzymes, leading to a decreased tumor incidence at higher doses. Secondly, inactivation of reactive metabolites requires other enzymes, such as the phase 2 enzymes. The efficiency of these enzymes may differ, both between individuals and species. Accordingly, enzyme capability may influence the dose-response curve. Enzymes that are involved in DNA repair also have an impact on the outcome of the toxic exposure, depending on both the quantity and quality of the particular enzymes.

Mechanistic Factors

High dose exposure to genotoxic agents commonly leads to cytotoxicity and cell death, followed by regenerative proliferation. Accordingly, enzymes involved in DNA repair have to compete with proliferation-dependent DNA synthesis in order to inhibit fixation of DNA damage. In contrast, low doses can induce cell cycle arrest, as a consequence of low levels of DNA damage. This may result in a lower tumor incidence, below the baseline for spontaneously induced tumors. In addition, the apoptotic frequency may differ depending on the proportions of exposure. It has been suggested that cell cycle progression and regenerative proliferation may represent the most relevant mechanisms resulting in thresholds for tumor incidence by carcinogens (for review see Hengstler *et al*. 2003).

In one of the largest studies of dose-response effects, 4080 rats were exposed to DEN in the drinking water. Lifetime exposure resulted in a linear dose response curve for tumor incidence, even at low doses (Peto *et al*.1991). However, in other studies, short-term DEN exposure at low doses demonstrated threshold doses (Williams *et al*. 1996; Williams *et al*. 2000). At low doses, the time of exposure may be a strong determinant for the subsequent outcome, which may explain these different results (Hengstler *et al*. 2003).

5. The p53 pathway

The p53 gene was first described in 1979 and was one of the first tumor suppressor genes to be identified. The p53 protein normally functions as a transcription factor and is involved in cell cycle regulation, DNA repair, and apoptosis. Abnormalities of the p53 tumor suppressor gene and related pathways are among the most frequent molecular events in human and animal neoplasia. In more than half of all human

tumors, mutational inactivation of the p53 gene has been observed (Oren *et al.* 2002; Vogelstein *et al.* 2000). The consequence of a non-functional p53 pathway would be loss of protection against harmful compounds and other types of cellular stress. Normal functional p53, on the other hand, protects cells by inducing cell cycle arrest or apoptosis.

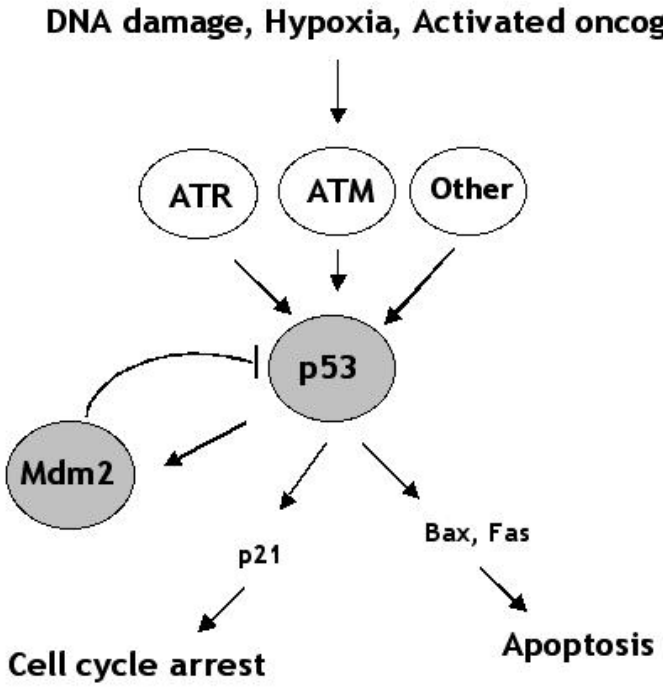

Figure 2. Some pathways involved in p53 signaling.

Different types of cellular stress can induce p53 activation and stabilization. These include different types of DNA damage, hypoxia, and heat shock (An *et al.* 1998; Lain and Lane 2003). Mutations of the p53 gene occur frequently at certain hotspots within the central domain of the gene. Inactivation of p53 by mutations usually results in loss of its specific DNA binding capacity. The mutated form of p53 is generally overexpressed in cells (El-Deiry *et al.* 1994; Levin 1997; Prives and Hall 1999; Vousden 2000).

Modifications of p53, such as phosphorylations and acetylations, regulate its activity. A large number of kinases have been found to phosphorylate p53 at different sites. The kinase ATM (Ataxia Telangiectasia Mutated) is an important regulator of p53 after DNA damage. It is activated upon DNA damage and phosphorylates p53 at serine15, resulting in stabilization of p53 (Banin *et al.* 1998; Canman and Lim 1998; Canman *et al.* 1998). Another kinase involved in the phosphorylation of p53 is ATR (ATM and Rad3-related) (Shiloh 2001; Tibbetts *et al.* 1999). ATM and ATR are both effectively and irreversibly inhibited by wortmannin and caffeine (Blasina *et al.* 1999; Sarkaria *et al.* 1999; Sarkaria *et al.* 1998). Since ATM is a positive regulator of p53 stabilization, mutations and dysregulation of the ATM signaling pathway may have serious consequences in tumor development.

A normal cell synthesizes and degrades p53 continuously. Consequently the cellular levels of p53 are very low. The Mdm2 protein is the negative regulator and also a transcriptional target of p53. After Mdm2 binding of p53, the formed complex is translocated from the nucleus to the cytoplasm where degradation occurs (Vousden 2000). Mdm2 is also known as an oncogene, since its overexpression results in decreased levels of p53, inhibition of p53-mediated transactivation, and eventually carcinogenesis (Momand *et al.* 1992). Just as for p53, the activity of Mdm2 is regulated by phosphorylation by different kinases after DNA damage, e.g. ATM (Maya *et al.* 2001). Increased stability of p53 by phosphorylation result in inhibition of its binding capacity to Mdm2, which further leads to increased nuclear levels of p53 (Banin *et al.* 1998; Chehab *et al.* 1999; Higashimoto *et al.* 2000; Sakaguchi *et al.* 1997; Shieh *et al.* 1997).

Following p53 activation, transcription of important target genes is induced, e.g. the cell cycle inhibitor p21/WAF1 that mediates p53-induced G1 arrest of the cell cycle. p53 also induces transcription of genes important for apoptosis, such as the Bax gene, that mediates an apoptotic response (El-Deiry *et al.* 1994; Wu and Deng 2002). In this regard, the transcription activity domain of p53 is necessary for its tumor suppressor function (Lain and Lane 2003).

Mutations of the p53 gene are, as mentioned above, widespread in tumors in general and are common events in human hepatocellular carcinomas. However, in rodent livers, p53 mutations, if induced, have been suggested to be part of the late events in carcinogenesis. Studies of early preneoplastic cellular changes in humans have previously been technically difficult to perform. Several studies have reported p53 mutations in human liver tumors, but it is still not clear whether such events appear early or late in the carcinogenic process (Feo *et al.* 2000; Su *et al.* 2000).

p53 alterations in EAF

As previously discussed, the tumor suppressor gene p53 has a central role in the regulation of cell growth and apoptosis. Exposure to toxic substances and chemicals will naturally induce a p53 response in liver cells as a consequence of cellular stress and DNA damage. Since p53 is commonly mutated in evolving tumors, several studies have focused on its role and expression in early preneoplastic lesions, including the liver.

In rat livers, a number of chemicals like DEN and aflatoxin B1, as well as radiation have been found to induce a p53 response. Exposure of cells to these substances also resulted in development of EAF lesions. Furthermore, upon exposure to genotoxic stress, these lesions demonstrated an attenuated p53 response (Finnberg *et al.* 2000; Lennartsson *et al.* 1998; Stenius and Högberg 1995; van Gijssel *et al.* 2000b). This phenotype has been termed p53-negative EAF. In contrast, EAF lesions initiated by DEN and promoted with phenobarbital retained a normal p53 response, suggesting that phenobarbital does not promote p53-negative lesions (Finnberg *et*

al. 2000) This also indicated that initiation by genotoxic agents is not enough for development of the p53-negative phenotype. Instead, it suggests an adaptation to repeated exposure to DNA damaging agents, eventually leading to downregulation of the p53 response (Finnberg *et al.* 2000; Stenius and Högberg 1995; van Gijssel *et al.* 2000b).

It has previously been shown that preneoplastic lesions contain higher levels of Mdm2 compared to surrounding, normal tissue (van Gijssel *et al*. 2000a). However, high levels of Mdm2 per se can not explain the lack of p53 response in DEN-induced EAF since lesions induced by phenobarbital possess both a high Mdm2 expression and a normal p53 response (Finnberg *et al.* 2000).

The Cell Cycle

The different phases of the cell cycle are commonly referred to as the G1 (Gap 1)-, S (Synthesis), G2 (Gap 2)- and M (Mitosis)- phase. A non-dividing cell is in the resting phase, termed the G0 phase. During the cell cycle, the cell proceeds through different stages characterized by cycling levels of proteins termed cyclins. Every phase is characterized by elevated levels of a specific cyclin, e.g. high cyclin D levels initiate the G1 phase. The cyclins are required for activation of specific kinases, called cyclin dependent kinases (cdk). Activation of cdks is important for the progression of the cell cycle, by inducing genes essential for entering the S phase (Stewart *et al.* 2003; Vermeulen *et al.* 2003).

Upon cellular stress the cell cycle will normally be stopped. This will delay proliferation and mitosis and give time for cellular repair mechanisms to occur. Cell cycle arrest commonly involves inhibition of the cdks/cyclin complexes. The tumor suppressor gene p53 has an important role in inhibition of the cell cycle since p53 increases transcription of the cdk-inhibitor p21. Other important inhibitors of cdks and the cell cycle are p27 and p16 (Coqueret 2003; Stewart *et al.* 2003).

6. Apoptosis

In order to maintain tissue homeostasis, an organism has to provide for a good balance between cell death and cell growth. Apoptosis has been described as a physiological form of cell death, with activation of apoptotic genes. This process has been found to be essential for embryogenesis, development and tissue homeostasis of all organisms. The morphological characteristics of apoptosis include membrane blebbing, chromatin condensation, fragmentation of the nucleus and formation of apoptotic bodies, which are rapidly phagocytosed by neighboring cells.

Apoptosis can be induced by a variety of stimuli, e.g. DNA damaging agents. The apoptotic process can be induced by the tumor suppressor gene p53. Another pathway for apoptosis induction is through stimulation of death receptors belonging

to the tumor necrosis factor (TNF) receptor superfamily (Wajant 2002; Yoon and Gores 2002).

The mitochondria is proposed to have an important role in the apoptotic process. During apoptosis, the mitochondria releases several proapoptotic factors, e.g. cytochrome c (cyt c). Moreover, the proteins of the Bcl-2 family play important roles in the apoptotic response. There are several different pro- or anti-apoptotic proteins belonging to this protein family. They are mainly located in the mitochondria. Bcl-2 has been found to inhibit the release of cyt c during apoptosis, thereby acting as an antiapoptotic factor.

Caspases are involved in the degradation of intracellular substrates. Cleavage by caspases results in inactivation of many substrates, e.g. enzymes involved in DNA repair or structural components of the cell. The caspases can be divided into two groups, the initiator and executor caspases. Caspase 3 belongs to the executor caspases and has a central role in apoptosis by cleavage of a large number of substrates (Geske and Gerschenson 2001; Pinton *et al*. 2001).

Cell Proliferation and Cell Death of the Liver

The liver is a regenerative organ, unlike many other organs in the body. Normally, in healthy livers, the apoptotic and mitotic frequency of hepatocytes is relatively low, i.e. around 1 to 5 apoptotic cells/10 000 hepatocytes (Leffert *et al*. 1988; Pitot, 1998; Schulte-Hermann *et al*. 1995). Apoptosis in the liver can be initiated by many different signals, including ligand-receptor interactions or through other stimuli involving sphingolipids or DNA damage (Kanzler and Galle 2000). Upon injury, the liver will regenerate to restore its important tissue-specific functions. However, compensatory regeneration may stimulate damaged cell to proliferate, which could further contribute to the progress of liver cancer and other diseases (Diehl 2002). Likewise, chemical exposure may induce massive cell death in the liver, followed by regeneration (Lutz and Kopp-Schneider 1999; Williams *et al.* 2000).

Preneoplastic liver lesions have an increased cellular turnover rate compared to normal livers. Even though the apoptotic rates in preneoplastic and tumor tissue are higher compared to normal tissue, the proliferation rate is even higher. This results in a net proliferation of preneoplastic cells. In experimental models, EAF lesions are stimulated to proliferate by promoting agents, whereas withdrawal of the promoter results in apoptosis and regression of induced lesions (Grasl-Kraupp *et al.* 1997; Schulte-Hermann *et al.* 2000). The change in growth control and apoptosis is believed to be an early alteration, maybe in association with initiation (Grasl-Kraupp *et al.* 2000).

EAF lesions have been found to be resistant towards a large number of apoptotic inducers, such as different chemical agents. Resistance to apoptosis triggered by the Fas receptor was demonstrated in EAF cells *in vitro*. The Fas receptor is a transcriptional target of p53, and since the normal p53 response is attenuated in

EAF hepatocytes, this may lead to apoptotic resistance triggered by Fas (Nordstrand and Stenius 1999). High expression of the antiapoptotic protein Bcl-2 has also been found in EAF (Van Gijssel *et al.* 2000a). Activated p53 normally represses Bcl-2, and consequently, lower p53 levels may result in higher Bcl-2 levels. However, the mRNA levels of Bcl-2 have been found to decrease during progression towards malignancy, which may explain the increased apoptotic rates found in tumors (De Miglio *et al.* 2000).

7. Sphingolipids

For many years sphingolipids were thought to be static components of the plasma membrane. During recent years however, many studies have shown an involvement of sphingolipids in important cellular events like apoptosis, proliferation, and differentiation (Hannun and Bell 1989; Merrill *et al.* 1997a; Merrill *et al.* 1997b; Merrill *et al.* 1995; Shayman 2000). The balance between pro- and anti-apoptotic sphingolipid metabolites is suggested to be more important for the cellular response than the absolute concentrations of any single sphingolipid (Radin 2001b).

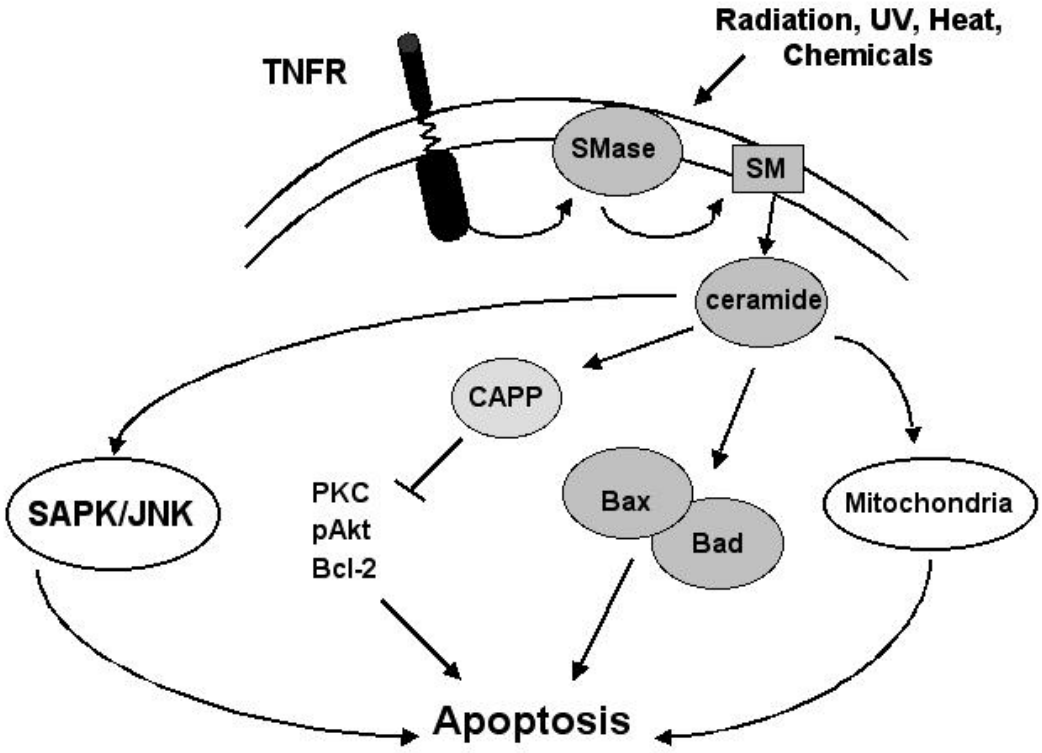

Figure 3. Schematic illustration of different mechanisms leading to cell death by ceramide.

Most focus has been on the apoptotic function of sphingolipids, which is mainly mediated by ceramide and sphingosine. Stimulation by TNF-alpha, Fas ligand, radiation and chemotherapeutic agents have been found to increase the cellular concentrations of ceramide and induce apoptosis (Kolesnick and Kronke 1998).

Sphingomyelin (SM) is the central sphingolipid in the plasma membrane, usually in close interaction with cholesterol. Upon apoptotic signaling, SM is cleaved by sphingomyelinases (SMases), followed by increased ceramide levels (Gulbins and

Kolesnick 2002; Pettus *et al*. 2002). Ceramide is mainly found localized in microdomains termed rafts, in lysosomes and in mitochondria (Grassme *et al.* 2001). The rafts contain signal transduction factors like receptor tyrosine kinases, Fas receptors, PI3-kinases, PKC, and sphingomyelin.

Several findings suggest a role for mitochondria in ceramide and sphingosine induced apoptosis (Pettus *et al.* 2002). Ceramide and sphingosine also activate the SAPK/JNK (Stress Activated Protein Kinases/c-Jun N-terminal Kinases) cascade, which is suggested to be another important pathway for apoptotic induction. However, in connection with apoptosis and cell cycle arrest, the most extensively characterized targets of ceramide and sphingosine are probably the "ceramide activated protein phosphatases" (CAPP). Protein phosphatase 2A (PP2A) induction results in inactivation of protein kinase C (PKC), Akt kinase, and Bcl-2, whereas protein phosphatase 1 (PP1) dephosphorylates the Rb protein, resulting in cell cycle arrest (Radin 2001b; Ruvolo 2003).

In contrast to apoptosis, sphingosine has proliferative effects on cells as well. This may possibly depend on the relative concentrations of different sphingolipids in the cells. The mechanisms of sphingosine-induced proliferation probably involve inhibition of certain isoforms of PKC, or by phosphorylation to form sphingosine-1-phosphate (Sph1P). Sphingosine has also been found to activate the oncogene ras. This results in induction of the MAP kinase cascade, which controls a wide range of cellular events, involving both proliferation and cell death (Cuvillier 2002).

Exogenously added ceramides and sphingosine have also been found to induce the biochemical and morphological changes characteristic of apoptosis (Merrill *et al.* 1997a; Cuvillier 2002; Ogretmen *et al.* 2002). Short-chain ceramides are often used for *in vitro* studies of ceramide-induced apoptosis due to increased solubility compared to long-chain ceramides. Studies have found that short-chain ceramide exposure leads to growth inhibition and apoptosis in many cells. However, natural, long-chain ceramides seem to be more potent inducers of apoptosis at much lower concentrations (Ji *et al.* 1995).

Ceramide can be glucosylated to form glucosylceramide (GluCer). Glucosylation of ceramide is the major pathway for ceramide clearance (Pettus *et al*. 2002). High levels of GluCer have been found in many tumor- and multidrug-resistant cells and patients exhibiting high GluCer levels in their tumors may fail conventional chemotherapy (Morjani *et al.* 2001; Radin 2001a). PDMP is a potent inhibitor of ceramide glucosylation, which is presently suggested as a novel therapeutic agent for treatment of certain tumors. PDMP irreversibly inhibits GluCer synthesis, resulting in accumulation of ceramide (Merrill *et al.* 1997a; Sietsma *et al.* 2000).

Cancerpreventive Properties of Sphingolipids

Sphingolipids are important components of food. High levels of Sphingomyelin (SM) are found in eggs, soybeans, and dairy products. Recently, sphingolipids have been shown to have tumor preventive effects on preneoplastic formations in the colon. Mice with 1,2 dimetylhydrazine-induced tumors were given a diet containing high amounts of SM. Compared to mice receiving a control diet, these mice demonstrated a reduced number of preneoplastic lesions in the colon and developed fewer tumors. Another study demonstrated a shift from more malignant colon tumors to more benign tumors in mice receiving SM diet, but no difference was found in the amount of tumors after longer feeding (Vesper *et al.* 1999). The first alteration found in the dimetylhydrazine-mice was a reduction of the Sphingomyelinase (SMase) activity, followed by loss of apoptosis by TNF-alpha. Sphingolipids in diet was found to bypass this signaling. In humans, studies have found that dairy products in the diet may reduce the risk for development of colon cancer (Glinghammar *et al.* 1997; Tsuda and Sekine 2000).

SM is digested in the small intestine and in the colon of rats and mice. The luminal contents of rat intestine contain SMase, glucoceramidase, and ceramidase activities. Some of the SM is excreted from the body, whereas a fraction of the metabolites are absorbed by the intestinal cells. Some of the metabolized SM is transported through the blood to the liver (Dillehay *et al.* 1994; Merrill *et al.* 1997b; Merrill *et al.* 1995; Schmelz *et al.* 1996; Schmelz *et al.* 2000). Not much is known about the human sphingolipid metabolism, but the pancreatic juice, bile, serum, urine, salivary, and tear fluid have been found to contain SMases (Takahashi *et al*. 2000; Vesper *et al.* 1999). Furthermore, the activity of SMases has been found to be markedly decreased in many human colorectal carcinomas (Hertervig *et al*. 1998; Lipkin *et al*. 1999).

8. The Akt Signaling Pathway

The Akt/PKB kinase family is widely implicated in the cellular survival and protection of cells from apoptosis. Overexpression of Akt kinase has been found in a number of cancer forms like ovarian, breast, and pancreatic cancer (Bellacosa *et al.* 1995; Cheng *et al.* 1996). Furthermore, activated Akt has been found to increase during tumor progression (Dhawan *et al.* 2002; Malik *et al.* 2002).

Akt is involved in mediating many anti-apoptotic biological actions and plays a key role in insulin and other mitogenic signaling from various growth factors. Activation of Akt involves phosphoinositide 3 kinase (PI3 kinase) and its mediators phosphatidylinositol-3,4,5-triphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate (PIP_2). Akt is phosphorylated on at least two different sites, threonine-308 and serine-473. This can be effectively prevented by treating cells with inhibitors of PI3-kinase, such as wortmannin and LY294002 (Downward 1998; Nicholson *et al.* 2003).

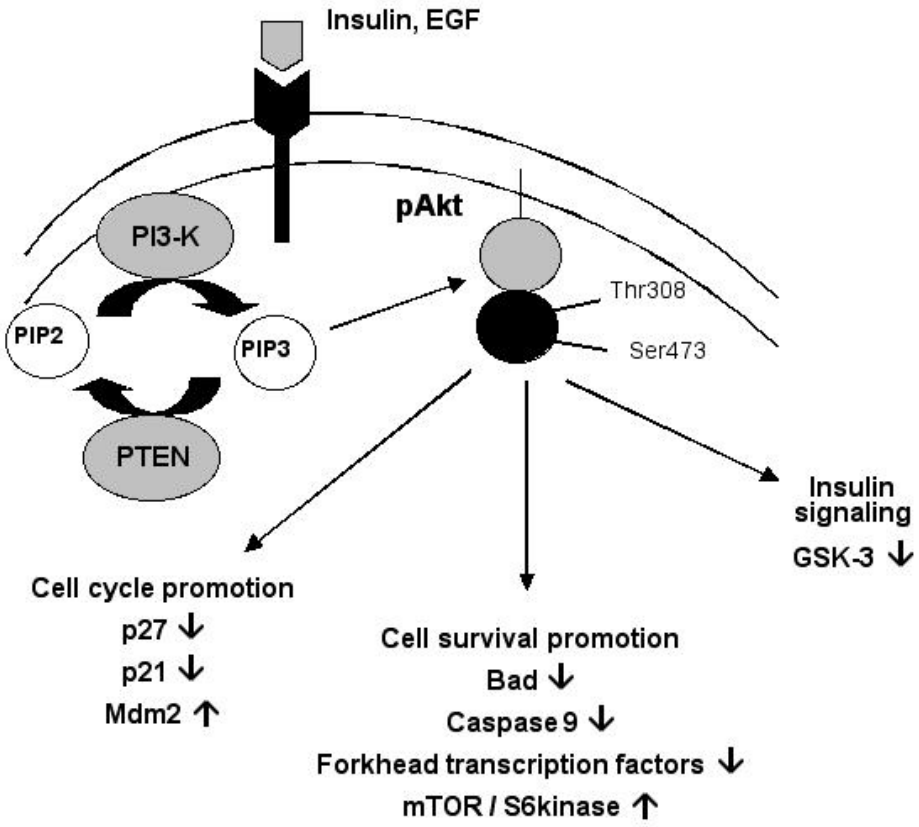

Figure 4. Schematic illustration of the Akt signaling pathway and pAkt targets.

Many targets of Akt have been identified. These include mTOR and p70 S6kinase that are involved in cellular growth signaling (Brazil and Hemmings 2001). Phosphorylated Akt also inhibits targets, such as glycogen synthase kinase 3 (GSK3), the forkhead transcription factors, the cdk inhibitor p27, and the pro-apoptotic factor Bad (Brazil and Hemmings 2001; Burgering and Medema 2003; Cantley *et al*. 2002). Furthermore, Mdm2 phosphorylation by Akt translocates Mdm2 to the nucleus, where it inactivates p53 (Gottlieb *et al.* 2002).

Recently, decreased pAkt levels were found to be important for mediating cell death in various models (Luo *et al.* 2003). Furthermore, several studies have found that both ceramide and sphingosine can decrease levels of pAkt in the cells. This has been suggested as a possible mechanism for sphingolipid-mediated cell death (Chang *et al.* 2001; Kim *et al.* 2001; Mimeault 2002; Mora *et al.* 2002; Salinas *et al.* 2000; Zhou *et al.* 1998). As previously mentioned, pAkt levels are decreased by activation of PP2A. Targeting of the Akt pathway has been suggested as a novel therapeutic way for inhibition of cancer (Tsuruo *et al.* 2003; West *et al*. 2002).

9. The Mevalonate Synthesis Pathway

Cellular DNA synthesis, proliferation, and cell growth is partly dependent on the mevalonate and cholesterol synthesis pathway (Soma *et al*. 1992). Products of this

pathway include isoprene incorporation into sterol and non-sterol compounds such as cholesterol, dolichol, and ubiquinone. In tumors, this pathway is commonly altered (Eggens *et al.* 1989; Siperstein and Fagan 1964).

Statins are inhibitors of the enzyme HMG-CoA reductase, the rate-limiting enzyme in the mevalonate and cholesterol synthesis pathway. They are widely used therapeutically to lower high cholesterol levels in the blood. During recent years, statins have also been found to inhibit survival of tumor cells, by inducing cell death (Kaushal *et al*. 2003; Chan *et al.* 2003). The mechanism underlying statin-induced cell death is still unclear (Brower 2003). The main mechanism probably includes inhibition of mevalonate synthesis. However, some studies have reported increased pAkt levels after statin exposure (Llevadot *et al.* 2001), whereas others report reduction of pAkt levels after statin treatment (Weiss *et al.* 1999). The outcome of statin exposure on pAkt levels seems to depend on time of exposure, concentration, and cell-type.

In hepatocytes of liver noduli, the gene for HMG-CoA reductase has been found to be hypomethylated and overexpressed (Coni *et al*. 1992; Eriksson and Andersson 1992). Higher levels of the HMG-CoA reductase enzyme may support high levels of mevalonate and cholesterol, which may be of importance during a state of proliferation.

The Present Study

10. Aims of the Present Study

Preneoplastic rat liver lesions are considered to be precursors of liver cancer. Consequently, studies of preneoplastic hepatocytes and their cellular alterations can provide important information. Considering the possibility that similar mechanisms are involved in the development of human liver cancer, the rodent liver model may be an important tool. Studies of human livers have identified dysplastic hepatocytes as an early alteration in carcinogenesis, corresponding to foci and noduli in rodent livers. Furthermore, many of the common morphologically alterations found in rat noduli have also been found in human preneoplastic lesions.

The aim of the present study was to investigate some of the signal transduction pathways involved in DNA repair, cell cycle control, and cell death in preneoplastic hepatocytes. Such studies may give general information about the early changes in hepatocarcinogenesis. Moreover, an understanding of how some of the signal transduction pathways are modified in these cells may result in therapeutic and chemopreventive strategies. Of specific interest was the p53 signaling pathway, previously shown to be downregulated in many EAF lesions. An overall goal was to understand the role of adaptations to genotoxic stress in EAF development and effects on resistance to toxicity.

Specific Aims of the Study

The specific aims were as follows:

1. To examine possible mechanisms underlying the attenuated p53 response in EAF hepatocytes after genotoxic exposure.
2. To determine if induction of p53-negative EAF was a consequence of acutely cytotoxic doses of DEN.
3. To compare low-dose induced EAF lesions with high-dose induced EAF with respect to the p53 signaling pathway.
4. To examine the susceptibility to cell death in EAF hepatocytes by sphingolipids.
5. To investigate the possible involvement of the Akt signaling pathway in the susceptibility of sphingolipid induced cell death in EAF hepatocytes.

11. Methods

Donor animals for cell isolation

Female Sprague-Dawley rats were injected with DEN (0.3 mmol/kg) within 24 hours after birth. Following three weeks of weaning, the animals received DEN 0.3 mmol/kg (i.p.) once every other week, 6-11 times. One week after the last injection, cells were isolated using collagenase perfusion technique (Moldeus *et al*. 1978). The primary cells were then cultured on collagen-coated plates in ENAT medium (ENAT *et al*. 1984). After 1.5 hours, the medium was changed to ordinary

RPMI medium without growth factor or serum supplementation. Cells were subsequently treated with different substances for different time periods, no longer than 48 hours. Finally, cells were scarped off for western blot analysis or fixed in formaldehyde for immunocytochemistry.

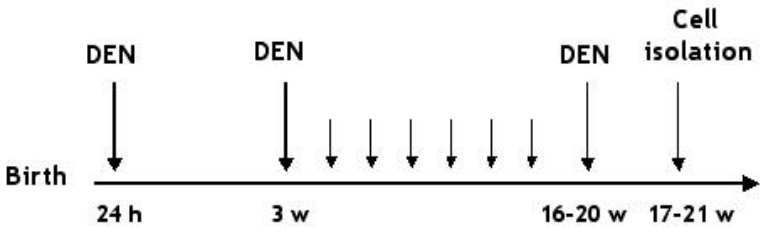

Figure 5. Schematic presentation of the treatment protocol used for induction of EAF by DEN and cell isolation. w=weeks.

The Sphingomyelin (SM) diet experiments

Female Sprague-Dawley rats, 8 weeks old, were given 6 injections of DEN (0.3 mmol/kg i.p.) once every week. One week after the last injection, the rats were divided into two groups, one receiving a control diet, the other receiving the same diet supplemented with 0.1 g sphingomyelin/100 g diet (0.1%). The diets were given for two weeks, after which the animals were sacrificed, and the livers were fixed in formaldehyde, embedded in paraffin, sectioned, and stained immunohistochemically.

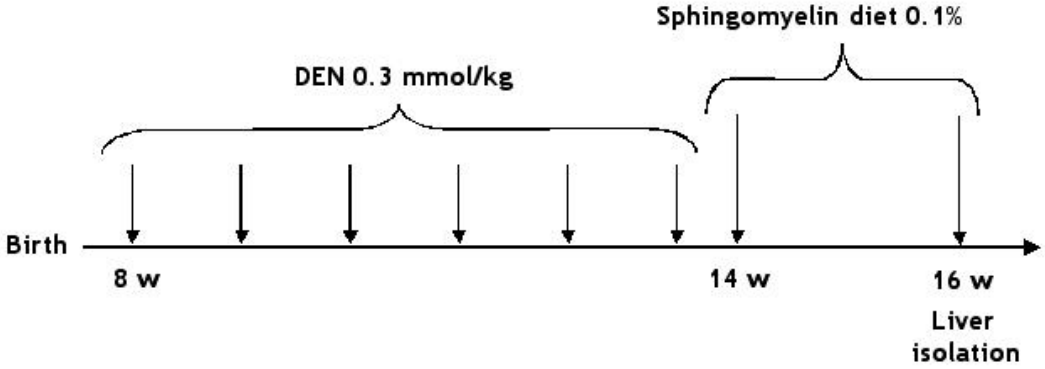

Figure 6. Schematic illustration of the treatment proocol used in sphingomyelin diet feeding experiments of DEN treated rats.

Low dose experiment

Female Sprague Dawley rats, 8 weeks old, were injected with DEN (0.025, 0.050, 0.1, 0.2, and 0.3 mmol/kg) for 10 weeks and (0.0125, 0.025, 0.05, and 0.1 mmol/kg) for 20 weeks. The rats in the two different dose rate-groups thus received the same cumulative doses, 0.25, 0.50, 1.0, and 2.0 mmol/kg. One week after the last injection, the animals received a final injection of DEN (0.6 mmol/kg) 24 hours before sacrifice, in order to induce a p53 response in the livers. The livers were then perfused with formaldehyde (Martens and Stenius 1999) and prepared for immunohistochemical staining.

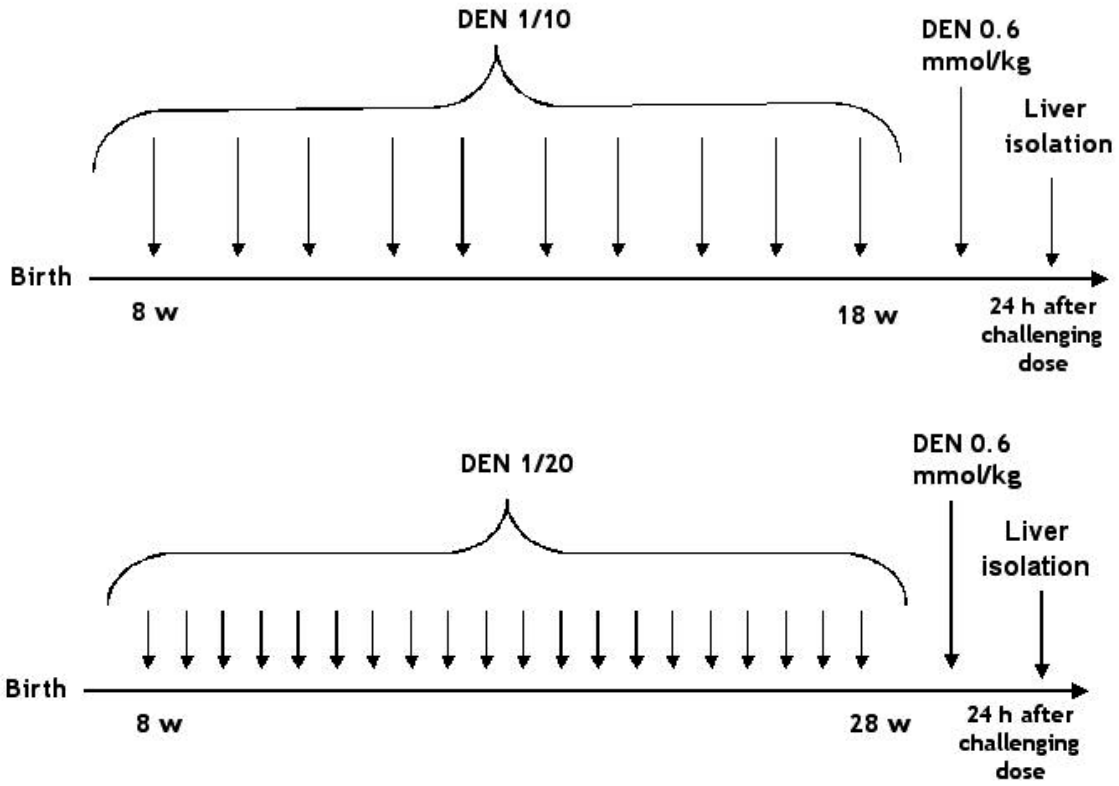

Figure 7. Schematic presentation of the experimental protocols used to induced EAF lesions at a low and a high dose-rate.

12. Results and Discussion

In this thesis data are presented that suggest that adaptive alterations might govern the early development of EAF and perhaps also their further transformation into malignancies. Thus, adaptations to genotoxic stress and end points such as cell death and proliferation have been studied. In these studies, *in vivo* models and primary cultures of isolated cells have been used. These comparatively complex models were chosen because it can be expected that they retain at least some aspects of the complexity in the interactions between cells in a tissue and their matrix. Furthermore, many genes and gene products might regulate such adaptations. Not only must the multiplicity of gene products involved in and constituting a single signaling pathway be considered, but also the notion that signaling pathways tend to form complex networks, in which they interact with each other. Signaling pathways may influence each other and minor changes in one pathway may have a profound impact on another pathway. Without a comprehensive picture of the network, data concerning a single pathway may be difficult to interpret.

A number of studies investigating the attenuated p53 response in EAF cells have previously been published (Finnberg *et al.* 2000; Lennartsson *et al.* 1998; Stenius and Högberg 1995, van Gijssel *et al.* 2000b). Since p53 normally functions to arrest cell growth and induce apoptosis upon DNA damage and other cellular stress, an

attenuated p53 response may suggest a downregulation of such events in preneoplastic cells. The consequences of this may result in unrepaired DNA damage and uncontrolled proliferation (Stenius and Högberg 1995). Furthermore, EAF heptocytes have previously been shown to resist different cell death inducing stimuli (Schulte-Hermann *et al*. 1995; Pitot 1990). This may possibly involve the attenuated p53 response in these cells.

Attenuated ATM expression in EAF (Paper I)

This study aimed to examine some of the possible mechanisms underlying the attenuated p53 response previously found in EAF lesions. Primary co-cultures containing a mix of normal (non-EAF) and EAF hepatocytes were used in these studies. GST-P was used as a marker for detection of EAF cells. EAF cells are commonly termed GST-P-positive cells, whereas non-EAF cells are termed GST-P-negative.

To induce p53 by a means alternative to DNA damage, cells were incubated with the hypoxia-mimicking chemical cobalt chloride (Wang *et al*. 1993). We found that in contrast to the lower p53 response in EAF cells after DEN treatment, cobalt chloride induced p53 both in normal and EAF hepatocytes. Approximately 20±5 % of GST-P-negative cells and 4±1 % of GST-P-positive cells stained positive for p53. The control values were 4.2±3 % for GST-P-negative cells and 0.3 ±0.2 % for GST-P-positive cells. By DEN treatment, the p53 staining normally increases up to 1-2 % in GST-P-positive cells. The p53 response induced by cobalt chloride indicated functionality, as shown by increased Mdm2 staining and decreased labeling index (cells in S-phase). In addition, the increased p53 response by cobalt chloride was effectively inhibited by treatment with antisense oligonucleotides to p53. This indicates that the p53 response to cobalt chloride exposure required *de novo* synthesis of p53. Furthermore, western blot analysis revealed increased levels of HIF1-alpha, as previously shown in the literature. Combination experiments using cobalt chloride, DEN, and the Proteasome Inhibitor (PSI) demonstrated an additive p53 response in non-EAF cells. In EAF hepatocytes only, PSI treatment resulted in an additive effect, whereas DEN treatment did not. This suggests that the attenuated p53 in EAF cells was not due to any threshold phenomenon in our detection system. Furthermore, control studies using Leptomycin B (an inhibitor of nuclear export, Wolff *et al*. 1997) demonstrated increased expression of nuclear p53 both in normal and EAF hepatocytes. This suggests that the import of p53 in the nucleus is functional, and furthermore that there is no difference in basal protein synthesis between the both cell-types. Altogether, our results suggest that p53 can be induced in EAF hepatocytes by stimuli other than DNA damage.

Modification of the p53 protein results in its activation. A large number of kinases have been found to phosphorylate p53 in response to different DNA damage (Banin *et al*. 1998; Woo *et al.* 1998; Prives and Hall 1999). In this study, we found that the levels of serine15-phosphorylated p53 were lower in EAF tissue compared to normal tissue. However, PCR analysis revealed no changes in p53 mRNA levels when comparing EAF and normal cells. This result suggested that the altered p53

response was regulated post-transcriptionally. This influenced us to further study the kinases upstream of p53. One important kinase that phosphorylates p53 N-terminally after DNA damage is ATM. N-terminal phosphorylation of p53 following DNA damage may result in inhibition of binding to its negative regulator Mdm2. This may further lead to stabilization of p53 in the nucleus.

Earlier studies have found that ATM is potently inhibited by low concentrations of wortmannin and caffeine (Sarkaria *et al*. 1998; Blasina *et al.* 1999). We found that incubation of cells with these inhibitors in combination with DEN decreased levels of both p53 and serine-15 phosphorylated p53 expression. This indicated that ATM might be involved in the regulation of p53 stabilization after DEN exposure. Furthermore, incubation with antisense oligonucleotides to ATM resulted in a reduced p53 response after DEN exposure. Moreover, western blot analysis demonstrated lower expression of ATM in EAF tissue compared to normal, surrounding tissue. This correlated with the reduced expression of ATM found in EAF using immunohistochemistry. Moreover, a kinase activity assay confirmed lower activity of ATM in EAF tissue compared to normal tissue.

In conclusion, the attenuated p53 response found in EAF tissue may partly be explained by lower expression of ATM. This is in line with previous data and supports the hypothesis that EAF hepatocytes have a downregulated response to DNA damage.

Sphingolipids suppress EAF hepatocytes *in vitro* and *in vivo* (Paper II)

Sphingolipids are involved in both apoptosis and proliferation of cells, partly depending on their relative cellular concentrations. The aim of this study was to examine the effect of cell death induction by sphingolipids on EAF hepatocytes.

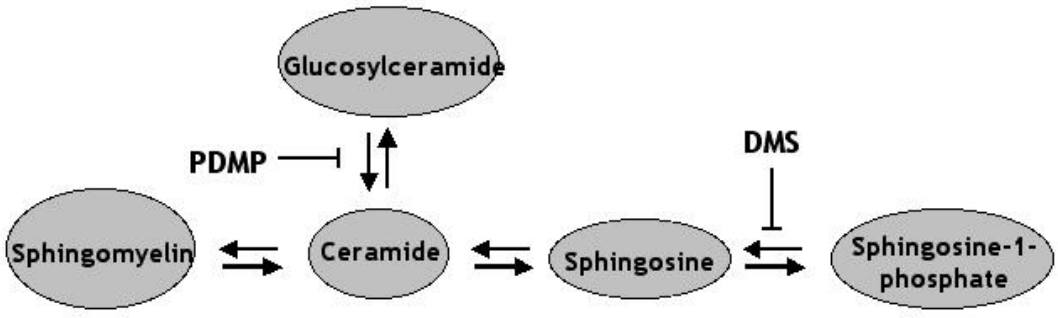

Figure 8 . Some metabolic pathways of sphingolipids

Incubation with C2- and C18-ceramide, sphingosine and an inhibitor of sphingosine kinase, DMS, resulted in a decreased number of EAF hepatocytes remaining on the culture plates. The effect of sphingosine was found to be relatively rapid; after 3 hours of exposure there was no further change of remaining cells on the plates. Furthermore, increased cytochrome c was found in the cytoplasm after sphingosine

exposure. This correlated with an increased staining of cleaved caspase-9 in cell cultures within 4 hours. These results indicate a selective, rapid onset of cell death after sphingolipid treatment, possibly involving apoptosis.

Previously, EAF hepatocytes have demonstrated resistance to Fas-mediated apoptosis (Nordstrand and Stenius 1999). In this study, cells were incubated with the Fas-activating agent GCDC, with or without sphingosine. Sphingosine exposure resulted in loss of EAF hepatocytes from the plates, and eliminated the previously demonstrated resistance to cell death mediated by Fas. TNF-α is a cytokine, which among other things can activate sphingomyelinase and sphingosine kinase, resulting in accumulation of sphingosine-1-phosphate (Sph1P). Treatment with TNF-α was found to protect the EAF hepatocytes from sphingosine-induced cell death. Moreover, sphingosine treatment alone was found to have effect on different cell cycle-regulating proteins such as p27, cyclin E, and cyclin D2.

Higher levels of glucosylated ceramide (GluCer) have been found in many multidrug-resistant cancer cells (Liu *et al*. 2001). Treatment of primary cells with an inhibitor of glucosylation, PDMP, potentiated cell death induced by ceramide, mainly by affecting EAF hepatocytes. Furthermore, immunohistochemical staining for GluCer demonstrated higher levels of GluCer in many EAF lesions from DEN-treated rats. Furthermore, TLC analysis of the lipid contents in EAF and non-EAF tissue revealed lower levels of both sphingosine and Sph1P in EAF tissue.

Two separate experiments with sphingomyelin (SM)- supplemented diet were performed. EAF-bearing rats received either a control diet or a diet supplemented with 0.1 g sphingomyelin/100 g diet. Following 2 weeks of feeding, the rat livers were prepared and stained immunohistochemically for GST-P (the marker for EAF) and the number of EAF/cm^2, the area fraction (%) and the number of single- and double- GST-P-positive cells/mm^2 was calculated. The rats receiving the SM-supplemented diet demonstrated a 40-50 percent reduction of all three parameters compared to control diet animals. These results suggest that the sphingomyelin diet had an effect on the liver, maybe by inhibiting cell growth or by inducing cell death predominantly in EAF lesions.

In summary, these results suggest a selective induction of cell death (apoptosis) in EAF hepatocytes by treatment with sphingolipids *in vitro*. Furthermore, data show that EAF hepatocytes have an altered sphingolipid metabolism compared to normal, surrounding tissue. This alteration may result in a selective sensitivity to sphingolipid induced cell death, also *in vivo*.

Induction of p53-deficient EAF by low doses of DEN (Paper III)

This study was designed to examine whether the altered p53 response in EAF hepatocytes was a consequence of acutely cytotoxic doses of DEN. For this purpose, rats were given relatively low cumulative doses of DEN (0.25, 0.50, 1.0, 2.0, and 3.0 mmol/kg), received as different high (10 weeks) or low (20 weeks) dose-rates. The livers were examined and the number of EAF/cm^2, the mean EAF

area (mm^2) and the area fraction (%) were calculated. A supralinear increase of all three parameters was seen in the livers from rats receiving the highest cumulative dose (3.0 mmol/kg). Furthermore, comparison of the livers from rats receiving the same cumulative doses at different dose-rates indicated that prolonged exposure of lower doses resulted in larger EAF.

The p53 response of the livers was studied using immunohistochemistry. In line with previous data, EAF tissue demonstrated lower p53 levels compared to surrounding tissue (Finnberg *et al*. 2000). The proportion of p53-positive cells in EAF was compared to the corresponding value in surrounding non-EAF tissue. When equivalent cumulative doses at different dose rates were compared, it was found that prolonged treatment of low doses resulted in more prominent p53-negative EAF. In addition, when dividing each dose-group into size categories, it was found that a high dose and long treatment time was important for development of p53-negative lesions. Importantly, these data also show that even the lowest doses induced p53-negative EAF.

In further experiments, control animals received single doses of DEN (12.5, 25, 50, 100, 200, and 300 μmol/kg). Both western blot analysis and immunohistochemical staining of the livers demonstrated a supralinear increase in p53, Mdm2, and TUNEL at the highest doses (200 and 300 μmol/kg). These results suggest that DNA damage and apoptosis, possibly due to cytotoxicity, was induced at these doses, but not at lower doses. However, even at the lowest doses, some hepatocyte nuclei stained positive for Mdm2 and were detected close to the central vein. This indicates that DNA damage is induced at these low doses. Nevertheless, limited DNA damage at such low doses may not lead to cytotoxicity and apoptosis.

To study the effect of another carcinogen on induction of p53-negative EAF, animals received aflatoxin B1 once weekly for 10 weeks. Two different doses were given 0.5 and 1.0 mg/kg (cumulative doses 5 and 10 mg/kg, i.e. 0.016 and 0.032 mmol/kg). In agreement with previous studies, aflatoxin B1 was found to induce EAF in rat livers. The p53 response in these EAF lesions was considerable lower compared to the surrounding tissue. In fact, almost all EAF lesions were p53-negative.

The localization of DEN and aflatoxin B1-induced EAF lesions was studied and found to be mainly in the midzonal region. It has previously been documented that large DEN-induced EAF are localized predominantly in the centrilobular region. However, since large EAF may occupy more than one zone, the exact localization of large EAF may be difficult to estimate. In this study a larger fraction of small DEN-induced EAF was located midzonally, compared to aflatoxin-induced lesions. Both DEN and aflatoxin B1 are metabolized by enzymes in the centrilobular region. Consequently, cells localized in this region will probably be most affected and damaged by the reactive metabolites formed in this region. Subsequently these cells could be replaced by EAF hepatocytes from the midzonal region during carcinogenesis.

In conclusion, these results indicate that the attenuated p53 response in EAF lesions is not only an effect seen after acute cytotoxic doses of DEN, but is induced also at low doses. Furthermore, p53-negativity could not explain the threshold reported for DEN by Williams *et al.* (1996; 2000). The duration of exposure is a strong determinant for the development of p53-negative EAF at non-cytotoxic doses. Furthermore, the reduced p53 response in EAF cells might have a large impact in the development of EAF and could be important for clonal expansion.

Susceptibility to Cell Death in Preneoplastic Hepatocytes; the role of Akt Kinase (Paper IV)

The aim of this study was to examine the susceptibility of EAF to sphingolipids and the investigation focused on the role of the Akt signaling pathway.

The kinase Akt is commonly phosphorylated as a consequence of growth factors binding to their receptors (Downward 1998). Sphingolipids have been suggested to exert their cell death inducing effect by decreasing cellular pAkt levels (Chang *et al*. 2001; Kim *et al*. 2001).

We found that pretreatment *in vitro* with antisense oligonucleotides towards Akt counteracted the selective cell death induced in GST-P-positive cells by sphingosine. This effect was probably caused by an increased sensitivity of normal cells to sphingosine. This finding suggest a role for Akt in sphingosine-induced cell death. Furthermore, using western blot analysis, it was found that sphingosine was able to decrease insulin-induced pAkt levels in primary hepatocytes *in vitro*.

Immunohistochemical staining revealed that at least 15 percent of all EAF lesions constitutively expressed lower levels of pAkt, compared to the surrounding tissue. In addition, the same amount of EAF expressed relatively high levels of ceramide. These markers were found to overlap; almost all EAF lesions expressing lower pAkt levels were ceramide positive and vice versa. Immunohistochemical staining of pAkt in EAF induced by high doses of DEN revealed that a larger fraction of the total lesion area expressed less pAkt, compared to EAF induced by low doses.

Livers from animals fed a control- and a sphingomyelin-diet for 2 weeks were stained for ceramide, pAkt, and the EAF-marker GST-P. None of the markers were selectively changed in the sphingomyelin-enriched livers compared to control livers. This suggests that relatively high ceramide or low pAkt levels in EAF lesions may not be the only criterion for sensitivity to sphingolipids. Alternatively, the SM diet feeding may induce the phenotype of EAF expressing lower levels of pAkt.

P27 and mTOR are targets of pAkt (Brazil and Hemmings 2001; Burgering and Medema 2003). p27 is negatively regulated by pAkt, and we found that many EAF lesions expressed higher levels of p27 compared to normal tissue. This could possibly be a consequence of lower pAkt levels in many EAF lesions. mTOR is positively regulated by pAkt and is potently and selectively inhibited by rapamycin.

Treatment of primary co-cultures with rapamycin resulted in a reduced labeling index (cells in S-phase), mainly in EAF hepatocytes. The selective sensitivity of EAF cells to rapamycin may be a consequence of the low pAkt levels in these cells. These results suggest that the pAkt-mTOR signaling pathway could have an effect on cell proliferation in EAF hepatocytes.

The HMG-CoA reductase is the rate-limiting enzyme in the mevalonate synthesis pathway (Soma *et al.*1992). *In vitro* treatment with inhibitors to this enzyme, pravastatin and simvastatin, was found to induce cell death primarily in EAF cells. Western blot analysis demonstrated decreased pAkt levels by pravastatin treatment induced by insulin. Low pAkt levels in EAF hepatocytes may increase the sensitivity to statins, resulting in cell death. However, other mechanisms of statin-induced cell death may also be involved.

In summary, our data suggest that the pAkt levels in many EAF lesions are lower. Reduced pAkt levels may result in increased sensitivity to sphingolipids. Data indicate that treatment with statins also induce cell death selectively in EAF cells, possibly due to already reduced levels of pAkt.

13. Conclusions

Hepatocytes of preneoplastic lesions possess alterations that possibly confer a growth advantage in a hostile and toxic environment. Development of EAF lesions may arise as a physiological adaptation to toxicological stress in some cells. In this study, we found lower levels and activity of the kinase ATM compared to surrounding tissue. Low levels of cellular ATM may partly explain the lower p53 response found in the majority of DEN-induced EAF. Furthermore, it was found that small EAF lesions, induced by relatively low DEN-doses, share the same characteristics of an attenuated p53 response as large high-dose-induced lesions. Since larger lesions are more “p53-negative”, a downregulated p53 pathway seems important for the clonal expansion of these lesions. Moreover, prolonged treatment of low doses resulted in more prominent p53-negative EAF lesions. Thus, p53 and ATM downregulation may be part of the adaptational events to genotoxic stress that result in growth advantages in EAF hepatocytes, as compared to surrounding cells.

Resistance to different types of cell death is a common property of many EAF hepatocytes, and may be a consequence of a downregulated p53 response. However, sphingolipids may induce cell death independently of DNA damage-induced p53 signaling. In this regard, EAF cells were found to be more sensitive to exogenously added sphingolipids both *in vitro* and *in vivo*. This susceptibility to sphingolipids could be explained by changes in sphingolipid metabolism or the lower pAkt levels found in EAF hepatocytes. Altered metabolism of sphingolipids may render EAF cells to be more sensitive to cell death induced by sphingolipids, statins, or agents affecting the mTOR pathway. Thus, alterations of pathways involved in regulating cell death and proliferation may increase the resistance

towards certain cell death stimuli, but at the same time increase the susceptibility to cell death induced by other stimuli.

14. Future Perspectives

After chemical exposure and during carcinogenesis, hepatocytes seem to alter the expression of different growth factor receptors, such as the EGF receptor, in the cell membranes (Carr *et al.* 1986; DeCicco *et al*. 1997; Levinovitz *et al.* 1990). Furthermore, expression of the growth factor TGF-α has been found to be increased in EAF lesions during carcinogenesis, and it has been suggested that it may be used as a marker for EAF progression (Dragan *et al.* 1995). However, exogenous stimulation of TGF-α was demonstrated to mainly affect DNA synthesis in normal cells but not in EAF cells (Lennartsson *et al.* 1999). It has also been suggested that preneoplastic cells may downregulate the influence of exogenous growth factors and increase their autonomous control of proliferation (Eriksson and Andersson 1992). It is thus possible that one of the findings in this thesis, i.e. low pAkt levels in many EAF, might be accounted for by alterations in membrane receptors. Thus, further studies may reveal relationships between the expression of the EGF receptor and pAkt levels in EAF hepatocytes.

During recent years, hypotheses regarding so called "bystander effects" have emerged. It has been suggested that responses to radiation-induced genotoxicity in one cell, via some type of cell communication such as gap junctions, may affect surrounding cells. Effects imposed on surroundings cells vary with the model used, but may include DNA damage, apoptosis and cell proliferation. Such bystander effect may have a large impact on how small doses of radiation can affect surrounding tissue (Djordjevic 2000). A recent publication (Rothkamm and Lobrich 2003) showed that cells with low levels of damage following radiation remained unrepaired for many days. Upon proliferative stimulation, these cells eliminated themselves in a delayed type of apoptosis and were replaced by non-damaged neighboring cells. Results presented in this thesis indicate that low DEN doses apparently induced DNA damage predominantly in centrilobular areas of the liver, but EAF development in midzonal areas. Assuming that bystander effects can also be induced by low doses of chemical carcinogens in the liver, the question may be raised of whether damaged centrilobular cells were replaced by undamaged midzonal hepatocytes, and whether EAF were initiated in this process. This question can be addressed by new studies focusing on the role of connexins in liver carcinogenesis. These gap junction proteins have previously been implicated in EAF development (Krutovskikh *et al*. 1991; Neveu *et al*. 1990).

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