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THE ATTENUATION OF THE P53 RESPONSE TO DNA DAMAGE IN RODENT LIVER PRENEOPLASTIC ENZYME-ALTERED FOCI

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ABSTRACT

In connection with life-time bioassays in rodents, the liver is one of the organs most frequently affected. During the carcinogenic process, single preneoplastic hepatocytes develop into hepatocellular adenoma or carcinoma (HCC). Preneoplastic hepatocytes are identified on the basis of their overexpression of the inactivating phase II enzyme glutathione-S-transferase Pi (GST-P). In the presence of continuos exposure to carcinogen, initiated hepatocytes expand clonally to form hepatic enzyme-altered foci (EAF). Such development of EAF might be considered to be an adaptive response and their pattern of gene expression may provide mechanistic information concerning the action of a putative carcinogen.

Our studies have focused on the response of the tumor suppressor p53 to DNA damage in EAF. Activated by several types of cellular stress, the p53 protein is involved in regulating cell cycle arrest and apoptosis. The overall aim of our project was to characterize the attenuated p53 response to DNA damage in preneoplastic EAF lesions and the possible role of this attenuation in the development of EAF by diethylnitrosamine (DEN).

To this end, after receiving an initiating neonatal dose of DEN, female Sprague-Dawley rats were exposed to DEN or the non-genotoxic agent phenobarbital (PB), which induced the development of EAF in both cases. A challenging dose of DEN was also administered 24 hours prior to sacrifice to elicit a p53 response in these EAF.

Only EAF arising from treatment with DEN exhibited an attenuated p53 response in comparison to that of surrounding, non-EAF tissue and PB-induced EAF. This attenuation was enhanced by prolonging the period of treatment, as well as in larger EAF. The attenuated p53 response to DEN-induced DNA damage was also present in primary co-cultures of hepatocytes isolated from EAF (GST-P-positive hepatocytes) and from non-EAF tissue (GST-P-negative hepatocytes).

Treatment of such co-cultures with $CoCl_2$, which mimics hypoxia, resulted in nuclear accumulation of p53 in the GST-P-positive cells. This finding demonstrates that a p53 response may be evoked by hypoxic stress, but not by genotoxic chemicals. Additional studies with the PI3 kinase inhibitors caffeine and wortmannin, as well as with ATM antisense oligonucleotides indicated that ATM is involved in signalling to p53 following DEN-induced damage of DNA. Immunohistochemical analysis of the livers of DEN-treated rats and Western blotting of macroscopic EAF tissue revealed lowered expression of ATM in these tissues. Thus, down-regulation of ATM may to some extent explain the attenuated p53 response to DEN exhibited by EAF.

Upon examining the combined p53-MDM2 response in rat liver at different time-points following a single injection of DEN, significant temporal and spatial variations were observed. Mid zonal areas demonstrated a transient combined p53 – MDM2 response 6 – 24 hours after the DEN challenge, whereas in centrilobular areas this response culminated 24 – 72 hours after injection. MDM2 was constitutively expressed in midzonal areas. Furthermore, following repeated treatment with low doses of DEN, GST-P-positive EAF were found to be particularly prevalent in this same zone.

Finally, the influence of the p53 gene dosage on the development of p53-negative preneoplastic lesions was investigated. Treatment of p53 (+/+) and (+/-) mice for 15 – 20 weeks with DEN revealed a genotype-dependent difference in the numbers of p53-negative preneoplastic hepatic lesions obtained, with p53 (+/-) mice developing significantly fewer p53-negative lesions than p53 wild-type (+/+) mice. However, the total number and average size of all preneoplastic lesions were similar in these two types of mice.

In conclusion, these findings indicate that an attenuated p53 response to DNA damage confers a growth advantage on preneoplastic focal lesions in the liver. The selective pressure for focal lesions exhibiting such p53 attenuation can be modulated by altering the p53 gene dosage or by exposure to xenobiotics. These observations indicate that the attenuated p53 response in preneoplastic lesions is an adaptive response to genotoxic stress.

Keywords: enzyme-altered foci, p53, ATM, diethylnitrosamine, phenobarbital, liver, gene dosage

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their roman numerals:

I. Finnberg N, Stenius U and Högberg J. Xenobiotics modulate the p53 response to DNA damage in preneoplastic enzyme-altered foci in rat liver; Effects of diethylnitrosamine and phenobarbital. *Toxicological Sciences* 2000, 54; 95 – 103.

II. 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* 2001, 22; 2023 – 2031.

III. Finnberg N, Stenius U and Högberg J. In vivo responses to diethylnitrosamine in rat liver: curtailed expression of p53 in midzonal areas and initiation of preneoplastic lesions. *Manuscript*

IV. Heterozygous p53-deficient (+/-) mice develop fewer p53-negative preneoplastic focal liver lesions in response to treatment with diethylnitrosamine than do wild-type (+/+) mice *Manuscript*

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LIST OF ABBREVIATIONS

Akt	v-Akt murine thymoma viral oncogene homolog 3 (PKB)
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad-3 related kinase
Bax	Bcl-2 associated protein x
Bcl-2	B-cell CLL/lymphoma 2
Chk	Checkpoint kinase
CK	Casein kinase
CYP2E1	Cythochrome P450 2E1
DNA-PK	DNA-dependent protein kinase
DEN	Diethylnitrosamine
EAF	Enzyme-altered foci
GST-P	Glutathione –S-transferase Pi
HCC	Hepatocellular carcinoma
HIF-1	Hypoxia-inducible factor - 1
IR	Ionizing radiation
KILLER/DR5	P53-inducible apoptotic death receptor 5
MDM2	Murine double minute 2
NES	Nuclear export signal
NOXA	A pro-apoptotic p53-inducible gene
P14ARF	The protein product from the alternative reading frame of CDKN2A
P21WAF1	The p53-inducible p21 cdk2/4 inhibiting protein
P53	The protein product of the tumor suppressor gene p53
pAkt	Akt phosphorylated on Ser-473
PB	Phenobarbital
PBRE	Phenobarbital response element
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog
PUMA	P53-upregulated modifier of apoptosis
RH	Resistant hepatocyte protocol
Ser	Serine
SAH	S-Adenosylhomocysteine (methyl group acceptor)
SAM	S-adenosylmethionine (methyl group donor)
Thr	Threonine
Tyr	Tyrosine

1 INTRODUCTION

1.1 CANCER

The primary factors involved in the etiology of cancer, one of the most common causes of death in Western countries, have not yet been fully elucidated. Accumulating epidemiological evidence suggests that a pronounced predisposition to develop cancer as a consequence of a mutation in a single gene is rare (approximately 1 – 5 %) [1]. One possible explanation for this finding is that carcinogenesis is a multi-stage process involving a number of different genes and environmental factors [2].

Hanahan and Weinberg [3] have argued that in connection with more than 100 distinct subtypes of cancer, six functional alterations are required for malignant transformation: self-sufficiency with respect to growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), the potential for unlimited replication, sustained angiogenesis, tissue invasion and metastasis. The exact number of distinct stages involved may vary from tumor to tumor, since some of these acquired characteristics probably interact with other processes. Indeed, the heterogeneity of tumors, both with regards to morphology and pattern of gene expression, may even indicate the participation of many more sequential steps.

The products of tumor suppressor genes and oncogenes play important roles in connection with carcinogenesis and, consequently, analysis of the functions of and possible mutations in these proteins in tumor cells can shed light on the carcinogenic process. Thus, new technologies in the fields of functional genomics and proteomics will be useful in improving our understanding of this processes, thereby allowing more precise diagnosis and individualized cancer therapy. In addition, transgenic "knock-out" and conditionally "knock-out"/"knock-in" strains of mice are continuously being developed in order to better understand the functions of genes of putative importance to carcinogenesis [4,5].

These genes are classically divided into oncogenes (whose products sustain tumor development) and tumor suppressor genes (inhibiting development). Oncogene products are considered to be involved in mitogenic stimulation and signal transduction, being primarily transcription factors and receptors for growth factors. The proteins encoded by tumor suppressor genes typically inhibit cell growth and/or, control genomic stability and/or the microenvironment of the cell in a direct fashion.

Oncogenes and tumor suppressor genes often form a complex network, delicately balancing one and another, directly or indirectly. “Nodes” in such networks, integrates the combined efflux of signals and execute a response to them. Such proteins are of particular importance for understanding the process of carcinogenesis. One such protein is the tumor suppressor p53.

1.2 THE LIVER, THE HEPATOCYTE AND HEPATOCELLULAR CARCINOMA

The liver is the largest single gland in the human body where it constitutes approximately 2.5% of total body weight. This organ deals with large quantities of nutrient amino acids, carbohydrates and vitamins as well as pollutant xenobiotics in

food and water that enter via the intestinal tract. Thus, the liver metabolizes xenobiotics including clinical drugs, primarily in the cytosol, endoplasmic reticulum and outer nuclear envelope of hepatocytes. Various monoxygenases, utilize oxygen to activate xenobiotic substrates and thereby render them susceptible to excretion following conjugation with glutathione, glucuronic acid and/or inorganic sulfate.

In addition to hepatocytes, the liver is populated by several other types of cells, including Kupfer cells, ITO (stellate) cells and endothelial cells. However, of the approximately 300 million cells in the human liver, approximately 78% (by volume) are hepatocytes, which exhibit a high degree of differentiation and slow rates of apoptosis and proliferation. Under normal physiological conditions, hepatocytes are responsible for the laminellar architecture of the liver, forming the microcirculatory unit as the acinus (Figure 1).

The hepatic acinus, often devided in zones 1, 2 and 3, receives approximately 75% of its blood supply from the portal vein, the remainder coming from the hepatic arteries. All of these vessels drain into the sinusoids, i.e., open channels passing through the acini. The sinusoids extend into the hepatic central vein, and are surrounded by a single layer consisting of approximately 20 hepatocytes, the so-called cell plate. The unidirectional perfusion of blood through the acinus may create a microenvironment near the inlet, which differs from that near the outlet.

In the scientific literature, the functional unit is sometimes referred to as a liver lobule. The liver lobule has a central vein in the middle and in the periphery, there are several periportal regions (i.e., the area surrounding the vessels draining into the sinusoids or, roughly, acinar zone 1). The centrilobular area surrounds the central vein. The area between these two regions is designated as the midzonal area.

Hepatic heterogeneity with respect to morphological, biochemical and functional properties may be a consequence of the organization of the microcirculatory unit described above. For example, periportal hepatocytes are seen to contain fewer numbers of larger mitochondria; whereas perivenous hepatocytes contain more extensive endoplasmatic reticulum [6]. Furthermore, in the mature mammalian liver, the expression of most genes appears to differ in the different regions, with an increasing or decreasing level of expression from the portal vein to the central vein in the acinus [6-8].

Two types of such zonated gene expression are observed in the liver, i.e., constitutive or related to gradients of low molecular-weight compounds. The latter type of zonation is usually dynamic, responding to fluctuations in levels of blood-borne signals. In this case, all of the hepatocytes in the acinus express the gene in question, but to different extents, and the steepness of the gradient thus created is different for different proteins.

Among the genes that are expressed in such a zonated fashion are those coding for the enzymes which catalyze phase I and phase II reactions in xenobiotic metabolism. In phase I reactions xenobiotics are metabolized to reactive intermediates, most often by the mixed function oxidase systems (MFO) in which isozymes of the cytochrome P-450 (CYP) participate [9]. These systems are also involved in the metabolism of

endogenous hydrophobic substances, such as fatty acids and with members of the CYP1, CYP2 and CYP3 families being of particular importance in the metabolism of xenobiotics. In general, CYP isozymes are expressed at higher levels in the perivenous region of the liver [6].

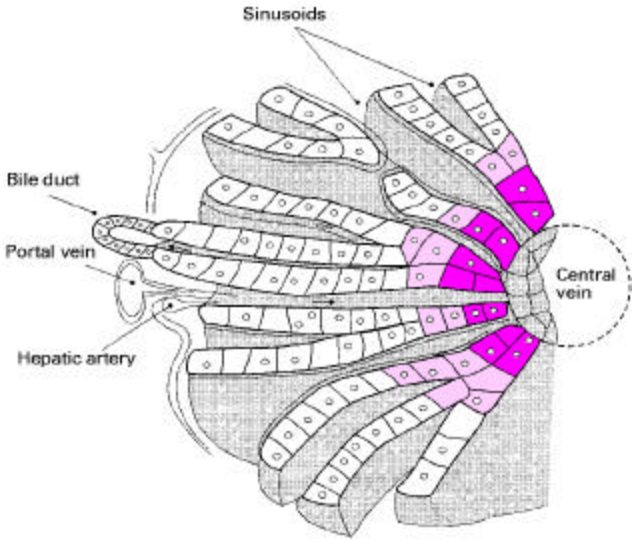

Figure 1. **Structure of the mammalian hepatic acinus.** Blood enters from the terminal portal venules and hepatic arteries in the periportal (zone) area and flows through the sinusoids to the terminal central vein in the perivenous (centrilobular) area (pink - purple). These latter cells generally express CYP isozymes at higher levels and participate more extensively in drug metabolism. In the bile canniculi bile flows in the opposite direction, towards the portal tract and into the bile ducts. (Slightly modified from [6])

As mentioned above, in phase II of xenobiotic metabolism the reactive intermediates produced by phase I are conjugated with glucuronic acid, sulfate and gluthathione to yield hydrophilic products that can be excreted in the urine or bile. As in the case for CYP isozymes, some of the phase II enzymes, such as certain isozymes of glutathione transferase and UDP-glucuronosyltransferase, are enriched perivenously [8]. These observations suggest that the major quantitative burden in connection with detoxification is borne by the perivenous hepatocytes. However, up-regulation of phase II detoxifying processes is often less potent than induction of phase I activation, an imbalance which may give rise to perivenous toxicity [6].

Globally, hepatocellular carcinoma (HCC) is one of the most common forms of cancer, with approximately 600 000 new cases being diagnosed world-wide in the year 2000 [10,11]. The common setting for this disease has a common nominator, linking conditions where many hepatocytes die, inflammatory cells invade the liver and connective tissue is deposited there, i.e., characteristic symptoms of chronic hepatitis or cirrhosis [12,13]. Unfortunately, HCC is associated with frequent metastasis, and consequently, a poor prognosis.

In animal models, HCC appears to develop directly from hepatocytes [14], but the molecular sequence of events underlying this process is unclear. The complexity of this ethiology can be explained in part by the heterogeneity of the molecular mechanisms involved in gene regulation and in part by the fact that several different signal

transduction pathways are affected in such manner as to lead to malignant transformation of the hepatocyte [12]. However, in some cases structural alterations in genes may initiate this etiology, for example mutations at Ser249 of p53 following chronic exposure to aflatoxin B [15,16], one of the xenobiotics which, in combination with viral infections, are major risk factors for human HCC. The development of HCC in humans spans over a time-period of 30 years and obviously occurs in steps with preneoplasia preceding the malignant disease.

1.3 CARCINOGENESIS

1.3.1 Stepwise accumulation of genetic and epigenetic alterations

In connection with carcinogenesis, a progressive, step-wise accumulation of genetic changes has been documented to occur [17]. Classical alterations in the primary structure of DNA (e.g., point mutations, deletions and translocations) leading to activation and/or inactivation of oncogenes and/or tumor suppressor genes, respectively, are considered to constitute typical "events" leading to or predisposing cells for subsequent transformation. However, in recent years it has been suggested that epigenetic alterations, resulting in decreased genomic stability, may precede such genetic alterations [13,18,19].

Alterations in gene expression that drive the carcinogenic process may be accomplished in several different ways. One such type of epigenetic event involves alterations in the packing of DNA facilitated by modifications in the methylation of promoter CpG islands in DNA and in histone acetylation. Indeed, most cancers do demonstrate abnormalities with respect to DNA methylation [20].

Abnormalities in methylation patterns may arise in response to forced proliferation [20,21]. Increased methylation (hypermethylation) of promoter CpG islands in tumor suppressor genes, leading to decreased expression, and decreased methylation (hypomethylation) of such islands in the promoters of oncogenes, leading to increased expression, predispose cells to tumorigenesis. Genes whose products are involved in maintaining of DNA integrity (e.g., MLH1, MLH2 and MGMT) are frequently found hypermethylated in e.g. colon cancers and lymphomas [22]. Decreased genomic stability may predispose cells to accumulation of mutations, which, in connection with tumorigenesis, may be selected for (generation of the “mutator phenotype”) [21].

Early in the development of human HCC, expression of transforming growth factor α (TGF-α) insulin growth factor-2 (IGF-2) is frequently found to be aberrant as a result of, e.g., a regenerative response to cell loss or inflammation. Such alterations are accompanied by abnormalities in CpG methylation and in the expression of DNA methyltransferases [23]. Microsatellite instability (MSI) has also been reported to occur in HCC [24,25], although infrequently.

In at least some cases, mutagenesis appears to proceed via non-mutagenic alterations, e.g., the c-myc promoter might be hypomethylated at an early stage leading to overexpression; whereas the c-myc gene is amplified in later stages of tumor development [12]. The opposite, i.e., promoter hypermethylation and loss of heterozygosity at later stages is observed for the tumor suppressor gene $p16^{INK4A}$ [26,27]. Structural alterations in DNA (e.g., mutations, deletions and amplifications)

accumulate slowly during the preneoplastic stage, whereas virtually all HCC have been shown to contain genetic alterations [28].

1.3.2 Chemically induced carcinogenesis

The initial evidence for a relationship between exposure to chemicals and development of cancers was provided by epidemiological observations (e.g., scrotal cancer in chimney sweeps, described by Percival Potts) and experiments in animals (e.g., tumors induced by painting coal tar on the ears of rabbits, described by Ishikawa and colleagues). The first carcinogens to be obtained in pure form were polycyclic aromatic hydrocarbons isolated in the 1920's, by Kennaway *et al.* [29] and considered to account for the carcinogenicity of coal tar. In the 1960's, Brookes and Lawley [30] discovered that carcinogens after metabolic activation bind covalently to DNA.

Induction of the hepatocarcinogenic process induced by administration of chemicals provides a system for characterizing alterations in the liver at early stages in the process. Human HCC and both spontaneous and chemically induced HCC in rodents exhibit considerable similarities with regards to morphology, genomic alterations and gene expression [31], despite their different etiologies. Therefore, investigation of the development of liver cancer in rats and mice might provide valuable insight into the human condition [12,32].

From a toxicological point of view, the liver is of particular interest, since in connection with lifetime bioassays of putative carcinogens in rodents, the liver is one of the organs most often affected [33]. Hepatocytes exhibiting altered morphological and biochemical properties (referred to here as initiated hepatocytes) arise in response to exposure to hepatocarcinogens and the frequency of such initiated hepatocytes can be estimated employing simple immunohistological techniques. There is substantial evidence indicating that initiated hepatocytes are the precursors of HCC [14,34-36].

The appearance, growth and transformation of these hepatocytes with altered patterns of enzyme expression into cancer are described by the terms *initiation*, *promotion* and *progression* (see below). It should be remembered that these terms are strictly operational, indicating that the hepatocarcinogenic process involves at least three stages, but do not necessarily reflect molecular modifications imposed on the enzyme-altered hepatocyte [37]. The terms *initiator* and *promoter* are also used (see below) to designate chemicals that act, more-or-less selectively, during the initiation and promotion phases, respectively.

1.3.2.1 Initiation

Although, the exact nature of molecular events underlying initiation are not yet known, experimental evidence suggests that alterations in DNA sequence (e.g., mutations) may play a causal role in this process [38]. This hypothesis receives support from the observations that most initiating compounds are genotoxic and that initiation occurs most frequently when alkylating agents are administered during the period of most rapid DNA replication [37,38], suggesting that the presence of unrepaired adducts during replication can result in mutations [36]. Furthermore, mice that lack the enzyme O6-methylguanine-DNA methyltransferase, which participates in DNA repair, demonstrate enhanced susceptibility to tumor formation following initiation with diethylnitrosamine [39]. In addition, treatment with antioxidants in combination with an

initiating agent decreases the frequency of cancer formation, whereas active cellular proliferation exerts a synergistic effect on this frequency. Such findings indicate that mutations, which activate proto-oncogenes and/or inactivate tumor suppressor genes, are involved in the carcinogenic mechanism. Indeed, it has been reported that preneoplastic hepatocytes exhibit an increased frequency of mutations in the Ras and beta-catenine oncogenes [40-42].

Evidence that initiation can also be induced by epigenetic mechanisms is now accumulating. For example, the poor correlation between the frequency of formation of GST-P-positive hepatocytes and rate of cell proliferation in rodent liver in response to N-nitrosomorpholine is consistent with the involvement of epigenetic mechanisms [43]. The appearance of such GST-P-positive hepatocytes may reflect an accumulation of endogenous ligands in association with DEN-induced initiation and subsequent up-regulation of GST-P in response to the resulting toxic stress [44]. There are even controversial suggestions that in connection with multi-stage skin cancer, initiating agents such as 7, 12-dimethylbenzanthracene (DMBA) might affect the extracellular matrix throughout the stroma, thereby altering the substrate to which the cells attach and grow. Obviously, such a situation would lead to alterations in the profile of gene expression in cells affected, which could then be selected for [45].

1.3.2.2 Promotion

Despite alterations of gene expression that might favor proliferation of initiated hepatocytes, these cells are unable to grow autonomously [46]. However, during the promotion phase following initiation, initiated hepatocytes can be stimulated to expand clonally to form Enzyme-Altered Foci (EAF, see below) [36]. The stimuli involved may be physiological (e.g., the growth of the neonatal liver) or provided by a variety of chemical compounds (promoters) [47-49] or by inhibition of the growth of unaltered hepatocytes. This step is considered reversible, since EAF "remodel/redifferentiate" to morphologically normal liver upon removal of the promoting agent [50]. Interestingly, when such "remodeled/redifferentiated" liver is treated with a tumor promoter, new EAF may appear [36,46], illustrating the irreversible nature of initiation, in contrast to the reversibility of tumor promotion.

Also, in contrast to initiation, the promotion process is slow and requires the continuos presence of appropriate stimuli. EAF are sometimes considered to be an adaptation to toxic stress and have been shown to exhibit increased resistance to hepatotoxic compounds such as CCl_4. EAF exhibit a variety of phenotypes [51].

EAF of a particular phenotype may be selected for during promotion. This phenotype might be present in the original pool of EAF hepatocytes in the initiated liver or arise by "remodeling" of EAF [49]. The phenotypic diversity of the EAF present in the liver following promotion may reflect the properties of the tumor promoter employed.

With regards to morphology and biochemistry, the phenotype of EAF hepatocytes differs from that of surrounding, non-EAF hepatocytes in several respects. As discussed above, epigenetic alterations that alter packing of the chromatin (e.g., methylation and/or histone acetylation/deacetylation) could be of significance in this context. EAF

hepatocytes exhibit an increased proportion of euchromatin, suggesting increased accessibility and transcription of genes [36]. Indeed, several hepatocarcinogenic procedures, including exposure to DEN and administration of methionine-deficient diet, cause wide-spread hypomethylation of the DNA in EAF, possibly as a result of a disturbance in the ratio between donors and acceptors of methyl groups [52-54]. Such hypomethylation could lead to increased expression of proto-oncogenes encoding for e.g., c-jun, ras and c-myc [53,55,56].

Characteristically, EAF hepatocytes demonstrate increased resistance to xenobiotics. One factor in this connection is presumably the down-regulation of activating, phase I enzyme systems (e.g., CYP) and concomitant up-regulation of inactivating phase II enzymes (GSH transferases, epoxide hydrolases). In addition, EAF hepatocytes in the carcinogen-treated rodent liver exhibit several distinct phenotypes with respect to xenobiotic metabolism [31]. Expression of the phase II enzyme glutathione-S-transeferase pi (GST-P) is enhanced in approximately 90% of all EAF, making this a valuable marker for initiated hepatocytes or EAF.

EAF hepatocytes proliferate at a slightly increased rate, suggesting alterations in the expression of proteins involved in growth and replication. Such alterations may confer a survival advantage, relative to non-EAF hepatocytes, in the exposed liver. Furthermore, the frequency of apoptosis is elevated among EAF hepatocytes but may not compensate for the increased proliferation (39). Proliferation of these cells accelerates dramatically following progression to malignancy.

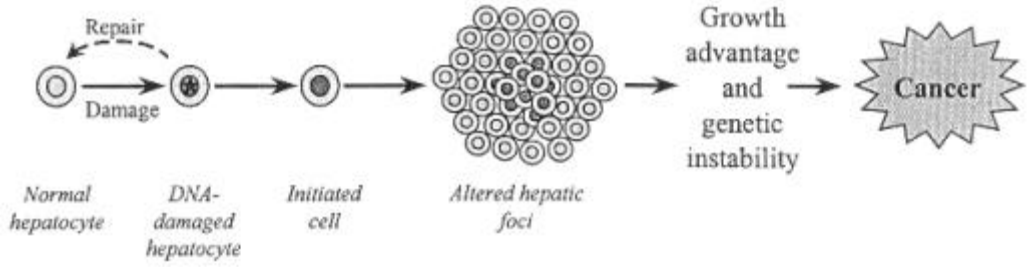

Figure 2. **Schematic representation of the major steps involved in hepatocarcinogenesis.** [37]

1.3.2.3 Progression

The progression stage involves irreversible development of benign and malignant neoplasms. After termination of the promotion phase, groups of EAF hepatocytes, if large enough, form (visible) noduli in the liver. In certain animal models, approximately 10% of these noduli fail to undergo "remodeling" when the promoting stimulus is withdrawn. Since such noduli have acquired the ability to grow autonomously, the progression stage is considered to be irreversible. Transplantation of such persistent noduli into the spleen of syngenic rats gives rise to cancer [36].

Various morphological and molecular hallmarks have been proposed to be associated with the transition from promotion to progression. Such characteristic changes include the appearance of neonoduli (i.e., noduli whitin noduli) in combination with structural alterations in genes, e.g., rearrangement of the c-myc oncogene and/or karyotypic aberrations of chromosomes [48]. Alterations of this sort are observed only rarely in EAF, consistent with the fact that only a small percenta ge of EAF have the potential to develop into carcinoma.

1.3.3 Hepatocarcinogens

Hepatocarcinogens possess a wide diversity of structures, but many share the ability (with or without metabolic activation) to bind covalently to DNA, which is one factor that determines the potency of carcinogens in general. Thus, numerous compounds that can react with DNA may act as initiators of carcinogenesis.

Typically, compounds acting as tumor promoters (e.g. barbiturates, dioxin and hormones) are classified as non-genotoxic [48,57]. However, there exists some controversy around this question, since these compounds may inflict a degree of DNA damage, below the limit of detection. It has also been argued that tumor promoters may induce DNA damage indirectly, e.g., via oxidative stress and/or via stimulation of proliferation.

By itself, the ability to damage DNA is not sufficient for a compound to be classified as a "complete" liver carcinogen, since stimulation of hepatocyte proliferation is also required. This rate of proliferation is normally low (1 of 10 000 hepatocytes undergoing mitosis at any given time) and must probably be enhanced in order to achieve hepatocarcinogenesis. Genotoxic compounds may be more potent carcinogens in other organs whose cells are proliferating more rapidly.

1.3.3.1 Nitrosamines, in particular diethylnitrosamine (DEN)

Human beings are exposed to nitrosamines from both exogenous and endogenous sources. Exogenous exposure may occur in the form of dietary components, occupational settings, tobacco and tobacco smoke, cosmetics, agricultural chemicals and pharmaceutical agents [58-60]. Endogenous exposure is a consequence of nitrosation of precursors taken up together with food, water and/or air. Such precursor includes amines, ureas and carbamates and the nitrosyl group (NO) added can be derived from nitrogen oxides or nitrate [60].

The biological effects of nitrosamines have been investigated extensively, i.e., approximately 300 different nitrosamines have been tested in about 40 animal species. Approximately 90% of the nitrosamines thus examined exert carcinogenic effects and none of the species tested are resistant to those effects [59-61]. Tumor induction by nitrosamines is most potent upon exposure to small quantities of the chemical over long periods of time [60,62].

Human liver appears to metabolize nitrosamines in a manner similar to that of rodent liver. Furthermore, both humans and rodents develop similar effects, such as necrosis and cirrhosis, upon exposure to nitrosamines [61]. Finally, nitrosamine-induced DNA adduct formation, is also similar in rodents and men [63-65].

Diethylnitrosamine (DEN) is a so-called complete liver carcinogen, a single dose of which can, under certain conditions, induce the formation of HCC. DEN inhibits mitosis in the liver, induces hypomethylation and promotes the development of EAF [37,64,66]. In one study involving 4080 rats, a linear relationship between the dose of DEN administrated and the frequency of tumor formation was observed [67]. However, other investigations on much fewer animals revealed the existence of threshold doses of DEN with respect to the formation of EAF and cancer [68,69]. It is, however, possible that the relatively short period of exposure employed in these latter studies was the origin of this apparent threshold [70].

In the liver DEN is metabolized to the reactive ethylcarbonium ion by the phase I CYP2E1. This intermediate reacts with hepatocyte DNA to form ethyl adducts, typically at the N^7 (69%) of the total and O^6 (3-6%) positions on guanine. However, the rate of repair for e.g. O^6-ethyldeoxyguanosine is 200-fold higher than the corresponding rate for O^4-ethyldeoxyguanosine and the level of O^6-ethyldeoxyguanosine is thus poorly correlated to the frequency of formation of initiated cells [71,72]. Persistent DNA adducts may be cell-type specific.

1.3.3.2 Tumor promoters, in particular phenobarbital (PB)

Most tumor promoters are food additives, hormones (e.g., diethylstilbestrol) or clinical drugs (e.g., PB and clofibrate) and are thought to act via epigenetic mechanisms [73]. Such agents can stimulate the growth of resistant preneoplastic cells in several different ways, e.g., by mitoinhibition of surrounding, non-resistant cells, by induction of adaptive growth by specialized cells, and/or inhibition of intercellular communication. A limited number of tumor promoters have also been reported to modify specific intercellular control mechanisms, both *in vitro* and *in vivo*.

PB exerts sedative effects on the central nervous system and is used today primarily as an antiepileptic agent [74,75]. This and structurally similar compounds also evoke other profound responses in living organisms, in some cases pleiotropic responses including hypertrophy of the liver. Furthermore, PB triggers a partially evolutionarily conserved induction of proteins involved in "sensing" the environment and detoxifying potentially harmful molecules [75-77]. These genes contain specific DNA sequences, such as the proximal promoter sequence in the CYP2B1gene, and PB response elements (PBRE) which are transcriptionally activated by the constitutively expressed nuclear receptor that binds androstane [78-81]. Recent data also indicate that the expression of PB-responsive genes can be modulated by histone acetyl transferases such as pCAF/p300 and that PB may evoke hypermethylation of GC-rich regions in DNA [82,83].

PB has been widely used as an antiepileptic drug in large numbers of individuals and there is no evidence that such usage is associated with harmful side effects, such as initiation or promotion of HCC [84]. However, in rodent models PB was the first

promoter to be characterized in connection with the 2-stage or initiation-promotion concept of hepatocarcinogenesis [47]. Thus, in rats and mice initiated with genotoxic carcinogens, administration of PB, which itself is not genotoxic increases the frequency of hepatocellular tumor formation approximately 5-fold [85]. In this context, it has been reported that PB enhances cell proliferation and reduces the rate of cell death in hepatic EAF [50,86-88].

1.4 APOPTOSIS

Two types of cell death, i.e., apoptosis and necrosis, have been extensively studied. Apoptosis mediates the normal turnover of cells and is considered to a physiological process. In contrast, necrosis results from damage produced by, e.g., hypoxia or exposure to xenobiotics. The term "apoptosis" was coined in analogy to "mitosis" and suggests a "falling-away" of cells [89].

The morphological features associated with apoptosis include nuclear condensation, loss of microvilli, cytoplasmic budding and nuclear fragmentation [90], processes requiring energy and the action of endogenous endonucleases. The apoptotic bodies thus formed are phagocytized by neighboring epithelial and mesenchymal cells, including macrophages, and subsequent lysosomal degradation of these bodies prevents the release of substances that might cause an inflammatory response like that associated with necrosis. Apoptosis is a frequent event in connection with tissue resorption, involution of endocrine organs, remodeling of hepatocellular hyperplasia and cell death in growing tumors [91,92].

It has been proposed that four major functional groups of proteins are involved in triggering and sustaining the apoptotic process, i.e., members of the superfamily of receptors for tumor necrosis factors (TNF), caspases, adaptor proteins and members of the Bcl-2 family. Certain TNF receptors (e.g., Fas and TNFR1) trigger apoptosis and are therefore referred to as death receptors [90]. These receptors are activated upon binding of their ligands (e.g., TRAIL, TNF-α, the Fas ligand) and the expression of certain of them (e.g. Fas, KILLER/DR5) is regulated by p53. In this way, p53 may influence an apoptotic response at the receptor level [93,94].

The death receptors contain a death domain (e.g., the FAS-associated death domain, FADD) and are associated via adaptor proteins with caspases, thereby providing a potential link through which DNA damage may activate caspases [95]. The complex formed between FADD, pro-caspase-8 and clusters of the Fas receptor is often referred to as the death-inducing signaling complex, or DISC [96]. Binding of pro-caspase-8 to FADD induces conformational changes and subsequential cleavage of the pro-caspase into the active protease. Expression of certain genes may prevent the assembly of DISC, one such gene being FLIP (FLICE inhibitory protein), variants of which are present in both viral (herpes virus) and cellular genomes [90].

Caspases are cysteine proteases that cleave their protein substrates next to an aspartic acid residue. Known critical target substrates for this enzymes include poly-(ADP-

ribose)-polymerase (PARP), DNA-PK and MDM2. In the case of certain substrates, the role played by this proteolysis in cell death is not clearly understood [97].

Another target for caspase action is the anti-apoptotic protein Bcl-2, which belongs to a family of pro- and anti-apoptotic proteins containing a BH3 domain. Bcl-2 is capable of interacting with Bax, NOXA and PUMA through this BH3 domain and this interaction may be responsible for preventing apoptosis. The genes encoding Bax, NOXA and PUMA are up regulated by p53, leading the accumulation of these proteins in mitochondria and subsequent release of cytochrome c, pro-caspase-9 and Apaf-1 from these organelle [98-100]. Upon activation, caspase-9 in turn activates caspase-3, thus giving rise to the morphological changes characteristic of apoptosis.

Death receptors play a prominent role in the induction of hepatocyte apoptosis, even through TRAIL-dependent death signaling is blocked under normal physiological conditions [101]. Hepatocytes express Fas constitutively and treatment of mice with anti-Fas antibodies may trigger the Fas receptor, thereby producing massive hepatocyte apoptosis and subsequent liver failure [102]. *In vitro,* EAF hepatocytes have been shown more resistant to apoptosis induced by anti-Fas antibodies than are non-EAF hepatocytes[103]. *In situ* these same cells have been reported to express the Bcl-2 protein at elevated levels, although other investigations suggest that the level of Bcl-2 mRNA is actually lowered in EAF [104,105].

1.5 THE TUMOR SUPPRESSOR GENE P53 AND CANCER

The p53 gene has been the focus of many studies by cancer researchers, due the fact that this gene is mutated in approximately 50% of all human cancers. About 80% of these mutations are missense mutations, which most often interfere with the ability of p53 to interact with DNA [106]. In addition, other protein components involved in the stabilization or degradation of p53 can be functionally altered in cancers containing no p53 mutations, further emphasizing the importance of p53.

Germline mutation in one allele of p53 is associated with the highly penetrant, autosomal dominant family of cancers known as the Li-Fraumeni syndrome[107]. Individuals suffering from this syndrome develop multiple tumors at several different sites, with the risk for developing an invasive tumor being nearly 50% for patients when reaching the age of 30. The corresponding value for individuals reaching the age of 70 is 90% [107].

Patients afflicted by the Li-Fraumeni syndrome, 50% of whom carry mutations in one p53 allele [108], exhibit early tumor development at several sites. Such mutations are associated with a narrow spectrum of different types of tumors, including osteosarcomas, soft tissue sarcomas, brain tumors, leukemias and carcinomas of the lung and breast. It is of interest to note that mice heterozygous for p53 (+/-) develop a similar spectrum of tumors [109,110].

The Li-Fraumeni syndrome is also associated with heterozygous germline mutations in the kinase CHK2 gene [111]. This kinase is associated with phosphorylation of Ser-20 on p53, which is proposed to disrupt the MDM2-p53 feedback-loop (se below) [112].

In connection with hepatocarcinogenesis induced by DEN in the rat, only a few mutations are actually found in the p53 gene [113]. Even exposure of rats to aflatoxin B1, a compound capable of causing mutations in codon 249 of human p53, does not give rise to p53 mutations in EAF. Thus, although p53 mutations may occur in HCC, they appear to be rare events during the development of preneoplastic foci.

The p53 gene encodes a 393 amino acid-long protein with an apparent molecular weight of 53 kDa (as determined on denaturating gels). This protein contains at least three major functional domains, i.e., the N-terminal, acidic transactivation domain (TAD); the central DNA-binding domain; and a third C-terminal domain involved in oligomerization. Moreover, p53 possesses several sites for post-translational modification, along with nuclear import/export signals and a basic domain functioning as a repressor of DNA-binding.

P53 is undetectable or present at relatively low levels in the cytoplasm of unstressed cells. The subcellular localization of p53 in the cytoplasm, which is an essential factor in its function, is influenced by the associated Parkin-like protein (Parc), which functions as a cytoplasm anchor for this protein [114,115]. In response to several types of cellular stress (i.e., double-strand breaks, nucleotide depletion, introduction of single-stranded DNA, loss of cell-matrix interaction, hypo/hyperoxia and hyperproliferative stimuli), p53 is stabilized post-translationally and accumulates in the nucleus of the cell.

P53 is considered a stress center or node where a number of post-translational signals converge. Stabilized p53 forms tetramers, which are involved in recruiting important factors for transcriptional activation (e.g., TAFII, p300/pCAF) to specific p53-responsive elements in DNA [116]. Such responsive elements are typically located within the open reading frame or proximal to gene promoter sequences, thus participating in activating the transcription of down-stream genes. In this manner p53 functions as an activating transcription factor and transactivates a subset of genes.

Target genes for p53-induced activation include the cyclin-dependent kinase inhibitor p21WAF1/CIP1 [117,118], GADD45 (a protein involved in DNA repair) [119] and pro-apoptotic proteins such as BAX, PUMA, KILLER/DR5, Apaf-1 and NOXA [98-100,120-122]. Thus, nuclear accumulation of p53 favors cell cycle arrest, DNA repair and cell death. P53 may also repress the expression of anti-apoptotic stimuli such as Bcl-2, thereby evoking apoptosis independently of transcriptional activation.

The overall physiological function of a p53 response appears to be maintenance of genomic integrity and thereby prevention of transformation of normal cells into malignant cells. Therefore, the gene encoding p53 is considered to be a tumor suppressor gene.

The absence of mutations in p53 during the hepatocarcinogenic process has led to the conclusion that inactivation of this protein is not of central significance in this context. However, recent findings have suggested that in neoplastic lesions in mouse liver, p53 may be inactivated by c-Jun [123]. Interestingly, expression of c-Jun is elevated in preneoplastic lesions [124] and this elevation is associated with hypomethylation of DNA, which can be counteracted by administration of methionine [125]. Furthermore, c-Jun has been found to be up-regulated in both chemically induced and spontaneous HCC [126-128]. Accordingly, although not mutated, p53 may be functionally inactivated by epigenetic mechanisms in EAF.

1.6 THE PROTO-ONCOGENE MDM2

1.6.1 MDM2 and cancer

The murine double minute gene 2 (MDM2) was originally isolated from a spontaneously transformed cell line carrying amplifications of this gene on extra chromosomal material in the form of double minutes [129-131]. Subsequently, this gene has been shown to be amplified in one-third of all human sarcomas examined; while analysis of a very large number of samples from 28 different types of tumors revealed an overall amplification frequency of 7% [132]. However, the involvement of MDM2 in carcinogenesis is probably not reflected accurately in these values, since, overexpression of this protein may occur without gene amplification, e.g., by enhanced stabilization and/or accelerated translation of mRNA [133,134].

Furthermore, MDM2 can also be expressed in the form of splice variants, the functions of which are poorly understood. It has been suggested that over-expression of such splice variants could eliminate the growth inhibitory action of the full-length protein. However, investigations on rodents suggest that the opposite in this case [135-139]. The possible presence of splice variants is seldom taken into account in clinical studies on a possible association between the level of expression of the MDM2 protein and tumor prognosis, which may help explain why the results reported are contradictory: overexpression of MDM2 appears to be associated with a poor prognosis in the case of certain types of tumors, but not others [139].

1.6.2 The central p53 – MDM2 autoregulatory loop

The most thoroughly characterized function of MDM2 is its involvement in the regulation of p53. Maintenance of low levels of p53 in unstressed cells by rapid turnover allows the cell to survive and the organism to develop properly. This conclusion is emphasized by the fact that MDM2 "knockout" mice die in an early stage of their embryonic development and can be rescued from this embryonic lethality by "knocking out" both p53 alleles [140-143].

The turnover of p53 is regulated by direct interaction between N-terminal regions of this protein and MDM2 in the nucleus of the cell. Binding of p53 induces the formation of a hydrophobic cleft in MDM2, into which the alpha helix of p53 inserts. Conserved

amino acid residues in both proteins then interact via van der Waals forces, resulting in stabilization of the p53 – MDM2 complex [144,145].

Although, the exact details remain unclear, MDM2 functions as an ubiquitin E3-ligase, joining ubiquitin moieties onto both MDM2 itself and p53 [146]. Ubiquitinylation is thought to be required for the subsequent export of these proteins from the nucleus, perhaps by exposing export signals (NES) contained in their structures [147,148]. The mechanism by which MDM2 promotes degradation of p53 is distinct from this E3-ligase function, as well as from the mechanism by which MDM2 promotes autoproteolysis. This degradation involves phosphorylation of serine residues within a conserved, acidic central core domain of MDM2, together with subsequent recognition and degradation by the 26S proteasome [149-151].

Finally, physical interaction between MDM2 and p53 conceals the transactivation domain on the latter protein, thereby functioning as a transcriptional repressor, which prevents transcriptional activation of p53-inducible genes involved in cell cycle arrest and apoptosis. [152].

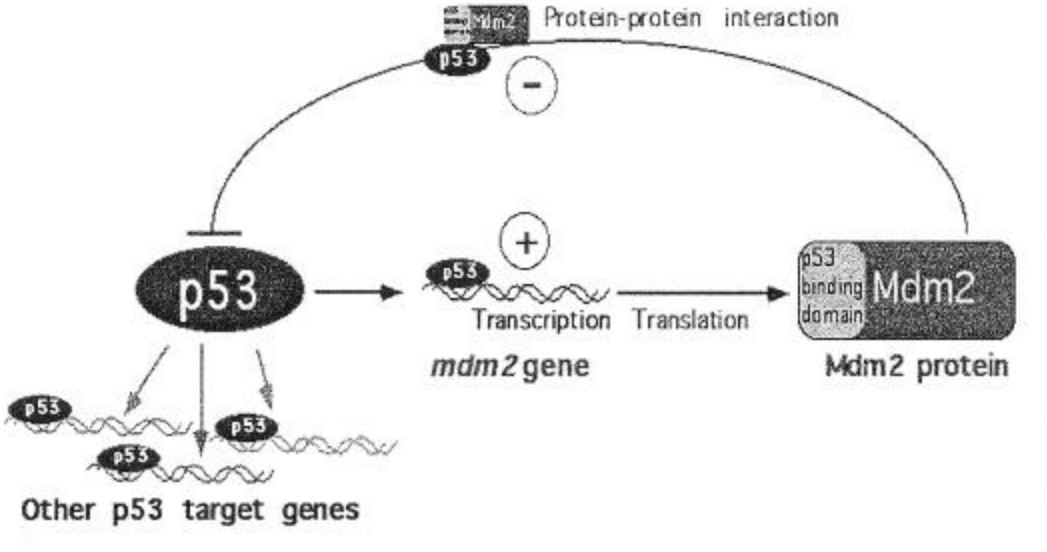

Figure 3. **The p53 – MDM2 autoregulatory loop** [142].

1.6.3 Escape from this loop

1.6.3.1 MDM2

Although a limited number of reports dealing with transcriptional inhibition of the MDM2 gene in response to UV irradiation or alkylating agents have appeared [153-155], the major mechanism by which p53 "escapes" regulation by MDM2 involves post-translational phosphorylation of either protein [156-159]. Such modification either abolishes the interaction between p53 and MDM2 (see above) [140,142-145,149,159-161], prolongs nuclear retention of these proteins and/or alters the ability of MDM2 to ligate ubiquitin to and/or degrade p53. Together, these changes p53 to escape degradation and transcriptional repression.

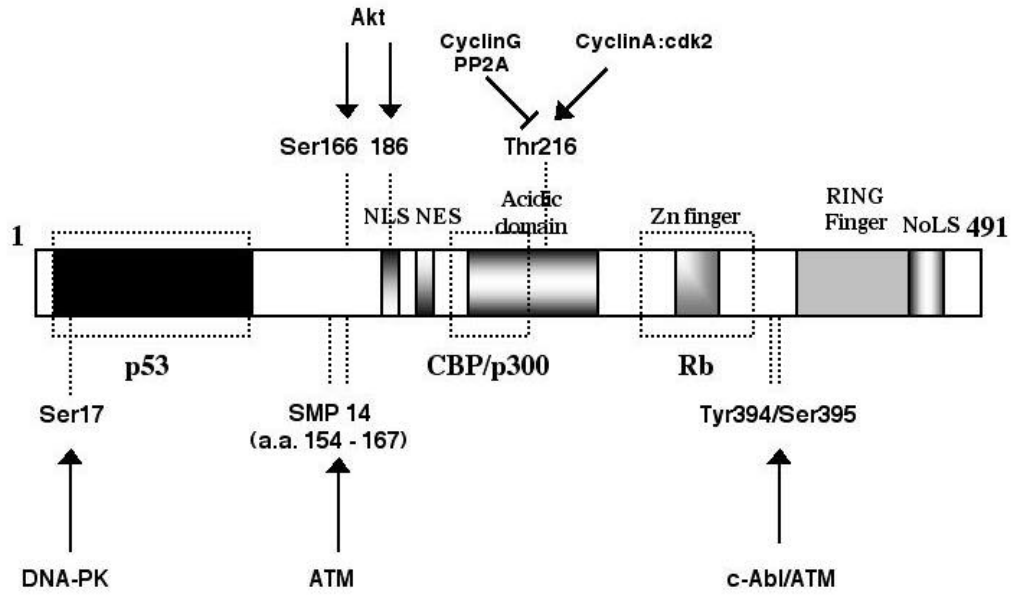

Figure 4. **Some of the sites and enzymes involved in phosphorylation of MDM2**. The bar represents the 491 amino-acid long MDM2 polypeptide with putative functional domains (not to scale). Phosphorylation of Ser17 on MDM2 by DNA-PK *in vitro* [162] prevents interaction between p53 and MDM2. Following irradiation, ATM rapidly forms a complex with MDM2, resulting in phosphorylation of the SMP14 epitope containing Ser and Thr residues [163], an event which precedes accumulation of p53. Phosphorylation of Ser166 and Ser186 (demonstrated by mass spectrometry) is dependent on activation of the Akt kinase and facilitates translocation of MDM2 into the nucleus [164]. Constitutive activation of Akt also results in lowered cellular levels of p53. Thr216 is phosphorylated by the cyclin A:cdk2 complex, predominantly after cyclin A accumulation during early S-phase, thereby enhancing interaction with p53 and slightly counteracting interaction with $p14^{ARF}$ [165]. A quaternary complex of cyclin G with the phosphatase 2A (PP2A) can dephosphorylate Thr216 and transcription of the gene encoding this cyclin is regulated by p53 [166]. In response to DNA damage, c-Abl interacts with and phosphorylates MDM2 at Tyr394 [167], a process that is independent of the ATM-catalyzed phosphorylation of Ser395 following irradiation although both events promote p53 accumulation by impairing its degradation [168].

ATM appears to be the primary regulator of the ability of MDM2 to interact with p53, although recent data indicate that c-Abl also plays an important role in this context[167]. Akt exerts an important influence on the subcellular localization of MDM2 [164]. Interestingly, down-regulation of Akt through PTEN sensitizes cells to etoposide treatment, suggesting that Akt may be a useful target for chemotherapy [169].

1.6.3.2 p53

The stabilization of p53 by post-translational phosphorylation is catalyzed by several different kinases (Figure 5). However, the pattern is complex and it is not known at present exactly which modifications occurring in response to DNA damage are detrimental to p53 stability. Characterization of the kinetics and thermodynamic properties of the p53-MDM2 interaction using a set of truncated p53 peptides has revealed that phosphorylation of Thr18 is responsible for eliminating this interaction [170], whereas phosphorylation of Ser15 and Ser20 does not affect the complex formation at all.

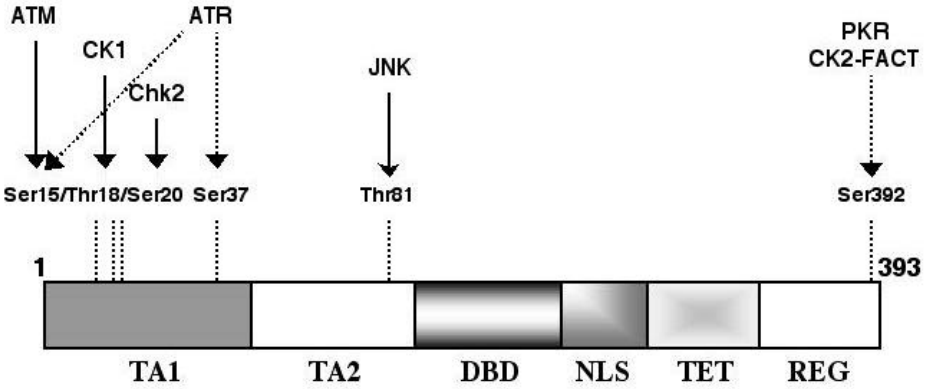

Figure 5. **A few of the known sites for phosphorylation of p53**. The bar represents the 393 amino acid-long p53 polypeptide; and regions associated with transactivation (TA), sequence-specific DNA binding (DBD), nuclear localization (NLS), tetramerization (TET) and DNA-mediated negative regulation of specific DNA binding (REG) are indicated (not drawn to scale). ATM is activated primarily by irradiation whereas UV irradiation activates ATR, and both of these proteins are able to phosphorylate p53 on Ser15. This modification may be followed by phosphorylation of Thr18 by Ck1 and of Ser20 by Chk2 [171], Thr81 is phosphorylated by the Jun N-terminal-kinase (JNK) in response to DNA damage. Inhibition of this phosphorylation results in reduced p53-mediated transcriptional activation, reduced inhibition of growth and induction of apoptosis [172].

The Ser15 and 18 residues are evolutionarily conserved and some findings suggest that Ser15 is phosphorylated prior Ser18 [171]. Phosphorylation of Ser15 has also been demonstrated to influence transcriptional activation via CBP/p300 [173]. Although not affecting the interaction between p53 and MDM2 directly, phosphorylation of Ser20

plays a role in the regulation of p53 turnover [174]. In response to UV irradiation *in vivo* ser392 is phosphorylated, which is also the case following ionizing radiation, although to a lesser extent. This latter phosphorylation stabilizes the p53 tetramer and thus exerts a synergistic effect on the binding of p53 to DNA [175]. Stress- and cell-specific patterns of phosphorylation and/or stabilization may also exist [176-178].

ATM-catalyzed phosphorylation of p53 on Ser15 facilitates subsequent phosphorylations of Thr18 by casein kinase 1 (CK1) and of Ser20 by the checkpoint kinase 2 (Chk2). Following UV-induced DNA damage, CK2 forms a multisubunit complex with the chromatin transcriptional elongation factor (FACT) [179], as a result of which the substrate specificity of CK2 is altered so as to exhibit a preference for Ser392 on p53. Thus, several kinases may form a cascade, which affects the stability and function of p53. This complex pattern of regulation, together with the fact that p53 may also be modified post-translationally in other ways, may explain why the p53 response to different types of stress and in different cell types is so heterogeneous [176-178].

1.7 THE P53 – MDM2 NETWORK

1.7.1 The ataxia-telangiectasia mutated (ATM) protein

Ataxia-telangiectasia (AT), an autosomal recessive disorder linked to mutations in the ATM gene, is characterized by cerebellar ataxia, telangiectases, defective immunological defenses and an enhanced predisposition for malignancy. Affected cells display an increased frequency of double-strand DNA breaks and are abnormally sensitive to the lethal effects of, but resistant to the inhibition of DNA synthesis caused by ionizing radiation [180]. AT patients develop lymphomas, leukemia and solid tumors such us medulloblastomas and gliomas with abnormally high frequencies [181,182]. Approximately 10 – 15% of these individuals are diagnosed as having lymphomas and leukemias at an early age and their relative risk for developing these types of tumors are several hundred-fold higher than that of the general population [183,184]. Interestingly, ATM and p53 knockout mice display a similar predisposition to develop the same type of thymic tumors, confirming the importance of interactions between these proteins in this tissue [109,185,186]. ATM, 370-kDa phosphoprotein belonging to the phosphatidylinositol-3 kinase family of proteins, phosphorylates several central protein regulators of the cell cycle (e.g., p53, Chk2 and MDM2) in response to DNA damage [163,168,187,188]. ATM is expressed in all tissues and localized primarily in the nucleus, particularly in proliferating cells. It has been suggested that ATM does not sense DNA damage by interacting directly with strand breaks, but rather by sensing alterations in chromatin structure [189]. Approximately 50% of all ATM molecules are activated by 5 minutes of exposure to 0.5 Gy [189]. ATM can also be activated by several other types of non-genotoxic and genotoxic agents, including radioactive substances and topoisomerase inhibitors. Some of these, including heat-shock and arsenite may evoke ATM-mediated phosphorylation of Ser15 on p53 [190,191]. Recently, UV irradiation was also reported to induce such phosphorylation.

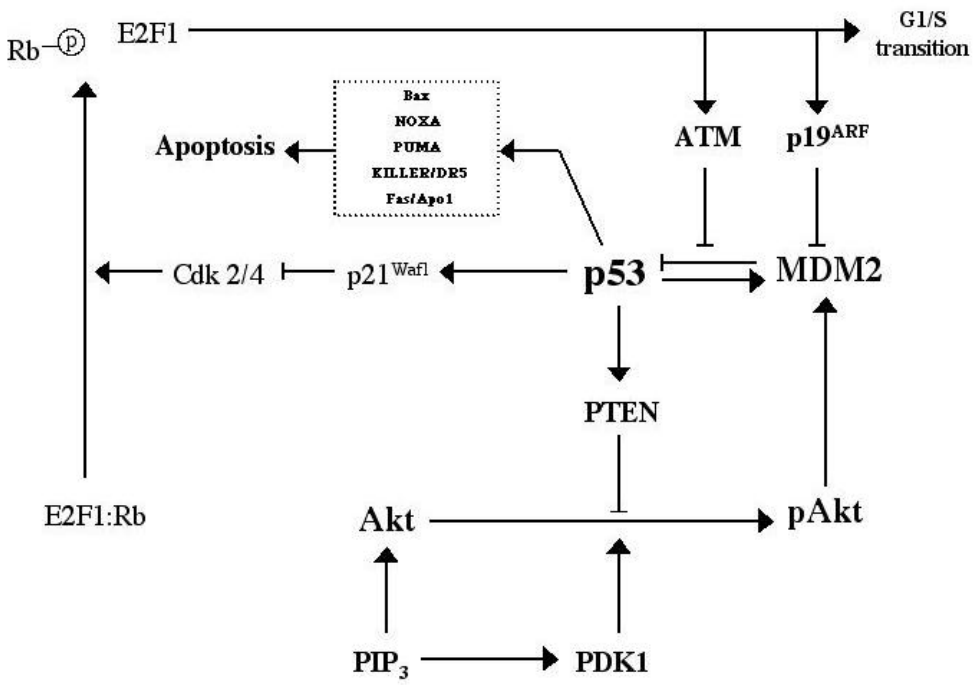

Figure 6. **The p53 – MDM2 network**. T-bars designate antagonistic, inhibitory effects, whereas arrows indicate a positive synergistic effect on, e.g., transcription, protein – protein interaction or phosphorylation. Transactivation of the MDM2 gene by p53 [192] results in increased synthesis of MDM2. MDM2 interacts directly with p53 [193], thereby antagonizing p53-mediated transcription and increasing p53 turnover [194,195]. Furthermore, p53 induces several apoptosis-mediating proteins [94] [99,100] [98] [186], the cyclin-dependent kinase inhibitor $p21^{WAF1}$ [196] and the tumor suppressor PTEN [197]. $P21^{WAF1}$ inhibits phosphorylation of Rb by cdK2/4 [198], which would otherwise disrupt the Rb-E2F1 complex [199]. In the presence of growth factors, the release of E2F1 from Rb promotes entry into the cell cycle. E2F1 may also up-regulate the expression of ATM and $p19^{ARF}$, thereby stabilizing p53 [200] [201]. By promoting dephosphorylation of membrane-bound PIP_3 (phosphatidylinositol triphosphate), PTEN indirectly inhibits Akt from becoming phosphorylated at Thr-308/Ser-473 [202,203]. Growth factors regulate the level of PIP3, which functions as a membrane anchor for PDK1 and Akt1 [204]. PDK1 catalyzes phosphorylation of Akt at Thr-308 and Ser-473 [205] and the p-Akt thus formed phosphorylates MDM2 on Ser-166 and Ser-186, in this manner facilitating translocation of MDM2 into the nucleus and subsequent degradation of p53 [164,206]. Thus, PTEN may form a "positive" feedback loop together with p53, increasing sensitization for DNA damage [169], in contrast to the "negative" feedback loop involving MDM2.

1.7.2 Akt1/Protein Kinase B

Phosphoinositide 3-kinases (PI3Ks) catalyze the formation of specific inositol lipids involved in the regulation of cell proliferation, survival and differentiation, as well as of certain alterations in the cytoskeleton. One of the most thoroughly characterized targets for these lipid regulators is the protein kinase Akt, also referred to as protein kinase B (PKB) [204]. In quiescent cells, PKB resides in the cytosol in relatively inactive conformation. Upon stimulation, of the cell, PKB is activated via recruitment to cellular membranes by PIP_3 and subsequent phosphorylation on Thr308 and Ser473 by 3-prime phosphoinositide-dependent kinase-1 (PDK1). In a manner not yet investigated in

details, phosphorylated and activated PKB is then translocated from the cytoplasm into the nucleus.

Akt phosphorylates a number of proteins, some of which (e.g., caspase-9 and Bad) are pro-apoptotic [207,208], and has been shown to delay cell death [209]. Furthermore, Akt may activate E2F and increase cellular levels of cyclin D1 [210]. Of particular interest is the observation that Akt is able to, form a complex with and phosphorylate at least two residues on MDM2, thereby facilitating translocation of this latter protein from the cytoplasm to the nucleus [164,206,211]. In this manner, Akt may influence p53 stability and, indeed, inhibition of Akt via PTEN sensitizes cells to the lethal effects of ionizing radiation [169]. Akt is also overexpressed in, e.g., breast cancers and lymphomas [212-215].

1.7.3 c-Jun

c-Jun is a component of the transcription factor AP-1. Fibroblasts derived from fetuses of c-jun knockout mice progress inefficiently from the G1 to the S-phase of the cell cycle and demonstrate enhanced expression of p53 and p21 even under non-stressed conditions. In other cells, overexpression of c-Jun was found to repress expression of p53 and p21, even under conditions of stress by UV radiation. Apparently c-Jun down-regulates transcription of the p53 gene by binding directly to a responsive element in its promoter. Additional experiments have revealed that deletion of p53 counteracts defects in the cell cycle of fibroblasts lacking c-Jun [216]. c-Jun may also regulate $p21^{WAF1}$ protein levels by inhibiting p53 binding to the p21 promoter. As discussed above this same research group later provided evidence that activation of c-Jun and subsequent inactivation of p53 may lead to the development of HCC in mice [123].

1.7.4 PTEN

The phosphatase and tensin homolog gene located on chromosome ten (PTEN) codes for phosphatase that is frequently lost by deletion or mutation of this gene in the advanced stage of many forms of cancer (e.g., glioblastomas, colon cancers, endometrial cancers and leukemias). PTEN counteracts the activities of several tyrosine kinases and has been discovered to dephosphorylate PIP_3, the membrane-bound substrate causing Akt phosphorylation and activation (see Figure 3). PTEN is also a down-stream target gene for p53.

Thus, p53 mediates a positive feedback loop, wherein PTEN prevents the nuclear translocation of its antagonist MDM2 by counteracting Akt. An analysis of neoplastic breast epithelium and stroma has revealed that mutation of the p53 or PTEN gene is mutually exclusive in these tissues [217], indicating a functional relationship between these two tumor suppressors. There is also a strong link between germ-line mutations in PTEN and the development of the Bannayan-Zonana Syndrome (BZS) and Cowdens disease, both of which predispose the afflicted individual to tumor development.

1.7.5 Hypoxia-inducible factor (HIF)

Hypoxic stress regulates gene expression via induction of the so-called hypoxia-inducible factor 1 (HIF-1) [218,219]. This regulation is mediated by a dimer consisting of the alpha (HIF-1alfa) and beta (HIF-1beta/ARNT) subunits of HIF-1, which functions as a basic helix-loop-helix transcription factor [220]. It is the alpha subunit that is regulated by oxygen and under non-hypoxic conditions, HIF-1alfa is subject to rapid ubiqutination and degradation, a process which is enhanced by the VHL1 tumor suppressor. Under hypoxic conditions such interaction with VHL1 is, however, blocked by dehydroxylation of conserved proline residues in HIF-1alfa, resulting in stabilization of the protein [221-223].

The target genes for HIF-1 include those encoding erythropoeitin (EPO), NIP3 and VEGF. Furthermore, HIF-1 can stabilize p53 in the nucleus and induce the transcription of several genes whose products are involved in cellular responses to hypoxia [224,225]. Through a mechanism, which remains to be fully clarified, the hypoxic state can also be mimicked by the addition of $CoCl_2$. It is known that exposure to either hypoxia or $CoCl_2$ enhances the generation of reactive oxygen species [226].

It has been reported that under conditions of hypoxic stress HIF-1α interacts with p53 and protects it from degradation [218]. However, that this interaction alone is not sufficient to stabilize p53 is suggested by the finding that cells lacking HIF-1α also exhibit a p53 response to hypoxia [227]. This observation may reflect the fact that ATR is activated by hypoxia and phosphorylates p53 on Ser15 [228], thereby promoting accumulation of p53 in the nucleus and cell cycle arrest.

Parts of tumors are often subjected to hypoxia due, e.g., to a reduced blood supply and in the case of some cancers elevated expression of HIF-1alfa is associated with a poor prognosis [229,230]. The increased expression of VEGF in response to hypoxia might enhance angiogenesis [231] and stabilization of p53 may increase the selective pressure on transformed cells to "lose" this protein [228,229].

1.7.6 $p16^{INK}/p19^{ARF}$

The CDKN2A gene codes for two different proteins, i.e., $p16^{INK}$, an inhibitor of the cyclin-dependent kinases CDK4/6, and $p19^{ARF}$, which stabilizes p53. The mRNA´s for these two different proteins are produced by using two distinct promoters and different first exons (1α in the case of $p16^{INK}$ and 1β for $p19^{ARF}$) in connection with transcription [232,233]. These different exons are spliced together with the common exons 2 and 3, resulting in the usage of an *a*lternative *r*eading frame and no more than 50% identity between the $p16^{INK}$ and $p19^{ARF}$ proteins [233].

Inhibition of CDK4/6 by $p16^{INK}$ decreases phosphorylation of the Rb protein and increases complex formation between Rb and E2F, resulting in cell cycle arrest [234]. $p19^{ARF}$ interacts with nuclear MDM2 to stabilize p53 in some as-yet-unknown manner. MDM2 has been found to co-localize with $p19^{ARF}$ in nucleoli and it is possible that sequestration of MDM2 in these structures is what stabilizes nuclear p53 [232,235-237].

Activation of $p19^{ARF}$ has been shown to occur in response to the on-set of transcription of cellular oncogenes such as c-myc or of viral oncogenes like E1A [238] [239]. In this way, the cell may utilize $p19^{ARF}$ to increase its level of p53 and counteract hyperproliferative stimuli. Thus, CDKN2A is involved in cell cycle control through effects on both p53 and Rb and alterations at the CDKN2A locus, (e.g., methylation of CpG islands in the promotor) are also frequently present in a number of cancers. Although less common than in the case of p53, germ-line mutations in CDKN2A are associated with an increased risk for skin cancer and melanoma.

1.8 PREVIOUS INVESTIGATION ON P53 AND MDM2 IN PRENEOPLASTIC HEPATIC LESION

Such *ex vivo* investigations have been performed employing primary hepatocyte cultures derived from EAF in rats subjected to repeated injections of DEN [240]. In response to an *in vitro* challenge with DEN, GST-P-negative hepatocytes (assumed to originate from surrounding, non-EAF tissue) responded with increased expression of p53; whereas GST-P-positive (i.e., EAF) hepatocytes, only rarely demonstrated such a response, as determined by immunohistochemistry. Furthermore, DNA synthesis was arrested in virtually all of the GST-P-negative cells, but in only a small fraction of the GST-P-positive cells. These observations indicate that most EAF hepatocytes lack functional expression of p53.

In a follow-up *in vivo* study on rats [241], a challenging dose of DEN was seen to induce a marked p53 response in most centrilobular hepatocytes, while most EAF hepatocytes remained unaffected. Furthermore, the larger the EAF, the greater was the lack of expression of p53. This study suggests that the attenuated p53 response by EAF hepatocytes confer resistance to genotoxic stress and growth advantages.

In similar experiments performed in another laboratory [242], *in vitro* treatment of hepatocytes with DNA damaging agents evoked a less pronounced p53 response in isolated EAF than in non-EAF hepatocytes. Even ultimate carcinogens (which do not require metabolic activation) induced an attenuated response in EAF hepatocytes, as did x-rays as well. Thus, the attenuated p53 response by EAF cells cannot be explained by alterations in xenobiotic metabolism.

A more recent investigation focused on p53 and MDM2 expression by EAF (induced employing a different protocol) *in situ* [104]. These lesions also exhibited relatively low nuclear levels of p53, with accumulation of this protein in the cytoplasm. In addition, most of the EAF hepatocytes expressed MDM2 and Bcl-2 at high levels. It was concluded that MDM2 inactivates p53 in EAF hepatocytes, thereby promoting their clonal expansion and malignant transformation.

1.9 TRANSGENIC MICE

1.9.1 p53 knock-outs and transgenes

Several mouse models designed to help elucidate the molecular relationship(s) between genes and, e.g., cancer has been developed. Most commonly, the aim of such models is to overexpress a certain gene, such as a proto-oncogene, or to knockout (eliminate) a tumor suppressor gene, either partially (allelic deletion) or completely ("null"). In addition, there are now more refined procedures available for generating "conditionally" transgenic or knockout mouse models, in which the alteration in the genome is strictly confined to a certain organ or cell type, thus providing insight into the spatial and temporal control of gene expression. Such conditional alterations may be achieved by, e.g., utilizing tissue-specific abundance of transcription factors in combination with the Cre-lox system [243].

Most p53 knock-out (-/-) mice undergo normal embryonic development, but these animals are prone to spontaneous development of a variety of neoplasms, most frequently lymphomas and sarcomas by 6 months of age [109]. However, p53 heterozygous (+/-) mice exhibit only limited spontaneous tumor formation up to 9 months of age and no more than 50% lethality after 18 months of postnatal life. In the case of humans, p53 has been found to be mutated in approximately 50% of all tumors. Mutation of one allele usually results in loss of the second allele (LOH) as well [244]. Some reports suggest that a low p53 gene-dosage accelerates tumorigenesis and that a "second hit" is not required for this process. One example of this is the finding that most tumors that develop in p53 +/- mice retain the wild-type, functional p53 allele.

Heterozygous p53 +/- mice are also considered be a relatively good model for the Li-Fraumeni syndrome. Patients with this syndrome typically have a germ-line mutation in one of their p53 alleles and are predisposed to develop osteosarcomas, lymphomas and soft tissue sarcomas, as are p53 +/- mice. The intact p53 allele is lost in 50% of the tumors in these patients, providing additional evidence that the gene dosage is of importance in this context [245].

Interestingly, "super p53" mice, whose cells contain an extra p53 allele in addition to the two endogenous ones, are less susceptible to cancer induced by chemicals than are the wild-type animals. Despite their enhanced p53 response to DNA damage, these mice show no signs of premature aging [246]. In another mouse model, there are two point mutations in p53, which prevent gene transactivation. These animals are predisposed to develop tumors, indicating the importance of p53-dependent transactivation in protecting against tumorigenesis [247].

1.9.2 The use of heterozygous p53 mice in risk assessment

C57BL/6 mice heterozygous (+/-) for p53 have been employed in testing for carcinogenicity. In addition to their intact p53 allele, the cells of these animals contain one allele, from which intron 4 and exon 5 have been deleted, preventing transcription [109]. p53 +/- mice exhibit increased sensitivity to tumor formation in response to genotoxic agents, so that it has been suggested that a 6-month period of testing (during which time these animals develop very few spontaneous tumors) might suffice.

However, the sensitivity of p53 +/- mice to development of tumors in organs such as the mammary glands, lungs or liver is not elevated compared to wild-type animals [248-250]. C57BL/6 mice are considered to be relatively resistant to tumor formation in the liver and skin [251], which may be one reason why p53 +/- mice are no more sensitive to the induction of HCC by genotoxic agents such as DEN, dimethylnitrosamine (DMN) and 6-nitrochrysene [248] [252]. However, C57BL/6 mice heterozygous (+/-) for TGF-beta receptor type II or overexpressing TGF-alfa are more susceptible to induction of liver tumors by DEN [253], suggesting that the choice of the gene to be altered may have a greater impact than the genetic background of the mouse strain involved. In summary, although they do not appear to be more susceptible to tumorigenesis in the liver, p53 +/- mice display an overall increased susceptibility to tumor formation in response to treatment with genotoxic agents.

2 THE PRESENT STUDY

In the present study, the attenuated p53 response exhibited by preneoplastic liver lesions has been characterized further. The general aim was to provide evidence that the early development of hepatic lesions is an adaptive response to genotoxic damage.

The specific aims were as follows:

- to characterize the relationship between the number and size of p53-attenuated EAF and the length of DEN treatment
- to compare the potencies of genotoxic DEN treatment and non-genotoxic tumor promotion with respect to the number and size of p53-attenuated EAF induced
- to examine upstream factors of possible importance for the attenuation of the p53 response
- to correlate the appearance of EAF hepatocytes with the spatial and temporal zonation of the MDM2-p53 response to DEN treatment
- to determine the influence of p53 gene dosage on the number and size of p53-attenuated focal hepatic lesions that develop in mice

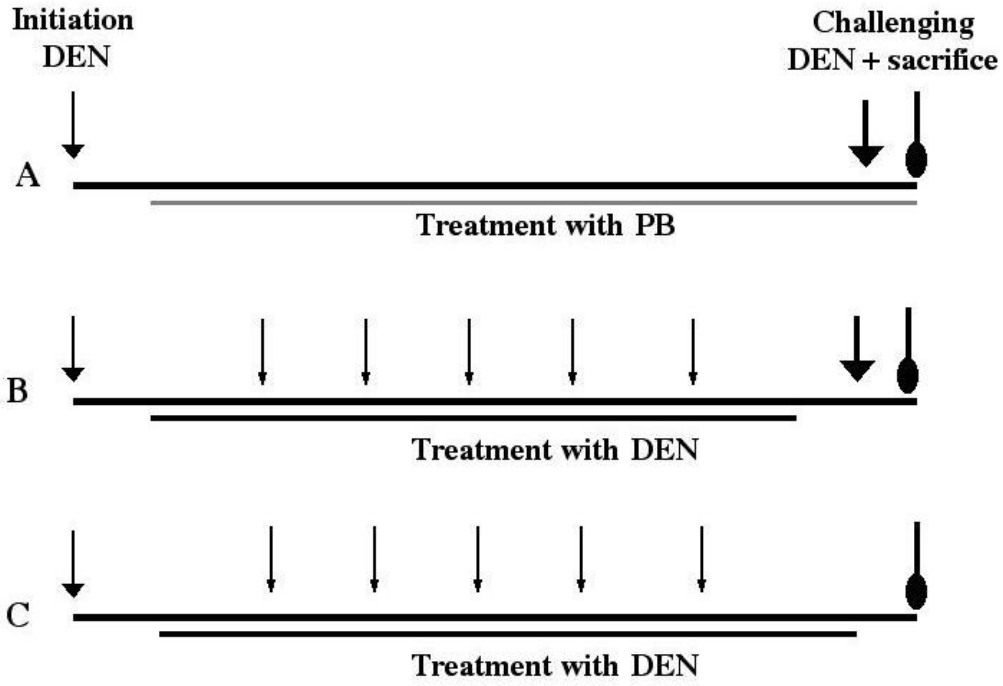

Figure 4. **Schematic representation of the treatment schedules employed to induce EAF in rats.** Female Sprague-Dawley rats were initiated neonatally (slim arrows) with DEN (0.33 mmol/kg bw). **A:** phenobarbital was administered (PB, 500 ppm in the drinking water) from three weeks of age **B, C:** DEN (0.33 mmol/kg bw, i.p.) was injected once each week (small arrows) and, in B, as a challenging dose (0.66 – 1.32 mmol/kg bw; bold arrows) 24 hr prior to sacrifice. Tissue from rats receiving a challenging dose of DEN (**A** and **B**) was employed for immunohistochemical and Western blot analysis. Rats not receiving such a challenging dose (**C**) were used for isolation of hepatocytes by collagenase perfusion and subsequent *in vitro* studies.

2.1 RESULTS AND DISCUSSION

2.1.1 Xenobiotics modulate the p53 response in EAF

These studies were designed to compare the non-genotoxic tumor promoter phenobarbital (PB) and the genotoxic complete carcinogen diethylnitrosamine (DEN) with respect to the their potencies in inducing EAF of similar sizes with an attenuated p53 response (analyzed both immunohistochemically and by Western blotting). Immunohistochemical staining for glutathione-S-transferase Pi (GST-P) was employed to identify EAF. Animals treated for 2 weeks with DEN or for 3 months with PB developed hepatic EAF of similar sizes, as did animals treated for 10 – 12 weeks with DEN or for 14 months with PB.

Not unexpectedly, the extent of attenuation of the p53 response to the challenging dose increased with increasing length of the period of DEN treatment. Thus, after two weeks of DEN treatment, 59 ± 6.0 % of the EAF were p53-negative; while the corresponding value following 10-12 weeks of treatment was 91 ± 3.8%. In contrast, no such increase was observed with prolonged PB treatment.

In both of the groups of rats injected once weekly with DEN (see Figure 7), the proportion of p53-positive cells in EAF compared to the corresponding value in surrounding, non-EAF tissue increased with the size of the EAF. Again, this phenomenon was not observed in the case of PB treatment. An increased frequency of cell death (as monitored by TUNEL staining) was detected in EAF from PB-treated rats administered challenging dose of DEN.

Based on these findings, we conclude that the p53 response to DNA damage in EAF can be modulated by exposure to other xenobiotics and, furthermore, that down-regulation of p53 may provide cells with a growth advantage. The altered p53 response detected in EAF from DEN-treated rats may act as an adaptation to the genotoxic effects of this compound. PB is considered to be non-genotoxic and, thus, not have a defensive role to play in connection with exposure to this compound.

We reason that mutation in the p53 gene is not the cause of the attenuation of the p53 response following treatment with DEN. Such mutations in unstressed cells often lead to accumulation of p53 in the nucleus, which is not consistent with the fact that the p53 response in EAF hepatocytes is attenuated after prolonged treatment. Furthermore, mutations in p53 are rarely observed in rat liver tumors [254,255]. The correlation between the extent of attenuation and the size of the EAF suggests that an attenuated p53 response provide cells with a growth advantage during continuous DEN treatment. These increasing proportions of p53-attenuated hepatocytes enhance the probability for further malignant conversion.

2.1.2 The expression of ATM is attenuated in EAF

These studies were designed to elucidate the mechanism(s) underlying the attenuated p53 response to DNA damage exhibited by EAF/GST-P-positive hepatocytes. Employing rats treated with weekly injections of DEN for 6 – 11 weeks (see Figure 7),

hepatocytes were isolated by collagenase perfusion and co-cultures containing both hepatocytes from EAF (GST-P-positive hepatocytes) and surrounding tissue (GST-P-negative hepatocytes) was established [256]. In such primary cultures, GST-P-positive hepatocytes retain the phenotype and demonstrate an attenuated p53 response to genotoxic compounds [240,242,256,257]. Here, culturing was performed on collagen coated dishes (35 mm in diameter) with 250,000 cells/dish, conditions found to be optimal for subsequent immunocytochemical staining.

An inhibitor of nuclear export, leptomycin B, was used to show that p53 enters the nucleus of GST-P-positive hepatocytes *in vitro*. Following treatment with Leptomycin B (5 ng/ml), 16.2 ± 1.9% of GST-P-negative hepatocytes accumulated p53 in the nucleus, while the corresponding value for GST-P-positive hepatocytes was 10.5 ± 1.5 %. This relatively high number of GST-P-positive cells that accumulate p53 in their nucleus gives an indication of the transcriptional activity of these cells.

We mimicked hypoxia with $CoCl_2$ in order to look for an alternative signal transduction pathway that might stabilize p53. We observed an increase in the expression of HIF-1α (proposed to bind to p53 and thereby increase its stability; see above) in response to incubation of our hepatocyte co-cultures in the presence of $CoCl_2$. Indeed, incubation of these co-cultures of GST-P-positive and -negative hepatocytes with subtoxic doses of $CoCl_2$ resulted in increased stabilization of p53 compared to incubation with DEN (0.33 mM) (approximately 15% versus 1 – 2%, respectively). $CoCl_2$ also caused a dose-dependent decrease in DNA synthesis and increased accumulation of MDM2 in the nuclei, indicating the presence of a transactivation competent p53 response.

Upstream mediators of p53 stability were also examined. Since several PI3 kinases have been proposed to stabilize p53 following DNA damage (e.g., ATR, DNA-PK and ATM) we treated our co-cultures with caffeine and wortmannin, which inhibit these kinases, in combination with DEN. 150 nM wortmannin inhibited the p53 response to DEN by approximately 50%; whereas 1 mM caffeine reduced this response from 12.5 to 5.0%. With the support of other published studies, ATM was identified as a potential mediator of the p53 response to DNA damage induced by DEN [258-260]. In contrast, neither wortmannin nor caffeine had any effect on the p53 response induced by $CoCl_2$.

Furthermore, prevention of the translation of ATM mRNA using the antisense technique resulted in a significant decrease in the p53 response to DEN *in vitro*, providing further evidence for the involvement of ATM in DEN-induced p53 stabilization. Immunohistochemical staining suggested that the level of ATM in EAF was abnormally low, a finding confirmed by Western blotting. In addition, the kinase activity of immunoprecipitated ATM was low. ATM has been shown one of the major kinases involved in the phosphorylation of p53 at Ser15 known to occur in response to DNA damage [187,188,191,261,262]. Accordingly, we detected enhanced phosphorylation of Ser15, but no significant alteration in the degree of phosphorylation of Ser20 following treatment with DEN.

Considered together, these findings suggest that the signal transduction pathway leading to stabilization of p53 following DNA damage is attenuated in EAF hepatocytes. A partial explanation for this phenomenon may be provided by the

lowered expression of ATM. Down-regulation of the phosphorylation of p53 induced by DNA damage may confer a growth advantage on EAF hepatocytes.

2.1.3 Expression of p53 in midzonal areas and initiation of preneoplastic lesions

In attempt to explain the observation that preneoplastic lesions in rodent liver often lack a p53 response to DNA damage, we looked for a liver-specific alteration(s) in the p53/MDM2 autoregulatory feedback loop. The majority of published reports in this area describe the co-regulation of p53 and MDM2 in *vitro* cultures, often employing transfection to obtain overexpression, rather than studying the endogenous proteins. Interactions between p53 and MDM2 *in vivo* have been investigated to only a very limited extent. However, the p53 response to ionizing radiation (IR) *in vivo* was recently found to exhibit both interorgan and intraorgan heterogeneity [176,263]. For example, IR elicits a weak p53 response in the liver, in contrast to the readily detectable response evoked by DEN.

In the present studies, the acute responses of p53 and MDM2 in rat liver to exposure to DEN were characterized employing immunohistochemistry, Western blotting, RT-PCR and *in situ* hybridization. Nuclear accumulation of MDM2 could be detected within 3 hours following exposure to DEN and in the majority of the hepatocytes in centrilobular and midzonal areas, this nuclear MDM2 was phosphorylated at the 2A10 epitope. These findings suggest that ATM and/or c-Abl is involved in signalling to MDM2 following DEN-induced damage [167,168,264].

Both p53 and MDM2 could be detected immunohistochemically in the nuclei of hepatocytes located in perivenous and midzonal regions of the liver 6 hours after treatment with DEN. During the period 12 – 24 hours after DEN treatment, the nuclear staining for p53 and MDM2 in midzonal areas was progressively lost and, in the case of MDM2, the cytoplasm became stained instead. Twenty-four hours after DEN treatment, nuclear staining only in hepatocytes in centrilobular areas, where somewhat fewer cells stained for p53 and MDM2, but with increased intensity. At this time-point, apoptosis (demonstrated by TUNEL-staining) was confined mainly to centrilobular regions of the liver, together with entrance of hepatocytes into the S-phase of the cell cycle (monitored by PCNA staining).

Analysis by Western blotting confirmed these immunohistochemical data, although using the former procedure the level of MDM2 was observed to peak earlier. RT-PCR analysis revealed no major increase in the corresponding mRNA levels prior to 24 hours after DEN treatment. *In situ* hybridization indicated that MDM2 mRNA was expressed primarily in the p53-positive centrilobular areas of the liver 24 hours after DEN treatment. All of these data are consistent with previously published findings on transcriptional activation mediated by p53 binding to responsive elements in DNA [265].

Considered together, the relevant data suggests that in midzonal hepatocytes, which constitutively express higher levels of cytoplasmic MDM2, DEN evoked a more

transient p53 response without inducing apoptosis. In the case of, rats pre-treated with only three weekly injections of DEN, the small preneoplastic lesions that developed were localized preferentially in midzonal areas and expressed high levels of cytoplasmic MDM2. We propose that the high constitutive level of MDM2 expression in midzonal areas attenuates the p53 response, thereby facilitating the development of preneoplastic lesions.

2.1.4 P53 (+/-) mice develop fewer p53-attenuated preneoplastic hepatic lesions

As discussed above, mice that are heterozygous (+/-) for p53 appear to exhibit an overall increased sensitivity to the effects of genotoxic agents and are considered to be a useful system for carcinogen testing [266-268]. However, these mice do not demonstrate increased susceptibility to the induction of HCC by DEN [248]. In order to investigate the impact of an altered p53 gene dosage on the development of preneoplastic lesions in mouse liver, C57BL/6 female mice which were +/+ or -/+ with regards to p53 received an injection of DEN once each week for 19 weeks (in most cases). The resulting preneoplastic lesions were identified employing a combination of markers (i.e., H/E staining, increased expression of cyclin D1 and decreased or increased expression of FABP) together with morphological examination.

The number and size of preneoplastic hepatic lesions induced by DEN in p53 +/+ and +/- mice were the same, in agreement with previously published data [248,269,270]. The p53 status of these lesions was tested for by administering a challenging dose of DEN 24 hours prior to sacrifice (in order to activate p53), after which tissue sections were stained immunohistochemically for p53 phosphorylated at Ser15. The percentage of preneoplastic lesions which were p53-deficient was found to be lower in p53 +/- than in +/+ mice (74% versus 17%, respectively; $p < 0.05$), suggesting that the p53-negative lesions do not arise as a result of a mutation in the p53 gene.

We also noticed that in p53 +/+ mice, the p53-deficient hepatic lesions were significantly larger than lesions expressing normal levels of p53, suggesting that the former possessed a growth advantage. Twenty-four hours after the challenging dose of DEN, western blotting revealed pronounced up-regulation of p53 in the surrounding, non-EAF hepatic tissue. However, a clear p53 response was detected only in macroscopic lesions dissected from the liver of p53 +/- mice.

Our present findings indicate that focal hepatic lesions exhibiting an attenuated p53 response do not possess the same growth advantage in p53 +/- as in p53 +/+ mice. This difference may be related to the described consequences of differences in p53 gene dosage [109,110,246,271,272]. Moreover, our results question the general belief that p53 +/- mice provide a system for testing potential hepatocarcinogens, which is more sensitive than the p53 +/+ mouse.

2.2 ADDITIONAL FINDINGS

The results documented in papers I, II and III and in a previous publication [104] indicate that both DEN-induced lesions and lesions elicited by other treatments express high levels of MDM2. In order to examine the relationship between the expression of MDM2 and GST-P (routinely used as markers for EAF), we reanalyzed the liver slices obtained from the rats described in paper I. Of the four groups of rats used in that study, two were treated with repeated (2 or 12) weekly doses of DEN and the other two received an initiating dose of DEN and were then exposed to phenobarbital for 3 or 14 months. The animals in all four groups received a challenging dose of DEN 24 hours prior to sacrifice (for further details, see Figure 7 and paper I).

As documented in Table 1, there was a convincing overlap in the staining for GST-P/p53 and for MDM2. Thus, cytoplasmic staining for MDM2 appears as reliable a marker for EAF as staining for GST-P. The small differences between the two patterns of staining can be attributed to the fact that the serial sections employed were not identical. In summary all GST-P-positive EAF, irrespective of the treatment protocol and length of treatment, also express high levels of cytoplasmic MDM2, which can thus be utilized as an alternative marker for EAF.

In addition, we found that in EAF containing a high proportion of hepatocytes staining for p53 (p53-positive EAF), many hepatocytes also exhibited both cytoplasmic and nuclear staining for MDM2. In the case of EAF, which stained positively for p53 in a discreet area, nuclear staining for MDM2 was observed in this same area. Thus, nuclear staining for MDM2 may be related to accumulation of p53, e.g., via p53-induced translocation of MDM2 into the nucleus.

These data indicate that p53 can accumulate in EAF hepatocytes even in the presence of high cytoplasmic levels of MDM2. Nuclear accumulation of p53 following DNA damage has also been observed in tumor cell lines that overexpress MDM2. Furthermore, our present findings suggest that MDM2 can be translocated to the nucleus of EAF cells in response to p53 expression. One possibility that cannot be excluded here is that EAF hepatocytes express two splice variants of the MDM2 protein, one of which accumulates in the nucleus and the other in the cytoplasm.

It is possible that this co-localization of GST-P and MDM2 in EAF reflects the involvement of these two proteins in a programmed response, which is activated in most EAF hepatocytes. However, the observation of a similar pattern of staining for MDM2, but not for GST-P, in midzonal hepatocytes in the liver of untreated animals (paper III) is also striking. This latter correlation may indicate that MDM2 is of particular functional significance for EAF and midzonal hepatocytes and/or that EAF derive from midzonal hepatocytes.

Staining of serial sections for PTEN and p-Akt (i.e., Akt phosphorylated at Ser-473) revealed enhanced staining for PTEN in many EAF compared to surrounding tissue, with concomitant decreased staining for p-Akt. This finding is not surprising, since PTEN is known to be a negative regulator of Akt. Our interpretation of the poorer

agreement between GST-P and MDM2 is that we missed smaller lesions in sections stained for PTEN or p-Akt and the data set is therefore incomplete.

The functional significance of these alterations in the expressions of PTEN and p-Akt in EAF remains to be elucidated. It is possible that the cytoplasmic localization of MDM2 is related to the low levels of activated Akt. Phosphorylation of MDM2 on Ser166 and 186, by pAKT facilitates translocation of MDM2 to the nucleus [164].

EAF induced by prolonged (14-mounth) treatment with PB contained levels of PTEN, which were higher than those in EAF arising in response to DEN treatment. In these PB-treated animals, the pattern of staining for PTEN correlated most closely with those for MDM2 and GST-P (see Table I). An inverse correlation between staining for PTEN and p-Akt, similar to that observed in EAF, was also seen in the hypertrophic perivenous areas of the liver following PB treatment. These hypertrophic zones demonstrate distinct morphological characteristics, including increased cell and decreased nuclear size, as is well-documented [273,274]. In summary, expression of PTEN was enhanced in EAF and exhibited a zonated pattern similar to the regional patterns of hypertrophic cell morphology and decreased expression of p-Akt [275].

Constitutive overexpression of the tumor suppressor PTEN at early stages of tumor development may increase the selective pressure for EAF to lose PTEN during the later stages of tumorigenesis, when the rate of cell proliferation in such lesions is elevated. The significance of PTEN as a factor in the development of HCC is supported by the observation that the PTEN gene is mutated in some HCC [276,277].

Table I: Comparison of the patterns of EAF staining for various markers

	No of EAF/cm^2 ± SD			
Treatment	**p53/GSTP**	**MDM2**	**PTEN**	**pAkt**
DEN (2 weeks)	10.0 ± 1.60	8.70 ± 1.30	5.70 ± 0.16	2.68 ± 1.80
PB (3 months)	9.72 ± 1.95	8.87 ± 1.26	8.53 ± 3.22	7.02 ± 1.55
DEN (12 weeks)	101 ± 2.13[1]	96.1 ± 31.9	49.2[2]	-[3]
PB (14 months)	42.7 ± 18.5	37.3 ± 9.08	34.4 ± 11.4	-[3]
	Mean EAF area (mm^2) ± SD			
Treatment	**p53/GSTP**	**MDM2**	**PTEN**	**pAkt**
DEN (2 weeks)	0.016 ± 0.005	0.016 ± 0.006	0.029 ± 0.009	0.028 ± 0.016
PB (3 months)	0.029 ± 0.008	0.029 ± 0.015	0.045 ± 0.024	0.040 ± 0.015
DEN (12 weeks)	0.27 ± 2.31[1]	0.31 ± 0.047	0.33[2]	-[3]
PB (14 months)	0.38 ± 0.15	0.43 ± 0.23	0.42 ± 0.22	-[3]
	Area fraction (%) ± SD			
Treatment	**p53/GSTP**	**MDM2**	**PTEN**	**pAkt**
DEN (2 weeks)	0.16 ± 0.026	0.14 ± 0.036	0.17 ± 0.049	0.090 ± 0,090
PB (3 months)	0.29 ± 0.13	0.25 ± 0.12	0.34 ± 0.053	0.25 ± 0.13
DEN (12 weeks)	27.8 ± 2.31[1]	36.2 ± 2.72	29.4[2]	-[3]
PB (14 months)	18.1 ± 15.2	16.9 ± 14.3	16.2 ± 13.7	-[3]

[1] two rats analyzed [2] one rat analyzed [3] not analyzed

2.3 CONCLUSIONS

Our present findings indicate that in rodent liver the p53 and MDM2 responses to DNA damage are heterogeneous with respect to the number of hepatoctyes affected, the level of expression of either protein, and the duration of the response. The initial response involves most of the hepatocytes in the midzonal and centrilobular areas, whereas at later time-points this response is sustained in centrilobular regions only. This pattern highlights the complexity of *in vivo* responses to genotoxic agents.

Small EAF, which develop in response to low doses of DEN, arise primarily in midzonal areas of the liver, which constitutively express cytoplasm MDM2 at relatively high levels. In these areas, the p53 response to DEN treatment is also transient. This relationship, as well as the observation of an almost complete overlap in the patterns of expression of GST-P and cytoplasmic MDM2 in EAF, suggests that MDM2 plays an important role in connection with the initiation of hepatocytes and development of EAF.

EAF, which develop following several weeks of DEN treatment, demonstrate progressive attenuation of their p53 response to a challenge by DEN. The correlation between the extent of this attenuation and the length of DEN treatment and size of the EAF suggests that clonal expansion of p53-attenuated EAF hepatocytes take place. In contrast, EAF whose formation is induced by treatment with PB show no such correlation. Thus, these two xenobiotics may promote clonal expansion of EAF hepatocytes with different phenotypes.

The lowered levels of ATM in EAF from DEN-treated rats may explain at least in part, the attenuation of the p53 response in these lesions. However, *in vitro* GST-P-positive hepatocytes express p53 in response to a hypoxia-mimicking agent. Down-regulation of ATM may therefore constitute a DNA damage-specific adaptation in EAF hepatocytes.

p53 (+/-) mice develop fewer preneoplastic lesions with an attenuated p53 response than do p53 wild-type (+/+) mice. This situation may reflect the presence of decreased selective pressure for p53-attenuated cells in a population of p53 +/- hepatocytes. Effects of p53 gene dosage on several genes have been documented and may explain this phenomenon.

Altogether, our present findings indicate that p53 is an important factor in connection with clonal expansion of putative preneoplastic EAF hepatocytes promoted by the genotoxic compound DEN. They lend further support to the hypothesis that EAF develop as an adaptation to toxicological stress. The attenuation of the p53 response to DNA damage in EAF appears to be explained at least partially by decreased expression of the ATM kinase.

It is often argued that genotoxic carcinogens initiate carcinogenesis by inducing mutations and that there is no threshold for this effect. The implication is that there is no “safe” level of exposure for many genotoxic carcinogens. Further studies providing more definitive evidence for epigenetic mechanisms of initiation may challenge this view.

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