

Review

Molecular and Cellular Mechanisms of Chemically Induced Hepatocarcinogenesis

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Abstract

Liver is the main organ of xenobiotic biotransformation. Since biotransformation may generate highly mutagenic active metabolites and reactive oxygen species, liver cells are both the producers and targets for these compounds. Xenobiotics can lead to liver tumour formation *via* genotoxic or non-genotoxic mechanisms. Non-genotoxic chemicals are often inducers of monooxygenase reactions depending on cytochrome P450 isoforms, active metabolites of which are potential carcinogens. Moreover, non-genotoxic xenobiotics influence expression of genes responsible for cell proliferation and apoptosis. Increased proliferation may lead to an increased number of cells mutated as a result of genotoxic effects. In animal models of hepatocarcinogenesis at least three steps of tumour development are characterized: initiation, promotion and progression, but it is still unclear what the cellular origin of the liver cancer is. It is likely that either cancer cells originate from differentiated adult hepatocytes or from undifferentiated liver stem cells. Better knowledge about cell changes in neoplastic transformation during hepatocarcinogenesis and gaining control over this process may lead to determination of therapy alternative to cytostatic treatment.

Keywords: cytochrome P450, genotoxicity, non-genotoxic carcinogens, proliferation, apoptosis, hepatocarcinogenesis, oval cells.

Abbreviations

AhR - aryl hydrocarbon receptor;
CC - cholangiocarcinoma;
CDK - cyclin-dependent kinases;
CYP - cytochrome P450;
HCC - hepatocellular carcinoma;
HRE - hormone response elements;
LCD - large cell dysplasia;
MAPK - mitogen-activated protein kinases;
PB - phenobarbital;
PB-like - phenobarbital-like;
PPAR - peroxisome proliferator receptor;
RB - retinoblastoma protein;
ROS - reactive oxygen species;
RXR - retinoid X receptor;

SCD - small cell dysplasia;
TCDD - 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

Introduction

Hepatic carcinogenesis is a multistep process most commonly induced by viruses and/or chemical compound action leading to mutations of oncogenes, tumour suppressor genes and DNA repair genes. Among risk factors of hepatocellular carcinoma (HCC) there are mainly chronic viral infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), metabolic disorders (genetic hemochromatosis, α 1-antitrypsin deficiency) and several genotoxic and non-genotoxic chemical carcinogens. These factors, and cofactors like alcoholic liver disease, cirrhosis, growth factors, maternal-infant transmission of HBV infection contribute to HCC spreading all over the world. Every year, about 1,000,000 new cases of HCC are diagnosed, out of which

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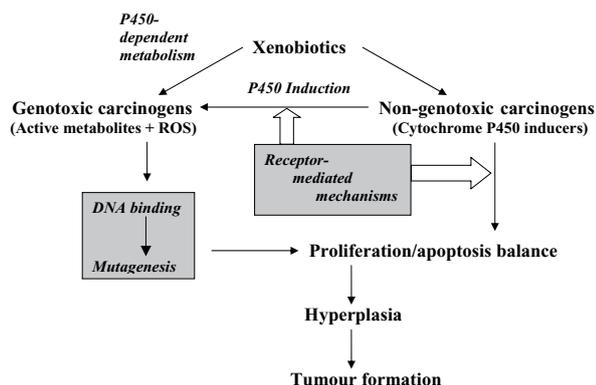


Fig. 1. Genotoxic and non-genotoxic mechanisms of chemically induced hepatocarcinogenesis. ROS- reactive oxygen species.

80% appears in men. Its spread is heterogeneous in different world regions because of exposure to the various risk factors. The incidence of HCC per 100,000 habitants is 5-40 times lower in western countries than in Southeast Asia and sub-Saharan Africa [1]. Poland belongs to the countries of low risk, in which the incidence does not exceed 5 in men and is slightly lower in women. In 1996, almost 2,000 new cases and over 2,300 deaths of primary malignant neoplasms of the liver were recorded. In Southern European countries, however, incidence of the disease increases two to sixfold [www.onkolink.pl].

Initiation of hepatocarcinogenesis by chemical compounds is strictly connected with metabolic activation of procarcinogens during monooxygenation reactions dependent on representatives of 1, 2, 3 and 4 families of cytochrome P450 (CYP). By-products of monooxygenation (biotransformation) are reactive oxygen species (ROS) [2]. Both activated carcinogens and ROS are able to produce genotoxic (mutagenic) effects by forming DNA-adducts or modifying bases of DNA, respectively (Fig. 1).

An important group of carcinogens are non-genotoxic carcinogens, which modify xenobiotics metabolism inducing cytochromes P450. They intensify in this way intracellular production of factors immediately binding and damaging DNA, consequently initiating multiple mutations (Fig. 1). Expression of cytochromes P450 may be induced either at the transcriptional or translational level [3]. The molecular mechanisms of CYP induction are mostly based on the ligand-cytoplasmic or ligand-nuclear receptor interactions. Among non-genotoxic hepatocarcinogens there are inducers affecting CYP1A subfamily (polychlorinated biphenyls, dioxins); CYP2B subfamily (phenobarbital-like - PB-like inducers: drugs; pesticides; solvents; plant products); and 4A subfamily (peroxisome proliferators: hypolipidemic fibrate drugs, plasticisers) [4, 5].

CYP1A inducers are ligands of the cytoplasmic aryl hydrocarbon receptor (AhR) characterized by a basic helix-loop-helix (bHLH) DNA-binding domain and a PAS (Per/AhR/Arnt/Sim) homology region for dimerisation [6]. In the absence of a ligand the AhR resides as a complex with Hsp90 (chaperoning heat-shock protein). AhR action in-

cludes ligand binding, release of heat shock protein Hsp90, receptor translocation to the nucleus, its dimerisation with transcription factor – nuclear translocator protein (Arnt), binding to DNA hormone (dioxin) response element and activation of gene transcription (Fig. 2A).

Action of structurally heterogeneous CYP2B and CYP4A inducers depends on nuclear orphan receptors: constitutive androstane receptor (CAR) and peroxisome proliferator receptor (PPAR), respectively (Fig. 2B). Liver specific PPAR is PPAR α [7]. These receptors, as well as pregnane X receptor (PXR) specific for CYP3A inducers, heterodimerise with the retinoid X receptor (RXR), bind appropriate DNA hormone response elements (HRE) and cause transactivation of several genes. PB is able to activate both CAR and PXR, and transactivate about 50 genes in the liver, which among the others is manifested by pleiotrophic effect and increased activities of xenobiotic metabolism of phase I and phase II [8, 9].

Many cytochrome P450 inducers, in addition to a range of monooxygenase active metabolites of genotoxic significance, are able to perturb rodent tissue homeostasis, leading to the proliferation of numerous mutated cells accompanied by programmed cell death repression (Fig. 1). These inducers are non-genotoxic carcinogens since they do not damage DNA, yet cause development of tumours during experimental hepatocarcinogenesis. These non-genotoxic carcinogens display both species and tissue specificity [10].

Interactions Between Xenobiotic and Endogenous Metabolism

It is important for the liver homeostasis that RXR is a dimerisation partner for several nuclear receptors mediating activity of endogenous ligands, e.g. vitamins A and D

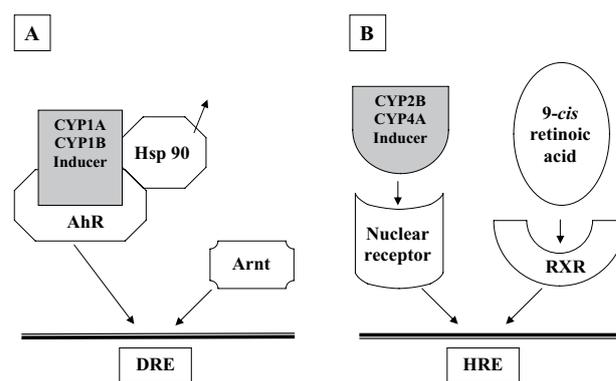


Fig. 2. The role of cytochrome P450 inducers in (A) cytoplasmic and (B) nuclear receptor-mediated transactivation. The inducers are also non-genotoxic carcinogens promoting development of liver tumours by signal transduction mechanism dependent on receptors being ligand-activated transcription factors and heterodimerising with Arnt protein or retinoid X receptor.

AhR- aryl hydrocarbon receptor; Arnt- AhR nuclear translocator protein; DRE- dioxin response element; Hsp90- heat shock protein 90; RXR- retinoid X receptor; HRE- hormone response element.

derivatives [11, 12]. Dimerisation of RXR with receptors presenting different signal transduction pathways dependent on both endo- and exogenous ligands, and having their own endogenous ligands by nuclear receptors, creates an opportunity for intercrossing those pathways and overlapping of physiological effects [13]. Using signal transduction paths that depend on receptors being at the same time transcription factors, non-genotoxic xenobiotics influence gene expression and physiological effects such as cell proliferation and apoptosis (Fig. 1). PB-like inducers and peroxisome proliferators stimulate hyperplasia and/or hypertrophy. Increased proliferation may lead to increased number of cells mutated as a result of genotoxic effects of xenobiotics metabolised *via* cytochrome P450. Hyperplasia dependent on phenobarbital (PB) action on the liver is one of the symptoms of the pleiotrophic effect resulting in increased organ mass and promotive action towards tumour development [8]. PPAR α mediating action of hypolipidaemic peroxisome proliferators is involved in regulation of hepatocyte growth and differentiation, it also mediates induction of peroxisomal enzymes responsible for fatty acid metabolism [14]. Discontinuation of hepatocytes mitotic stimulation results in restoring the normal status by induction of hepatocytes apoptosis. In this way, the pro-proliferative effect of non-genotoxic compound action may be reversible. Disruption of elimination of mutated cells *via* apoptosis can lead to proliferation/apoptosis disbalance and promotes uncontrolled cell proliferation [15]. There is evidence in literature that non-genotoxic carcinogens (TCDD, PB-like inducers, peroxisome proliferators) induce S-phase and suppress apoptosis in rodent hepatocytes *in vivo*. Some PB-like inducers similarly change the balance between proliferation and apoptosis in hepatocytes *in vitro*. Similarly, an increase in DNA synthesis, cell proliferation and hepatocarcinogenesis is observed after administration of organochlorine pesticides in mouse liver. Dieldrin, in principle, numbered among CYP2B inducers can also induce CYP1A and 1B subfamilies by unknown mechanism and its action is species specific. In contrast to rodent hepatocytes, disturbing the proliferation-apoptosis balance in human hepatocytes or other types of rodent cells is infrequent [10].

Xenobiotic Modifications of Proliferative Activity

Increased DNA synthesis and cell proliferation are the results of binding the receptors for non-genotoxic carcinogens to DNA and profile modification of cell cycle key genes. Physiologically, in the presence of mitotic signaling, the cell enters the G₁ phase (Fig. 3), which increases expression of the D cyclins. Their association with cyclin-dependent CDK4 and 6 kinases results in phosphorylation and activation of the CDKs. The activated CDKs then phosphorylate the retinoblastoma protein (RB). Control of the phosphorylation/dephosphorylation status of RB seems to be crucial for the cell moving from early G₁, and passing through restriction point (R) to late G₁ and S. Hypophospho-

phorylated (active) RB binding with E2F transcription protein inhibits transcription, whereas the hyperphosphorylated (inactive) RB allows E2F to dissociate and promote E2F-mediated transcription of genes required for S phase. The proteins functioning as a RB phosphorylation status regulators – CDK4/6 – are immediately negatively regulated by the INK4 proteins (p16^{INK4a}), while the Cip/Kip family inhibitors (p21) are indirectly involved in CDK4/6 inhibition through regulatory pathway including INK4a transcript - p19, murine double-minute 2 protein - MDM2, and transcription factor - p53. The latter protein inhibits RB phosphorylation *via* p21 activation and interrupts the cell cycle. In the absence of p53, RB and then E2F activation may be continued. For the passage from G₁ into S phase, an increased expression of cyclin E and cyclin E/CDK2 complex formation is required. CDK2 activity is under negative control of the p27^{Kip1} protein representing the Cip/Kip cyclin kinases inhibitors family [16, 17, 18].

The retinoblastoma protein is known as an important tumour suppressor factor. Chromosomal mutations and methylation status of retinoblastoma gene may be a molecular mechanism regulating its potential for expression and cell proliferation. Genetic alterations of the RB gene mapped on chromosome 13q have been described in one fourth of HCC cases [19].

Evidence implicates Ah receptor influence on normal cell proliferation and differentiation. It was shown in 2001 [20] that the LXCXE motif located in the Ah receptor's PAS homology domain, involved in protein dimerisation, takes part in RB protein binding. Evidence points to hypophosphorylated RB binding only to the AhR, because phosphorylation of RB Thr821 and Thr826 residues represses RB/LXCXE

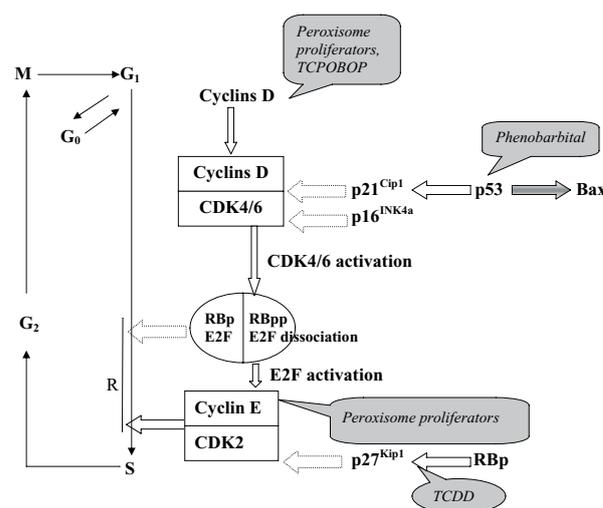


Fig. 3. The cell cycle regulators influenced by non-genotoxic carcinogens.

Solid arrows- induction; dashed arrows – inhibition; R – restriction point of the G₁ phase; CDK- cyclin-dependent kinases; RB- retinoblastoma protein; TCPOBOP- 1,4-bis[2-(3,5-dichloropyridoxy)]benzene (PB-like inducer); RBp- hypophosphorylated RB; RBpp- hyperphosphorylated RB.

interaction. This proves that carcinogens acting through AhR dependent mechanism may modify cell cycle course in quiescent G_0 and in early G_1 phase. The studies show that cells respond to TCDD differently in particular phases of the cell cycle. RB/AhR interaction appears necessary for maximal transcriptional CYP1A1 expression. Late G_1 cells accumulate 3-fold more CYP1A1 mRNA than do G_2/M cells and AhR-dependent activation of CYP1A1 by TCDD is markedly suppressed in G_2/M phase [21].

Several alternative models have been proposed concerning TCDD-induced AhR-mediated G_1 regulation. At first, it was suggested that Ah receptor contributes to G_1 cell cycle arrest mediating induction of CDK2/cyclin E activity inhibitor p27Kip1. Suppressed CDK2/cyclin E activity prevents cells from entering phase S [22]. Secondly, it was proposed that AhR and RB form a ternary complex with E2F and play a corepressor role which potentiates repression of E2F-dependent transcription and cell cycle arrest [23]. Most recently, Hines et al. [24] suggested that AhR-mediated G_1 arrest is a result of p27Kip1 induction preventing RB hyperphosphorylation and E2F→CDK2/cyclin E action. On one hand, AhR-mediated expression of p27Kip1 inhibits cell division and in turn prevents RB phosphorylation (inhibitory positive feedback). It seems likely that AhR should be connected with an agonist to obtain transcriptional activity towards the p27Kip1 gene, whereas cancel-

lation of p27Kip1 inhibiting action requires AhR inactivation. On the other hand, transition through the G_1 restriction point relies on RB hyperphosphorylation, E2F dissociation and cyclin E gene expression. CDK2/cyclin E activity facilitates RB phosphorylation and cell cycle progression (stimulatory positive feedback). In summary, G_1/S phase transition requires the E2F transcriptional activity and also AhR inactivation, which is under common regulation by RB phosphorylation (binary switching mechanism). Authors conclude that the carcinogenic effect of TCDD favors mutations promoting cell proliferation directly - interfering with AhR-mediated cell cycle regulation or indirectly - disrupting the RB pathway. There is a little discrepancy between described above *in vitro* anty-proliferatory, and *in vivo* pro-proliferatory effect of TCDD action observed after chronic exposure. Moreover, a cytostatic effect of TCDD observed in a variety of cell lines, e.g. rat hepatoma 5L cells, was not reported in murine hepatoma 1c1c7 cells and several others, non-hepatic lines of cells [21]. Hence, the exact role of TCDD in the cell cycle regulation on the transcriptional level is not decisively determined.

Xenobiotic Modifications of the Programmed Cell Death

The crucial role in control of proliferation/apoptosis balance is played by multifunctional transcription factor p53 [25, 26]. In normally replicating cells the expression of p53 is very low, which activates transcription at the RB promoter. In high concentrations p53 protein binds to the RB promoter and represses transcription. In cells with DNA damage or oncogene activation cycling is interrupted by phosphorylated p53 to allow time for repairs. When genomic damage makes DNA repair impossible, activated p53 induces the expression of the pro-apoptotic bax protein and cell cycle is arrested [27, 28]. Thus, p53, known as a tumour suppressor protein, controls the G_1/S restriction point in the cell cycle, inducing cell proliferation arrest and/or programmed cell death (Figs. 3 and 4).

Mutations of tumour suppressor p53 gene resulting in increased cell proliferation have been found in more than 80% of HCC. They often accompany RB gene mutations and together may contribute to hepatocarcinogenesis [19]. In humans G:C to T:A transversions leading to a substitution Arg/Ser were identified in African patients, and moreover, G:C to C:G substitutions in codon 249 of the p53 gene were described in HCC patients from China. Similar mutations are caused by aflatoxin B1 in rodent experimental hepatocarcinogenesis and it seems likely that they may be responsible for the high incidence of HCC in areas with high endemic exposure to this food contaminate. The aflatoxin B1 metabolism is dependent on cytochrome P450 (Fig. 5) and occurs as an example of genotoxic effect of the active metabolites (exo-8,9-epoxides). The results of DNA-epoxides interaction are N7 guanine adduct formation and the DNA depurination or, alternatively, formation of aflatoxin B1 formamidopyrimidine. Both effects preferentially produce G→T mutations [29].

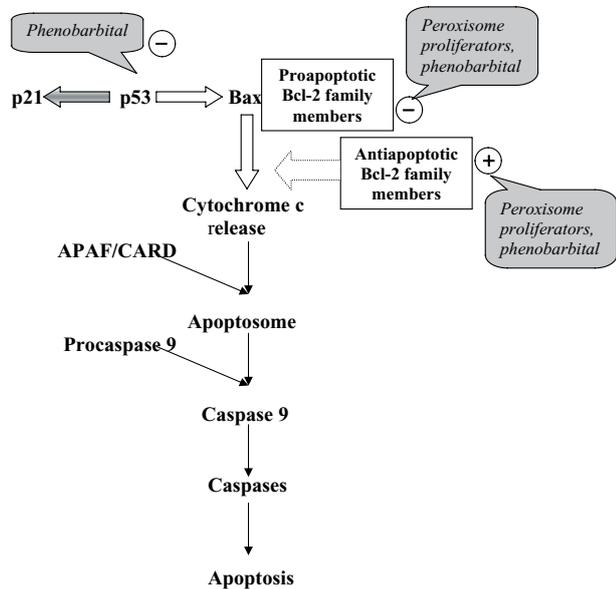


Fig. 4. Control of intracellular pathway of apoptosis by hepatic non-carcinogens. In cytoplasm, cytochrome c joins an apoptosis protease activating factor (APAF) containing caspase recruitment domain (CARD) and form apoptosome. This complex recruits procaspase 9, activates it causing in the same time activation of caspase cascade. Alternatively, extracellular signals (TGF β , TNF α , Fas-ligand, TRAIL) can activate caspases cascade through appropriate receptors, adaptors and procaspase 8. \ominus , dashed arrow - negative regulation; \oplus , solid arrow - positive regulation.

p53 is a link in endogenous apoptosis path, in which pro-apoptotic proteins from bcl-2 family translocate to the external mitochondrial membrane and cause cytochrome c and apoptosis inducing factor (AIF) release from intermembranous space. Data on the influence of non-genotoxic carcinogens on p53 expression are not uniform. For example, lower expression of p53 protein was observed after phenobarbital, but not peroxisome proliferators treatment [10].

On the other hand, non-genotoxic carcinogens have been described as factors which can cause, after acute treatment, both induction of antiapoptotic bcl-2 proteins (bcl-2, BAG1), and inhibition of proapoptotic members of bcl-2 family (bax, bak), what can be a reason for apoptosis arrest (Fig. 4). It was confirmed that the expression of several bcl-2 family members is altered both in a number of cancers, primary hepatocytes and mouse liver after PB-like inducers and peroxisome proliferators administration [30]. The mechanisms of P450 inducers-mediated inhibition of apoptosis may be different, and carcinogen- and cell-specific. The latter means that non-genotoxic carcinogens have an ability for selective modulation of gene expression depending on their location within hepatic lobules. As a consequence, it can result in a selective survival advantage for cells representing particular lobular zones. In mice, PB-induced bcl-2 expression and bax inhibition were localized to centrilobular zone 3, which is also a place of increased cell proliferation after PB administration. It suggests that neoplasms could potentially arise from this cell population. In comparison, peroxisome proliferators promote cancer development arising from cells located in periportal zone 1 in rodent liver.

Xenobiotic Control of the Cell Signalling Pathways Regulating Proliferation/Apoptosis Balance

Both proliferation and apoptosis are under control of cell signalling pathways, in which signal molecules are growth factors and cytokines used for signal translocat-

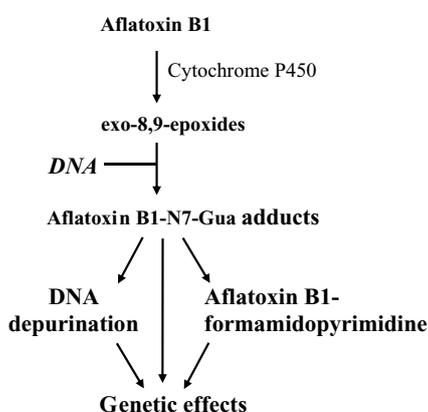


Fig. 5. Genotoxic effects of aflatoxin B1. Aflatoxins are known food contaminants in endemic areas of Asia and Africa which increase the risk of HCC in human and experimental animals. N7-Gua - N7 guanine.

tion different tyrosine kinases (Fig. 6). Receptor tyrosine kinase is a cytoplasmic part of the receptor, which auto-phosphorylates the receptor and through adaptor proteins activates Ras proteins and cascade of mitogen-activated protein kinases (MAPK). Kinases Src may be activated by receptor kinase or by other receptor types. This results in secondary messengers synthesis by effector enzymes. Finally, receptor tyrosine kinase-dependent and Src-dependent signal transduction paths activate proper transcriptional factors.

MAP kinases-regulated signals both for proliferation and apoptosis are activated by peroxisome proliferators. It was also found by Schwarz et al. [31] that AhR/HSP90 complex joins in cytoplasm Src, which under the conditions of TCDD induction, separates and activates the Ras-dependent transduction, AP-1 transcription factor and occurs pro-proliferative effect. Activation of *ras* by mitogens induces, among the others, cyclin D expression triggering the pathway of RB hyperphosphorylation and p27Kip1 suppression [32]. Genetic mutations of *ras* genes lead to activation of Ras-mediated signal transduction resulting in changes in expression of antiapoptotic bcl-2 and bcl-XL proteins, and enhances cell survival [30]. It must be pointed out, however, that genetically activated (mutated) genes of *ras* family, which are cellular oncogenes, as well as other oncogenes, have been found in several animal models of hepatocarcinogenesis, but not in human hepatocellular carcinoma. It can be different if compared to other human tumours.

The third group of tyrosine kinases, kinases Jak/Tyk, directly phosphorylates and activates STAT transcriptional factors (Fig. 6.). Similarly to receptors activating tyrosine kinases Jak/Tyk, act receptors for TGF β connected with serine-treonine kinases, directly phosphorylating and activating Smad transcriptional factors. It was found that the latter pathway is involved in apoptosome formation and caspases activation resulting in apoptosis. TGF β (and TGF α) regulates apoptosis as well as cell proliferation and bcl-2 proteins expression. Treatment of mice with PB leads to inhibition of TGF β expression in zone 3 of the liver acinus [33].

The Role of Oval Cells and Hepatocytes in Hepatocarcinogenesis

Increased proliferation of hepatocytes having enzymatic potential able to activate xenobiotics to toxic metabolites may be an organ reaction to intralobular necrosis, but also to damages caused by partial hepatectomy or viral infection. Afterwards, it appears compensative hyperplasia involving hepatocytes, creating about 90% of mature liver mass; cells which are highly differentiated and rarely divide in normal liver of adult humans and animals. Within the liver of the grown rat only one hepatocyte out of 10-20,000 may divide, activated mitotically, however, hepatocytes participate in quick and effective regenerative response happening simultaneously in different liver lobules. Hepatocytes localized near but not

directly adhering portal zone (zone 1 of the liver acinus) replicate earlier and more frequently than cells localized within the surroundings of the central vein (zone 3). Compensative hyperplasia, apart from hepatocytes, also includes (but to the significantly lower grade) non-parenchymal cells, but time for DNA synthesis to appear as well as that of mitoses is delayed from several to tens of hours in comparison to the hepatocytes [34, 35, 36].

Significant damages of hepatocytes done by hepatotoxic compounds, inflammatory statuses or liver cirrhosis stimulate proliferation and differentiation of multipotent cells during the regeneration process of human and animal liver. Similar cell reaction is observed in hepatocarcinogenesis [34, 37]. During extrafetal life, undifferentiated multipotential cells are probably presented in the periportal space and/or in the intrahepatic bile ducts, at first in intralobular canals of Hering and small interlobular ducts. Multipotent stem cells give the origin for heterogenous population composed of bipotential cells and blastic cells from hepatocytic or cholangiocytic lineages, being about 1-3% of cells of normal liver. Their common feature is small size (12-15 μm), relatively few cytoplasmic organelles and oval-shaped euchromatic nucleus (oval cells) [38].

Biotransformation-Proliferation Relations

Cause and effect relationships between hepatic necrosis, or hepatitis B, and HCC incidence in humans show that frequency of HCC incidence is strictly associated with intensified liver cell regeneration stimulated by their earlier destruction. Hepatocytes and oval cells share in reparative processes and in hepatocarcinogenesis significantly depend on impulse-causing organ damage and biotransformation-detoxication potential of the cells

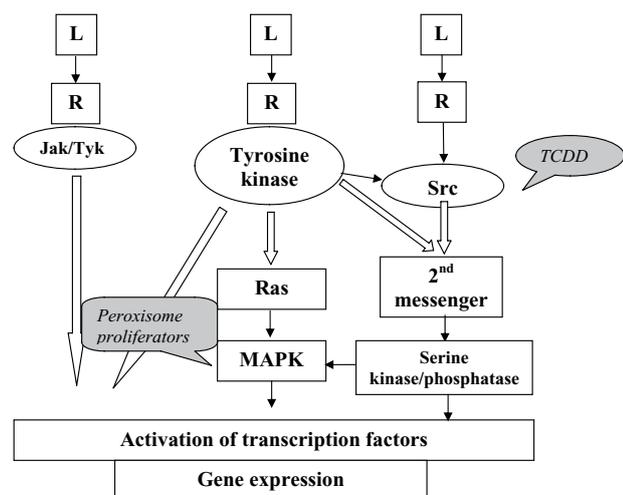


Fig. 6. Xenobiotic control of cell signalling pathways dependent on Jak/Tyk, receptor and non-receptor (Src) tyrosine kinases. L- ligand; R- membrane-bound receptor; MAPK- mitogen-activated protein kinases.

[34, 39]. As opposed to normal hepatocytes, hepatocytes initiated by chemical factor action, oval cells and biliary ducts cells exhibit weak expression of phase I enzymes of xenobiotic metabolism and strong expression of phase II enzymes. This promotes detoxication if compared to hepatocarcinogens activation, and may explain why oval cells in culture are resistant to toxic effects of exposure to procarcinogens, and also better than normal hepatocytes proliferate in experimental hepatocarcinogenesis [40]. *In vitro*, hepatocytes sampled from neoplastic nodules often do not show intensified tendency for proliferation and further transformation, whereas in cultures of oval cells the creation of cell clusters and spontaneous mitotic activity was observed. Therefore, there is potentially high probability that proliferatively activated oval cells become the source of the neoplasm.

Stimulated proliferative activity within the regenerating organ may effectively influence the increase of carcinogenesis initiation frequency through increased probability of mutation appearance. Single application of carcinogenic compound does not have to mean 'carcinogenesis', but repeatedly induced liver cell proliferation by mitogenic factors leads to accumulation of mutations, providing neoplastic phenotype manifestation and its promotion [41]. Lack of enzymes biotransforming hepatotoxic compounds and activating procarcinogens consequently would not be a sufficient factor protecting the cell from neoplastic transformation if probability of neoplasm incidence within the organ is proportional to the frequency of cell divisions. On one hand, within the liver exposed to toxic compounds, resistance of some cells to hepatocarcinogens is parallel to increased proliferative activity underlying the regenerative processes. On the other hand it creates the danger of neoplastic transformation, which can originate from, not participating in xenobiotics metabolic activation, oval cells and/or initiated hepatocytes.

Differentiation or Dedifferentiation?

The role of oval cells in histogenesis of primary liver neoplasms hasn't been precisely determined yet. Still, the question exists if neoplastic cell origin, within the damaged and regenerating liver, from abnormally differentiating maternal cells, as the effect of "maturation arrest", or from mature hepatocytes, "dedifferentiating" as a result of mutations cumulation [42]. Sometimes during experimental carcinogenesis the proliferation of oval cells without simultaneous hepatocytes mitotic activation, and transformation of oval cells transduced *in vitro* by an oncogene, leading to phenotypically various neoplasms, were observed. According to "stem cell" hypothesis, undifferentiated cells are stimulated by carcinogens, intensively proliferate into the adjacent periportal parenchyma, then form cords and ductular structures which have been shown to be connected to the portal bile ducts [43]. Initially, they show expression of intermediate cyokeratine filaments characteristic for biliary duct epithelium and also fetal α -fetoprotein and hepa-

toxic albumin. Part of ductular cells, expressing, among the others, bile duct-type cytokeratin CK7 and CK19, remains within the biliary ducts as cholangiocytes; part, expressing, among the others, hepatocyte-type cytokeratin CK8 and CK18, migrates from the ductules and becomes maternal population for parenchymal cells; part undergoes apoptosis. Proliferation abilities and direction of differentiation of oval cells are under control of both auto- and paracrine factors (HGF, TGF α , TGF β , FGF, IGF-I, IGF-II) which, among other things, appear to enhance the survival of carcinogen-activated cells by increasing the number of proliferating cells and suppressing apoptosis. They also depend on the modifying influence of mesenchyma, as well as on animal species and sex, on applied, in experimental model, hepatotoxic factors and on individual abilities for its metabolic activation, which reflects heterogeneity of individual metabolic sensitivity to xenobiotics action [34, 35, 39]. Cells with similar to the oval cells development characteristics are observed within the embryonal liver [44].

The longevity of oval cells seems to be sufficient to accumulate mutations under carcinogenic exposure, and some of the oval cells, as a result of multiple mutations, change direction of development. They maintain, however, division ability, and therefore create groups of descendant cells, from which, in turn, may originate neoplastic cells of hepatocytes or cholangiocytes characteristics [45]. The possibility of the coexistence of those differentiation directions is shown by the similarity of antigen markers of oval and cancer cells (eg. CK7 and CK19), and the presence of mutations in tumour suppressor gene Tg737, responsible for oval cell differentiation [46]. A significant role in the transformation into cancer cells seems to be played by an extracellular substance in the hepatocytes microenvironment, which apparently reduces probability of oval cell development towards neoplasm. The "stem cell" theory provides a satisfactory explanation for the occurrence of different liver cancers, apart from HCC, mixed hepatocellular and cholangiocarcinomatous tumours [47].

A different mechanism for hepatocarcinogenesis induced by peroxisomal proliferator WY-14643 was suggested, because neither oval cell proliferation nor enzyme alterations were defined [41]. Thus, alternatively we can assume that cells observed in intermediate stages of carcinogenesis, and finally cancer cells are succeeding dedifferentiation stages of mature hepatocytes, or that presented model of liver cells participation in neoplasia does not exclude each other.

Transformative changes observed in animal models for hepatocarcinogenesis were so far classified as 3 independent steps on hepatocytes dedifferentiation: initiation, promotion and progression [see lit. in: 19]. It was suggested that in a carcinogenic environment most hepatocytes undergo cytotoxic action of applied carcinogens. If carcinogen-induced DNA damages leading to initiation are irreversible, the cells presenting those damages may be eliminated from the liver through apoptosis. Some of the initiated cells as a result of mutation obtain resistance

to applied carcinogens owing to detoxifying enzyme activation. Result of this resistance is maintenance of division ability and creation of pre-neoplastic hepatic foci. More recently, these foci have been classified in humans as "small" and "large cell dysplasia" (SCD and LCD, respectively). Libbrecht et al. [46] showed that more than 50% of SCD foci contain not only hepatocytes, but also cells immunohistochemically relative to progenitor cells and intermediate hepatocyte-like cells. On the contrary, LCD foci were composed of fully differentiated hepatocytes of limited proliferation capacity. It was suggested that in foci of SCD, undifferentiated cells become mature hepatocytes, which is characteristic for regenerative events both in human and rodent liver. The differentiation process is preceded by an intensive proliferation of these cells. Because liver progenitor cells are a target either for chemical carcinogens or for viruses, it is possible that cells in SCD foci will also transform into cancer cells.

Probably different subpopulations of initiated cells clonally expand into dysplastic foci under the influence of additionally administered to the animals factors promoting hepatocarcinogenesis and/or performance of partial hepatectomy. In a number of different experimental models of hepatocarcinogenesis, simultaneous proliferation of oval cells and hepatocytes, was observed. The effects of chemical promoter depend on its dosage, time and continuity of exposure. Promoting factor at the same time inhibits divisions of hepatocytes surrounding foci. Altered hepatic foci exhibit increased rates of cell proliferation and cell death relative to surrounding hepatocytes, which demonstrate lower levels of DNA synthesis. Thus, changed balance of proliferation/apoptosis that can be influenced also by non-genotoxic carcinogens and cooperative interaction between certain activated oncogenes in initiated selected cells may determine clonal expansion, accelerate progression of cancer and contribute to determining the lesion phenotype.

The cells of preneoplastic foci potentially may return to the normal phenotype or undergo apoptosis. 1-5% of hepatic foci may exhibit progression to neoplastic or hyperplastic nodules built of hepatocytes characterized by increased basophilia, showing different expression of genes (oncogen activation, tumour suppressor genes inactivation) and activity increase of γ -glutamyl transpeptidase and glutathione transferase. Analysis of cell markers in hepatocarcinogenesis shows that migrating oval cells infiltrate neoplastic nodules and there undergo neoplastic transformation [48]. Every tenth preneoplastic focus and every fifth neoplastic nodule show in their cells expression of antigens typical for the oval cells and hepatocytes. Furthermore, as far as sizes, expression of some markers and polyploidy (2n) are concerned, cells appearing within many nodules soon after administration of a carcinogen resemble more the oval cells than twofold bigger, tetraploid hepatocytes, numerous within the normal liver.

Few neoplastic nodules create persistent nodules being cells groups showing progression of neoplastic transformation as a result of gene changes accumulation.

In summary, based on ultrastructural, morphological, biochemical and immunocytochemical investigations, it is not clear whether the cells in the persistent nodules take part in carcinogenesis as differentiating cells or arise from regenerating/dysplastic mature hepatocytes. Imperfection of the latter hypothesis results, among the others, from experimental evidence that despite the fact that composition of the extracellular matrix in foci of SCD promotes this direction of cell transformation, *in vivo* proliferating mature rat hepatocytes are not able to dedifferentiate. This process was observed only *in vitro*.

For the purpose of histopathological practice, in 1995, cytological criteria of liver nodules diagnosis were published according to The International Working Party of the Classification of Liver Nodules and, later, in the literature [19, 49]. It was mentioned that the liver nodules should be classified into "regenerative" and "dysplastic" lesions of low or high grade. Useful criteria in the differential diagnosis of liver nodules and hepatic carcinomas are:

1. Characteristic reticulin fiber arrangements outlining islands of abnormal hepatocytes or trabeculae more than 3 cells in thickness.
2. Increased CD34 (vascularization marker) expression in the endothelium of periportal and pericentral sinusoids depending on nodule capillarizations.
3. Transdifferentiation of tumour cells into neoductular cells expressing cytokeratines CK7 and CK19 (negative in HCC but positive in cholangiocarcinoma).
4. Expression of carcinoembryonic antigen (CEA) and MOC-31 antibody.
5. α -fetoprotein, a marker of undifferentiated liver cells expressed in about 48% of hepatocellular cases.
6. α -1-antitrypsin expression – reported in 18-73% of HCC.
7. Telomerase activity.

Genetic Studies on Hepatocellular Carcinoma and Cholangiocarcinoma origin

Earlier *in vitro* studies based on insertion of an oncogen into genetically marked hepatocytes, transplantation of those cells into the animal liver *in vivo*, and then observation towards neoplastic transformation showed that hepatocytes may be maternal cells for HCC, but they were not the source of cholangiocarcinomas (CC) [43]. The oval cells in similar studies revealed transformation ability towards both mentioned neoplasm types [50]. As part of the observed neoplasm types was morphologically undifferentiated, and taking under consideration the close relationship between hepatocytes and oval cells, which are a heterogenous population, and also strong, modifying influence of culture conditions on cell phenotype, those results should not be treated as the final ones. To avoid culture environment influence on experiment result and decisions about the hepatocytes role in CC genesis, oncogen transfer (e.g. activated k-Ras) to rat hepatocytes *in vivo* using a retroviral transduction was performed, strengthening the mutagenic effect by administering di-

ethylonitrozoamine to the animals [51]. In all zones of the liver acinus the creation of big groups of descendent cells presenting morphologic and enzymatic features of hepatocytes were observed. From those cells, after 3-5 months, arose HCC and CC. The fact, that hepatocyte may be the source of CC creation indicates its ability to dedifferentiate, under the influence of the oncogen, to the more primitive phenotype. If hepatocyte dedifferentiation is not the condition for proliferative activation during regeneration, or even neoplastic transformation towards HCC, it seems probable as the stage proceeding the change of cell differentiation direction, at least in animals.

Practical Aspects of Studies on Undifferentiated Cells

The oval cells present a population of undifferentiated cells able to generate new parenchymal cells in conditions when proliferative abilities of hepatocytes would be exceeded. This situation distinguishes functional maternal liver cells from other "stem" cells, present in epithelia regenerating in a continuous way [41]. Multipotential cells present in mature organs, having an ability for directional, tissue- and organ-specific differentiation may be used as cell vectors in widely applied gene therapy in humans and animals, e.g. in the treatment of congenital, metabolic diseases, requiring reparation of gene deficiencies [43]. One of the conditions providing its efficacy is to maintain, within the recipient's organ, genetically modified cells many years after a gene transfer was performed. Control over liver cells proliferation process should help to obtain permanent correction of genetic defects and restore liver function impaired because of cirrhosis. There is the possibility of sampling, through the biopsy, and running cultures of animal and human cells from biliary ducts epithelium providing a possibility for gene therapy *ex vivo* of maternal liver cells descendants obtained after their implantation into the liver or the spleen. The advantage of *ex vivo* therapy and transplantation of autologous liver cells is risk exclusion of graft rejection and no immunosuppression. Also, transplantation of mature, allogenic hepatocytes turned out to be an efficient way for correction of congenital metabolic defects in experimental animals and humans [34, 38].

Evidence shows that neoplastic cells may originate from undifferentiated liver cells, although after maturation this organ doesn't show proliferative activity comparable to digestive tract or skin epithelium or bone marrow cells. The oval cells, as target cell population for liver carcinogens, may be useful tool for neoplasm gene therapy, because they proliferate in most if not in all models of hepatocarcinogenesis, and stimulated towards neoplastic transformation *in vitro* and *in vivo* show multipotentiality and the ability to transform into HCC and CC, as well as into series of other neoplasm types e.g. hepatoblastoma, CC with intestinal differentiation of epithelium, intestinal adenocarcinoma and anaplastic tumours, which probably reflects the mutations appearing in different protooncogenes [39]. Getting to know the control of differentiation

process of "stem" cells during carcinogenesis may lead to determination of therapy alternative to cytostatic treatment.

Currently, in studies on carcinogenesis, in which the assumed source of the neoplasm are the oval cells, the fact must be considered that they create a population of not clearly defined intermediate stages. Usually we are not sure which of those stages began the development of the neoplastic cell. It is calculated, that only one out of 10^5 - 10^6 oval cells obtained from the culture and transplanted into the liver, not necessarily representative for all the population, may divide. Therefore, basic meaning for studies on hepatocarcinogenesis and liver regeneration present achievements of final evidence on multipotential cells existence, and determination of differentiation precisely describing their developmental stages. Further possibilities for practical application of undifferentiated cells result from studies proving that epithelial liver cells may originate from maternal cells sampled from bone marrow. A series of common markers for both cell groups has been identified. It indicates potentially very big plasticity of "stem" cells, whose limitations still require determination [52, 53].

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