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Ah receptor- and TCDD-mediated liver tumor promotion: clonal selection and expansion of cells evading growth arrest and apoptosis

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Abstract

The Ah receptor (AhR) has been characterized as a ligand-activated transcription factor which belongs to the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family of chemosensors. Transgenic mouse models revealed adaptive and developmental functions of the AhR in the absence of exogenous ligands. Use of persistent agonists such as dioxins including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds demonstrated that the AhR mediates a plethora of species- and tissue-dependent toxicities, including chloracne, wasting, teratogenicity, immunotoxicity, liver tumor promotion and carcinogenicity. However, molecular mechanisms underlying most aspects of these toxic responses as well as biological functions of the AhR are currently unknown. Previous studies of liver tumor promotion in the two-stage hepatocarcinogenesis model indicated that TCDD mediates clonal expansion of 'initiated' preneoplastic hepatocytes, identified as enzyme-altered foci (EAF) by inhibiting apoptosis and bypassing AhR-mediated growth arrest. In contrast, the Ah receptor has been shown in cell models to stimulate growth arrest and apoptosis. Possible underlying mechanisms of these AhR responses are discussed, including enhanced metabolism of retinoic acid which attenuates TGFβ-mediated apoptosis and interaction of the Ah receptor with the hypophosphorylated retinoblastoma tumor suppressor protein. The discrepancy between in vivo findings in EAF and AhR functions may be solved by hypothesizing that sustained activation of the Ah receptor generates a strong selective pressure in liver treated with genotoxic carcinogens leading to selection and expansion of clones evading growth arrest and apoptosis. Models are discussed which may facilitate verification of this hypothesis.

Keywords: Ah receptor; Apoptosis; Cell contact inhibition; Liver tumor promotion; Retinoblastoma protein; Retinoids

1. Introduction

Historically, the Ah receptor (AhR) was discovered in efforts to understand the induction of xenobiotic metabolizing enzymes, such as CYP1A1, and the toxicity of dioxins, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent dioxin and persistent AhR receptor agonist [1–4]. The AhR was characterized as a ligand-activated transcription factor which belongs to the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family of chemosensors. Transgenic mouse models established the role of the AhR in both bioactivation and detoxification of xenobiotics [5], and also revealed functions of the AhR in the absence of exogenous ligands, affecting both metabolism of endobiotics and cell cycle regulation [4,6–10]. Sustained activation of the AhR by TCDD mediates a plethora of species- and tissue-dependent toxicities, including chloracne, wasting, teratogenicity, immunotoxicity, liver tumor promotion and carcinogenicity [1–4]. However, molecular mechanisms underlying most aspects of these toxic responses as well as biological functions of the AhR are currently unknown.

TCDD-mediated liver tumor promotion in multistage rat hepatocarcinogenesis has been studied in detail because of its relevance for regulatory toxicology [11]. Rodents expressing mutant AhR suggest a key role of the AhR in liver tumor promotion [12,13]. In particular, study of a toxicity-resistant rat strain which bears a point mutation in the AhR that results in loss of amino acids from the transactivation domain revealed that the ability of the

Abbreviations: AhR, aryl hydrocarbon receptor; RA, retinoic acids; CYP, cytochrome P450; EAF, enzyme-altered foci; Rb, retinoblastoma protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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AhR to induce enzyme activities, induce thymus atrophy and promote liver tumors is not interrelated [12]. Hence, TCDD-mediated toxic responses critically depend upon tissue-specific factors, and – in the case of liver tumor promotion – on differential effects of AhR signaling on surrounding liver cells and on evolving genetically deregulated preneoplastic hepatocytes, identified as enzyme-altered foci (EAF). Recently, major advances have been made in AhR-controlled metabolism of endobiotics such as retinoic acid and in its linkage to apoptosis via the cytokine TGFβ [8]. In addition, cell cycle arrest via interaction of the AhR with the retinoblastoma tumor suppressor protein (Rb) was characterized [9,10]. Studies in the two-stage model of hepatocarcinogenesis revealed TCDD-mediated antiapoptosis in EAF while in many cell models in vitro the activated AhR stimulated growth arrest and proapoptotic signaling. The commentary tries to reconcile these discrepancies by hypothesizing that non-genotoxic TCDD generates a strong selection pressure in the liver treated with genotoxic carcinogens leading to the outgrowth of 'initiated' cell clones evading growth arrest and apoptosis.

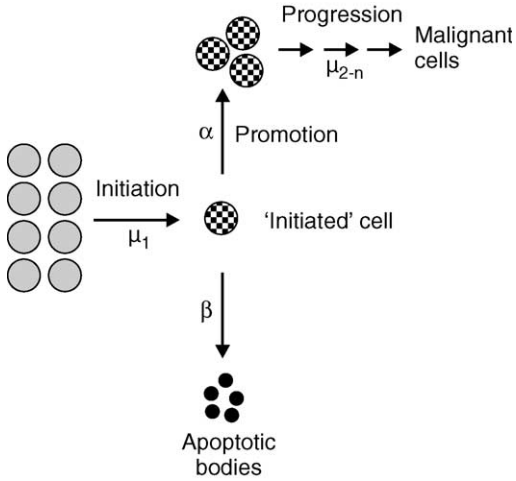

Fig. 1. Multistage model of hepatocarcinogenesis used for mathematical modelling [22,23]. Genotoxic chemicals may generate 'initiated' cells at a rate termed μ_1. Initiated cells may either divide at a rate of α to generate enzyme-altered foci (EAF) or undergo apoptosis at a rate of β. Tumor promoters increase $\alpha - \beta$ and decrease β/α. Progression includes further genotoxic lesions at rates termed μ_{2-n}.

2. Two-stage model of hepatocarcinogenesis: deregulated cell cycle control and apoptosis in enzyme-altered foci (EAF)

Accumulating evidence suggests multiple genotoxic insults in genes responsible for proliferation control, such as activation of oncogenes and loss of tumor suppressor genes, as the basis of carcinogenesis [14,15]. Genotoxic insults may be generated either by endogenous or exogenous factors, the latter including radiation, viral disease and chemicals. In experimental models, carcinogenicity can be divided into three distinct stages, termed initiation, promotion and progression (Fig. 1). Genotoxic chemicals such as diethylnitrosamine (DEN) may lead to irreversible genetic alterations and to the generation of 'initiated' cells. Initiated cells may either be removed by apoptosis or may be selected by the action of tumor promoters for clonal expansion to form preneoplastic foci, frequently identified histologically as GSTP1-positive EAF. Hence, non-genotoxic tumor promoters facilitate carcinogenesis by selecting and expanding 'initiated' cell clones. A variety of mechanisms may be involved, including stimulation of regenerative growth by cytotoxicity, by hormone-mediated growth stimulation and by actions of classic liver tumor promoters, such as phenobarbital- and dioxin-like compounds [16,17]. Recently, it has been shown that the latter compounds are often persistent agonists of nuclear receptors, including the AhR and its most potent agonist TCDD. During progression, additional genetic alterations occur leading to autonomous, invasive and metastatic growth.

The two-stage model of rodent hepatocarcinogenesis has been scientifically attractive because early preneoplastic hepatocytes can be identified as EAF, and the actions of genotoxic carcinogens and of non-genotoxic tumor promoters can be studied separately. Recently, direct lineage between hepatocytes, EAF, hepatic nodules and hepatocellular carcinoma has been demonstrated [18]. It should be noted that – in addition to hepatocytes – liver stem cells (often recognized as proliferating 'oval' cells) are also discussed as key targets of genotoxic lesions leading to liver cancer [18].

2.1. TCDD-mediated inhibition of apoptosis in EAF

Liver tumor promotion by TCDD was first shown by Pitot et al. [17], who based their dosing conditions on the 2-year TCDD feeding study of Kociba et al. [19]. These investigators who probably promoted spontaneously generated 'initiated' hepatocytes detected EAF already at a dose-schedule of 0.01 μg TCDD per day, and preneoplastic nodules and hepatocellular carcinomas at 0.1 μg TCDD per day in female but not male rats. Ideally the two-stage model allows to separate the actions of initiators and tumor promoters. Therefore in our own studies treatment with DEN (10 mg/kg daily by stomach tube for 10 days) was followed by a recovery period of 10 weeks before TCDD-treatment. Cell division was visualized by the incorporation of bromodeoxyuridine into hepatocyte nuclei (labelling index) and apoptosis by counting apoptotic bodies. Interestingly, early EAF were characterized by high rates of both cell division and apoptosis [20,21]. Hence, early focal cell populations did not expand but revealed a much higher cell turnover than surrounding liver cells. High proliferation of EAF necessitates that cell–cell contacts and gap junctional communication are reduced. Cell division in EAF was found to be unchanged to moderately enhanced by TCDD (1.4 μg/kg subcutaneously, given

biweekly for 17 weeks) [20,21]. Interestingly, the high rate of apoptosis in EAF was significantly inhibited by TCDD [21]. Therefore, the tumor promoting action of TCDD appears to be mainly due to inhibition of apoptosis, a finding which was substantiated by mathematical modelling of mitotic and apoptotic indices obtained histologically [22,23]. In support of TCDD-mediated antiapoptotic responses, UV light-mediated apoptosis and the resulting accumulation of p53 was found to be inhibited by TCDD in hepatocyte primary cultures [24].

2.2. TCDD-mediated survival of initiated cells by inhibition of apoptosis

In addition to substantiating inhibition of apoptosis as major factor responsible for TCDD-mediated liver tumor promotion, mathematical modelling of the two-stage hepatocarcinogenesis model also suggested that the non-genotoxic TCDD may influence the initiation process [22,23]. Surprisingly, recovery from DEN-mediated genotoxic stress required more than 10 weeks, the time period selected for the start of TCDD-treatment. TCDD accelerated the appearance of EAF, presumably by stimulation of the select outgrowth of cell clones that respond to the inhibitory effects of TCDD on apoptosis. TCDD does not directly initiate EAF but modifies the formation of EAF in the wake of genotoxic insult. It is conceivable that some mutated cells which are apoptotically removed in untreated controls, may survive in TCDD-treated animals. The sequence of initiation (mutation) and promotion (clonal expansion) shows an interesting similarity to neo-Darwinian theory of evolution. The described >10 weeks time period after DEN treatment to complete initiation hints at complex cell-specific events influencing the fate of multiple genetically injured cells struggling for survival. Tumor promotion-mediated clonal selection is supported by observations in DEN-initiated and phenobarbital-promoted mouse liver (given the caveat that selection may be somewhat different in TCDD-promoted liver): while in DEN-treated young mice ras- but not β-catenin-mutated tumors were found, additional phenobarbital-promotion of DEN-treated mice suppressed ras-mutated but strongly increased β-catenin-mutated tumors [25]. To what extent a 'negative selection mechanism' [26] adds to TCDD-mediated antiapoptotic effects due to decreased retinoic acid levels (discussed later), or to reduced adhesion factor-mediated mitoinhibition remains to be elucidated.

3. AhR signaling: metabolism and cell proliferation

Most of the unliganded AhR is known to reside in the cytoplasm in a complex with a dimer of Hsp90 and additional chaperones such as ARA9 (also known as AIP1 or XAP2) and p23 [4]. Upon ligand binding the complex translocates to the nucleus and the AhR associates with its partner protein Arnt. This heterodimer binds in the enhancer region of target genes to DNA elements, termed xenobiotic response elements (XREs; also termed DREs or AhREs). DNA binding of the enhancer leads to the recruitment of coactivators and corepressors, to remodelling of the chromatin structure and activation of the transcription machinery of target genes. Early findings with mice or hepatoma cell lines expressing mutated AhR have shown that AhR-responsive genes include enzymes of phase-I and -II of endo- and xenobiotic metabolism [1–4]. However, studies with rodent cell lines expressing mutated AhR implied that the AhR has additional functions, including cellular growth, development and differentiation [1,4]. Recent global expression studies revealed a bewildering array of TCDD-responsive genes [27,28]. Most of the altered genes are probably due to secondary responses. Table 1 lists some AhR-responsive genes, where functional XREs in their regulatory region have been characterized [3,7,29]. It is obvious that the gene battery not only includes genes of detoxification but also cyclin-dependent kinase inhibitors, such as $p21^{CIP1/WAF1}$, involved in cell cycle control [9]. Furthermore, in some cases the AhR interacts with other proteins, such as the G1 checkpoint regulator Rb [9,10]. It is also noteworthy that the AhR level may be regulated differently in vivo and in cell cultures; for example, early down-regulation and subsequent up-regulation of AhR expression was found in vivo, in contrast to sustained depletion of AhR caused by TCDD in cell cultures, underlining the importance of in vivo models [30,31]. In addition, it was found incidentally that only a small fraction of the AhR pool appeared in the nucleus after TCDD-treatment.

The commentary focuses on two AhR functions, biotransformation of endo- and xenobiotics (termed adaptive mechanism [4]) and cell cycle control [9,10]. Studies with AhR-null mice established a key role of the AhR in adaptive, developmental and toxic reactions [4]. Generation of mice carrying a mutation in the nuclear localization sequence of the AhR indicated resistance to TCDD-mediated toxicity [32]. Because of their possible involvement in liver tumor promotion three of these processes

Table 1
Selected AhR-responsive genes where XREs in their regulatory region have been characterized

Gene	Species	References
CYP1A1	(r, m, h)	[3,29]
CYP1A2	(h)	[3,29]
UGT1A1	(h)	[3,39]
UGT1A6	(r, h)	[3,29]
GSTA1/2(GSTYa)	(r)	[3]
NQO1	(h)	[3]
ALDH3A1	(m)	[3]
$p21^{CIP1/WAF1}$	(h)	[9]

Abbreviated detoxification enzymes: CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; GST, glutathione S-transferase; NQO, NAD(P)H: quinone oxidoreductase; ALDH, aldehyde dehydrogenase. Abbreviated species: r, rat; m, mouse; h, human.

(AhR-controlled retinoic acid metabolism, AhR interaction with Rb and AhR-mediated release from cell contact inhibition) are subsequently discussed in detail. It is understood that other mechanisms may also contribute to liver tumor promotion, including cytotoxicity due to oxidative stress.

3.1. AhR-controlled retinoic acid metabolism modulating TGFβ and apoptosis

Various isomeric retinoic acids (RA) are ligands of retinoic acid receptors (RAR, RXR) which play important roles in many aspects of life, including development and maintenance of numerous epithelial tissues. RAR and RXR are members of the nuclear receptor supergene family. All-*trans* retinoic acid activates RAR which binds to enhancer regions of target genes as homodimer; 9-*cis* retinoic acid activates RXR which functions as heterodimeric partner for many orphan receptors such as CAR (constitutive androstane receptor) and PXR (pregnane X receptor), mediating phenobarbital-type induction of drug metabolizing enzymes (for references see [29]). Because of its life-saving functions RA homeostasis has to be rigorously controlled. It has been known for a long time that in general RA levels are decreased in TCDD-treated animals. In support of a role of the AhR in homeostasis, RA levels were found to be enhanced in AhR null mice [33]. Evidence has been obtained that increased RA levels may be due to down-regulation of mouse Cyp2C39 expression leading to decreased RA 4-hydroxylase activity [34]. Enhanced RA levels are known to stimulate transglutaminase II and thereby stimulate the conversion of latent TGFβ to the active, secreted form of this important cytokine (Fig. 2). TGFβ receptor signaling is known to activate apoptosis [8]. Hence, it is tempting to speculate that decreased retinoid levels in TCDD-treated liver may attenuate TGFβ receptor signaling and favour antiapoptotic signaling pathways. Recently, it was found that the level of all-*trans* RA was elevated in TCDD-treated rats while levels of retinyl esters and of RA metabolites were decreased [35]. A variety of drug metabolizing enzymes including CYPs, UGTs and ALDHs (Table 1) appear to be involved in the synthesis and catabolism of RA [35]. However, the key retinoids and RA-metabolizing enzymes still need to be identified.

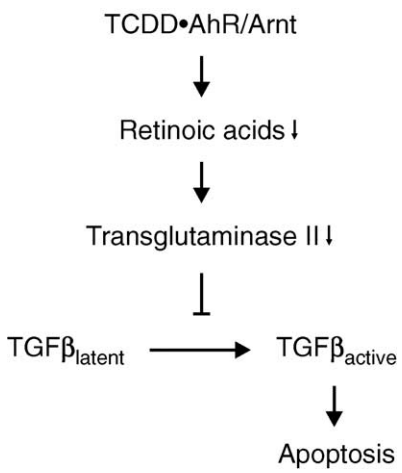

Fig. 2. Involvement of AhR in retinoic acid metabolism, leading to decreased retinoic acid level, decreased activation of TGFβ and to antiapoptosis [8]. Retinoic acid levels critically determine transglutaminase II expression, an enzyme which is involved in the activation of apoptosis-mediating TGFβ.

3.2. AhR interaction with Rb tumor suppressor protein: E2F-dependent repression of S-phase progression

Evidence has been obtained that the AhR directly interacts with hypophosphorylated Rb [9,10]. Interaction between AhR and Rb has been shown to affect a major G1 checkpoint of the cell cycle (Fig. 3). Mitogenic signaling via protein kinases and cell contact-mediated mitoinhibition via protein phosphatases affect phosphorylation of Rb, a key G1 restriction point of the cycle. AhR–Rb interaction appears to be restricted to the hypophosphorylated Rb [9,10]. Two different mechanisms have been proposed to explain TCDD-mediated growth arrest: (i) coactivation via a ternary complex of Rb with the AhR/Arnt dimer [10] leading to induction of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [7], and (ii) corepression via a quaternary complex of AhR–Rb with E2F (which transcriptionally activates genes required for entering S-phase) and DP (the E2F-binding partner in transactivation) [9]. Evidence was obtained that both mechanisms are possibly operating [10]. Interestingly, while being required for S-phase progression, E2F appears to be able to trigger both apoptotic and antiapoptotic signaling [36].

Several recent reports have shown that AhR activity in the absence of exogenous ligands can affect G_1-phase progression of cultured cells [6,9,10]. Interestingly, addition of serum to serum-starved (G_0) 5L rat hepatoma cells triggers transient AhR activation and CYP1A1 expression concomitant with the G_0/G_1-to-S-phase transition [referred to in 10]. In contrast, sustained AhR activation in response to TCDD-treatment increases $p27^{Kip1}$ expression, resulting in G1-phase cycle arrest. These findings suggest that CYP1A1 negatively regulates the duration of AhR action through the metabolic removal of an unknown endogenous receptor agonist, thereby preventing AhR-mediated G_1-phase arrest. Hence, CYP1A1 may fulfil an important role in cell cycle regulation together with the AhR, a hypothesis which may explain its preferential inducibility among AhR-responsive genes.

3.3. TCDD-mediated release from cell contact inhibition

Release from cell contact inhibition was observed in several cell lines, such as liver stem cell-like rat WB-F344 cells [37,38]. Liver stem cells, identified as 'oval cells', are able to proliferate under conditions of strong

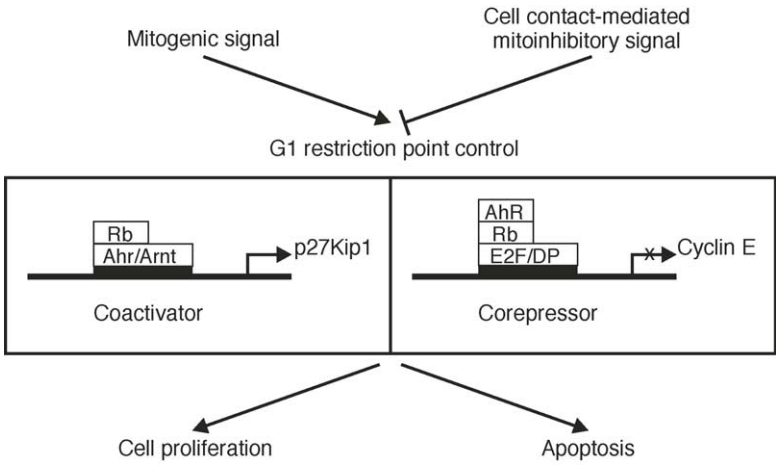

Fig. 3. Interaction of AhR with retinoblastoma tumor suppressor protein (Rb), and its possible consequences for cell cycle regulation [9,10] and apoptosis [36]. Mitogenic signaling via protein kinases and cell contact-mediated mitoinhibition via protein phosphatases affect the Rb protein, a key G1 restriction checkpoint. The hypophosphorylated Rb has been shown to interact with the AhR. Two mechanisms may be operative in TCDD-mediated growth arrest, coactivation and corepression, as discussed in the text. While being required for S-phase progression, E2F may be involved in both progression of the cell cycle and the apoptosis machinery of the cell [36].

mitoinhibition. It has been proposed that under these conditions preneoplastic liver cells may originate from stem cells. In confluent WB-F344 cells, TCDD-treatment led to translocation of the tyrosine kinase c-Src from the cytosol to the plasma membrane [38]. Under these conditions phosphorylation of the EGF receptor was found to be enhanced. These effects may explain TCDD-mediated release of WB-F344 cells from cell contact inhibition. In support of the importance of released contact inhibition, cell–cell contact was found to be low in cultures of nodular hepatocytes compared to normal hepatocyte cultures [39]. For example, while increasing cell density led to enhanced contact inhibition of normal hepatocyte cultures, we observed density-dependent enhanced proliferation of nodular hepatocyte cultures. TCDD-mediated release of cell contact inhibition may be one factor responsible for cell cycle progression in EAF while in surrounding hepatocytes TCDD may lead to cell cycle arrest.

4. Conclusions

TCDD-mediated hepatocarcinogenesis is of importance for regulatory toxicology [11]. Hepatocarcinogenesis models of TCDD-mediated tumor promotion are scientifically attractive since the toxic reaction is mediated by a defined nuclear receptor, the AhR, since early preneoplastic cell clones can be identified as enzyme-altered hepatic foci (EAF), and clonal expansion of these foci by tumor promoters can be investigated. However, tumor promotion differs from other toxic reactions since multiple genetically deregulated target cells are selected and expanded. It has been established in cell lines that the persistently activated AhR triggers G1 growth arrest and apoptosis. In contrast, cell cycle progression and inhibition of apoptosis has been observed in EAF in the in vivo model. The discrepancy can be reconciled by assuming that sustained AhR signaling creates a strong selection pressure in genotoxin-treated liver leading to outgrowth of clones evading apoptosis and growth arrest.

Hepatocarcinogenesis models have been established in rats and mice [16,17]. A mutant rat strain is available that is 100-times less responsive to TCDD-mediated liver tumor promotion [12]. AhR-mutated mice [4,32] have been generated as well as mice expressing a constitutively active AhR mutant (Ca-AhR) [13]. These mutant rodent strains may be invaluable to test the proposed hypothesis and to identify the key AhR signaling pathways involved in liver tumor promotion. In addition, current efforts to generate corresponding ‘humanized’ mouse models, such as the ‘humanized AhR mouse’ [40], may facilitate human risk assessment.

Acknowledgements

We regret that not original articles but reviews and recent publications often had to be cited due to limitations in the number of references. We thank Michael Schwarz for helpful discussions.

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