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Title: Aryl hydrocarbon Receptor and liver fibrosis

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Abstract

The concept of toxicant-associated liver disease (TALD) has recently emerged linking exposure to pollutants to the development of chronic liver diseases (CLD) including fibrosis. Among such pollutants, ligands of the Aryl hydrocarbon Receptor (AhR), a transcriptional factor involved in detoxification processes, have been suspected to trigger multiple mechanisms (oxidative stress, epithelial and mesenchymal transition) associated with the occurrence of such pathologies. However, AhR knockout-mice also could develop liver fibrosis, an observation which might first be described as a paradox. In this review, we will present the different mechanisms linking AhR ligands and CLD and provide a hypothesis to solve this paradox.

1. Introduction

Liver fibrosis is defined as a response to a sustained cell injury and chronic inflammation that leads to the activation of pro-fibrotic cells and the excessive deposition of extracellular matrix. While reversible, it often leads to fatal complications or increases the risk of hepatocellular carcinoma (HCC). To study the mechanisms which lead to this pathology, experimental animal models have been developed mostly implicating rodents, and can be chemically-induced, surgically-based, diet-based or genetically-modified [1]. Several types of etiologic factors have been identified such as alcohol consumption or viral exposure. However, the contribution of environmental causes such as exposure to persistent organic pollutants (POPs), has been recently documented (TALD: toxicant-associated liver disease) [2]. In this review, we will focus on the specific contribution of the Aryl hydrocarbon Receptor (AhR), which can bind some of these POPs, on the occurrence of liver fibrosis.

2. AhR ligands, mechanism of action and functions (Figure 1)

The AhR has been identified in the 90's as a xenobiotic receptor which detects many organic pollutants (some of them persistent) such as dioxins, furans, some polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons and triggers a transcriptional program leading to the expression of xenobiotic metabolizing enzymes such as cytochromes P450 (CYP) or UDP-glucuronosyltransferases (UGT), that metabolize the pre-cited ligands, favoring their elimination in wasting fluids (e.g. urine). This elegant mechanism of action (detection/metabolism/elimination) historically strengthens the idea that the AhR displayed only one unique function, dedicated to the regulation of xenobiotic metabolism. However, the development of knock-out models in vertebrates and invertebrates led to the suspicion of alternative functions of this receptor. Endogenous ligands including tryptophan metabolites started to be identified [3]. During the 00's with the emergence of large-scale omics-based technologies, several teams started to decipher other AhR functions such as the regulation of the epithelial-mesenchymal transition (EMT associated to cell adhesion/migration, and to the regulated expression of epithelial/mesenchymal markers) [4-6]. Non-genomic pathways and complex interactions with nuclear receptors were then identified. Thus, the AhR appears to be a far more complex protein than initially expected [4,6].

3. Chemically-activated AhR and liver fibrosis

3.1 Epidemiological and animal models-based evidence

Epidemiological studies reported previously a potential link between environmental or accidental exposure to dioxins or AhR ligands and CLDs. Studies on US veterans (Operation Ranch Hand) exposed to TCDD (2,3,7,8-TetraChloroDibenzo-p-Dioxin, a contaminant of Agent Orange), did not show any increase of the expected mortality compared to the overall population but they identified an increased number of deaths due to digestive diseases especially caused by CLDs such as cirrhosis (the highest grade of fibrosis) [7–9]. This was also observed in cohorts of workers potentially-exposed to dioxins (e.g. resulting from manipulation of pentachlorophenol) [10–12] or on accidentally-exposed horses, which displayed centrilobular fibrosis as the most predominant liver lesion [13].

Animal models exposed to TCDD (and subsequently activating the AhR) were also reported to develop liver fibrosis [14–16]. For example, the US National Toxicology Program (NTP), a 2-years study on TCDD or binary mixtures of dioxin-like compounds such as PCBs or furans, reveals a hepatotoxicity in several female rats, including liver fibrosis (mostly portal, sometimes centrilobular) [17–23].

3.2 TCDD-activated AhR and oxidative stress (Figure 1)

One of the first legitimated mechanisms that could link AhR ligands with liver fibrosis, is oxidative stress [24]. Indeed, TCDD is known to produce reactive oxygen species (ROS) through transcriptional and translational induction of CYP1 enzymes which catalyze uncoupling reactions [24]. Such a CYP-related oxidative stress mechanism is already firmly suspected in the chemically-induced CCl_4 - experimental model of liver fibrosis as CCl_4 is metabolized notably by cytochrome P450 2E1 (CYP2E1), leading to lipid peroxidation by the production of ROS. In addition, TCDD promotes a decreased glycolytic flux with a switch of pyruvate kinases, favoring the production of NADPH, H^+ used by CYP-based catalytic reactions [25,26]. Furthermore, TCDD represses the level of expression of hepcidin (involved in the regulation of intracellular iron levels) which indirectly contributes to oxidative stress through iron-related Fenton reactions [27]. **Moreover, a tight and complex interplay between the AhR and the Nrf2 signaling pathways (known to activate antioxidant enzymes) has been described, with direct consequences on TCDD-induced oxidative stress and fibrosis in the liver [28].**

Over-production of ROS induces cell death (apoptosis/necrosis) and as a consequence, the release of pro-inflammatory and pro-fibrotic mediators by dying cells activates hepatic stellate

cells (HSCs) which acquire a myofibroblastic phenotype (expressing alpha-smooth muscle actin (alpha-SMA), producing pro-fibrotic factors, e.g. TGF- β , and components of the extracellular matrix such as collagens type I, III and IV; fibronectin and proteoglycans). Additionally, TCDD favors the recruitment of inflammatory cells and the development of a local inflammation in the liver through AhR interaction with inflammatory pathways further promoting ROS production as well as the activation of HSCs [29–31]. Beside, TCDD disrupts lipid metabolism in the liver promoting the production of triglycerides, cholesterol esters and phospholipids and the increased uptake and packaging of lipids [14,25,32]. Such deregulations associated with a local inflammation promote NASH and probably fibrosis [14,16,33]. In addition, alterations in lipid metabolism can lead also to lipotoxicity that has been shown to contribute to oxidative stress, local inflammation and subsequent liver damages [34].

3.3 TCDD-activated AhR and EMT (Figure 1)

We and others [4–6,14,15,35,36] reported that the AhR regulates EMT through the induction of Slug and Snail, two transcription factors which down-regulate the expression of E-Cadherin, a critical transmembrane protein essential for the proper architecture of epithelia. Over-expression of such EMT regulators is reported for TCDD-treated cells but also *in vivo*, where such processes are demonstrated in parallel to the occurrence of liver fibrosis in mice, after only 2 weeks of sub-chronical exposure to high doses of TCDD (25 $\mu\text{g}/\text{kg}$, once a week) [15]. While EMT is a fundamental process for embryonic development (e.g. gastrulation), it is also suspected to participate to pathological processes such as the formation of metastatic cells or organ fibrosis (kidney and liver). The involvement of EMT in hepatocytes and biliary cells during the progression of CLDs, is nowadays challenged by several authors and it is legitimate to think that those cells might not undergo a complete EMT but still, could contribute to the activation of HSCs through perturbation of the liver microenvironment.

3.4 TCDD-activated AhR and hepatic stellate cells (Figure 1)

TCDD can activate HSCs both *in vitro* and *in vivo* with an over-expression of pro-fibrotic genes or pro-ECM-remodeling genes (matrix metalloproteinases, procollagens); however since the period of exposure was too short and despite the induction of pro-fibrotic markers, the authors did not observe a liver fibrosis [31,37]. Interestingly, *in vitro* and *in vivo*, the effect of TCDD on HSCs might be due to the activation of several signaling pathways (not only the

classically-described AhR-ARNT signaling pathway); TCDD-induced proliferation of HSCs was indeed blocked by an inhibitor of the PI3K, but not the production of monocyte chemoattractant protein-1 (MCP-1), which contributes to the development of a local inflammation [29,30]. Besides, TCDD might also favor intrahepatic coagulation and collagen deposit through the action of Protease Activated Receptor 1 (PAR-1), a coagulation receptor expressed by HSCs, whose activation promotes myofibroblast differentiation. Indeed, PAR-1-deficient mice displays a less pronounced collagen deposit in the liver upon TCDD exposure [38].

4. A contextual role of POPs

The role of the AhR in the occurrence of liver fibrosis is also probably contextual depending on the influences of many parameters such as diet composition [14,27]. Indeed, iron promotes AhR-mediated hepatotoxicity as an iron-enriched diet potentiates TCDD-induced hepatotoxicity [27]. Recently, we demonstrated that a low dose of TCDD which do not lead to liver fibrosis *per se* (under a 6-week exposure protocol, 5 µg/kg once in a week), was able to trigger such phenomena in a high fat diet-induced NASH context [14]. Those studies highlight two important points to consider regarding the influence of POPs on the occurrence of liver fibrosis: 1) the length of the POP exposure which is highly relevant to consider for persistent organic pollutants and 2) the combination of the POP exposure with other risk factors of liver fibrosis. Moreover, human western diets not only represent a significant source of fat but also contaminants (lipophilic organic pollutants). The view that xenobiotic AhR ligands promote liver fibrosis over a long period of exposure suggests that the AhR needs to be continuously activated to trigger such phenomena. This is consistent with the mode of action of POPs which can be stored in the liver (TCDD binds with a very high affinity in the catalytic site of CYP1A2 without any conversion) and exert their biochemical effects over long periods of time (the half-life of TCDD in humans is comprised between 7-8 years). Moreover, the adipose tissue slowly releases POPs, a phenomena which can impact the liver chronically [39].

5. AhR and liver fibrosis in the absence of sustained activation by chemical ligands

5.1 AhR-KO mice and liver fibrosis

The first experimental reports mentioning a potential link between the AhR and liver fibrosis, came from studies on AhR-knockout (KO) models. As initially demonstrated [40–42], a large

majority of AhR-KO mice (81%) develops hepatic portal fibrosis. Subsequent molecular studies showed that the absence of the AhR during mice development, leads to local increased expression of pro-fibrotic effectors such as transforming growth factor- β 1 and 3 (TGF- β 1 and 3) or TGF- β regulators such as Latent TGF-beta-binding protein-1 (LTBP-1) [36,43]. An increased number of apoptotic hepatocytes is also observed in AhR-KO mice [43]. Beyond the involvement of these hepatocytes, the role of vitamin A has been quickly underlined as its depletion in the diet of AhR-KO mice impairs the development of the fibrosis [44,45] suggesting that HSCs (which store vitamin A and release it following their activation) could play a role in the development of fibrosis. Moreover, a down-regulation of cytochrome P450 2C39, a retinoic acid 4-hydroxylase, is observed in AhR-KO mice, contributing probably to the accumulation of vitamin A in the mouse liver and a subsequent higher activation of HSCs [44-46].

The portal fibrosis described in AhR-KO mice could be analyzed as a paradox, as TCDD commonly described as an AhR agonist also promotes fibrosis (see §4). However, a decreased expression of the AhR at both mRNA and protein levels (and subsequently of the induction of CYP1), is observed in parallel to the progression towards CLDs in cirrhotic rats and human patients [47,48], in support for a protective role of the AhR regarding liver fibrosis.

5.2 Endogenous ligands of AhR and liver fibrosis: the paradox resolution

Actually, a hypothesis can be easily drawn which reconciles this first-sight paradox: the AhR has been identified as a xenobiotic receptor but several recent studies demonstrated the existence of several endogenous ligands (like tryptophan metabolites) [3]. Such ligands are probably involved in liver development and homeostasis. Beyond this developmental role, the AhR has evolved as a xenobiotic receptor whose transient activation participates in the regulation of xenobiotic metabolism, an important detoxifying function which is significantly different from the ones regulated by the endogenous ligands and probably not toxic over short periods of activation. However, as both transcriptional programs are probably clearly distinct between both types of ligands as suggested by ChIP-on-ChIP experiments performed by the team of A. Puga, sustained xenobiotic-related AhR activation could disrupt homeostatic programs regulated by endogenous ligands as for example TCDD can be seen as a high affinity ligand, then highly competitive (figure 2) [49].

The diet could then represent an important contextual parameter as such endogenous ligands could be amino acids metabolites (e.g. tryptophan). Interestingly, alteration of the levels of amino acids has been associated with the promotion of liver fibrosis [50]. For instance, a high-methionine diet induces experimentally hyperhomocysteinemia (HHCy) and liver fibrosis in mice. Yao L *et al.* have shown that HHCy activates the AhR leading to an increase expression of CD36, the fatty acid (FA) transporter and subsequently to liver uptake of FA and steatosis. The authors suggest that this activation is linked to the production of lipoxin A4, an endogenous ligand of the AhR and a metabolite of arachidonic acid [50].

6. Conclusion

Strong evidence supports a crucial role of a sustained AhR activation by pollutants in the development of liver fibrosis through direct regulation of pro-fibrotic processes such as oxidative stress, EMT or HSC activation. CLDs highly depend also on metabolic disruptions which can be triggered by TCDD (and other pollutants) [33], putting the dietary context as a central aspect in studying the deleterious effect of TCDD on fibrosis development.

Persistent organic pollutants are not the only “new” etiologic factors which lead to liver fibrosis. Other factors need to be considered such as gender or housing temperature [51]. Another interesting question is related to the models used to study liver fibrosis, mostly rodents; indeed, the use of complementary *in vitro* models could also be useful to define precisely the molecular mechanisms of action since new relevant hepatocellular models (HepaRG, 3D cell cultures) are now available [52]. Also, a better assessment of the role of the AhR in fibrosis with the development of new methodologies of quantification such as PicroSirius Red could be undertaken [53]. Finally, other animal models such as zebrafish, display liver metabolic alterations upon exposure to TCDD and could become relevant regarding their costs, to better understand the progression of CLDs [54].

While preventing or therapeutic protocols are now well documented, the identification of the xenobiotics and their mechanisms of action might represent an interesting outcome to decrease the incidence of CLDs including liver fibrosis. The persistence of molecules which could sustain the activation of the AhR in hepatocytes and of HSCs, appears as one important criteria to take into account. As such contaminants are also part of Western type diets (a “double-edge sword”), the prevention to eat such diets might be even more potent if taking

into account the “contaminant factor” and a good way to change our habits regarding food consumption.

Figure legends

Figure 1: The AhR regulates a diversity of signaling pathways in hepatocytes and in hepatic stellate cells which can contribute to the development of liver fibrosis including epithelial-mesenchymal transition, oxidative stress, secretion of extracellular matrix components and local inflammation.

Figure 2: The AhR is activated by endogenous ligands whose action can be potentially disrupted by the competitive binding of xenobiotics.

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Conflict of interest:

The authors declare no conflict of interest.

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