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Aflatoxin B1 induces persistent epigenomic effects in primary human hepatocytes associated with hepatocellular carcinoma

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ABSTRACT

Chronic exposure to aflatoxin B1 (AFB1) has, in certain regions in the world, been strongly associated with hepatocellular carcinoma (HCC) development. AFB1 is a very potent hepatotoxic and carcinogenic mycotoxin which is frequently reported as a food contaminant. Epigenetic modifications provoked by environmental exposures, such as AFB1, may create a persistent epigenetic footprint. Deregulation of epigenetic mechanisms has actually been reported in HCC patients following AFB1 exposure; however, no attempts have yet been made to investigate early effects on the epigenome level which may be persistent on longer term, thereby possibly initiating carcinogenic events. In this study, we aim to identify methyl DNA-mRNA-interactions representative for a persistent epigenetic footprint associated with the early onset of AFB1-induced HCC. For this, primary human hepatocytes were exposed to 0.3 μM of AFB1 for 5 days. Persistent epigenetic effects were measured 3 days after terminating the carcinogenic exposure. Whole genome DNA methylation changes and whole genome transcriptomic analysis were analyzed applying microarray technologies, and cross-omics interactions were evaluated. Upon combining transcriptomics data with results on DNA methylation, a range of persistent hyper- and hypo-methylated genes was identified which also appeared affected on the transcriptome level. For six of the hypo-methylated and up-regulated genes, namely TXNRD1, PCNA, CCNK, DIAPH3, RAB27A and HIST1H2BF, a clear role in carcinogenic events could be identified. This study is the first to report on a carcinogen-induced persistent impact on the epigenetic footprint in relation with the transcriptome which could be indicative for the early onset of AFB1-related development of HCC.

1. Introduction

Exposure to the highly potent carcinogenic and hepatotoxic mycotoxin aflatoxin B1 (AFB1) through the food chain presents a significant risk factor for the development of hepatocellular carcinoma (HCC) worldwide (Liu and Wu, 2010; Wild and Gong, 2010). Especially populations in regions such as sub-Saharan Africa, Southeast Asia, and China are at risk of developing HCC due to the combination of high hepatitis B virus prevalence and largely uncontrolled dietary AFB1 exposure (Liu and Wu, 2010). Of the 550,000–600,000 new HCC cases worldwide each year, about 25,000–155,000 may be attributable to AFB1 exposure (Liu and Wu, 2010). Therefore, AFB1 exposure may have a causative role in 4.6–28.2% of all global HCC cases (Liu and Wu, 2010). Against this background, a better understanding of the AFB1 mechanism-of-action may be of value for underlining policy measures aiming at reducing exposure to this foodborne carcinogen.

Specific cytochrome P450 enzymes in the liver metabolize AFB1 into the highly reactive AFB1-8,9-epoxide, which may then bind to proteins and cause acute toxicity (aflatoxicosis) or to DNA to initiate mutations that over time increase the risk of HCC (Groopman et al., 2008). Especially, the transversion mutation (G → T) in codon 249 of the p53 tumor suppressor gene has been associated with AFB1 exposure (Soini et al., 1996). A mutation in

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this gene may cause silencing of its tumor suppression function and allow uncontrolled cell proliferation.

Besides inducing genetic effects, AFB1 exposure also has been reported to affect the epigenome (Zhang et al., 2002, 2003, 2006, 2012).

"Epigenetic memory" has been proposed as an indicator of prior exposure (Mirbahai and Chipman, 2014) to for example AFB1, ultimately leading to the development of HCC. Epigenetic alterations induced by environmental stressors, such as AFB1, including changes of the normal DNA methylation pattern and microRNA expression alterations, can create a persistent memory of the received signal. It is hypothesized that each class of toxicants (with a specific "mode of action") can induce class-specific alterations in the normal pattern of DNA methylation or microRNA expression pattern (also called an "epigenetic foot-print") (Goodman et al., 2010; Koufaris et al., 2012; Legler, 2010; Mirbahai and Chipman, 2014; Moggs et al., 2004). These changes will thereupon induce alterations in the gene expression profile, which may promote changes in an organism's traits either immediately or at a later stage (e.g. development of HCC) (Izzotti and Pulliero, 2014; Mirbahai and Chipman, 2014).

Epigenetic changes in liver tissue from HCC patients following AFB1 exposure, have been demonstrated, e.g. gene-specific hypermethylation (for example of RASSF1A, p16 (Zhang et al., 2006) and MGMT) as well as genome-wide hypo-methylation (Zhang et al., 2012). This appears frequently accompanied by a mutation in the p53 gene (Hsu et al., 1991; Hussain et al., 2007; Soini et al., 1996). In addition, specifically altered microRNA expression patterns (e.g. microRNA-221 and microRNA-122) have been observed in HCC patients (Fornari et al., 2008; Fornari et al., 2009; Gramantieri et al., 2008; Karakatsanis et al., 2013; Sun et al., 2013).

The underlying mechanisms by which AFB1 induces DNA methylation changes and microRNA expression changes important in chemical carcinogenesis, are complex, however some potential mechanisms have been proposed. Altered microRNA expression changes occur in response to DNA damage induced by a range of environmental stressors (Izzotti and Pulliero, 2014). Another potential mechanisms by which DNA methylation changes occur, is suggested to involve the preferential binding AFB1 to methylated CpG regions within the DNA, thereby inducing damage leading to conformational changes which may have an impact on the degree of methylation (e.g. causing genome-wide hypomethylation) (Zhang et al., 2003). Hyper- and hypo-methylation in especially the promotor region may impact on gene expression, while specifically hypo-methylation may also affect genome stability. In addition, DNA damage may be generated by AFB1-induced reactive oxygen species production, which may reduce the binding affinity of methyl-CpG binding protein 2 (MECP2), thus resulting in epigenetic changes (Valinluck et al., 2004). The MeCP2 protein binds to forms of DNA that have been methylated. The MeCP2 protein then interacts with other proteins to form a complex that turns off the gene. Once bound, MeCP2 will condense the chromatin structure, form a complex with histone deacetylases (HDAC), or block transcription factors directly (Yasui et al., 2007). The exact mechanisms by which AFB1-induced DNA methylation alterations and microRNA expression changes occur, however still remain complex and therefore need to be investigated in more detail. Furthermore, the effects of AFB1 exposure on whole genome DNA methylation and microRNA expression is not clear and therefore needs to be further explored.

However, neither in experimental studies on AFB1 carcinogenicity, nor in HCC patient studies in relation to AFB1 exposure, the persistence of epigenomic effects (including microRNA expression changes) of AFB1 exposure after removal of the exposure has yet been investigated. In addition, no attempts were made to integrate such a persistent epigenetic "footprint" with the transcriptome.

Consequently, in this present study, we hypothesize that by investigating the persistence of the AFB1-related epigenetic "footprint" together with perturbations of the transcriptome, we will be able to identify novel methyl DNA-microRNA-mRNA-interaction networks which are associated with early AFB1-induced onset of HCC. Simulation of chronic AFB1 exposure, leading to HCC, was initiated by repeated daily dosing. This is crucial since the half-life of AFB1 is 30 min and that of AFB1-DNA adducts in animals is less than 24 h (Lai et al., 2014; Qian et al., 2013). For that reason, primary human hepatocytes (PHH) were exposed to 0.3 μM of AFB1 for 5 days. Persistence of epigenetic effects was evaluated after 3 days of withdrawal of the carcinogen exposure. A 3-day wash-out period will permit the full removal of AFB1 and AFB1-DNA adducts from the PHH, in order to prevent effects taking place due to the presence of the carcinogen or its respective DNA adducts. Whole genome DNA methylation changes were measured by means of NimbleGen 2.1 deluxe promoter arrays, microRNA expression changes were measured through Agilent microRNA microarrays while whole genome transcriptomic analysis was performed using Affymetrix whole genome gene expression microarrays. Persistent hyper- or hypo-methylated genes with a concomitant alteration in their gene expression were investigated in more detail for their biological role within AFB1-induced onset of HCC using ConsensusPathDB (Kamburov et al., 2009) and when of relevance, by GeneCards (Rebhan et al., 1997).

2. Materials and methods

2.1. Cell culturing, dose determination and aflatoxin B1 exposure

Cryopreserved primary human hepatocytes (PHH) were purchased from Life Technologies. Cells were cultured in pre-coated 24-well plates (700,000 cells/ml) in a 2-layer collagen sandwich (A11428-02, Gibco), according to the supplier's protocol (Invitrogen). The following culture media were used: Hepatocyte Thawing Medium (HTM) for thawing (CM7500, Gibco), Williams' Medium E (1x, no phenol red) (A1217601, Gibco) + Cell Maintenance supplement B kit (CM4000, Gibco) for plating and incubation. After quick thawing in a water bath at 37 °C, viability of the cells was checked by a Trypan blue (CAS no. 72-57-1, Sigma–Aldrich) exclusion test as instructed in the supplier's protocol (Invitrogen). All viability scores after thawing were in agreement with those listed by the supplier. Before AFB1 dosing, cells were allowed to acclimatize for 48 h. This is needed for the hepatocytes to restore an *in vivo* like cellular configuration and enzyme expression levels as optimally as possible. An AFB1 dose causing minimal (IC20) cytotoxicity after 5 days of repetitive daily exposure was established by means of the MTT assay (Mosmann, 1983). Furthermore, crucial liver function enzymes such as lactate dehydrogenase (LDH) and alanine transaminase (ALT) were measured. LDH and ALT were spectrophotometrically determined on a Cobas 8000 Modular Analyser (Roche Diagnostics, Basel, Switzerland). Based on this data, a dose of 0.3 μM of AFB1 (CAS no. 1162-65-8, Sigma-Aldrich), was selected for the main experiment. Control cells were exposed to 0.5% of the vehicle solvent dimethyl sulfoxide (DMSO, CAS no. 67-68-5, Sigma-Aldrich).

To diminish the possible impact of inter-donor variability, PHH from 3 donors of different sex, ethnicity, age, body mass index and behavioral status concerning smoking, alcohol and drug use (see Supplementary-Table-1) were pooled prior to culturing. After pooling, cells were cultured in triplicate in 24 wells (RNA) or 6 wells (DNA) in a collagen sandwich layer. One part of the cells was harvested following 5 days of repetitive daily exposure to AFB1 or the vehicle control and the other part of the cells was harvested following an additional 3 day wash-out period. After all cell lysates were properly collected, total RNA was isolated.