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Influence of sex and developmental stage on acute hepatotoxic and inflammatory responses to liver procarcinogens in the mouse

Daniel Hanna, Ariane Emami Riedmaier[1], Kim S. Sugamori, Denis M. Grant*

Department of Pharmacology & Toxicology, Faculty of Medicine, University of Toronto,1 King's College Circle, Toronto M5S 1A8, Canada

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ABSTRACT

The incidence of liver cancer is higher in men than in women. This sex difference is also observed in murine tumor induction models that result in the appearance of liver tumors in adult mice following their exposure on postnatal days 8 and/or 15 to carcinogens such as 4-aminobiphenyl (ABP) or diethylnitrosamine (DEN). Previous studies performed in adult mice showed that acute hepatotoxic and inflammatory responses to high-dose DEN exposure were greater in males than in females, leading to the suggestion that these responses could account for the sex difference in tumor development. We also recently observed that female but not male mice exposed postnatally to ABP had slightly increased expression of the antioxidant defense genes *Nqo1* and *Ggt1*, which are regulated by the oxidative stress response protein nuclear factor erythroid 2-related factor 2 (NRF2), while expression of *Hmox1* was increased in both sexes. The goal of the present study was therefore to compare selected acute hepatotoxic, inflammatory and oxidative stress defense responses to ABP, DEN, or the prototype hepatotoxicant carbon tetrachloride (CCl_4), in male and female mice exposed to these chemicals either postnatally or as adults. Exposure of adult mice to ABP, DEN or CCl_4 produced a 2-fold greater acute elevation in serum levels of the hepatotoxicity biomarker alanine aminotransferase (ALT) in males than in females, while levels of the inflammatory biomarker interleukin-6 (IL-6) showed no sex difference. However, treatment of immature mice with either ABP or DEN using standard tumor-inducing postnatal exposure protocols produced no increase in serum ALT or IL-6 levels in either males or females, while CCl_4 produced a 40-fold ALT elevation but with no sex difference. Basal expression of the NRF2-responsive gene *Nqo1* was higher in adult females than in males, but there was no sex difference in basal expression of *Ggt1* or *Hmox1*. Sexually immature animals showed no sex difference in basal expression of any of the three genes. Postnatal DEN exposure modestly increased the expression of *Ggt1* only in male mice and *Nqo1* in both sexes, while CCl_4 slightly increased expression of *Ggt1* in both males and females and *Nqo1* only in females. Taken together, our results make it unlikely that acute hepatotoxic, inflammatory or NRF2-activated gene responses account for the male predominance in liver tumor growth following postnatal carcinogen exposure in mice. Our findings also suggest that acute toxicity studies performed in adult mice should be interpreted with caution when extrapolating potential mechanisms to liver carcinogenesis models that commonly use postnatally exposed mice.

1. Introduction

Liver cancer is the third most common cause of cancer-related death and the fifth most common malignancy worldwide (Bosch et al., 2004). Hepatocellular carcinoma (HCC) makes up roughly 80% of all liver cancer cases (Farazi and DePinho, 2006). Risk factors for liver cancer include hepatitis B and C virus infection, chronic heavy alcohol consumption and exposure to foreign chemicals such as the fungal-derived food contaminant aflatoxin B_1 (AFB_1) (Bosch et al., 2004; El-Serag, 2012).

There is a significant sex bias in human liver cancer with men having a 2- to 4-fold higher incidence than women, even after

Abbreviations: ABP, 4-aminobiphenyl; AFB_1, aflatoxin B_1; ALT, alanine aminotransferase; APAP, acetaminophen; CCl_4, carbon tetrachloride; CYP, cytochrome P-450; DEN, diethylnitrosamine; DMSO, dimethyl sulfoxide; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ggt1, γ-glutamyltransferase-1; HCC, hepatocellular carcinoma; H3, histone 3; γH2AX, Ser^{139}-phosphorylated histone variant H2AX; Hmox1, heme oxygenase-1; IL-6, interleukin-6; Nqo1, NAD(P)H dehydrogenase quinone 1; NRF2, nuclear factor erythroid 2-related factor 2.

* Corresponding author.
E-mail address: denis.grant@utoronto.ca (D.M. Grant).
[1] Current address: Simcyp Limited (a Certara company), Blades Enterprise Centre, John Street, Sheffield S2 4SU, UK.

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adjusting for differences in risk factor exposure (El-Serag, 2011; Guy and Peters, 2013). Male predominance is also seen in models of liver tumor induction in mice following exposure to chemical carcinogens such as AFB_1 (Vesselinovitch et al., 1972), 4-aminobiphenyl (ABP) (Sugamori et al., 2012) and diethylnitrosamine (DEN) (Nakatani et al., 2001), as well as in transgenic mouse models of hepatitis virus infection (Kim et al., 1991).

ABP is an environmental contaminant of the aromatic amine chemical class, found predominantly in cigarette smoke and as a trace component in the dyestuff industry (Vineis and Pirastu, 1997). As a procarcinogen, ABP undergoes enzymatic bioactivation to reactive electrophiles that can form protein and DNA adducts and result in acute cytotoxicity and mutations, respectively. Protein and DNA adducts of bioactivated ABP have been used as biomarkers of carcinogen exposure (Gyorffy et al., 2008). In C57BL/6 mice, exposure to ABP on postnatal days 8 and 15 leads to the appearance of liver tumors by one year of age predominantly in male mice (Kimura et al., 1999; Sugamori et al., 2012). This 'neonatal' tumor induction protocol, which involves carcinogen exposure prior to sexual maturity followed by tumor enumeration well into adulthood, provides high sensitivity for genotoxic chemicals (McClain et al., 2001). In previous studies we observed that only male C57BL/6 mice developed liver tumors by one year after postnatal ABP exposure, while levels of the potential ABP-bioactivating enzymes CYP1A2, CYP2E1 and NAT, acute DNA damage (ABP-DNA adducts) and ABP-induced *in vivo* mutation frequencies were no different between male and female mice (Sugamori et al., 2012; Wang et al., 2015a; Wang et al., 2012). A lack of correlation between ABP bioactivation capacity, DNA adducts and liver tumor incidence has also been observed by others (Chen et al., 2005), and it supports the idea that although DNA damage is necessary for tumor initiation, it may not account for the observed sex difference in ABP-induced tumor growth. Thus other factors that occur during the classically defined tumor 'promotion' stage subsequent to DNA damage are likely to be sexually dimorphic.

The nitrosamine DEN is the most widely used model liver carcinogen in rodents and other species (Verna et al., 1996), with genetic and histological signatures that are similar to those found in human HCC (Lee et al., 2004). DEN tumorigenicity is also sexually dimorphic in mice, and it can be altered by manipulation of sex hormone status (Nakatani et al., 2001). One suggested mechanism for this effect relates to hormone-dependent differences in hepatotoxic and inflammatory responses between male and female mice. Chronic inflammation is now recognized as a general hallmark of many cancers (Hanahan and Weinberg, 2011), including that of the liver (Bishayee, 2014), and it is associated with many of the known liver cancer risk factors (Berasain et al., 2009). A previous study showed that exposure of sexually mature adult mice to 100 mg/kg DEN resulted in greater acute hepatotoxicity and inflammation in male than in female mice (Naugler et al., 2007), leading to the theory that sex differences in acute chemical-induced liver damage and inflammatory responses are causally related to the sex differences in eventual tumor growth. However, in protocols that use DEN to induce tumors by 9–12 months of age, a single low dose of DEN (typically 2–25 mg/kg) is given to mice on postnatal day 15 (Kang et al., 2007; Naugler et al., 2007; Oganesian et al., 1997). Thus it is not known whether mice exposed on postnatal day 15 to low but ultimately tumorigenic doses of DEN exhibit similar acute hepatotoxic and inflammatory effects to those seen at high doses in adult mice, nor whether such effects are also found with other tumorigenic chemicals such as ABP.

We recently observed that postnatal exposure of mice to an ultimately tumorigenic dose of ABP causes an acute increase in the Ser^{139} phosphorylation of histone H2AX to produce γH2AX, which is an early marker of double-stranded DNA breaks (Kuo and Yang, 2008). The increase was greater in males than in females, which may be related to the greater nuclear factor erythroid 2-related factor 2 (NRF2)-mediated antioxidant gene expression responses that we observed in females than in males (Wang et al., 2015b). NRF2 nuclear accumulation is an important defense mechanism against liver oxidative stress, and it upregulates a battery of antioxidant proteins that can reverse or prevent oxidative damage (Nguyen et al., 2009; Slocum and Kensler, 2011), as well as protect against carcinogen-induced liver tumorigenesis in mice (Kitamura et al., 2007). NRF2 also plays an important role in the innate immune system, assisting in mounting responses to infection by preserving redox balance and preventing dysregulation of inflammatory signalling cascades (Thimmulappa et al., 2006). NRF2 can also directly influence the expression of pro-inflammatory cytokines such as IL-6 by binding to antioxidant response elements (AREs) within the IL-6 promoter (Wruck et al., 2011).

The goals of the present study were therefore to compare selected acute hepatotoxic, inflammatory and NRF2-dependent oxidative stress defense gene responses to ABP and DEN, as well as to the well-established and potent hepatotoxicant carbon tetrachloride (CCl_4), in male and female mice exposed to these chemicals either as adults or using typical postnatal tumor-inducing carcinogen exposure protocols.

2. Materials & methods

2.1. Chemicals and reagents

Except where indicated, all chemicals and reagents were purchased from Sigma-Aldrich Ltd (Oakville, ON). DMSO was used as the vehicle for ABP and CCl_4 exposures and saline (0.9% NaCl) was used as the vehicle for DEN exposures.

2.2. Animal handling

All animal procedures were performed in compliance with the Canadian Council on Animal Care guidelines for the use and care of animals, and all experimental protocols were approved by the University of Toronto Animal Care Committee. Adult mice (strain C57BL/6N) were purchased from Charles River Laboratories (Montreal, QC), and were housed in sterile microinsulator cages with *ad libitum* access to food and water on a 12-hour light/dark cycle. Litters generated from housed breeding pairs were used in postnatal chemical administration studies. In all studies mice were anesthetized by isoflurane inhalation prior to sacrifice and blood was collected by cardiac puncture. Livers were perfused *in situ* with phosphate-buffered saline, then removed and sections were either fixed in 10% neutral buffered formalin or snap-frozen in liquid nitrogen. Collected blood was placed into serum-gel tubes (Sarstedt Inc., Montreal QC) and allowed to clot for 2 hr at room temperature before centrifugation for 10 min at 10,000 x g for serum isolation using the manufacturer's instructions. Serum was aliquoted and stored at −80 °C for subsequent analysis.

2.3. ABP plasma pharmacokinetics

Adult (8 wks of age) male and female mice were injected i.p. with 50 mg/kg ABP as described previously (Sugamori et al., 2006), and serial blood samples were drawn from the saphenous vein 2 h and 6 h later using heparinized microvette capillary tubes (Sartstedt Inc., Montreal, QC). For postnatal pharmacokinetic studies, male and female postnatal day 15 mice were injected i.p. with 50 mg/kg ABP, blood was collected by cardiac puncture either 2 h or 6 h later, placed into heparinized microvette capillary tubes and plasma was isolated following the manufacturer's instructions. ABP plasma levels were quantified by HPLC as previously described (Sugamori et al., 2006).