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Glutathione-S-transferase A3 knockout mice are sensitive to acute cytotoxic and genotoxic effects of aflatoxin B1

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ABSTRACT

Aflatoxin B1 (AFB1) is a major risk factor for hepatocellular carcinoma (HCC) in humans. However, mice, a major animal model for the study of AFB1 carcinogenesis, are resistant, due to high constitutive expression, in the mouse liver, of glutathione S-transferase A3 subunit (mGSTA3) that is lacking in humans. Our objective was to establish that a mouse model for AFB1 toxicity could be used to study mechanisms of toxicity that are relevant for human disease, i.e., an mGSTA3 knockout (KO) mouse that responds to toxicants such as AFB1 in a manner similar to humans. Exons 3–6 of the mGSTA3 were replaced with a neomycin cassette by homologous recombination. Southern blotting, RT-PCR, Western blotting, and measurement of AFB1-N^7-DNA adduct formation were used to evaluate the mGSTA3 KO mice. The KO mice have deletion of exons 3–6 of the mGSTA3 gene, as expected, as well as a lack of mGSTA3 expression at the mRNA and protein levels. Three hours after injection of 5 mg/kg AFB1, mGSTA3 KO mice have more than 100-fold more AFB1-N^7-DNA adducts in their livers than do similarly treated wild-type (WT) mice. In addition, the mGSTA3 KO mice die of massive hepatic necrosis, at AFB1 doses that have minimal toxic effects in WT mice. We conclude that mGSTA3 KO mice are sensitive to the acute cytotoxic and genotoxic effects of AFB1, confirming the crucial role of GSTA3 subunit in protection of normal mice against AFB1 toxicity. We propose the mGSTA3 KO mouse as a useful model with which to study the interplay of risk factors leading to HCC development in humans, as well as for testing of additional possible functions of mGSTA3.

Introduction

We report the development of a mouse model for testing of relationship of the sensitivity of aflatoxin (AFB1) carcinogenesis to the level of a glutathione S-transferase (GST) subunit mGSTA3. AFB1 is metabolized by the P450 enzyme system to active epoxide intermediates, which bind to DNA and induce carcinogenic mutations. These epoxides are detoxified by GST to inactive forms, and are then degraded. There are significant differences in sensitivity to acute (adduct formation, cytotoxic damage) and chronic (carcinogenic) effects of AFB1 among species (Croy and Wogan, 1981; Degen and Neumann, 1981; Gorelick, 1990; Hayes et al., 1991; Hengstler et al., 1999; O'Brien et al., 1983; Wong and Hsieh, 1980). Thus, humans, rats, ducks (Sell et al., 1998) and trout (Sinnhuber et al., 1977) are very sensitive to low doses of AFB1, whereas adult mice are resistant (Wong and Hsieh, 1980). Adult mice can tolerate high doses of AFB1 (up to 60 mg/kg) without manifesting toxic or carcinogenic effects (Wogan, 1969). However, mice are prone to AFB1 toxicity if the toxin is administered during the first week after birth, with males being significantly more sensitive than females (Ueno et al., 1991; Vesselinovitch et al., 1972). In addition, the susceptibility to AFB1 increases in adult mice if hepatocyte proliferation is stimulated, such as following partial hepatectomy or CCl_4-induced injury (Arora, 1981; Shupe and Sell, 2004), or during chronic regenerative hyperplasia (Ghebranious and Sell, 1998; Sell et al., 1991). The enhanced sensitivity to AFB1 hepatocarcinogenicity in newborn mice and in adult mice after liver injury is closely related to a lower level of GST activity in the liver, as compared to the hepatic activity in normal adult mice (Sell, 2003).

The resistance of adult mice to AFB1 has been suggested to be due to constitutive expression in mouse liver of the A3 subunit of GST (mGSTA3; also known as Yc or Ya_3), which exhibits high catalytic activity toward AFB1 (Buetler and Eaton, 1992; Hayes et al., 1992; McDonagh et al., 1999). In support of this suggestion are the findings that mGSTA3 confers protection against 8,9-epoxide when transfected

Abbreviations: AFB1, aflatoxin B1; DMSO, dimethyl sulfoxide; HCC, hepatocellular carcinoma; KO, knockout; LC-ESI/MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; mGSTA3, mouse glutathione S-transferase A3 subunit; WT, wild-type.

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into hamster cells (Fields et al., 1999) and that substitution of the five critical mGSTA3 amino acid residues into the rat GSTA3 sequence increases the conjugation activity over 200-fold (van Ness et al., 1998). A 25,700 Da protein, mGSTA3 accounts for about 35% in male mice, and 47% in female mice, of all GST subunits (Mitchell et al., 1997). Besides AFB1, mGSTA3 appears to have narrow substrate specificity, and is largely unresponsive to drugs (Hayes and Pulford, 1995). However, it may have a function in an antioxidant defense mechanism (Hayes et al., 2000; McWalter et al., 2004; Yang et al., 2002), an idea which is further supported by its possession of a functional antioxidant response element (ARE) within its promoter. Nrf2 transcription factor binds to this promoter and, at least in part, controls its expression (Jowsey et al., 2003a).

In order to test the hypothesis that mGSTA3 is critical for protecting mice from AFB1 toxicity, we have generated an mGSTA3 knockout (KO) mouse. Following a single AFB1 injection, the KO mice show more than a 100-fold increase in AFB1-N^7-DNA adduct levels in their livers, relative to the levels in wild-type (WT) control mice. Thus, the KO mice show levels of adducts similar to those of AFB1-sensitive newborn mice. We propose the mGSTA3 KO mouse as a pre-clinical model with which we can more accurately study the interplay of risk factors contributing to development of HCC in humans.

Methods

Targeting vector. For preparation of the targeting vector used to generate the mGSTA3−/− mice, GSTA3 BAC clone RP23-80N7 (Invitrogen, Carlsbad, CA) was grown and DNA extracted and purified using "NucleoBond BAC Maxi Kit" according to the manufacturer (BD Biosciences Clontech, Palo Alto, CA). Primers specific to mGSTA3 exon 4 and exon 5 (forward primer: 5′-GGA GTT CAA CCA GGG CAA TA-3′; reverse primer: 5′-GGC GGA TCT GGA GAT AAT GA-3′) were used to PCR amplify this region, and the resulting amplicon was sequenced to confirm that the purified BAC DNA was mGSTA3. The purified BAC DNA was then digested with *Bgl*II and *Spe*I in separate reactions to generate 2-kb and 6.7-kb fragments, respectively; these DNA fragments were excised and gel purified. The 6.7-kb fragment was ligated into the EmbryoMax ESTV-Neo targeting vector (Specialty Media, Phillipsburg, NJ). This vector contains a floxed neomycin resistance selection marker. Subsequent screening and sequencing identified a clone with the proper GST fragment in the correct orientation. The 2-kb fragment was then inserted upstream of this and correct insertion and orientation was again confirmed by sequencing. We then proceeded to produce a null mGSTA3 by homologous recombination, as described in Results.

Breeding of mGSTA3 KO mice. The mGSTA3 KO mice, generated as described in the Results section, have been bred with C57Bl/6J WT mice (Jackson Laboratory, Bar Harbor, ME) and maintained on a C57 background. Routinely, homozygous mGSTA3KO males are bred with mGSTA3 homozygous KO females so that all offspring are expected to be KO mice. For some purposes, KO mice are bred to WT mice, or heterozygous (+/−) mice are mated together. For all offspring, we use PCR screening of the genomic DNA that involves three separate reactions: exon 4 and exon 5 (present only in the WT mice) and Neo (present only in the KO mice). The accuracy of the screens is 100%, since the results of the reactions are always consistent with the expected distribution of the WT and KO mGSTA3 gene.

Measurements. The activity of total hepatic GSTs was measured on liver homogenates, with DCNB as a substrate, by the method of Habig et al. (1974). The AFB1–DNA adducts were measured by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). This triple-quadrupole mass spectrometry (Thermo-

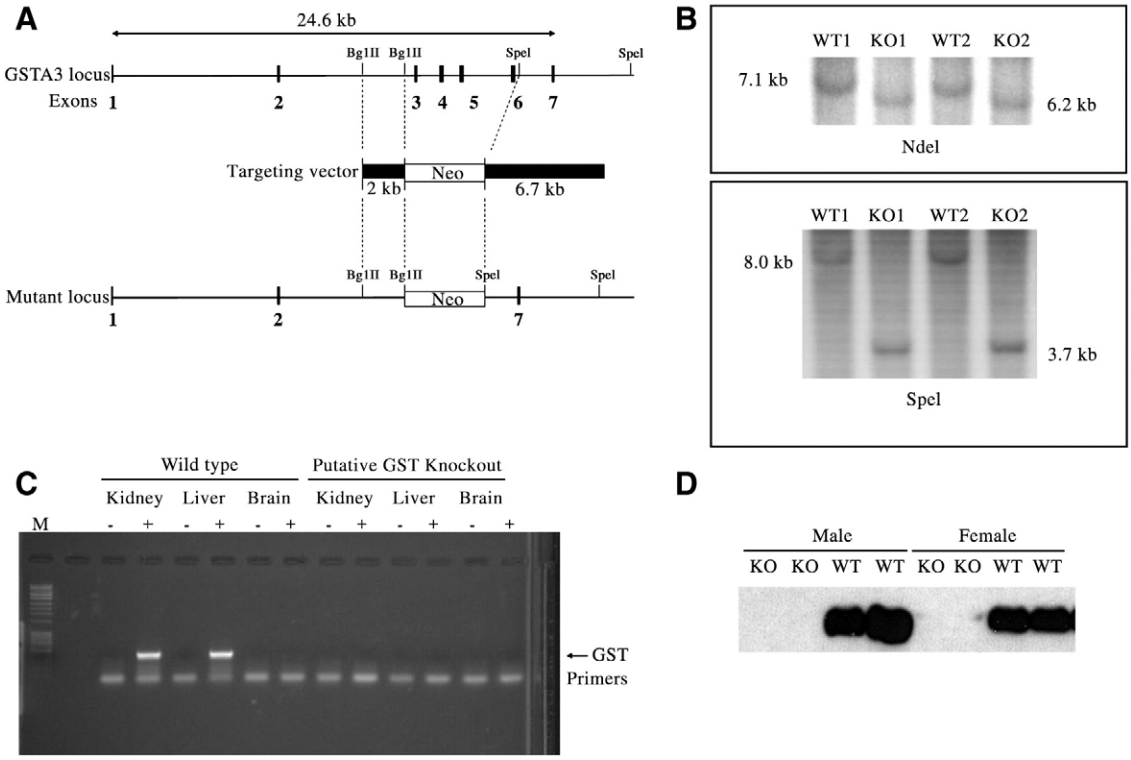

Fig. 1. Generation and characterization of mGSTA3 knockout (KO) mouse. (A) Scheme of mGSTA3 gene deletion. The targeting vector containing a neomycin resistance cassette is shown, along with restriction enzymes on the left and right arms used to generate it from genomic DNA. (B) Detection of mGSTA3 DNA by Southern blotting on two mGSTA3 KO and two WT DNA samples isolated from liver. The DNA was digested with either Nde1 or Spe1 and probed with the 2-kb Bgl2 fragment. (C) Determination of mGSTA3 MRNA by RT-PCR in liver, kidney, and brain of four mGSTA3 KO and four WT mice. (D) Determination of mGSTA3 protein by Western blotting using anti-mGSTA3 specific antibodies, in livers of four mGSTA3 KO and four WT mice. (B), (C), and (D) show the absence of targeted DNA and lack of mGSTA3 mRNA and protein expression for the KO mice.