Tumor formation in liver of conditional β-catenin-deficient mice exposed to a diethylnitrosamine/phenobarbital tumor promotion regimen

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The antiepileptic drug phenobarbital (PB) is a potent tumor promoter in mouse liver, where it stimulates the selective outgrowth of tumor populations harboring activating mutations in Ctnnb1, encoding β-catenin. A tumor initiation–promotion study was conducted in mice with conditional hepatocyte-specific knockout (KO) of Ctnnb1 and in Ctnnb1 wild-type controls. Mice received a single injection of N-nitrosodimethylamine (DEN) at the age of 6 weeks followed by continuous administration of PB given in the diet (0.05%) for 27 weeks. Metabolic activation of DEN in hepatocytes from both Ctnnb1 wild-type and KO mice was demonstrated. PB strongly enhanced liver tumor formation in Ctnnb1 wild-type mice, and 90% of the PB-promoted tumors were Ctnnb1-mutated. A similar increase in carcinogenic response was seen when using glucose-6-phosphatase and glutamine synthetase as tumor markers. The prevalence of tumors in Ctnnb1 KO mice was ~7-fold higher than in wild-type mice, suggesting an enhancing effect of the gene KO on liver tumor development. However, in strong contrast to wild-type mice, PB did not promote tumor formation in the Ctnnb1 KO mice. Livers of KO mice, particularly from the PB treatment group, demonstrated fibrosis and massive infiltration of immune cells, an effect not seen in wild-type mice. In summary, our data demonstrate that (i) liver tumor promotion by PB requires functional β-catenin signaling and (ii) absence of β-catenin enhances carcinogen-induced hepatocarcinogenesis and induces a pre-cirrhotic phenotype in mouse liver.

Introduction

Chemical hepatocarcinogenesis in mice is a useful experimental tool to study molecular alterations relevant for tumor initiation, promotion and progression. Single treatment with an initiating liver carcinogen like N-nitrosodimethylamine (DEN) and subsequent chronic administration of a tumor promoter leads to the development of liver tumors, which harbor, depending on the promoting agent, mutations in different oncogenes. In DEN-initiated mice treated with the model tumor promoter phenobarbital (PB), ~80% of liver tumors carry mutations in the Ctnnb1 gene (encoding β-catenin), whereas 30–50% of liver tumors are mutated in the Ha-ras or B-raf oncogenes in the absence of PB (1). Therefore, the promotional activity of PB and other PB-like tumor promoters appears to consist in a selection for hepatocytes harboring an activating mutation in Ctnnb1 that leads to constitutive activation of the Wnt/β-catenin signaling pathway (1,2).

β-Catenin is a multifunctional protein which constitutes a component of the cellular adhesion complex and also acts as a central component in the canonical Wnt/β-catenin signaling pathway, an evolutionarily highly conserved signal transduction cascade regulating differentiation, proliferation and morphogenesis (3,4). Cellular levels of β-catenin are tightly controlled by a cytosolic multi-protein complex (5); in the absence of Wnt signaling, formation of this complex results in phosphorylation and subsequent proteolytic degradation of β-catenin via the ubiquitin–proteasome pathway. Following stimulation of Wnt-signaling, degradation of β-catenin is inhibited and the protein translocates into the nucleus where it associates with transcription factors of the T-cell-specific transcription factor family and activates target gene transcription (6,7). Deregulation of the β-catenin pathway is frequently observed in human and rodent tumors of the liver and other organs (8). Inappropriate activation of β-catenin signaling is often caused by point mutations or small deletions affecting the phosphorylation sites in exon 3 of the Ctnnb1 gene, resulting in stabilization of the oncprotein. As a consequence, Wnt/β-catenin target genes such as glutamine synthetase (GS) are overexpressed in Ctnnb1-mutated hepatocellular tumors (8).

In the present paper, we made use of a genetically manipulated Ctnnb1 knockout (KO) mouse (9), in which the β-catenin signaling pathway is specifically inactivated in hepatocytes (10). This model allowed us to further test our previous hypothesis that PB-mediated tumor promotion is mechanistically linked to the Wnt/β-catenin pathway (1).

Materials and methods

Animals and treatment

Alb-Cre mice (purchased from The Jackson Laboratory, Bar Harbor, ME) expressing Cre recombinase under the control of the hepatocyte-specific albumin promoter and transgenic mice with loxP sites inserted in the introns flanking exons 3 and 6 of Ctnnb1 (9) were backcrossed for five generations into mice of the C3H/He strain leading to a genetic background which is highly susceptible for PB-mediated promotion. Mice with hepatocyte-specific KO of Ctnnb1 were obtained by interbreeding transgenic mice with Alb-Cre mice. Polymerase chain reaction-based genotyping was performed as recently described (10). Homozygous Ctnnb1/KO mice carrying a Cre allele, and therefore in hepatocyte-specific KO of Ctnnb1, are referred to as ’Ctnnb1 KO mice’ in the following. The respective wild-type mice are called ‘WT’ hereafter. Mice were kept on a 12 h dark/light cycle and had access to food and tap water ad libitum. Animals received humane care and protocols complied with institutional guidelines.

To analyse metabolic activation of DEN in hepatocytes, male 14-week-old WT and KO mice were intraperitoneally injected with a single dose of DEN (90 μg/kg body wt). Animals were killed 4 h later and livers were frozen on dry ice and stored at ~70°C.

For induction of liver tumors, male WT and KO mice were intraperitoneally injected with a single dose of DEN (90 μg/kg body wt) at 6 weeks of age. After a treatment-free interval of 3 weeks, mice were randomly assigned to four experimental groups (15 mice in each of the WT groups and 16 mice in each of the KO groups), which were either kept on a standard diet or on a diet containing 0.05% PB until killing. Eleven mice died before the end of the experiment (1 WT and 10 KO mice) and the remaining 51 mice were killed at an age of 36 weeks, i.e. 27 weeks after the start of PB treatment. Killing was between 9 and 11 a.m. to avoid circadian influences. Livers were excised, inspected for macroscopically visible tumors and the number and size of tumors were recorded. Aliquots of liver were frozen on blocks of dry ice and stored at ~70°C or were fixed in Carnoy’s solution and embedded in paraffin.

Visualization of O6-ethylguanine adducts in DNA

Frozen sections were prepared from livers of short-term DEN-treated WT and KO mice and stained immunohistochemically for the presence of O6-ethylguanine adducts in DNA using a rabbit antibody kindly provided by Dr Jürgen Thomale (University of Duisburg-Essen, Essen, Germany). A non-DEN-treated control animal was also included. We adapted the staining procedure described in ref. 11: liver sections were fixed in methanol (~20°C, 30 min), briefly washed with phosphate-buffered saline and incubated for 5 min in alkaline solution (70 mN NaOH, 140 mM NaCl, in 40% methanol). After incubation with proteinase-K (100 μg/ml each) for 10 min at 37°C each, sections were washed in phosphate-buffered saline containing 0.2% glycine, blocked with 5% skim milk and incubated with the primary antibody.
Glucose-6-phosphatase-activity staining and immunohistochemical demonstration of GS

Serial sections (10 µm thick) were prepared from frozen livers of the animals of the tumor initiation/promotion experiment. One section was stained enzyme-histochemically for glucose-6-phosphatase (G6Pase)-activity as described previously (12). A parallel section was stained immunohistochemically for GS using a mouse anti-GS monoclonal antibody (1:2000; BD Biosciences Europe, Heidelberg, Germany) in combination with a horseradish peroxidase-conjugated secondary antibody (1:20; Dako, Glostrup, Denmark) and 3-aminoo-9-ethylcarbazole/H2O2 as substrates.

Stainings for histopathological examination

Sections (5 µm) were prepared from paraffin-embedded liver blocks. The Masson–Goldner trichrome stain was applied for demonstration of fibrosis using a commercially available staining kit (Roth, Karlsruhe, Germany) according to the manufacturer’s instructions. Glycogen content was demonstrated by use of the Periodic Acid Schiff’s reaction (13).

Quantification of tumor response from stained tissue sections

The sizes of enzyme-altered liver lesions in G6Pase- and GS-stained sections were quantified by means of a computer-assisted digitizer system consisting of a Zeiss Axio Imager microscope and a Wacom Cintiq 21UX pen display. Areas of liver sections and of enzyme-altered tumor intersections therein were determined and the volume fractions of altered lesions (which are equivalent to the area fractions) were calculated.

Mutation analysis

For detection of point mutations in Ha-ras codon 61 and B-raf codon 637 (formerly referred to as codon 624), a restriction fragment length polymorphism analysis was performed with polymerase chain reaction-amplified DNA as recently described (14). For detection of hot spot mutations in the N-terminal part of Ctnnb1, we followed the procedure recently described by our group (1).

Statistical analysis

Kruskal–Wallis with Dunn’s multiple comparison test was performed using GraphPad Prism V5.03 for Windows (GraphPad Software, San Diego California). Differences were considered significant when \( P < 0.05 \).

Results

Demonstration of \( O^6 \)-ethylguanine formation

DEN, a potent liver carcinogen in mice, was applied for tumor initiation. DEN requires metabolic activation through cytochrome P450 (CYP)-mediated \( n \)-hydroxylation to exert its carcinogenic effect. CYP2E1 is involved in the catalysis of this reaction since Cyp2e1 KO mice develop less liver tumors than WT mice (15). To investigate activation of DEN in liver, we used an antibody directed against \( O^6 \)-ethylguanine, a promutagenic adduct formed in liver DNA of DEN-exposed mice (16). Frozen liver sections from WT and KO mice treated with a single injection of DEN were double-stained for the presence of \( O^6 \)-ethylguanine and GS. The latter staining was used for the identification of central venules that are exclusively surrounded by GS-positive hepatocytes (17). The results shown in Figure 1 convincingly demonstrate that \( O^6 \)-ethylguanine adducts from DEN are formed in hepatocytes from both WT and KO mice. The major difference between the two genotypes is that \( O^6 \)-ethylguanine staining demonstrated zonality in WT mice, which was not present in KO mice. In WT mice, perivenous hepatocytes stained strongly positive, whereas perportal hepatocytes displayed only very faint staining. In contrast, KO mice showed staining of nuclei in all hepatocytes, albeit with somewhat weaker intensity than in WT mice. The loss of \( O^6 \)-ethylguanine-zonality in KO mice was not unexpected since various CYP isoforms lose zonal expression in liver in the absence of \( \beta \)-catenin; for review, see ref. 18. The result clearly demonstrated that DEN is metabolized to its ultimate carcinogenic form in liver of KO mice and, as a side result, it also showed that CYP2E1 cannot be the only CYP-isoform responsible for this metabolic step since expression of CYP2E1 is strongly reduced in liver of Ctnnb1 KO mice (10,19).

Tumor initiation–promotion study

The carcinogenicity study was conducted using DEN as initiator and PB as tumor promoter according to the well-established scheme illustrated in Figure 2A. At the beginning of the experiment, KO mice had a slightly reduced body weight when compared with WT mice, which remained lower during the entire study period (Figure 2B). Ten of the KO mice (six on control diet and four on PB-treated mice) died during the experiment, in contrast to only one in the WT groups, indicating a much higher vulnerability of the KO mice.

PB treatment led to an increase in the liver/body weight ratio both in WT and in KO mice, but the effect was somewhat smaller in KO mice (Figure 2C). The more pronounced increase in mean liver/body weight ratio in PB-treated WT mice was not solely due to the well-known liver-enlarging effects of PB but also to a much stronger liver tumor burden in this group. After termination of the experiment, tumors visible on the surface of the liver were counted. Tumor multiplicity (mean number of tumors per mouse) in the four treatment groups is given in Figure 3A. The results clearly demonstrated that (i) the tumor response was, in the absence of PB, much higher (although not statistically significant at \( P < 0.05 \)) in KO than in WT mice.
(1.0 versus 0.14 tumours per mouse, respectively) and (ii) PB was very active as a tumor promoter in WT but not in KO mice. These findings are illustrated in detail in Figure 3B where the size class distribution of tumors in the four groups is shown. Although only two tumors with a diameter between 1 and 2 mm were observed in the 14 mice of the WT minus-PB group, KO mice had tumors in all three diameter classes, both in the PB and in the non-PB treatment groups. Moreover, tumor response was strongly enhanced in PB-treated WT mice in all three diameter classes.

Morphometric analyses

Serial sections were prepared from frozen livers and stained for G6Pase and GS. Tumors were identified in the stained sections by an alteration in G6Pase-activity (mostly negative) and/or alteration in GS staining-intensity (mostly positive) relative to the surrounding normal tissue. In most instances, compression of the surrounding normal liver tissue was also seen. Representative examples of tumors identified by the two markers in the liver of a PB-treated WT mouse are shown in Figure 4.

The two-dimensional sizes of intersections of tumors identified by alterations in G6Pase-activity and/or GS-content were determined thus allowing the quantification of the percentage of tumor tissue in the animals. The volume fraction, which is equivalent to the area fraction, is the most reliable parameter since it can be determined without bias-sensitive stereological procedures. The results of this analysis are summarized in Table I, showing that the by far strongest carcinogenic response occurred in livers of PB-treated WT mice and that most of the lesions were G6Pase negative and also GS positive. Non-PB-treated KO mice were somewhat more susceptible with respect to the formation of enzyme-altered lesions than WT mice. A promoting effect of PB, however, was not seen in KO mice.

Mutation analyses

Tumors with diameters of >3 mm were isolated and analyzed for the presence of mutations in codon 61 of Ha-ras, codon 637 of B-raf and exon 3 of Ctnnb1. In total, we analyzed 14 tumors from non-PB-treated mice, of which three (two from KO mice and one from a WT mouse) were Ha-ras-mutated, all of the type c.181C>A. B-raf- or Ctnnb1-mutations were not detected in these tumors. Of the 10 tumors analyzed from PB-treated WT mice, 9 showed activating point-mutations in Ctnnb1, all affecting the well-known hot spot positions in the gene region encoding exon 3 (1). Surprisingly, two of six tumors isolated from PB-treated KO mice were also Ctnnb1-mutated. A consistency check unequivocally demonstrated the hepatocyte-specific Ctnnb1 KO in these mice (for a possible explanation for this finding, see discussion).
Histopathological findings in non-tumorous tissue

In routinely prepared haemalum/eosin-stained sections, we detected clear signs of a fibrotic process in livers from KO mice, which was accompanied by massive infiltration of cells of the immune system. To further characterize and specify these effects, we performed a histological Masson–Goldner trichrome stain that allows the discrimination between hepatocytes (Figure 5A and C, red staining) and fibrotic tissue (Figure 5A and C, greenish staining). Some of the livers showed areas consisting of hepatocytes demonstrating massive lipid storage (Figure 5E) and other areas consisting of strongly enlarged hepatocytes of a preneoplastic phenotype (Figure 5F). The hepatocytes, arranged in nodular structures surrounded by fibrotic tissue (Figure 5A), appeared to store glycogen as indicated by the Periodic Acid Schiff’s stain but in a somewhat scattered manner (Figure 5B and D).

These histopathological abnormalities were only seen in KO but not in WT mice. The response, however, was very variable and differed between PB-treated and non-PB-treated groups. Some KO mice showed almost no signs of liver fibrosis/cirrhosis, whereas this abnormality was very evident in others. Strong differences in the degree of fibrotic appearance were observed even between different lobes of one and the same animal (data not shown). Notably, KO mice of the PB treatment group were more frequently affected: half of the mice of this group showed more or less pronounced signs of fibrosis (6 of 12), whereas only 1 of 10 of the non-PB-treated mice showed (albeit in this case very strong) liver fibrosis.

Fig. 4. Enzyme and immunohistochemical localization of liver tumors in G6Pase- and GS-stained tissue sections. The example shows two serial sections from a PB-treated Ctnnb1 WT mouse. Arrows indicate tumors identified by the two markers.

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<th>Table 1. Quantification of enzyme-altered liver lesions in G6Pase- and GS-stained sections</th>
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<td><strong>Group</strong></td>
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Relative fractions (%) of tumor tissue of the respective phenotype are given as mean ± SD.

*Kruskal–Wallis test is significant with \( P < 0.0001 \).

Significantly different from groups 1,4 \( P < 0.001 \) and 3 \( P < 0.01 \); Dunn’s multiple comparison test.

Significantly different from groups 1.3 \( P < 0.0001 \) and 4 \( P < 0.01 \); Dunn’s multiple comparison test.

Significantly different from group 1 \( P < 0.05 \) and 4 \( P < 0.01 \); Dunn’s multiple comparison test.

Significantly different from groups 1,3 and 4 \( P < 0.001 \); Dunn’s multiple comparison test.

Discussion

The present study aimed to investigate the effect of β-catenin deficiency in liver on tumor promotion by PB. The results then demonstrated unexpected additional alterations that occurred in non-tumorous liver of the gene KO animals. In the following, we will first discuss the tumor response observed in the absence and presence of PB, then our observations made in non-tumor liver tissue.

Tumor response

In the absence of PB treatment, the DEN-induced liver tumor response was ~7-fold higher in KO mice than in WT mice. A similar finding has very recently been reported during the preparation of this manuscript (20). This increase in tumor response in KO mice was somewhat surprising since one might assume that metabolic activation of DEN is attenuated in KO mice due to reduced expression of several CYP isoforms (10,19,21) required for DEN metabolism. The following considerations may explain this finding: DEN is metabolized to its ultimate reactive form by defined CYPs, which are preferentially localized in perivenous hepatocytes. As a consequence, formation of Oπ-alkylguanine adducts in DNA occurs preferentially in perivenous hepatocytes in WT mice (Figure 1A). Perivenous CYP expression is regulated by β-catenin-dependent signaling and is therefore partially lost in liver of KO mice, where some CYPs are homogeneously expressed throughout the liver lobule (18). This explains the homogenous staining pattern for Oπ-ethylguanine adducts in livers of KO mice (Figure 1B). Oπ-ethylguanine adducts are promutagenic but, to fix the mutation, cell proliferation is a prerequisite, which is much higher in periportal than in perivenous hepatocytes (22). Therefore, a shift of metabolic activation of DEN to midzonal and perportal hepatocyte populations, showing higher rates of replicative DNA synthesis, might enhance tumor initiation in KO mice leading to the increased tumor prevalence of these mice observed in our study.

PB is a strong tumor promoter in C3H mice when given subsequentially to a tumor-initiating dose of DEN administered to 5- to 6-week-old animals (23). In the present study, the multiplicity of tumors visible on the surface of the livers was increased ~80-fold by PB in WT mice when compared with the respective non-PB-treated controls, but no promoting effect of PB was observed in KO mice. Similarly, the fraction of liver occupied by lesions showing an alteration in G6Pase activity (mostly decreased) or GS (mostly positive) was strongly increased by PB treatment in WT mice but not in KO mice. The vast majority of G6Pase-altered tumors in PB-treated WT mice were also GS positive, suggesting that they harbor activating mutations in Ctnnb1, which is involved in the upregulation of GS in liver (8). In accordance with this, 90% of tumors isolated from PB-treated WT mice harbored Ctnnb1 mutations, which is close to the 80% observed in a previous study from our group (1). This finding
underscores our previous notion that PB selectively promotes the proliferation of Ctnnb1-mutated hepatocytes (1), strongly suggesting that there exists some sort of cross talk between PB-mediated signaling and β-catenin-dependent signal transduction. PB-mediated tumor promotion depends on the presence of constitutive androstane receptor (24) and on the presence of the gap junction protein connexin32 (23). The finding that PB is inactive as a tumor promoter in Ctnnb1 KO mice adds β-catenin to the list of proteins required for tumor promotion by the barbiturate. The molecular link between the three proteins, however, remains enigmatic.

A surprising finding of the present study was the presence of two Ctnnb1-mutated tumors in PB-treated KO mice. The easiest explanation for the existence of tumors of this phenotype is that the KO of Ctnnb1 mediated by the albumin promoter-driven Cre recombinase was not complete, sparing a small number of Ctnnb1 WT β-catenin-positive hepatocytes in liver. Such partial leakiness of the albumin promoter has been in fact reported by others (25). We have also observed small patches of β-catenin-positive hepatocytes in 8-week-old Ctnnb1 KO mice (21). We therefore hypothesize that the initial Ctnnb1 mutation occurred in hepatocytes that had escaped Alb-Cre-driven Ctnnb1 inactivation and were then stimulated to proliferate under the promoting influence of PB.

**Histopathological changes in non-tumor liver tissue**

The Ctnnb1 KO produced a precirrhotic phenotype in liver, which was more severe in the PB treatment group in the sense that more mice were affected. The observed effects in the hepatocytes point to disturbances in the intermediary metabolism with changes in carbohydrate (glycogen storage) and lipid metabolism (fat storage). In some of the animals, there were clear indications of liver fibrosis accompanied by infiltration of immune cells and a beginning nodulation of the hepatocytes, all signs of a cirrhotic process in liver. This is in accordance with the changes that have very recently been reported to occur in livers of DEN-treated Ctnnb1 KO mice (20). The nature underlying these pathological changes is unknown. Signaling through β-catenin is one of the key factors regulating the perivenous differentiation patterns of hepatocytes. The expression of several enzymes of drug metabolism, normally preferentially expressed in perivenous hepatocytes, is decreased in the liver of KO mice; these include detoxifying enzymes of phase-II, in particular the glutathione S-transferases (GST) (10,26). GSTs play a decisive role in the defense against electrophilic compounds and oxidative stress (27). The expression of the GST-isozymes m2, m3 and m6 is strongly induced by PB in liver of WT mice but to a much lesser extent in liver of KO mice (10). PB-exposure of hepatocytes leads to increased oxidative stress (28,29), which might produce hepatocellular degeneration and subsequent changes in liver architecture under the condition of a decreased GST expression. In addition, electrophilic intermediates generated by PB-induced CYPs from xenobiotics present in the diet may not be detoxified in the absence of optimal GST activity and produce hepatocellular damage, particularly in the PB-exposed Ctnnb1 KO mice.

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**References**


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