Tumor Promotion in Liver of Mice with a Conditional Cx26 Knockout

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Received January 21, 2008; accepted February 19, 2008

Connexin (Cx) 26 and 32 are the major gap junction proteins in liver. We recently demonstrated that Cx32 is essential for phenobarbital (PB)–mediated tumor promotion in mouse liver. To investigate whether Cx26 plays a similar role, an initiation-promotion experiment was conducted using mice with a liver-specific knockout of Cx26. Control and Cx26-deficient mice were injected a single dose of N-nitrosodiethylamine (DEN, 90 μg/g b.wt.) at 6 weeks of age and groups of mice were subsequently kept on a PB (0.05%) containing or control diet for 35 weeks. At the end of the experiment, the carcinogenic response in the liver was monitored. Mice from PB treatment groups showed strongly increased liver weights compared with mice treated with DEN alone, which was mostly due to a much higher tumor burden. The tumor response in PB-treated mice of both strains was quite similar, but the number of smaller tumors and of enzyme-altered tumor derived cell lines (Mesnil, 2002; Trosko and Ruch, 2002).

In a previous study we could show that Cx32 is essential for PB-mediated tumor promotion in mouse liver (Moennikes et al., 2000). To investigate, if Cx26 plays a similar role we conducted a tumor initiation-promotion experiment with N-nitrosodiethylamine (DEN) and PB using wild-type and Cx26-deficient mice. Because mice with a constitutive Cx26 knockout (Cx26 KO) are not viable, we used Cx26fl/fl mice coexpressing the Cre recombinase under the control of the albumin promoter (alb-cre) to generate a liver-specific conditional knockout of Cx26 (Cohen-Salmon et al., 2002; Ott et al., 2006). These mice were bred into the genetic background of C3H mice, which are highly susceptible to PB-mediated tumor promotion.

Promotion of mouse hepatocarcinogenesis by PB consists in a selection for hepatocytes carrying activating Ctnnb1 mutations (Aydinlik et al., 2001). Ctnnb1 encodes β-catenin which, upon activation by upstream signaling molecules or by mutation of the gene, acts as a transcriptional regulator together with proteins of the T cell factor family of transcription factors. One of the transcriptional targets of β-catenin is glutamine synthetase (GS), which may serve as a marker for the identification of Ctnnb1-mutated hepatocytes (Loeppen et al., 2002). In the present study, we have used the GS marker as a means to screen for liver tumors with activated β-catenin. As a second marker glucose-6-phosphatase (G6Pase) was employed, which is generally absent or lowered in activity in neoplastically transformed hepatocytes (Wachstein and Meisel, 1957). We have also analyzed the expression and intracellular localization of Cx32, which was previously shown to be absent from membranes of GS-positive mouse hepatomas (Moennikes et al., 2000). As we observed differences between GS-positive tumors from Cx26 wild-type (Cx26 Wt) and Cx26 KO mice, we conducted a phosphoproteome microarray experiment to elucidate changes in Wnt-signaling between tumors from the...
two genotypes. In addition, we analyzed the expression and intracellular localization of Cx26 in GS-positive tumors from Cx26 Wt mice.

MATERIALS AND METHODS

Generation of C3H Cx26fl/fl/alb-Cre mice. Cx26fl/fl/alb-Cre mice (Ott et al., 2006) were backcrossed into a C3H/HeJ background because C3H mice are highly susceptible for PB-mediated promotion. To this end, mice containing at least one Cx26 allele and/or the alb-Cre allele were mated with C3H/HeJ mice (Charles River Laboratories, Sulzfeld, Germany) for five generations to yield mice with a C3H background of > 96%. After the fifth backcross generation, Cx26 mice were mated with Cx26/fl-/alb-Cre mice; mice of the resulting Cx26/fl-/alb-Cre, Cx26FL/FL-/ and Cx26fl/fl-alb-Cre genotypes were brother/sister mated for two generations for expansion of the respective strains. For the tumor promotion experiment, Cx26fl/fl-- mice were mated with Cx26fl/fl- alb-Cre mice to yield both the Cx26FL/FL-/ and Cx26FL/FL-alb-Cre genotypes; only males were used for the experiment. Genotyping was performed by PCR using DNA from digests of ear punches. For detection of the Cx26fl genotype, C3H/HeJ mice (Charles River Laboratories, Sulzfeld, Germany) were made alb-Cre transgenic (Wittchen et al., 2002). Printing and blocking of chips as well as assay procedures followed the standard protocols provided by the manufacturer. Briefly, tissue samples were homogenized in a Braun Micro-Dismembrator at −80°C and lysed under denaturing conditions using Cel/LyA Lysis Buffer CLB1 (Zeptosens). Protein concentration was determined using a Bradford assay kit (Pierce, Rockford, IL), protein content was adjusted to 4 mg/ml and tissue lysates were subsequently diluted 1:10 in Cel/LyA Spotting Buffer CSBL1 (Zeptosens). Tissue lysates were arrayed using an ink-jet spotter (NP2, GeSiM, Großkarlsmünd, Germany) on ZeptoMARK chips (Zeptosens). Antibody incubations were done in Cel/LyA Assay Buffer CAB1 based on BSA according to standard protocols (Zeptosens). After incubation with secondary fluorescence-labeled anti-species antibodies the fluorescence imaging of assay signals was performed with the ZeptoREADER instrument (Zeptosens). Microarray images were analyzed using the microarray analysis software ZeptoVIEW Pro 2.0 (Zeptosens). Used antibodies are listed in the supplementary table.

Analysis of apoptosis. Frozen liver sections from Cx26 KO, Cx32 KO or WT mice were analyzed in two independent experiments (three animals per group) by the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit following the instructions of the manufacturer (Roche). The number of apoptotic nuclei per area was counted in 40 visual fields per section under a Leica Alpavox fluorescence microscope. The total area analyzed was 6 mm² per section (0.15 mm² per visual field).

Statistical analysis. Statistical analyses were performed by Students t-test for comparison of two groups and ANOVA analysis for comparing all four groups. Data were collected in MS-Excel, calculations were performed with MS-Excel (t-test) or the software-package R version 2.5.1 (ANOVA analysis) (R-Development-Core-Team, 2003). Differences were considered as significant if p < 0.05 and a trend was assumed if p < 0.1. Three different types of effects were analyzed by ANOVA: (1) effect of genotype (Cx26 Wt vs. Cx26 KO), (2) effect of treatment (plus PB vs. minus PB), and (3) an interaction effect, meaning that the genotypes are affected differently by treatment.

RESULTS

Characteristics of Cx26 KO Mice

The knockout of functional Cx26 in mice of the present study was achieved by Cre recombinase-mediated elimination of part of exon 2 of Cx26 in mice of the Cx26fl/fl-alb-Cre genotype. Because the expression of the Cre recombinase is under the control of the albumin promotor and because albumin is expressed in virtually all hepatocytes, Cx26 should be...
knocked out throughout the liver. To prove the functionality of
the genetic manipulation, liver sections from Cx26<sup>fl/fl</sup>/−
and Cx26<sup>fl/fl</sup>/alb-Cre mice were immunostained for Cx26. Although
Cx26 was localized in Cx26FL/FL/− (Cx26 Wt) mice to the
outer cell membrane forming characteristic Cx26-positive
plaques, the specific membrane staining was completely lost
in Cx26<sup>fl/fl</sup>/alb-Cre (Cx26 KO) mice in virtually all hepatocytes, in agreement with the expectation (Fig. 1).

At the end of the initiation-promotion experiment, liver and
body weights of the animals were determined. In animals
without PB treatment, there were no significant differences in
liver/body weight ratios between Cx26 Wt and Cx26 KO mice.
PB treatment induced significant increases in relative liver
weights (p < 0.001), both in Cx26 Wt and Cx26 KO mice
(Fig. 2), largely due to a strong increase in tumor burden of
PB-treated animals. Although relative liver weights of PB-
treated Cx26 KO animals were slightly smaller than those of
PB-treated wild-type animals, this effect was just above the
significance level (ANOVA, p = 0.057).

### Carcinogenic Response in Liver

Strong differences in tumor response were apparent upon
gross inspection of the livers from animals of the PB-plus and
PB-minus treatment groups (for typical examples see Fig. 3).

To quantify the promotional effect of PB, macroscopically
visible tumors with diameters larger than ∼5 mm were counted.
This cut off was chosen because the tumor response in the two
PB treatment groups was too strong to allow an exact
quantification of all tumors. As shown in Table 1, the average
number of tumors per mouse was 0.4 in Cx26 Wt controls,
4.7 in PB-treated Wt mice, 0.2 in Cx26 KO controls and 4.8 in
PB-treated KO mice. The PB treatment effects were highly
significant (p < 0.001), whereas the effect of genotype was
not. Moreover, tumors were stratified according to their size
into 2 size classes (Table 1). The largest tumor with a diameter
> 20 mm was observed in the Cx26 KO group, and the average
number of tumors > 9 mm was slightly larger in the PB-treated
Cx26 KO group than in the PB-treated wild-type group.

To further analyze the promotional effect of PB, enzyme-
altered liver lesions were quantified in the four treatment
groups. For this purpose, serial cryostat sections were prepared
from frozen liver blocks and stained for the marker enzymes
G6Pase and GS. As shown in Figure 4, the numbers of lesions
per cm<sup>3</sup> liver tissue were strongly increased in mice of the two
PB treatment groups, for both marker enzymes. It should be
mentioned that most GS-positive lesions were also G6Pase-
negative. Therefore, particularly in the PB-plus groups, the two
markers identified the same entities of lesions in the vast

**FIG. 1.** Immunohistochemical staining of livers from Cx26<sup>fl/fl</sup> mice without
(wild-type) and with (knockout) expression of Cre recombinase; frozen sections
were stained with a mouse anti-Cx26 primary and a Cy3-labeled secondary
antibody. Wild-type mice show Cx26 containing gap junctions, whereas
knockout mice do not. Bar: 0.05 mm.

**FIG. 2.** Relative liver weights of DEN-initiated Cx26 Wt and Cx26 KO
mice without and with PB treatment. The PB-mediated increase in relative liver
weights (ANOVA, p < 0.001) is largely due to a strong increase in liver tumor
mass (e.g., see Fig. 3).
majority of cases. In the PB-minus groups, however, the few G6Pase-negative lesions observed were mostly GS-negative. Upon statistical analysis by use of the ANOVA test, the increases in lesion numbers mediated by PB were highly significant \((p < 0.001)\), whereas there was no significant influence of genotype of mice (Cx26 Wt versus Cx26 KO, \(p = 0.097\) for G6Pase and \(p = 0.075\) for GS) on tumor response. However, a significant interaction effect could be detected (\(p = 0.003\) for G6Pase and \(p = 0.029\) for GS), giving evidence, that PB treatment led to somewhat larger numbers of GS-positive and G6Pase-negative lesions in PB-treated knockout mice when compared with the respective wild-type mice. We also analyzed the size distribution of G6Pase-negative and GS-positive lesions. The results are shown in Figure 5. It is quite obvious from the Figure that PB treatment of mice mediated a strong increase in lesion numbers in all size classes, but without obvious difference between mice of the two genotypes. Lesions in the largest size class (> 10 mm\(^2\)) were exclusively from PB-treated mice. GS-positive lesions were very rarely detected in non-PB-treated mice but very frequent in their PB-treated counterparts. The size class distribution also elucidates a tendency of PB-treated knockout animals to have smaller lesions (significant for lesions of less than 1 mm\(^2\) in area size). These results also suggest a slight interaction effect as seen for other parameters by ANOVA analysis (see below), suggesting that PB treatment affects genotypes slightly differently.

Data on the volume fractions of G6Pase-negative and GS-positive liver lesions are presented in Figure 6. The data clearly indicate a strong enhancing effect of PB on this parameter, which was highly significant for both markers (ANOVA, G6Pase, \(p < 0.001\); GS, \(p < 0.001\)), but not significantly affected by the genotype of the mice. Again, an interaction effect of treatment and genotype was significant for the volume fraction of GS-positive lesions (ANOVA, \(p = 0.026\)) and just above significance level for G6Pase-negative lesions (ANOVA, \(p = 0.096\)). To sum these interaction effects observed for the number of lesions per cm\(^3\) liver tissue, the size class distribution and the volume fraction of two marker-proteins up, one could state, that there is a tendency of PB-treated Cx26 KO animals to have more but smaller tumors when compared with their wild-type counterparts.

**Connexin Levels in Liver Tumors**

We also analyzed the expression and localization of the two major liver connexins in GS-positive tumors from PB-treated Cx26 Wt and KO mice. About 80% of tumors (total number investigated, \(n = 66\)) from PB-treated Cx26 Wt mice showed a reduction or absence of Cx32 immunostaining at the outer cell membrane, which is in accordance with earlier observations (Moennenikes et al., 2000). Surprisingly, however, Cx32 was localized to the cell membrane, very similar to the surrounding normal liver tissue in 42% of GS-positive tumors (\(n = 48\)) from Cx26 KO mice. The Cx26 membrane stain was decreased or absent in ~80% (\(n = 52\)) of GS-positive tumors from PB-treated Cx26 Wt mice, corresponding to what was seen with Cx32. There was no corresponding decrease, however, in the levels of Cx26 and Cx32 mRNAs (data not shown).

**TABLE 1**

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<th>Cx26 Wt</th>
<th>Cx26 KO</th>
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<tr>
<td></td>
<td>No. of tumors (tumor multiplicity)</td>
<td>No. of tumors (tumor multiplicity)</td>
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<td>PB treatment(^a)</td>
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<tr>
<td>Minus</td>
<td>15</td>
<td>6 (0.4)</td>
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<tr>
<td>Plus</td>
<td>14</td>
<td>66 (4.7)</td>
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\(^a\) Only tumors with diameters larger than ~5 mm were counted.

\(^b\) All mice received a single application of DEN (90 \(\mu g/g\) b.wt.) at 6 weeks of age; PB treatment (0.05% in the diet) was for 35–36 weeks.
Effect of Cx26 Deficiency in Normal Liver

A protein expression profiling experiment using RPP microarrays (Pawlak et al., 2002) was performed to analyze the effect of the Cx26 gene knockout on the levels of selected proteins in normal liver tissue: For this purpose, five liver samples from Cx26 KO mice were compared with seven tissues from Cx26 Wt mice. In the same experiment 15 ctnnb mutated liver tumors from a Cx26 KO background were compared with 14 tumors generated in a Wt background. Focus of the analysis was on proteins known to be related to the Wnt pathway and the detection of changes in their phosphorylation status. Using the 38 analyte specific antibodies listed in the supplementary table we could not detect any significant differences in protein expression or in the phosphorylation status of the examined proteins in Cx26 KO as compared with Cx26 Wt mice.

Finally, the effect of Cx26 deficiency on apoptosis of hepatocytes was studied in normal liver tissue. To this end, the frequency of occurrence of apoptotic hepatocytes was analyzed by use of the TUNEL assay. For comparison, livers from Cx32 mice were also studied. The following apoptotic frequencies (number of TUNEL-reaction positive apoptotic figures/section...
The area \(\text{[mm}^2\) of liver tissue) were determined: Wt mice, \(2.5 \pm 0.5\); Cx26 KO mice, \(1.2 \pm 0.2\); Cx32 KO, \(10.8 \pm 2.1\). The increase in apoptotic figures in livers from Cx32 KO mice was highly significant (t-test, \(p = 0.0003\)).

**DISCUSSION**

Connexins are regarded as tumor suppressor proteins involved in the regulation of fundamental cellular processes including differentiation, proliferation, and probably cell death (Kumar and Gilula, 1996; Trosko and Ruch, 1998). In rodent liver, Cx32 has been shown to play an important role in chemically induced carcinogenesis. Elimination of functional Cx32 by knockout of the gene or expression of a dominant-negative form of Cx32 leads to a considerable enhancement of the carcinogenic response toward the liver carcinogen DEN (Moennikes et al., 1999; Evert et al., 2002; Temme et al., 1997). Moreover, Cx32 plays an important role in tumor promotion mediated in mouse liver by PB because mice deficient in the gap junction protein are resistant to the promotional effect of the barbiturate (Moennikes et al., 2000). Less is known about the function of the second hepatocellular connexin, Cx26, as modulator of hepatocarcinogenesis. Previously, we could demonstrate that ablation of hepatocyte gap junctional communication in Cx32/Cx26 double knockouts does not increase spontaneous liver tumor formation nor strongly affect tissue homeostasis (Ott et al., 2006). Whether inactivation of Cx26 would affect PB-mediated tumor promotion, like its relative Cx32, was not known, however, prior to this investigation.

In synopsis the results of our study demonstrate that a deficiency in Cx26 in mouse liver produces only minor effects on hepatocarcinogenesis, if at all, which are by no means comparable to the effects that were seen in our previous studies in which Cx32 was eliminated from hepatocytes. Apparently, Cx26 plays a less important role in liver than Cx32, which has also been argued earlier (Traub et al., 1989). This finding is in accordance with results reported by one of us (T. O.) on a lack of effect of Cx26 deficiency on hepatocarcinogenesis in mice (Ott et al., 2006) but contrasts findings on tumor formation in other organs including mamma (Lee et al., 1991) and lung (Chen et al., 2005). Inactivating Cx26 mutations in humans are associated with deafness but we are not aware of any report on an increase in tumor incidence in these patients.

The results of our present study clearly demonstrate that elimination of functional Cx26 does not abolish promotability of tumor formation in liver by PB, dissimilar to the effect seen with elimination of Cx32. This difference may be a result of several factors: (1) The major gap junction protein in mouse liver is Cx32 and the concentration of Cx26 in hepatocytes is only about one-third of that of Cx32 (Traub et al., 1989), suggesting that Cx26 may be much less important for regulation of tissue homeostasis and hepatocarcinogenesis than Cx32. In fact, determination of cell coupling via gap junction revealed no decrease in GJIC in Cx26 KO mice, whereas coupling was decreased by more than 90% in Cx32 deficient mice (Ott et al., 2006). (2) Another explanation relates to a major structural difference between Cx26 and Cx32. Although the cytosolic C-terminal domain of Cx32 consists of 75 amino acids, amongst which are 9 serine, 1 threonine and

**FIG. 6.** Volume fractions of enzyme-altered lesions in liver. Liver sections from DEN-initiated Cx26 Wt and Cx26 KO mice with and without PB treatment were stained for G6Pase and GS, and the volume fractions of lesions were calculated as described under "Materials and Methods." For animal numbers and treatment conditions see Table 1. PB treatment significantly (ANOVA, \(p < 0.001\)) increased the volume fraction of liver lesions in mice of both genotypes and for both stainings as compared with non-PB-treated controls.
2 tyrosine residues, this domain is reduced to 18 amino acids in Cx26 containing only 2 serine and 2 tyrosine residues as potential targets for phosphorylation (for length of C-terminus see (Willecke et al., 2002) the number of amino acid residues was obtained from www.expasy.org). There exist at least two phosphorylation sites within the C-terminus of Cx32 which are missing in Cx26 (Locke et al., 2006; Moreno and Lau, 2007; Traub et al., 1989). Among these is Cx32 serine 233 which is targeted by protein kinase C and cAMP-dependent protein kinase (Lampe and Lau, 2000; Saez et al., 1990). Phosphorylation of Cx32 appears to play a role in regulating opening and closure of the channel which may be triggered, for example, by the activity of upstream regulatory molecules including oncogene products. It is very tempting to speculate that this C-terminal regulatory domain within Cx32 is also targeted by signaling proteins activated during the process of tumor promotion evoked in DEN-initiated hepatocytes chronically exposed to the tumor promoter PB. (3) A third possible explanation is that homotypic Cx26 and Cx32 containing gap junctional channels have a different conductivity for low molecular molecules (Goldberg et al., 1999; reviewed in Goldberg et al., 2004) which may be relevant for PB-mediated tumor promotion.

Cx26 and Cx32 were not localized to the outer cell membrane in the majority of tumors from Cx Wt mice in the PB treatment groups. These tumors were generally GS-positive. GS is a reliable marker for the presence of a mutation in the β-catenin gene (Ctnnb1) in the tumor cells (Loeppen et al., 2002). As the expression levels of the two connexins were not changed at the mRNA and protein level (data not shown), a transport problem may exist or the proteins are not correctly integrated into the membrane in the liver tumors with constitutively activated β-catenin.

A previous microarray analysis of liver tissues from Cx26-null mice had shown that a deficiency in the gap junction protein has only a very minor effect on the transcriptome of hepatocytes (Ott et al., 2006). We have extended this earlier study by investigating the phosphorylation status of 38 signaling proteins important for Wnt/β-catenin related signaling in hepatocytes from Cx26-null mice in comparison to their wild-type relatives in order to detect possible changes in the activity of selected cellular signaling pathways. The fact that there were no detectable alterations in the phosphorylation status of these signaling proteins in Cx26-deficient mice, neither in normal nor in tumor tissues, suggests that the deficiency in the gap junction protein is of minor relevance in liver which is in accordance with the finding of our present study that the deficiency in Cx26 does not appear to have a major impact on hepatocarcinogenesis in mice.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**FUNDING**

Deutsche Forschungsgemeinschaft (SCHW 329/3-2); and Studienstiftung des Deutschen Volkes (PhD program).

**ACKNOWLEDGMENTS**

We thank Mrs Elke Zabinsky for excellent technical support.

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