WY-14,643-mediated promotion of hepatocarcinogenesis in connexin32-wild-type and connexin32-null mice

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Abbreviations: Cx32, connexin32; DEN, N-nitrosodimethylamine; G-6-Pase, glucose-6-phosphatase; GS, glutamine synthetase; PB, phenobarbital.

Introduction

Connexins are subunits of gap junctional channels, through which directly neighbouring cells exchange low molecular weight molecules. Intercellular communication mediated via gap junctions plays an important role in tissue homeostasis and in cancer (for recent reviews see refs 1–5). The major gap junction proteins in liver are connexin32 (Cx32) and connexin26. Targeted disruption of the Cx32 gene in mice is associated with increased susceptibility to hepatocarcinogenesis (6,7). Moreover, we recently demonstrated that phenobarbital (PB), a prototype promoter of hepatocarcinogenesis in rodents, accelerates liver tumour formation in Cx32-null mice, demonstrating that functional Cx32 protein is required for tumour promotion by the barbiturate (8).

Treatment of rats and mice with the peroxisome proliferator WY-14,643 results in enlargement of the liver, which is associated with an increase in expression of peroxisomal enzymes required for catalyzing β-oxidation of fatty acids and of microsomal enzymes catalyzing ω-oxidation of long-chain polyunsaturated fatty acids (9). Chronic administration of peroxisome proliferators to rodents induces liver cancer (for review see ref. 10). The underlying mechanisms are not entirely clear; they do not appear, however, to represent direct mutagenic effects. In the two-stage initiation/promotion experiment, WY-14,643 acts as a tumour-promoting agent in rodent liver (11,12). Both, the biochemical changes observed in livers of peroxisome proliferator-treated rodents and the carcinogenic effect are mediated through the peroxisome proliferator-activated receptor-α (PPARα), a member of the nuclear receptor superfamily: PPARα-null mice are resistant to WY-14,643-induced peroxisome proliferation and hepatocarcinogenesis (for review see refs 9,13).

In the present study, we have addressed the question whether the gap junction protein Cx32 is relevant for liver tumour promotion by WY-14,643, in analogy to what was observed earlier with PB as tumour promoter. We observed that liver lesions induced in WY-14,643-treated mice displayed quite divergent phenotypes associated with strong differences in growth behaviour. Since mutation of β-catenin is a hallmark of liver tumours produced by a treatment regimen including PB as promoter, the presence of such mutations in WY-14,643-induced lesions was also investigated.

Materials and methods

Treatment of mice

Cx32-deficient mice used in the present study were generated by standard methods of targeted homologous recombination (14) and backcrossed into C3H-background for five generations prior to the experimental study. Cx32 heterozygous female mice were then crossed with male C3H/He mice to yield male Cx32-wild-type and Cx32-null F0 offspring. Cx32 genotyping was performed by standard PCR as recently described (7). Mice were i.p. injected with a single dose of N-nitrosodimethylamine (DEN) (90 μg/g of body wt) at 6 weeks of age. After a treatment-free interval of 3 weeks, DEN-treated Cx32-wild-type...
and Cx32-null mice were randomly assigned to groups, which were either kept on a standard diet or on a diet containing WY-14,643 until death. Fifteen weeks after start of treatment with WY-14,643 the diet concentration of the peroxisome proliferator was reduced from 0.05 to 0.025% because of toxic effects. The number of mice in the various treatment groups is listed in Table I. Mice were killed 38 weeks after initiation, livers were frozen on blocks of dry ice and stored at −70°C.

**Histology, enzyme- and immuno-histochemistry and stereology**

Frozen sections were taken from each liver and stained with haematoxylin/ eosin (H&E) for subsequent histopathological evaluation. Additional sections were stained enzyme-histochemically for glucose-6-phosphatase (G-6-Pase) activity (15) and immuno-histochemically for glutamine synthetase (GS) (16), using anti-GS-rabbit antiserum (Zymed, San Francisco, CA; 1:300). Binding of antibody to frozen liver sections was visualised using horseradish peroxidase-conjugated anti-rabbit-IgG secondary antibody (Sigma-Aldrich, Taufkirchen, Germany; 1:20) and 3-amino-9-ethylcarbazole/H2O2 as a substrate.

G-6-Pase- and GS-altered lesions were quantified by means of a computer-assisted digitizer system (17). The volume fraction of enzyme-altered tissue in liver was calculated using standard stereological techniques.

**Statistical analysis**

Statistical analysis of data regarding enzyme-altered lesions was performed with two-way ANOVA with interaction terms for genotype and diet of the liver was calculated using standard stereological techniques.

**Results**

In the present study, Cx32-wild-type and Cx32-null mice were treated sequentially with DEN and the peroxisome proliferator WY-14,643 according to a two-stage initiation–promotion protocol. WY-14,643 was administered via the diet at an initial concentration of 0.05%. This dose was chosen according to literature data where diet concentrations of up to 0.1% were used for subchronical or chronic studies in rats and mice (e.g. see refs 20,21). The diet concentration of 0.05%, however, displayed significant toxicity in the present study in C3H-mice of both genotypes, with increasing length of treatment, as indicated by a retardation of body weight gain and weight loss in individual mice. We therefore reduced the diet concentration to 0.025% after 15 weeks of treatment. Although this dose was tolerated by the animals, the mean body weight of WY-14,643-treated Cx32-wild-type and Cx32-null mice was still significantly \( P < 0.0001 \); calculation based on combined genotypes) decreased at the end of the experiment (Table I).

Livers of peroxisome proliferator-treated mice of both genotypes were found to be tremendously enlarged in size, showing a ~3-fold increase in weight in mice of both genotypes \( (P < 0.0001) \), demonstrating the potency of WY-14,643 as a liver-enlarging drug (Table I).

Frozen liver sections were prepared and stained enzyme-histochemically for G-6-Pase, which is decreased in activity in hepatocellular pre-neoplastic foci and neoplasms induced by the majority of hepatocarcinogens in rodent liver and frequently employed as a marker for identification of these lesions (22). G-6-Pase-negative liver lesions were also observed in the present study, both in mice treated with DEN alone and in DEN/WY-14,643-treated mice. In addition, large focal and nodular lesions were observed in livers from DEN/ WY-14,643-treated animals, which showed increases rather than decreases in G-6-Pase activity, often with somewhat inhomogeneous staining patterns (for representative examples see Figure 1A).

An increase in G-6-Pase activity was also seen in the normal-appearing liver tissue as a response to the peroxisome proliferator, although the preferential periportal expression of the enzyme, seen in controls, was preserved. In comparison with the untreated controls, the strongly G-6-Pase-positive periportal zones were frequently extended in the liver of mice exposed to the peroxisome proliferator.

GS, which is sometimes up-regulated in rodent liver lesions (17,23) was used as a second independent marker for

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**Table I. Effect of Wy-14,643 on mouse body and liver weights**

<table>
<thead>
<tr>
<th>Cx32 genotype</th>
<th>No. of mice</th>
<th>Wy-14,643 treatment</th>
<th>Mean body wt (g ± SD)</th>
<th>Mean liver wt (g ± SD)</th>
<th>Relative liver wt (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>13</td>
<td>Minus</td>
<td>28.44 ± 2.11</td>
<td>1.24 ± 0.11</td>
<td>4.37 ± 0.30</td>
</tr>
<tr>
<td>Wild-type</td>
<td>11</td>
<td>Plus</td>
<td>26.41 ± 1.49</td>
<td>3.94 ± 0.56</td>
<td>14.94 ± 1.19</td>
</tr>
<tr>
<td>Null</td>
<td>12</td>
<td>Minus</td>
<td>29.63 ± 3.86</td>
<td>1.24 ± 0.17</td>
<td>4.19 ± 0.35</td>
</tr>
<tr>
<td>Null</td>
<td>12</td>
<td>Plus</td>
<td>24.62 ± 2.87</td>
<td>3.50 ± 0.48</td>
<td>14.23 ± 0.87</td>
</tr>
</tbody>
</table>

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**Fig. 1. Phenotype of liver lesions.** Mice were given a single injection of DEN at 6 weeks of age and were subsequently kept on WY-14,653-containing or control diet; 38 weeks after initiation, mice were killed. (A) G-6-Pase-positive tumours in DEN/Wy-14,643-treated mice. Frozen liver sections were stained enzyme-histochemically for G-6-Pase activity. Arrows point towards large tumour transections showing positive G-6-Pase staining; larger tumours demonstrated diffuse basophilia in H&E-stained parallel sections. (B) Example of a GS-positive liver lesion, demonstrated immuno-histochemically in liver of a WY-14,643-treated Cx32-wild-type mouse; bar, 0.2 mm.
identification of (pre)-neoplastic lesions; a representative example of a GS-positive liver lesion is shown in Figure 1B. In H&E-stained liver sections, all of the G-6-Pase-positive focal and nodular lesions present in liver parenchyma of WY-14,643-treated mice (exemplified in Figure 1A) displayed an amphophilic phenotype, which is characterized by a more or less pronounced, diffuse or scattered, basophilia on an acidophilic background (24). The majority of the amphophilic lesions represented foci, which were well integrated into the normal liver parenchyma, frequently even when these lesions were wide-spread. In addition, 10 of 13 Cx32-wild-type and 9 of 11 Cx32-null mice had developed large expansively growing amphophilic cell nodules, most of which showed merely features of benign neoplasms and were classified as hepatocellular adenomas. Only in one case, a focal malignant transformation with an incipient infiltrative growth was observed. In the extrafocal liver tissue of animals exposed to the peroxisome proliferator, the liver parenchyma appeared predominantly acidophilic, and often exhibited single cell necrosis (apoptosis) or larger groups of necrotic cells, lipofuscin deposits indicating earlier cell death and small reactive inflammatory cell infiltrates. Similar changes were sometimes also detected within the amphophilic focal or nodular cell populations. The G-6-Pase-negative focal lesions observed in the livers of mice treated with WY-14,643, were only rarely identifiable as basophilic cell foci in the H&E-stained frozen sections. A few small foci with a similar phenotype were found in the livers of mice exposed to DEN alone, while parenchymal cell necroses, lipofuscin deposits and inflammatory infiltrates were almost absent from the liver of these animals.

Using the enzyme-histochemical preparations, we quantified lesions presenting the different phenotypes by means of standard stereological procedures (16). The most dramatic effect was seen with respect to lesions showing increased activity in G-6-Pase: while these were absent in mice treated with DEN alone, lesions of this particular phenotype occupied ~20% of liver tissue in DEN-plus WY-14,643-treated animals (P < 0.0001). With respect to this parameter, however, there were no significant differences between Cx32-wild-type and Cx32-null mice (P = 0.6367) (Figure 2). The volume fraction in liver occupied by G-6-Pase-negative lesions was ~7-fold higher in DEN-only treated Cx32-null mice as compared with the respective Cx32-wild-type mice. Combined treatment with DEN and WY-14,643 led to a ~14-fold increase in the volume fraction of G-6-Pase-negative lesions in Cx32-wild-type mice (P < 0.05) while there was no significant increase in their Cx32-null counterparts (Figure 2). Although the number of G-6-Pase-negative lesions in livers of DEN/WY-14,643-treated mice was approximately three times higher than the number of G-6-Pase-positive lesions, the latter were much larger in size (data not shown). GS-positive lesions were not detected in mice treated with DEN alone, but very small GS-positive lesions might have eluded their identification, as they cannot be discriminated by immuno-histochemistry from normal GS-expressing hepatocytes surrounding the central venules. Liver lesions strongly over-expressing GS were present, although low in number, in mice treated with DEN plus the peroxisome proliferator. Interestingly, there were significant differences in frequency and size of lesions between Cx32-wild-type and Cx32-null mice (P < 0.0001); in total, 35 GS-positive lesions were observed in liver sections (21.1 cm² total area) from Cx32-wild-type mice in contrast to only six lesions in liver sections (22.9 cm² total area) from Cx32-null mice. The volume fraction occupied in liver by GS-positive lesions was very small in WY-14,643-treated Cx32-null mice while it was significantly increased by the peroxisome proliferator in Cx32-wild-type mice (P < 0.0001), presumably reflecting the fact that lesions with GS-positive phenotype grew faster in mice of the latter genotype (Figure 2).
To zero-demonstrating much lower levels of Cx32 protein at intensity of staining was strongly decreased—in some mice, Cx32 localized to gap junctional plaques at the hepato-cytes. This staining pattern was also seen in liver sections from DEN plus WY-14,643-treated mice, but the cell membrane (Figure 3). Cx32-membrane staining was absent in liver tumours from Wy-14,643-treated mice and was only observed in the liver of rats treated with peroxisome proliferators (24–26). Although low in number, G-6-Pase-positive focal and nodular lesions were often very large in size occupying a considerable fraction of liver tissue in the peroxisome proliferator-treated animals (Figures 1 and 2). This demonstrates, that lesions of this latter phenotype are most efficiently selected during treatment with the peroxisome proliferator, probably by a mechanism involving PPARα: gene-knockout mice deficient in this receptor do not develop liver cancer upon treatment with peroxisome proliferators (for review see ref. 13).

Peroxisome proliferators have been reported to negatively regulate the expression and/or induce aberrant membrane localization of Cx32 in liver cells (27,28). In accordance with the earlier data, treatment of Cx32-wild-type mice with WY-14,643 led to a strong reduction or complete absence of Cx32-membrane staining both in the normal appearing liver tissue and in liver tumours (exemplified in Figure 3), which should result in a strong decrease in gap junction inter-cellular communication (GJIC) between neighbouring hepatocytes. A large number of chemicals, including PB, certain polyhalogenated biphenyls, and insecticides like endosulfane, chlordane, dieldrin or DDT, all known liver tumour promoters in rodents, have been shown to inhibit GJIC and this latter effect has been suggested to mediate their tumour promotional activity (for review see refs 3,4). This hypothesis, even though substantiated by several lines of evidence, is at variance with present findings on the development of G-6-Pase-positive liver lesions (16), four out of four GS-positive lesions showed base substitutions in the known mutational hot spots of β-catenin: all four base substitutions affected codon 33 and were of the type TCT → CCT and TCT → GCT. In contrast, no β-catenin mutations were detected in 24 G-6-Pase-positive lesions, analyzed for this genetic alteration.

Discussion

In the present study, pre-neoplastic and neoplastic liver lesions of differing phenotypes and growth behaviour were observed. In accordance with results obtained earlier, lesions displaying decreased activity in G-6-Pase were more frequent and larger in DEN-treated Cx32-null mice than in Cx32-wild-type mice, demonstrating the enhanced susceptibility of Cx32-null mice towards DEN-induced hepatocarcinogenesis (6–8). Treatment of mice with Wy-14,643 did not further increase the volume fraction of G-6-Pase-deficient lesions in Cx32-null mice, while lesions of this phenotype were significantly promoted by the peroxisome proliferator in Cx32-wild-type mice. Similarly, PB was shown previously to lack an enhancing effect on the evolution of G-6-Pase-deficient lesions in Cx32-null mice while it was active as a tumour promoter in the respective wild-type mice (8). Liver lesions that were selected by the barbiturate in our previous study in Cx32-wild-type mice over-expressed the enzyme GS (16). GS-positive lesions were also observed in the present study in Wy-14,643-treated mice and they were more frequent and larger in Cx32-wild-type than in Cx32-null mice. Cx32 therefore appears to play a role during promotion of G-6-Pase-negative and GS-positive lesions by both PB and Wy-14,643.

Lesions displaying an increase in the activity of G-6-Pase were only observed in the liver of Wy-14,643-treated mice. All of these lesions displayed an amphiphilic phenotype, similar to lesions seen in the liver of rats treated with peroxisome proliferators (24–26). Although low in number, G-6-Pase-positive focal and nodular lesions were often very large in size occupying a considerable fraction of liver tissue in the peroxisome proliferator-treated animals (Figures 1 and 2). This demonstrates, that lesions of this latter phenotype are most efficiently selected during treatment with the peroxisome proliferator, probably by a mechanism involving PPARα: gene-knockout mice deficient in this receptor do not develop liver cancer upon treatment with peroxisome proliferators (for review see ref. 13).
lesions: promotion of these lesions occurred to a very similar extent in Cx32-wild-type and in Cx32-null mice, without significant differences between genotypes. Therefore, inhibition of Cx32-mediated GJIC by the peroxisome proliferator as a cause of their selection appears unlikely. G-6-Pase-negative and GS-positive lesions, on the other hand, were promoted by Wy-14,643 exclusively in the liver of Cx32-wild-type mice, in agreement with the lack of promotional effect of PB in Cx32-null mice observed earlier (8,16).

Subpopulations of initiated cells containing different mutations may be able to grow selectively under the permissive conditions established by a tumour promoter of a certain class (29). Our previous and present results with PB and Wy-14,643, respectively, substantiate this hypothesis and add some new information on the molecular clues. We recently showed that the GS-positive phenotype is linked to mutation of the proto-oncogene β-catenin, also termed Ctnnb1 (16). β-Catenin codes for a protein associated with E-cadherin, a component of the adherens junction; it is also essential in the Wnt/Wingless signalling pathway mediating nuclear responses to Wnt signals by interacting with T-cell factor transcription factors (30). Mutation of the conserved serine/threonine residues at the N-terminal region of β-catenin abolishes glycosen synthase kinase 3β phosphorylation sites relevant for β-catenin degradation and leads to oncogenic activation of the protein (30). The fact that promotion by PB and Wy-14,643 of β-catenin-mutated, GS-positive lesions requires Cx32 may indicate of a functional link between Cx32- and β-catenin-dependent signalling pathways (31). The mechanistic basis of this link is under investigation.

Acknowledgements

The excellent technical assistance of Mrs J.Mahr and Mrs E.Zabinsky is acknowledged. We thank Dr K.Willecke for the gift of Cx32-null mice, Dr C.Gembardt, BASF, Ludwigshafen, for the gift of WY-14,643, Dr R.Gebhardt, University of Leibzig, for the gift of GS antibody. Dr A.Kopp-Schneider, DKFZ Heidelberg, is acknowledged for statistical analysis of tumour data. This study was supported by the Deutsche Forschungsgemeinschaft (SCHW 329/3-1) and by a grant of the Tübingen Fortune-program (908-0-0).

References


Received January 21, 2003; revised June 2, 2003; accepted June 3, 2003.