A Constitutively Active Dioxin/Aryl Hydrocarbon Receptor Promotes Hepatocarcinogenesis in Mice

Oliver Moennikes, Sandra Loeppen, Albrecht Buchmann, Patrik Andersson, Carina Ittrich, Lorenz Poellinger, and Michael Schwarz

Abstract

The dioxin/aryl hydrocarbon receptor (AhR) functions as a ligand-activated transcription factor regulating transcription of a battery of genes encoding enzymes involved in drug metabolism. Known ligands include polycyclic aromatic hydrocarbons, certain polychlorinated biphenyls, and the polyhalogenated dioxins including 2,3,7,8-tetrachlorodibenzo-p-dioxin. Both polyhalogenated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin are potent promoters of rodent hepatocarcinogenesis in two-stage initiation-promotion experiments. Although several lines of evidence indicate the involvement of the AhR in toxic effects mediated by polyhalogenated biphenyls and dioxins, its involvement in tumor promotion has not been unequivocally proven. In the present study, a transgenic mouse line expressing a constitutively active AhR (CA-AhR) has been used to investigate the role of the AhR in hepatocarcinogenesis. Male AhR wild-type and CA-AhR-transgenic B6C3F1-mice were treated with a single injection of the hepatocarcinogen N-nitrosodimethylamine at 6 weeks of age and were subsequently kept untreated on control diet. Thirty five weeks after carcinogen treatment, mice were sacrificed, and the prevalence and multiplicity of liver tumors were determined. Whereas only 1 small liver tumor was observed in 15 AhR-wild-type mice, 19 tumors (two >1 cm in diameter) were present in 18 CA-AhR-transgenic mice. This result demonstrates the oncogenic potential of the activated AhR and implicates an important role of the receptor in promotion of hepatocarcinogenesis. A microarray-based gene expression-profiling analysis revealed down-regulation in the CA-AhR-transgenic mice of a cluster of genes encoding heat shock proteins, including GRP78/Bip, Herp1, Hsp90, DnaJ (Hsp40) homologue B1, and Hsp105, which are important for protein folding and quality control.

Introduction

The dioxin/aryl hydrocarbon receptor belongs to a specific class of transcription factors, the basic helix-loop-helix/Per-Arnt-Sim domain transcription factors that appear to have been designed to respond to various classes of environmental stimuli (1). The ligand-activated aryl hydrocarbon receptor (AhR) mediates transcriptional activation of a battery of genes encoding enzymes such as cytochrome P450 (CYP) 1A1, CYP1A2, and glutathione S-transferase Ya that function in the metabolism of xeno- and endobiotics (2). A variety of environmental pollutants including polyhalogenated biphenyls and dioxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are well-characterized AhR ligands (1).

TCDD induces a variety of adverse biological responses, including reproductive and developmental defects, immunotoxicity, thymus atrophy, chloracne, wasting syndrome, liver toxicity, and cancer (2). There is strong evidence to suggest that these pleiotropic effects are all mediated via the AhR. A link between the AhR and chemical carcinogenesis has been established in an experiment using benzo(α)pyrene, which was shown to lack carcinogenic effects in AhR gene-knockout mice (3). In two-stage initiation-promotion models of carcinogenesis, TCDD has proven to be particularly effective during the promotional phase of carcinogenesis (reviewed in Ref. 4). Tumor promoters are believed to increase the probability of cancer by accelerating the clonal expansion of cells transformed during tumor initiation. In the case of promotion of hepatocarcinogenesis in rats initiated with the liver carcinogen N-nitrosodimethylamine (DEN), this effect appears to be primarily mediated via suppression of apoptosis of tumor precursor cells (5). It has been suggested that promotion of hepatocarcinogenesis by TCDD and other AhR ligands is because of activation of AhR-dependent signaling, primarily based on results from studies using mice strains with characteristics differences in AhR phenotype, i.e., TCDD-responsive and nonresponsive strains (6). Unequivocal proof, however, of the involvement of the AhR in dioxin-mediated promotion of hepatocarcinogenesis is still missing because results from initiation-promotion experiments using AhR gene-knockout mice are not yet available. In the present study, we have used a transgenic mouse model expressing an Ah receptor-mutant (7) that is constitutively active attributable to deletion of the PAS-B domain of the receptor harboring the ligand binding domain (8). Transgenic mice of this strain [constitutively active AhR (CA-AhR mice)] show increased expression of AhR-dependent genes such as CYP1A1 in several organs including liver (7). Interestingly, they also have a strongly elevated risk for spontaneous development of stomach tumors (7).

Materials and Methods

The generation of CA-AhR-transgenic mice has been described recently (7). CA-AhR (8) was subcloned into pEμSR containing the mouse immunoglobulin heavy chain promoter and the simian virus 40 polyadenylation site. Transgenic mice were created by injection of a 5.5-kb KpnI fragment encompassing the EμSR-CA-AhR construct into fertilized C57BL/6 × CBA eggs. Mice of a founder line were backcrossed into the C57BL/6 background for six generations before the experimental study. CA-AhR-genotyping was performed by standard PCR using primer pairs AhRP1n (forward) 5′-GCAAT-GTACATCCACCTCAG-3′ and AhRP2n (reverse) 5′-GTACAGCATCAT-GAGGAACCT-3′. CA-AhR heterozygous male mice were crossed with female C3H/He mice to yield AhR-wild-type (WT-AhR) and CA-AhR-transgenic B6C3F1-offspring. All male mice received injection i.p. with a single dose of N-nitrosodimethylamine (90 μg/g of body weight) at six weeks of age. The effective number of mice in the two experimental groups is listed in Table 1. Mice were killed 35 weeks after initiation, livers were removed, and the number of nodular lesions and tumors visible on the surface of the liver along with their individual size was recorded. Larger tumors were excluded and subjected to histopathological examination. Fisher’s exact test was used for...
statistical analysis of tumor data. For biochemical analyses (see below), aliquots of liver were frozen in liquid nitrogen and stored at −70°C.

Total RNA was prepared from approximately 250 mg of frozen liver tissue from three CA-AhR and three AhR mice using TRIzol reagent (Life Technologies, Inc.). Further purification of the isolated total RNA was performed using the RNeasy Mini protocol for RNA cleanup. Total RNA was reverse transcribed to produce double-stranded cDNA. A subsequent in vitro transcription step produced biotin-labeled antisense cRNA, which was used for gene array hybridization using Affymetrix GeneChip mouse expression array 430A containing 22,690 probe sets. Six chips were hybridized with the cRNA from each of the six mice.

The statistical analysis was conducted using the software package R, version 1.8.1 (9). The data preprocessing steps, background-adjustment, normalization, and computation of gene expression measures, were carried out using the affy software library (version 1.3.26) of the BioConductor Project. For each of the six arrays the log2 scale robust multi-array expression measures were obtained for each probe set following the recommendations in reference (10). The comparison of these expression measures between the two groups (CA-AhR and WT-AhR) was performed by two-sided t tests. We selected a threshold of 0.015 for the unadjusted P values and included only those genes that showed log2 expression ratio ≥ 0.5, whereby the log2 expression ratios were estimated by the difference between the means of log2 expression values in the CA-AhR and the WT-AhR group.

Liver homogenates were prepared and the activity of ethoxysresorufin-O-dealkylase (EROD) was determined as described previously (11). The expression of CYP1A1 and CYP2E1 mRNAs was determined by quantitative reverse transcription PCR (RT-PCR). Total RNA was prepared from livers of WT-AhR and CA-AhR mice (seven randomly selected animals per group) by standard procedures as described recently (12). PCR reactions were performed using a LightCycler and the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Molecular Biochemicals). The following primers were used: CYP1A1, forward, 5′-TGTCCCTCGTGATCTGCTA-3′; CYP1A1 reverse, 5′-GTTGCTAAACCCAGTCAAAA-3′; CYP2E1, forward, 5′-TCCCTAAGTGTCCTCCTGTA-3′; CYP2E1, reverse, 5′-GTAATGGAAGCGTTTGTTGTA-3′; and GPDH, forward, 5′-ACCACAGTCCATGCATCAC-3′. All PCR reactions included 3 μM MgCl2 and were run at annealing temperatures of 55°C (CYP1A1), 55°C (CYP2E1), and 70°C (glyceraldehyde-3-phosphate dehydrogenase).

The real-time PCR efficiencies (E) were determined for each transcript and were estimated using LightCycler software 3.5.3 (Roche Molecular Biochemicals) to be 1.87 (CYP1A1), 1.88 (CYP2E1), and 1.78 (glyceraldehyde-3-phosphate dehydrogenase). Crossing points (CP) were subsequently determined by use of the same software for each of the samples and transcripts. We used the Hedges-Lehrmann procedure (StatXact-5.0.3; Cytel Software Corp., Cambridge, MA) to obtain median point estimates and 95% confidence intervals for the magnitude of the shift (PCR cycles) between the two groups (WT-AhR and CA-AhR). On the basis of the median point estimates, relative mRNA expression ratios between WT-AhR mice and CA-AhR mice (CYP1A1 and CYP2E1, and reference, glyceraldehyde-3-phosphate dehydrogenase) were estimated by use of the following equation (13): Ratio = E(target)−E(reference) / E(target) . The Wilcoxon rank-sum test was used for statistical analysis of RT-PCR and EROD data.

Results and Discussion

At the end of the experiment, the mean body weight of CA-AhR and WT-AhR mice did not significantly differ. The mean liver to body weight ratio (%) of CA-AhR and WT-AhR mice was 5.22 ± 0.97 and 4.67 ± 0.33 (P = 0.045; Student’s t test, 2-sided). Three CA-AhR mice, however, showed relative liver weights >6% because of tumor burden. If these mice were excluded from the analysis, no significant differences in the relative liver weight existed between the two groups.

The liver tumor prevalence (number of mice with tumors) differed significantly (P = 0.0038, Fisher’s exact test) between WT-AhR and CA-AhR mice. Whereas only one small tumor was detected in 15 wild-type mice, 10 of the 18 CA-AhR mice showed one or multiple tumors on the surface of their livers (Table 1). Tumors with diameters >1 mm were only detected in CA-AhR-transgenic mice, and two of the animals had developed very large tumors exceeding 1 cm in diameter, which were diagnosed as adenomas (Table 1). In addition, the tumor multiplicity (average number of tumors per mouse) differed considerably between mice of the two groups.

The transgene driven by the mouse immunoglobulin heavy chain promoter, which was introduced into CA-AhR mice, was designed to allow tissue-specific expression to avoid overt toxic effects in multiple organs, potentially resulting from the use of a potent global expression vector. Earlier studies (7) demonstrated that CA-AhR mRNA was predominantly expressed in the thymus and spleen but also at low levels in a number of nonlymphoid tissues including liver. Moreover, all tissues that showed CA-AhR expression also demonstrated at various levels induced expression of CYP1A1 mRNA, indicating that CA-AhR is transcriptionally active and mimics the action of the ligand-activated AhR (7). Given the background that the AhR is a transcription factor, it is a plausible scenario that dysregulation by the CA-AhR of specific clusters of target genes in the liver is critical for tumor promotion in this tissue. The CA-AhR model is an important tool that might allow the identification of these genes in the absence of any confounding tumor promoting agents that may perturb the analysis by gene regulatory effects, which are irrelevant for the process of tumor development itself. We therefore used high-density oligonucleotide microarray assays for global gene expression profiling with the aim to identify genes differentially expressed in liver of CA-AhR and AhR wild-type mice. The analysis was originally performed with three mice per group using the Affymetrix platform and included a statistical analysis of data. In the initial analysis we observed, however, that one of the CA-AhR mice behaved abnormally: 44 genes were exclusively elevated in expression in this animal (see inset in Fig. 1). Theses genes code for lipases and proteases such as trypsin and chymotrypsin and are normally expressed in pancreas. The reason for the abnormal behavior of the animal is unknown, but potentially an occult tumor within the otherwise normal looking liver tissue could have produced this effect. This led us to exclude the mouse from further statistical analysis. It is worth mentioning, however, that most of the genes listed in Table 2 could still be identified without exclusion of the animal (not shown).

Changes in expression were regarded as significant if the following two criteria were met: log2 expression ratio ≥ 0.5 (20.5 -1.41-fold difference) and P < 0.015. A total of 28 genes met these criteria, of which 11 were up-regulated, and 17 were down-regulated in livers of CA-AhR mice (Fig. 1). In general, the effects were very small: Only

Table 1. Effect of CA-AhR on liver tumor response

<table>
<thead>
<tr>
<th></th>
<th>WT-AhR</th>
<th>CA-AhR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor prevalence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size class</td>
<td>1 mm</td>
<td>&gt;3 mm</td>
</tr>
<tr>
<td>Number of tumors</td>
<td>1/15 (7%)</td>
<td>&gt;3 mm</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td>0.067 (1/15)</td>
<td>10.056 (19/18)</td>
</tr>
</tbody>
</table>

NOTE. P = 0.0038 (Fisher’s exact test).

CA-AhR, constitutively active aryl hydrocarbon receptor; WT-AhR, aryl hydrocarbon receptor-wild-type.
The microarray data are presented in Table 2. Genes differentially expressed in liver of CA-AhR and AhR mice are listed. Each cross represents 1 of the 22,690 probe sets analyzed by microarray. The red and blue dots represent genes with fold changes of 1.4 above and below, respectively. The horizontal lines indicate discriminators used. Genes located within gray areas are listed in Table 2. The inset shows the respective volcano plot including mouse CA-AhR7, which showed exclusive expression of several genes normally expressed in pancreas (circled points).

CYP1A1 showed an ~4-fold increase in expression in CA-AhR mice when compared with their wild-type cousins (Table 2). It is remarkable, however, that among the genes negatively regulated in CA-AhR mice is a cluster of genes coding for heat shock proteins, including GRP78/BiP, Herp1, Hsp90, DnaJ (Hsp40) homologue B1, and Hsp105. We are aware of the fact that some false-positives may be among the 28 genes identified as differentially expressed, for simple statistical reasons because of the many transcripts analyzed simultaneously, and that some of the detected alterations may represent underestimated changes. It appears unlikely, however, that all five genes mentioned above represent false-positives, because they all belong to a group of proteins with similar function, i.e., heat-shock proteins. Heat shock proteins, which are located in the cytosol or the endoplasmic reticulum, play an important role as molecular chaperons in the folding of newly synthesized proteins, their assembly and disassembly and their translocation into organelles. It is also remarkable that most of the genes detected in the present screen were not detected in comparable screens aimed to identify TCDD-responsive genes in mouse liver (14 and 15 and references therein). The relevance of the observed changes for the process of cancer development in CA-AhR mice, however, remains to be elucidated.

Microarray analysis may underestimate changes in gene expression (10). We therefore analyzed the expression of CYP1A1 mRNA by quantitative RT-PCR using this gene as a biomarker for CA-AhR activity. As shown in Table 3, CYP1A1 mRNA expression in liver from CA-AhR mice was elevated about 60-fold as compared with WT-AhR mice. By contrast, CYP2E1 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels (the latter used for normalization) did not significantly differ between mice of the two genotypes (Table 3). In addition, the activity EROD, a marker of CYP1A-dependent enzyme activity, was determined in liver homogenates from mice of the two genotypes (five per group) and was found to be significantly (P = 0.043, Wilcoxon rank-sum test) increased in CA-AhR mice (7.78 ± 2.41 versus 17.62 ± 2.96 pmol/mg protein × min in WT-AhR and CA-AhR mice, respectively). These data demonstrate that the Ah-receptor mutant was constitutively activated in liver of the CA-AhR mice used in the present study.

Using CYP1A1 mRNA as determined by quantitative RT-PCR and EROD activity ratios, we estimated TCDD-equivalency factors based on published TCDD dose-effect relationships in C57BL/6 mice (16,
Table 3 Effect of CA-AhR\(^\text{a}\) on liver CYP1A1 and CYP2E1 mRNA expression as determined by quantitative reverse transcription-PCR

<table>
<thead>
<tr>
<th>WT-AhR-CA-AhR</th>
<th>CA-AhR/WT-AhR (fold-change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔPCR cycles</td>
<td></td>
</tr>
<tr>
<td>95% confidence intervals</td>
<td></td>
</tr>
<tr>
<td>( P ) values are given in brackets</td>
<td></td>
</tr>
<tr>
<td>CYP1A1</td>
<td>6.93 (4.92–11.45; ( P = 0.0006 ))</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>1.45 (1.03–3.33; ( P = 0.32 ))</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.42 (0.66–1.33; ( P = 0.48 ))</td>
</tr>
</tbody>
</table>

\( \text{a} \) CA-AhR, constitutively active aryl hydrocarbon receptor; CYP, cytochrome P450; WT-AhR, aryl hydrocarbon receptor-wild-type.

\( \text{a} \) Not significantly different.

17). These were found to range between \( \sim 1 \)–2 and \( \sim 6 \) ng TCDD/kg body weight/day, based on EROD activity and CYP1A1 mRNA levels, respectively. Interestingly, the promotional activity of TCDD in rat liver appears to require somewhat higher doses of TCDD because significant increases in carcinogenic response were only seen at doses equivalent to 10 and 100 ng/kg body weight/day (e.g., see Refs. 18–20). In conclusion, these data demonstrate that the AhR plays an active role in liver tumor promotion in mice exposed to a single tumor-initiating dose of a liver carcinogen and that the constitutively activated receptor works at least as efficiently, if not more, when compared with its ligand-activated wild-type counterpart.

Acknowledgments

We thank J. Mahr, E. Zabinsky, and I. Voith for excellent technical assistance; Dr. Christoph Koehle for help in real-time RT-PCR analysis; Dr. Peter Bannasch for histopathological evaluation of tumors, and Dr. M. Bonin, microarray facility, Tuebingen.

References

A Constitutively Active Dioxin/Aryl Hydrocarbon Receptor Promotes Hepatocarcinogenesis in Mice

Oliver Moennikes, Sandra Loeppen, Albrecht Buchmann, et al.

Cancer Res 2004;64:4707-4710.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/14/4707

Cited articles
This article cites 18 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/14/4707.full.html#ref-list-1

Citing articles
This article has been cited by 37 HighWire-hosted articles. Access the articles at:
/content/64/14/4707.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.