Characteristic Expression Profiles Induced by Genotoxic Carcinogens in Rat Liver

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When applied in toxicological studies, the recently developed gene expression profiling techniques using microarrays, which brought forth the new field of toxicogenomics, facilitate the interpretation of a toxic compound’s mechanism of action. In this study, we investigated whether genotoxic carcinogens at doses known to induce liver tumors in the 2-year rat bioassay deregulate a common set of genes in a short-term in vivo study and, if so, whether these deregulated genes represent defined biological pathways. Rats were dosed with the four genotoxic hepatocarcinogens dimethylnitrosamine (4 mg/kg/day), 2-nitrofluorene (44 mg/kg/day), aflatoxin B1 (0.24 mg/kg/day), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK, 20 mg/kg/day). After treatment for up to 14 days, the expression profiles of the livers were analyzed on Affymetrix RG_U34A microarrays. Among the significantly upregulated genes were a set of target genes of the tumor suppressor protein p53, indicating a DNA damage response. Such a response was expected and, therefore, confirmed the validity of our approach. In addition, the gene expression changes suggest a specific detoxification response, the activation of proliferative and survival signaling pathways, and some cell structural changes. These responses were strong throughout the 14 day time course for 2-nitrofluorene and aflatoxin B1; in the case of dimethylnitrosamine and NNK, the effects were weakly detectable at day 1 and then increased with time. For dimethylnitrosamine and aflatoxin B1, which caused observable inflammation in vivo, we found a corresponding upregulation of inflammatory genes at the same time points. Thus, by the toxicogenomic analysis of short-term in vivo studies, we identified genes and pathways commonly deregulated by genotoxic carcinogens, which may be indicative for the early events in tumorigenesis and, thus, predictive of later tumor development.

Key Words: toxicogenomics; microarray; genotoxic carcinogens; rat liver; characteristic profiles.

The recent advent of genomic sciences comprising large-scale cloning and sequencing efforts resulted in several genomes being completely sequenced with different grades of accuracy, including the genomes from human (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001), mouse (Mouse Genome Sequencing Consortium, 2002), and rat (Rat Genome Sequencing Consortium, 2002). This enabled the development of DNA microarrays that contain the information of more or less all genes of an organism, allowing quantitative comparisons of the expression levels of potentially all expressed genes between different biological samples. Previously developed differential gene expression profiling techniques classified as open systems, such as serial analysis of gene expression (SAGE), are not restricted to a preselected gene set as present on a microarray but are more labor-intensive to process (Storck et al., 2002). DNA microarrays are available in different formats with either cDNA fragments or oligonucleotides immobilized on a solid support. Although they are by definition closed systems, permitting only the immobilized genes to be analyzed, whole genome microarrays can, in principle, measure all genes like open systems. However, the technology is still improving; for example, the currently commercially available microarrays do not allow the analysis of specific splice variants.

The application of such technology to toxicology, known as toxicogenomics, has the potential to both enhance our understanding of the mechanism of a compound’s toxic effects and to identify a characteristic set of genes from a database of reference profiles, which may allow the prediction of a compound’s toxicity (Fielden and Zacharewski, 2001; Pennie and Kimber, 2002; Ulrich and Friend, 2002). Several examples have been reported supporting the concept of predictive toxicogenomics. Waring et al. (2002) could pinpoint the hepatic toxic mechanism of a drug lead by comparison of the gene expression profile obtained from treatment of rats for 3 days with a small compendium of reference profiles. Thomas et al. (2001) could identify a toxicologically predictive gene set for liver using cDNA microarrays after treatment of mice with model compounds of known toxicological mechanisms. Hamedeh et al. (2002a) were able to correctly classify 22 of 23 samples by applying statistical algorithms, first to derive discriminant genes from rat liver after treatment with four known hepatotoxicants and then to predict blinded samples. Currently, even more promising are studies reported in mechanistic tox-
cogenomics. Hamadeh et al. (2002b) found chemical class-
specific profiles for several liver toxicants in rats, which cor-
roborated histopathological findings and known metabolic,
pharmacologic, and toxicologic effects of these compounds.
Similarly, treatment of rats with carbon tetrachloride led to
hepatic upregulation of genes involved in stress response and
DNA and protein damage/repair, in accordance with the known
necrotic effects of carbon tetrachloride (Fountoulakis et al.,
2002). In addition to the well-studied rodent liver, toxic-
genomics has been applied to other experimental systems,
including neurons in vitro (Bouton et al., 2001), brain (Russell
et al., 2002), fetal rat testes (Shultz et al., 2001), and the
developing (Naciff et al., 2002) or prepubertal (Naciff et al.,
2003) female reproductive system.

In this study, we sought to examine whether well-known
genotoxic hepatocarcinogens, namely dimethylnitrosamine,
2-nitrofluorene, aflatoxin B1, and 4-(methylnitrosamino)-1-(3-
pyridyl)-1-butane (NNK), increase or decrease the expres-
sion of genes in the rat liver that represent defined cellular
pathways. Traditionally, assessing the carcinogenic potential
of chemicals with rodent models has been expensive and lengthy,
as it requires the use of many animals and a vast quantity of
compound during usually 2-year studies. Efforts to reduce the
time and cost required were initiated by the International
Conference on Harmonization (ICH) in the early 1990s (Sills
et al., 2001). Later, the International Life Science Institute (ILSI)
took over the task of validating numerous new models for
carcinogenicity testing (Robinson, 1998), including transgenic
and knock-out mouse models. These genetically altered mice
model major processes of carcinogenesis, like oncogene acti-
vation and tumor suppressor gene inactivation, and show in-
creased sensitivity to tumor induction, thus reducing the time
of testing to about 6 months. Employing toxicogenomics in
nongenetically altered animals may reduce the duration of
dosing even more substantially, if carcinogenic substances
would deregulate a characteristic set of genes in the first weeks
of treatment. Also, defining the pathways influenced by car-
cinogens through functional analysis of the affected genes may
reveal common mechanisms of action specific for different
classes of carcinogens.

To begin to investigate this hypothesis, we treated rats with
four genotoxic carcinogens for up to 14 days, and analyzed the
gene expression profile of the livers with Affymetrix
RG_U34A microarrays. The doses used for 2-nitrofluorene
(Cui et al., 1995), dimethylnitrosamine (IARC, 1978),
4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK; Hecht
et al., 1980), and aflatoxin B1 (Neal and Legg, 1983) have been
described in the literature to induce liver tumors in a long-term
study. At these doses, tumors were detected in all treated
animals (20/20) after 11 months for 2-nitrofluorene, in 10 of 12
animals for dimethylnitrosamine, in 22 of 24 animals after 20
weeks for NNK, and in 5 of 6 animals after 11 months for
aflatoxin B1. 2-Nitrofluorene is a nitroarene that is activated
most likely by cytochrome P450–mediated oxidation to a
reactive intermediate capable of binding DNA and other mac-
romolecules (Wierckx et al., 1990). Also, acetylation catalyzed
by phase 2 enzymes may be involved in metabolic activation of
2-nitrofluorene (Watanabe et al., 1994). Dimethylnitrosamine,
as an N-nitroso derivative, is oxidized by phase 1 metabolism
to the proximate carcinogen, which then decomposes to the
methyl carbonium ion resulting in methylation of DNA bases
(Pegg, 1984). NNK, also an N-nitroso derivative, is similarly
oxidized at the α position to a reactive intermediate, which can
decompose to two different types of carbonium ions, leading to
alkylation of both DNA bases and the phosphate backbone
(Haglund et al., 2002). One of the most potent hepatocarcino-
genons is aflatoxin B1, which is produced by certain strains of the
mold Aspergillus flavus. Its activation by cytochrome P450–
mediated oxidation leads to epoxidation of the terminal furan
ring, which primarily forms adducts with the N7 position of
guanine (Wild et al., 1986). Thus, all four genotoxic carcino-
genons induce chemical modifications and, thereby, mutations
and physical distortion of the DNA. Gene expression changes
following treatment with these chemically diverse carcinogens
were then analyzed to find similarities and differences among
them and to relate them to defined cellular pathways.

MATERIALS AND METHODS

Animal treatment. Male Wistar Hanover rats (Crl:WI/Gl/Brl/Han)GHS
BR from Charles River Laboratories, Inc. (Raleigh, NC) were maintained on
certified rodent chow (Certified Rodent Diet 5200; Purina Mills, St. Louis,
MO) ad libitum in individual suspended stainless steel wire-mesh cages. The
animals were kept under controlled temperature (18–26°C), humidity (30–
70%), and lighting (12 h light/dark cycle) and were acclimated for a minimum
of 6 days. Animals (8–10 weeks old) were assigned to dose groups (five rats/group) by weight using a weight stratification-based computer program.
Substances were administered by gastric gavage for up to 14 days (in a volume
of 5 ml/kg body weight/day) based on the group mean weekly body weight for
each dose group. Test substances were suspended (w/w) in either corn oil or a
0.5% (w/v) carboxymethyl cellulose (CMC)/DI water preparation (5 g CMC/1
DI water). To maintain a homogenous suspension during dosing, a magnetic
stir plate was used if needed. Dimethylnitrosamine (Sigma Chemical Co., St
Louis, MO; CAS 56-23-5; purity > 98%; 4 mg/kg/day) and 2-nitrofluorene
(Sigma Chemical Co.; CAS 607-57-8; purity 97.9%; 44 mg/kg/day) were
prepared using corn oil as vehicle; aflatoxin B1 (Sigma Chemical Co.; CAS
1162658; purity 98%; 0.24 mg/kg/day) and 4-(methylnitrosamino)-1-(3-pyri-
dyl)-1-butane (NNK; Toronto Research Chemicals, North York, Canada;
CAS 64997-91-4; purity > 98%; 20 mg/kg/day) were prepared using carboxy-
methyl cellulose. The animals were observed at least once daily for signs of
ovarian toxicity and readily apparent clinical symptoms. The experimental pro-
tocol was reviewed by the Institutional Animal Care and Use Committee
(IACUC) for compliance with the Federal Animal Welfare Act (1988): 7
U.S.C.2131 as well as the National Research Council’s (NRC) Guide for the
Care and Use of Laboratory Animals (National Academy Press, 1996).

Sample collection and histopathological and serum analysis. Following
1, 3, 7, and 14 days of treatment, the first surviving five animals/dose/test
group were anesthetized in a CO2 chamber and terminated by exsanguination
(and cutting the diaphragm) between 8:00 AM and 12:00 PM the morning
following the last dose. Animals were chosen sequentially for necropsy (i.e.,
the first of five from each group was sacrificed, then the second from each
group, and so on). After weighing the entire liver, a cross-section from the left
lobe was preserved in 10% buffered formalin. The remaining liver tissue was
divided into 0.5–1.0 g pieces, snap-frozen directly in isopentane cooled in
liquid nitrogen (within 2 min of removal of the liver), and stored at –80°C
prior to RNA isolation. As the sections from left and right lobes were pooled
in one container, randomly selected liver lobes were used for microarray analysis. Serum parameters from nonfasted blood subject to evaluation included aspartate aminotransferase (ALT), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), cholesterol, direct bilirubin, and total bilirubin. The liver tissue preserved in formalin was embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histopathologic examinations of the liver sections were conducted by a pathologist and peer-reviewed.

**RNA isolation.** Frozen liver sections were grinded in a Mixer Mill MM 200 (Retch GmbH and Co. KG, Haan, Germany) using precooled stainless steel balls. After adjusting the volume to 4 ml with RLT lysis buffer, total RNA was isolated using Qiagen RNAeasy 96-well kits on a Biomake 2000 robot (Beckman Coulter, Inc., Fullerton, CA), including a DNAse digestion step according to the manufacturer’s instructions. A volume of lyase corresponding to 5 mg of tissue was loaded per well. The final product was quality controlled by gel analysis using RNA 6000 Nano chips on an Agilent 2001 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with a peak area ratio >2.0 of 28S to 18S rRNA were used. RNA concentrations were determined with Ribogreen (Molecular Probes, Eugene, OR).

**Oligo microarray hybridization.** Biotin-labeled cRNA samples for hybridization on Affymetrix GeneChip RG_U34A arrays were prepared according to the protocol supplied with the GeneChip Sample Cleanup module (P/N 900371, Affymetrix Inc., Santa Clara, CA). Briefly, 5 µg total RNA was used for cDNA synthesis with the SuperScript Choice System (Invitrogen Life Technologies, Carlsbad, CA) employing a modified T7 primer with 24 thymidines at the 5′ end of the following sequence: 5′-GCCAGTGAATTGTAATACGACTCACTATAGGGAGCCGGG-dT24-3′ (Genset Corp., Evry, France). After spin column purification, biotin-labeled cRNA was synthesized from the cDNA using the ENZO RNA Transcript Labeling Kit (Affymetrix Inc.). Spin column-purified cRNA was quality controlled using an Agilent 2100 Bioanalyzer and spectrophotometrically quantified. The cRNA (15 µg) was then fragmented in buffer supplied with the Cleanup Module and hybridized for 16 h at 45°C. The microarrays were washed and stained with streptavidin-phyceroerythrin (SAPE, Molecular Probes) on the Affymetrix Fluidics Station 400 including an amplification step according to the manufacturer’s instructions. Fluorescent images were read using the Agilent Gene Array Scanner.

The raw data image files (DAT) were converted into CEL files using Affymetrix Microarray Suite (MAS) 5.0. In CEL files, the scan data from the 36 pixels per oligo set are averaged. For 2-nitrofluorene, dimethylnitrosamine, and the corresponding time-matched controls, pooled RNA from five identically treated animals per time point was processed and hybridized in triplicate (technical replicates); for NNK, aflatoxin B1, and the corresponding time-matched controls, the RNA from 3 animals was analyzed individually (biological replicates).

**Microarray quality control.** With GeneData Expressionist Quality Control Manager v2.1 software, dark and white spots, gradients, and distortions were detected and corrected using the CEL file data. Microarrays that were classified as being of bad quality were excluded from further analysis. The Affymetrix RG_U34A microarrays contain 8740 probe sets corresponding to about 7000 annotated rat genes and 1740 expressed sequence tags (ESTs). Each probe set is represented by 16 pairs of 25mer oligonucleotides. Each probe pair consists of a perfect match oligo (PM) complementary to the cRNA target sequence and a mismatch oligo (MM) with a single base change in the middle to control for background and nonspecific hybridization. Using the MAS 5.0 statistical algorithms implemented in the Quality Controller software, the intensities of all 16 probe pairs were condensed to one intensity value per probe set associated with a statistical detection p value calculated from the intensity differences of the PM and corresponding MM oligos. This p value indicates how reliably a transcript is detected. Transcripts with p ≤ 0.06 were designated present, whereas those with a p > 0.06 were designated absent. After condensing, which also included overall microarray background correction, the microarrays were scaled to an average signal intensity of 100 after excluding the highest and lowest 2% of the data.

**Microarray data analysis.** The microarray data were analyzed using the GeneData Expressionist Analyst v4.0.5 software. First, using the present genes, those significantly deregulated between treated and time-matched control samples were selected using a two-sample t-test with a p value cutoff of 0.001 (2-nitrofluorene, dimethylnitrosamine, aflatoxin B) or 0.002 (NNK) in combination with n-fold regulation/ratio of means. This requires that the mean intensity of a gene has to be at least n times different between the treated and control sample group to be included. The cutoff was set at 1.7 to 2 times, depending on the strength of the responses. The applied significance level for the analysis of the NNK data was somewhat decreased compared to the other compounds, as very few genes were found deregulated for the NNK samples at a t-test p value cutoff of 0.001.

For 2-nitrofluorene, dimethylnitrosamine, and time-matched controls, two to three technical replicates were employed, meaning that the pooled RNA from five animals of a treatment group was split up before cRNA synthesis followed by separate chip hybridizations. For NNK, aflatoxin B1, and time-matched controls, RNA of three single animals per treatment group was processed and hybridized separately.

Secondly, genes generally differentiating between the treated and control samples were obtained by applying a Welch test (p < 0.0001) on all genes including the low-expressed ones, with all treated or all control samples in one group each. This test was chosen as it does not require the same variance within both sample groups.

Thirdly, genes designated present in treated samples but absent in controls, or vice versa, were determined, as these could be genes induced from or repressed to background expression levels, respectively, after treatment. From these genes, those discriminating between treated and control samples were again selected with a two-sample t-test (p < 0.001) combined with the requirement of an at least two-fold difference of the mean intensities for a given gene.

Finally, all genes selected as described above were combined and divided into gene groups with characteristic expression profiles using self-organizing map analysis employing positive correlation as distance metric. Genes showing inconsistent expression profiles like variable expression between different control time points were excluded from further consideration. All others were categorized with respect to biochemical or cellular function. Taking into account both the biochemical category and the direction of deregulation, each gene was further assigned to a toxicological category. For Figure 1, selected gene groups were subjected to one-dimensional hierarchical cluster analysis using Euclidian as distance metric and complete linkage to define the similarity between clusters. As expression values, relative intensities were used that were obtained by dividing the absolute gene intensities of the replicates from the treated samples through the mean intensities of all time-matched control samples. These ratios were then visualized with a heat map.

**Real-time PCR (Taqmam®) experiments.** Total RNA was isolated, quantified, and quality checked as described above. RNA was reverse transcribed into cDNA following the manufacturer’s instructions using the “Superscript First-Strand Synthesis System for RT-PCR” kit (Invitrogen Life Technologies) and a mixture of oligo-dT and random hexamers as primers. Real-time PCR was performed with an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using template cDNA corresponding to 5 ng/µl of RNA and SYBR Green PCR Mastermix (Eurogentec, Seraing, Belgium), as recommended in the manufacturer’s protocol. Primers were designed for selected genes (Table 1) with Primer Express Software v2.0 (Applied Biosystems), following the manufacturer’s instructions for optimal primer design, and checked for specificity by blast searches. Also, primers were only used when they gave rise to a single amplicon as revealed by melting curve analysis after PCR. Sequences of forward and reverse primers, which were purchased from Invitrogen Life Technologies, are listed in Table 1. Samples were amplified in quadruplicate. Each run included a standard curve of four points with known amounts of template RNA, which was used to calculate relative amounts of gene-specific mRNAs in each sample via the Ct values. Results represent average ratios of treated versus time-matched control samples, employing the same five (2-nitrofluorene, dimethylnitrosamine) or three (NNK, aflatoxin B1) animals per group as used for microarray analysis; 18S rRNA primers served as normalization controls to correct for possible differences in input RNA amounts between the samples.
Supplemental materials. Ratios of gene expression levels in treated versus control samples for the 194 probe sets selected to be significantly deregulated by at least one compound are available online at www.toxsci.oupjournals.org.

RESULTS

Histopathology

Male Wistar Hanover rats were dosed daily via gavage for up to 14 days with four model genotoxicants using doses documented to induce liver tumors in rats, as described in the Materials and Methods. To check for additional pathological lesions induced during this subacute study, the livers were examined histologically. We found that 2-nitrofluorene induced modest hypertrophy from day 3 to day 14. Dimethylnitrosamine treatment resulted in increasing necrosis at day 7 and day 14, accompanied by reactive inflammation, as expected from the literature data for the dose used (Reynolds, 1972). Also, some transient mitosis was observed at day 7. After treatment with NNK, weak apoptosis was detected at day 14.

Gene Expression Analysis

To determine gene expression changes associated with short-term exposure to genotoxic carcinogens, RNA from the dosed animals and their time-matched vehicle controls was isolated after 1, 3, 7, and 14 days of treatment. As described in the Materials and Methods, two to three replicates of pooled RNA from five identically treated rats were subjected to microarray hybridization with Affymetrix RG_U34A Genechips.
in the case of 2-nitrofluorene and dimethylnitrosamine. For NNK and aflatoxin B1, the RNA from three animals was hybridized separately onto three Genechips each. Both ways of using replicates have their advantages and disadvantages. Technical replicates from pooled RNA samples can cover a wider range of animals but cannot detect outlier animals whose gene expression changes are very dissimilar to the remainder and, thus, bias the results. For biological replicates the converse is true, unless a large number of microarrays is used, which may be uneconomical in times. As shown later, both methods lead to comparable results for the samples used.

To extract characteristically deregulated genes, various tools provided by the GeneData Expressionist Analyst software were employed for samples from one compound at a time, as described in Materials and Methods. These included statistical testing between treated and control sample groups in combination with the requirement of at least 1.7 to 2 times deregulation with respect to the mean intensity of the samples groups, using either so-called present genes only or all genes represented by the Genechip. These significantly deregulated genes were then divided into gene groups with distinct expression profiles using self-organizing map (SOM) analysis, an algorithm that extracts prominent expression patterns from a set of profiles. SOM analysis also allowed the deselection of genes showing inconsistent expression between the controls at different time points. The numbers of genes are given either as probe sets or as nonredundant genes to account for the fact that one gene can be represented by several probe sets on the RG_U34A Genechip.

The number of probe sets and the corresponding numbers of up- and downregulated genes obtained by these analyses for each compound are given in Table 2. There were about 70–80 nonredundant genes for 2-nitrofluorene, dimethylnitrosamine, and aflatoxin B1, but only 16 for NNK, probably reflecting the weaker histopathological lesions found with NNK compared with the other compounds. Nevertheless, the direction of deregulation of genes selected for statistical significance after treatment with the other three carcinogens was largely comparable in the NNK samples (Fig. 1 and Table 3), even though they did not reach the thresholds to be selected from the NNK samples. In all four cases, the number of upregulated genes exceeded the number of downregulated genes. Altogether, 194 probe sets representing 162 nonredundant genes were significantly deregulated by at least one of the four compounds, with 118 genes being increased and 44 genes being decreased in their expression.

### Functional Analysis

To gain insight into the pathways depicted by these genes, a biochemical context and subcategory was assigned to each gene describing its main cellular or organismal function. The necessary information to categorize each gene was obtained from searching several databases, including NetAffx from Affymetrix, SwissProt, and Pubmed, among others. The biochemical category, the direction of deregulation, and information about upstream pathways, if described in the literature, were then considered to define a toxicological category for each gene and its encoded protein in the context of the direction of deregulation after treatment, helping in the identification or description of at least some of the affected toxicological pathways. The number of nonredundant genes assigned to these toxicological categories, for genes either significantly deregulated by each carcinogen separately or the union thereof,
are depicted in Table 2. Representative genes for these toxicological categories are listed in Table 3 together with their direction of deregulation. Expressed sequence tags (ESTs) or genes whose function could not be reconciled with a toxicological pathway were filed as other/unknown.

Several genes known to be induced by the transcription factor and tumor suppressor p53 (Amundson et al., 2001) were upregulated by all four carcinogens, including the apoptosis inducer BAX, the cyclin-dependent kinase inhibitor p21, the cell growth regulatory gene 11, the B-cell translocation gene 2, cyclin G1, and the ubiquitin E3 ligase MDM2. As p53 is known to be activated by DNA damage (Amundson et al., 2001), the upregulation of these genes was interpreted as DNA damage response. Another p53 target gene, PAG608, which was induced by 2-nitrofluorene, dimethylnitrosamine, and aflatoxin B1 but not by NNK, also fits into this category. Also, the DNA repair enzyme O6-methylguanine-DNA methyltransferase, whose expression was increased by all four carcinogens, was assigned to the DNA damage response pathway as it is known to be induced by genotoxic stress (Grombacher et al., 1998).

The toxicological category detoxification response/MDR was used for genes coding for biotransformation phase I and phase 2 enzymes, including aldehyde dehydrogenase 1A1, aflatoxin B1 aldehyde reductase, epoxide hydrolase 1, carboxyl-esterase CES2, cytochrome P450 1A1, UDP-glucuronosyltransferases 1A6 and 1A7, and glutathione-S-transferases A2 and A5, and genes encoding proteins conferring the multidrug resistance (MDR) phenotype, like the drug transporter MDR1b and the VATD subunit of the vacuolar proton pump (Broxterman et al., 1995; Simon et al., 1994). These genes were upregulated by all four carcinogens, sometimes quite strongly with a maximal induction of MDR1b by two orders of magnitude, suggesting an effort of the cells to metabolize and excrete these compounds. The observed increase in the transcript levels of the glutathione metabolism enzymes glutathione reductase and γ-glutamyl-cysteinyln-synthetase, the heat shock proteins GrpE-like 1 and HSP84, and the enzyme NADPH quinone oxidoreductase, especially after treatment with 2-nitrofluorene, implies an oxidative or general stress response evident mainly for this compound.

Downregulation of the genes encoding the mitochondrial proteins ornithine aminotransferase, a subunit of the ATP synthase F0 complex (ATP5G1), and mitochondrial cytochrome B was interpreted as mitochondrial damage. In addition to suggesting mitochondrial damage, the diminished expression of these mitochondrial enzymes could also be interpreted as dedifferentiation. Since metabolism is the major function of the differentiated liver, the decreased expression of genes encoding the mainly cytosolic or microsomal metabolic enzymes, including methionine adenosyltransferase 1a, betaine-homocysteine S-methyltransferase, glutathione-S-transferase M3, diaphorase, L-gulono-γ-lactone oxidase, serine dehydratase, glutaminase, and glycine-N-methyltransferase, was therefore classified as dedifferentiation. This treatment-related effect was most pronounced for 2-nitrofluorene but was also quite clear for aflatoxin B1. The downregulation of the metallothionein genes 1 and 2 was described as reduced stress response competence, as metallothioneins are proteins induced in response to various forms of stress (Sato and Kondoh, 2002).

**Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2-Nitrofluorene</th>
<th>Dimethylnitrosamine</th>
<th>NNK</th>
<th>Aflatoxin B1</th>
<th>Union</th>
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<tr>
<td>Probe sets</td>
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<td>84</td>
<td>18</td>
<td>86</td>
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<td>Detoxification response/MDR</td>
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<td>10</td>
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<td>6</td>
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*Note.* The number of probe sets and the corresponding number of nonredundant genes that were found to be characteristically deregulated by each compound or by all compounds together (union) are indicated in rows one and two, respectively. In rows three and four, the numbers of upregulated and downregulated nonredundant genes are given. The list of nonredundant genes is further subdivided into groups of genes assigned to different toxicological categories.
### TABLE 3
Partial List of Genes Assigned to Different Toxicological Categories

<table>
<thead>
<tr>
<th>Toxicological category</th>
<th>Accession ID</th>
<th>Gene name</th>
<th>Biochemical pathway</th>
<th>Deregulation</th>
<th>2-NF</th>
<th>DMN</th>
<th>NNK</th>
<th>AB1</th>
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<td>DNA damage response</td>
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<td>BAX Bcl2-associated x protein</td>
<td>Apoptosis</td>
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<td>DNA repair</td>
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<td>competence</td>
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TABLE 3—Continued

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<td>X62952</td>
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<td>ECM</td>
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<td>Innate immunity</td>
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<td>Antigen presentation</td>
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**Note.** For each toxicological category, representative genes are listed by their name together with the Genbank accession IDs. The main biochemical functions or pathways in which these genes are involved are described in column four. The last 4 columns indicate the direction of deregulation after treatment with 2-nitrofluorene (2-NF), dimethylnitrosamine (DMN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and aflatoxin B1 (AB1). ↑ indicates two-fold or higher upregulation for at least one time point and ↓ stands for two times more downregulation for at least one time point, as calculated using the mean intensities each of the treated and control replicate samples of a given time point. Parentheses around ↑ or ↓ denote 1.5- to 2-fold deregulation.

Survival signaling. Several genes that encode regulators of proliferation were also upregulated by all four compounds, including those for neuregulin I, growth- and transformation-dependent protein, and cyclin D1. Together with increased expression of the genes encoding thymidylate synthase, an enzyme involved in nucleotide synthesis, and the ribosomal proteins S19 and S27, these genes imply increased survival and proliferation signaling.

Cell structure is mainly determined by the cytoskeleton. The genes encoding a cytoskeletal constituent, spektrin α chain, and several cytoskeletal regulators, α B-crystallin, RhoB, and thymosin β-10, were induced by all four carcinogens, suggesting alteration or stabilization of the cell structure. Dimethylnitrosamine and aflatoxin B1 also elevated the expression of vimentin, an intermediate filament, of Annexin II and of a component of the ARP2/3 complex, which may be reconciled with the fact that these two compounds lead to observable necrosis, a process connected with cell structural changes. The deregulation of these cytoskeletal proteins was summarized as cell structure remodeling.

In contrast to these commonly affected pathways, an inflammatory response at the gene expression level was seen only for two compounds, strongly for dimethylnitrosamine with 31 genes deregulated in this category and modestly for aflatoxin B1 with the deregulation of 10 genes. This agrees well with the inflammation observed histologically, which was stronger for dimethylnitrosamine, weaker for aflatoxin B1, and hardly detectable for NNK and 2-nitrofluorene. The time dependence of the gene expression changes also paralleled the inflammation seen in vivo, being increased only at days 7 and 14, or throughout the time course for dimethylnitrosamine or aflatoxin B1, respectively. Among the genes whose upregulation indicated an inflammatory response were those for allograft inflammatory factor-1, cathepsin S, the immunoglobulin γ Fc receptor III, the small inducible cytokine B10, the transcription factor STAT1, and several genes involved in antigen presentation.
including MHC class Ib and class II genes, those for interferon regulatory factor 1, the two proteasome subunits PSMB8 and PSMB9, and the antigen peptide transporter Tap-1.

To visualize the expression changes upon treatment with the genotoxic carcinogens relative to vehicle controls, these were shown in Figure 1 as a heat map, using gene groups representing the toxicological categories of DNA damage response, detoxification response/MDR, survival/proliferation, and inflammation. To simplify the diagram somewhat, ratios were calculated between the expression values of single treated replicates and the mean expression value of the corresponding vehicle control samples. Overall, for the genes belonging to the categories of DNA damage response, detoxification response/MDR, and survival/proliferation, the response was qualitatively similar after treatment with all four carcinogens that were analyzed. The extent of deregulation for these genes was strongest with 2-nitrofluorene and aflatoxin B1, quite strong with dimethylnitrosamine, and weaker with NNK. With regard to the time course, the effects on gene expression were quite clear already at day 1 in the 2-nitrofluorene and aflatoxin B1 samples but showed an increase with time, from day 1 to day 7, in the dimethylnitrosamine samples and, from day 1 to day 14, in the NNK samples. The enlarged region in Figure 1 shows these findings in more detail for genes representing the DNA damage response and the detoxification/MDR response. While these genes are at least two-fold upregulated at any time after treatment with aflatoxin B1, BTG2, BAX, and GSTA2/5 reach the two-fold threshold only after 7 days with NNK. The heat map also clearly shows the strong upregulation of inflammatory genes after 7 and 14 days with dimethylnitrosamine, the weaker but still obvious response of genes in this category with aflatoxin B1, and the more sporadic effects on these genes with 2-nitrofluorene and NNK; these data correlate well with the observed histopathology.

In summary, a DNA damage response, a detoxification response, survival/proliferation signaling, and cell structure remodeling or stabilization were commonly affected by all four carcinogens, suggesting that these may be characteristic pathways targeted more or less specifically by genotoxic carcinogens in the liver, at least by those belonging to the chemical classes represented by the examined compounds.

Gene Expression Verification with Quantitative Real-Time PCR

The expression profile of selected genes found by microarray analysis to be characteristically deregulated (Table 1) was reanalyzed by quantitative real-time polymerase chain reaction (QRT-PCR), using Taqman® technology, with the day 3 and day 7 samples from all four genotoxicants. Liver RNA from the same animals as used for chip hybridization was employed, and mean expression ratios of all samples from identically treated animals versus time-matched controls were compared between Affymetrix and Taqman® technology. As shown in Fig. 2 for the day 7 samples from dimethylnitrosamine-treated rats, the QRT-PCR analysis corroborated the results obtained with microarray hybridization. As reported previously for comparable studies (Hamadeh et al., 2002b; Naciff et al., 2002), the induction ratios measured by QRT-PCR were mostly higher than those measured by microarray analysis, which may be considered as a semiquantitative measurement. With 120 such ratio comparisons between Affymetrix and Taqman® technology currently completed in our laboratory, which included the day 3 and day 7 samples described in this study, we obtained a regression coefficient of 0.83 between the natural log of the ratios (unpublished observations), confirming that the gene expression changes measured in our study by Affymetrix microarray technology represented real deregulations. Overall, the QRT-PCR results strongly supported the interpretation of the microarray data.

DISSCUSSION

The aim of this study was to investigate whether genotoxic carcinogens lead to deregulation of genes involved in common pathways at an early time point during exposure, which could allow greater insight into the mechanisms leading to tumor formation and eventually be employed to classify compounds with regard to their carcinogenic potential. Therefore, we dosed rats with four well-characterized genotoxic hepatocarcinogens belonging to three different chemical classes, and we examined the gene expression changes relative to vehicle-treated control animals after 1, 3, 7, and 14 days with microarrays from Affymetrix representing most known rat genes. Categorization of the characteristically deregulated genes then allowed a mechanistic analysis of the early responses after carcinogen application, which comprised both documented gene expression changes after genotoxicant treatment and new observations. Overall, a DNA damage response, a detoxification response, and survival/proliferation pathways were commonly affected by all four carcinogens, whereas a necrotic/
inflammatory response at the gene expression level correlated with the histologically detectable necrosis and subsequent inflammation in the case of dimethylnitrosamine and aflatoxin B1.

DNA Damage Response

The DNA damage response that is activated by chemically or irradiation-induced damage to the DNA can lead to cell cycle arrest, cell death, or malignant transformation, depending on the severity of the damage and the cellular context (Zhou and Elledge, 2000). Primary signal transducers are members of a family of PI3 kinase–like protein kinases (PIKK), which phosphorylate and thereby activate downstream effectors. Among them, the ATM kinase responds primarily to double-strand breaks and activates downstream effectors, including further kinases and the tumor suppressor p53. p53 is a transcription factor that induces the expression of genes involved in cell cycle checkpoint activation, DNA repair, and apoptosis (Amundson et al., 2001). Several of these p53 target genes were found to be induced by the four genotoxicants tested in this study, strongly implicating a DNA damage response. Such a response was actually expected in light of the known DNA-modifying capabilities of these compounds, thus demonstrating the validity of this microarray approach. Figure 3 shows the DNA damage response–related genes observed to be upregulated in this study. DNA damage induced by any of the carcinogens tested likely activated the transcriptional activation function of p53, either via phosphorylation by the ATM kinase or other pathways. Activated p53 then increased the expression of the cyclin-dependent kinase inhibitor p21, which could induce cell cycle arrest, of BAX, which could lead to apoptosis of cyclin G1 and B cell translocation gene 2, potentially inducing a replication arrest, and of the Mdm2 E3 ubiquitin ligase, which leads to proteasomal degradation of the p53 protein by ubiquitinylation in a negative feedback loop

![DNA Damage Response Diagram](https://example.com/diagram.png)

**FIG. 3.** Genotoxicant-induced DNA damage response. The genes found upregulated in this study are shown with rectangles. Lanes with arrows indicate induction of gene expression, and dotted lanes represent transcription-independent activation (with arrow) or repression (with bar) of a protein or a cellular pathway (in italics). DNA damage is known to activate the transcription factor p53 via the ATM protein kinase or via other pathways, resulting in upregulation of p53 target genes. See the text for further details.

Detoxification Response

The biotransformation of lipophilic compounds like steroids or xenobiotics occurs mainly in the liver and is divided into three phases. Phase 1 reactions result in the introduction of hydrophilic residues, which are conjugated with polar groups in phase 2 reactions, resulting in a large increase in hydrophilicity to facilitate excretion. Enhanced export of both xenobiotic conjugates and unconjugated lipophilic substances is then mediated by drug transporters in phase 3 (Parkinson, 2001). In the case of chemical carcinogens, phase 1 reactions can also activate the original compounds to the ultimate carcinogens, which are able to covalently interact with DNA. Phase 2 reactions can also lead to either detoxification or activation (Pitot III and Dragon, 2001). The xenobiotics themselves can increase the expression of biotransformation proteins. Phase 1 enzymes and drug transporter expression can be induced via specific binding to and activation of ligand-dependent transcription factors, including the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane x receptor (PXR), and the peroxisome proliferator-activated receptor (PPAR) (Rushmore and Kong, 2002).

In this study, the expression of genes encoding several phase 1 and phase 2 enzymes was specifically elevated, indicating an attempted detoxification response. The elevation of *aldehyde dehydrogenase 1A1* was remarkable, with a 10- to 20-fold increase at day 14 in all four cases. *Aldehyde dehydrogenase 1* expression has been shown to be increased by phenobarbital-type inducers (Pappas et al., 1998) known to act via CAR (Rushmore and Kong, 2002). CAR has also been shown to activate UDP-glucuronosyltransferase 1A1 and 1A6 transcription (Xie et al., 2003). UDP-glucuronosyltransferase 1A6 and 1A7 expression was found increased in this study, indicating an activation of the nuclear transcription factor CAR by the four carcinogens examined here. The expression of *aflatoxin B1 reductase*, microsomal epoxide hydrolase, glutathione-S-transferase A5, and NADPH quinone oxidoreductase was reported to be induced by chemopreventive agents and phenolic antioxidant, possibly by activating the antioxidant response element (ARE) that has been localized in the promoters of the
glutathione-S-transferase A5 and NADPH quinone oxidoreductase genes (Hayes and McMahon, 2001; Kelly et al., 2000). Electrophilic compounds that are capable of reacting with sulphydryl groups can stimulate ARE-driven transcription. This electrophilicity can also be obtained following oxidative metabolism. We found increased expression of aflatoxin B1 reductase, microsomal epoxide hydrolase, glutathione-S-transferase A5, and to some extent also NADPH quinone oxidoreductase after treatment with all four carcinogens, suggesting activation of the ARE pathway. We also observed an induction of cytochrome P450 1A1 after treatment with 2-nitrofluorene and aflatoxin B1, which is known to be regulated by aromatic hydrocarbons via the AhR (Rushmore and Kong, 2002). Oxidation of these compounds by CYP1A1 may convert them to ARE inducers. The human ortholog of rat carboxylesterase CES2 has been identified as a primary p53 target gene in a human lung cancer cell line that expresses a conditionally active form of murine p53 (Kannan et al., 2001). CES2 was found to be upregulated by treatment with all four carcinogens, which could be due to p53-mediated activation in response to DNA damage.

Human MDR1, also known as P-glycoprotein, is a member of the ABC family of membrane transporters that function in intestinal efflux and biliary excretion of many xenobiotics and endogenous compounds. Rodents express two isoforms of MDR1, MDR1a and MDR1b, encoded by distinct genes. DNA damage as well as oxidative stress have been reported to activate MDR1b expression in rodents, and responsive elements in the promoter of the rat MDR1b gene have been identified that mediate transcriptional activation, including binding sites for the transcription factors p53, AP-1, and NF-κB (Muller, 2000). Thus, the 10- to 100-fold elevation of MDR1b expression observed with the four genotoxic carcinogens in this study could have been induced by DNA damage via p53 or by an oxidative or general stress response with the activation of AP-1 and NF-κB. MDR1b was reported to be highly expressed in preneoplastic lesions and after partial hepatectomy in the rat liver (Teeter et al., 1993). In addition to enhanced efflux via membrane transporters, regulation of intracellular pH may play a role in MDR, for instance by sequestration of protonated xenobiotics in intracellular organelles. A subunit of a vacuolar H+-ATPase has been shown to be responsible for non-P-glycoprotein-mediated MDR (Simon et al., 1994). We found increased expression of the D subunit of vacuolar H+-ATPase, implying an MDR response possibly regulated in the same way as MDR1b expression. Overall, the induced expression in the rat liver of a subset of enzymes and transporters involved in the metabolism and export of xenobiotics by the four genotoxic carcinogens examined here suggests a detoxification stress response, which may be characteristic for genotoxicants or carcinogens in general and which could both decrease or increase the activity of the compounds with respect to DNA reactivity.

**Dedifferentiation**

The main function of the liver is to maintain the metabolic homeostasis of the body, including the metabolic conversion of nutrients, the synthesis of major serum proteins, and the detoxification of xenobiotic substances. A hallmark of the differentiated liver is, therefore, the constitutive or inducible expression of a diverse set of enzymes and proteins involved in these processes. Exposure to toxicants can dramatically alter the profile of expressed genes and protein in the liver. As described above, the increased expression of specific sets of biotransformation enzymes as an effort to detoxify certain xenobiotics is one response. The downregulation of genes encoding metabolic enzymes functioning in amino acid, carbohydrate, cofactor, and xenobiotic metabolism, as observed in this report (Table 3), suggests a dedifferentiation as a result of a stress response intended to repair possible damage, which may prevent the hepatocytes from accomplishing their normal metabolic functions. As a result of losing some of the differentiated state, the hepatocytes may be more prone to engaging into proliferative actions, which occur later during carcinogenic progression in neoplastic nodules. This hypothesis lends some support from the fact, that serine dehydratase enzyme activity (Kitagawa, 1976) and ornithine aminotransferase expression (Matthaei and Williams, 1987) were reported to be downregulated in areas of hyperplasia during rat hepatocarcinogenesis and in rat kidney and liver tumors, respectively.

**Survival and Proliferation Signaling**

All four carcinogens consistently induced the expression of tissue inhibitor of metalloprotease 1, a secreted metalloprotease inhibitor that has also been reported to inhibit apoptosis of hepatic stellate cells (Murphy et al., 2002), of growth differentiation factor 15, which inhibits apoptosis at least in neuronal cells (Subramaniam et al., 2003), of growth-potentiating factor, which has been shown to both potentiate the growth of vascular smooth muscle cells and protect them from apoptosis after growth arrest (Nakano et al., 1996), and of neuregulin 1, reported to induce proliferation and promote survival of neuronal cells (Canoll et al., 1996) and cardiac myocytes (Zhao et al., 1998). Common to these secreted polypeptides is their ability to promote cell survival; their induction could reflect an adaptive response of the cells after toxicant exposure to prevent unnecessary cell loss. Yet expression of these factors could also allow the survival of cells whose DNA had been damaged to an extent inevitably leading to mutations, a prerequisite for neoplastic transformation. The increased expression of the gene encoding growth- and transformation-dependent protein, whose mRNA has been found to accumulate following growth factor–induced transition of rat fibroblasts from a quiescent to a proliferative state (Glaichenhaus and Cuzin, 1987) but whose function is otherwise unknown, may have similar consequences.

The expression of the gene coding for cyclin D1, an activator of cyclin-dependent kinase 4 that regulates entry into and
progression through the G1 phase of the cell cycle (Martin-Castellanos and Moreno, 1997), was elevated at least two-fold at day 14 with all four carcinogens tested. Cyclin D1 is overexpressed in several types of carcinomas and, therefore, suggested to play an important role in tumorigenesis and tumor progression including hepatocellular carcinoma, a hypothesis that is corroborated by a report that transduction of antiseNSE cyclin D1 inhibits the growth of rat hepatoma cells (Uto et al., 2001). Another gene upregulated by the four carcinogens with a function in cell cycle progression was thymidilate synthase, which is a rate-limiting enzyme for DNA synthesis by providing precursors for DNA replication and which is a target for the anticancer drug 5-Fluorouracil (Paradiso et al., 2000). Also, its expression was found to be increased in primary human colorectal tumors (Paradiso et al., 2000) and in regenerating liver (Ozeki and Tsukamoto, 1999), both being in a proliferative state. In addition to suggesting entry into the cell cycle from the quiescent state, increased DNA precursor synthesis may be necessary for DNA repair.

Two mRNAs encoding ribosomal proteins, rpS27 and rpS19, were also elevated about two-fold in rat liver by all four carcinogens tested. The mRNA rpS27, also called metallopanstimulin-1, was reported to be highly expressed in a wide variety of actively proliferating cells and tumor tissues (Ganger et al., 2001). With respect to liver, rpS27 was suggested to be involved in both progression toward malignancy in regenerating cirrhotic nodules and in subsequent steps of hepatocarcinogenesis. Higher levels of rpS19 mRNA were found in primary colon carcinoma tissue compared to normal colon tissue of patients (Kondoh et al., 1992), proposing a connection between rpS19 expression and carcinogenesis in at least some cases. Thus, the upregulation of these two genes may be characteristic for carcinogens in general or for genotoxic carcinogens in particular. Together with increased expression of cyclin D1 and thymidilate synthase, this may describe a condition allowing hepatocytes with nonrepaired mutations to more easily proliferate later on.

Cell Structure Organization

Several genes encoding proteins that function in cytoskeletal organization were induced by all four carcinogens, which could indicate a stress response to toxicant exposure resulting in cell structure remodeling or stabilization. Alpha B-crystallin has been described as a small heat shock protein with a role in the stability of cytoskeletal organization (Iwaki et al., 1994), which may be related to its cytoprotective function in response to cardiac stress (Hoover et al., 2000). The cytoskeletal actin-binding protein spectrin α chain is cleaved during apoptosis into characteristic fragments by caspases (Brown et al., 1999). Therefore, the upregulation of its mRNA reported here could indicate a protective response against the loss of this structural protein. The small GTPase RhoB functions as a cytoskeletal regulator that integrates signals from integrins and cytokine receptors with cell shape via the actin cytoskeleton. It may have a role in cancer cell pathophysiology, as it has been linked to the antineoplastic effects of farnesyl transferase inhibitors, which block cancer cell proliferation and which originally were developed as a strategy to inhibit oncogenic Ras (Prendergast, 2001). Importantly, RhoB was reported to be inducible by genotoxic stress via transcription factors other than p53 (Fritz and Kaina, 2001). This correlates well with our data showing early elevation of RhoB mRNA in rat liver by all four carcinogens. Another cytoskeletal regulator whose mRNA was upregulated by the four carcinogens was thymosin β-10, which controls actin dynamics by sequestering actin monomers. Thymosin β-10 gene expression is related to cell growth in proliferating tissues, and its overexpression was detected in several neoplastic human tissues (Santelli et al., 1999), which led the authors to propose its investigation as a diagnostic marker for cancerogenesis. Also, human thymosin β was identified as a putative primary p53 target gene by Kannan et al. in a microarray study (2001). The upregulation of these genes involved in cytoskeletal organization after treatment with the four genotoxic carcinogens implies a stress response to preserve cell structure and, thus, enhance cell survival. Increased thymosin β-10 expression could also suggest a preparation for neoplastic progression.

Three additional genes encoding proteins functioning in cell structure organization were mainly upregulated by dimethylnitrosamine and aflatoxin B1, which may be correlated with the necrosis observed in these cases. Annexin A2 is an intracellular protein that binds to F-actin and to negatively charged phospholipids in cellular membranes in a Ca2+-regulated manner. It thereby plays a role in the organization of membrane domains and their association with the actin cytoskeleton (Gerke and Moss, 2001). The expression of the genes coding for a subunit of the ARP2/3 complex, which is involved in organization of the actin cytoskeleton, and for vimentin, an intermediate filament, was similarly elevated. Vimentin expression has been reported to be elevated after the induction of necrosis by denervation in muscle (Winter and Bornemann, 1999) and by toxicant treatment in the kidney (Wallin et al., 1992), probably indicating a regenerative process. Changes in the cytoskeleton are likely both induced by and have an influence on the necrotic process, as necrotic cells usually detach from the cellular network and round up before final lysis. Thus, elevation of the mRNA levels for annexin A2, the ARP2/3 complex 41kDa subunit, and vimentin after treatment with dimethylnitrosamine and aflatoxin B1 suggests either an effort of damaged cells to prevent necrotic death or a regenerative response of the surrounding cells.

Inflammatory Response

In agreement with the observed pathology, deregulation of genes mediating an inflammatory response was extensive after treatment with dimethylnitrosamine and quite prominent after treatment with aflatoxin B1, where the responses were parallel to the histologically detected inflammation during the time
The upregulation of genes mediating antigen presentation, including the proteasome subunits PSMB8 and PSMB9, which are normally induced by inflammatory signals, the antigen peptide transporter Tap-1, the interferon regulatory factor 1, a transcription factor regulating the expression of PSMB9 and Tap-1 (White et al., 1996), and several MHC genes, suggests that debris from necrotic cells were taken up, processed, and presented to inflammatory cells by parenchymal and nonparenchymal liver cells. Inflammation as a reaction to cell necrosis is similarly indicated by increased expression of the genes encoding cathepsin S, a lysosomal protease reported to contribute to MHC processing in phagocytic cells (Maurer et al., 1998), allograft inflammatory factor 1, which is a calcium and actin binding protein involved in phagocytosis and macrophage activation (Ohsawa et al., 2000), and the small inducible cytokine B10, a chemokine induced by various forms of liver injury and suggested to have a hepatoprotective function (Bone-Larson et al., 2001). Taken together, the set of genes encoding proteins with inflammatory functions, whose expression was found to be elevated in this study after treatment with dimethylnitrosamine and aflatoxin B1, suggests a reactive inflammation in response to necrosis.

Conclusions

In summary, this report provides evidence that genotoxic carcinogens deregulate characteristic sets of genes, which allows for interpretations of their mode of action. Whether all the deregulated genes are specific for genotoxic carcinogens, however, has to be further substantiated as soon as data for compounds with different modes of action, e.g., nongenotoxic carcinogens, direct-acting hepatotoxicants, and nontoxic controls, become available. According to published data, many of the genes or their encoded proteins identified here have previously been found to be deregulated in tumor tissues or cancer models, which supports the validity of our approach. By using short-term in vivo studies with well-known genotoxic carcinogens, we were able to derive a combination of genes that may be distinctive for genotoxic carcinogens and may represent the main pathways affected early after exposure to this compound class. The overall influence of the four carcinogens examined here on these pathways is shown in Figure 4, which gives the normalized mean deregulation of all genes belonging to a certain toxicological category at the four time points analyzed.

The DNA damage response was most strongly affected by aflatoxin B1 and quite strongly by 2-nitrofluorene, in both cases from day 1 to day 14. Dimethylnitrosamine and NNK also induced a DNA damage response, but the responses increased from being just detectable at day 1 to a maximum reached at day 7 and day 14, respectively. A similar expression profile was seen for genes involved in survival or proliferation signaling. The detoxification response, which is characterized by upregulation of genes encoding biotransformation enzymes,

FIG. 4. Main biological processes affected by treatment with the four genotoxic carcinogens. To illustrate the influence of 2-nitrofluorene (2-NF), dimethylnitrosamine (DMN), NNK, and aflatoxin B1 (AB1) on the overall expression of genes belonging to the most prominent toxicological categories identified here, the expression of each single gene (n-fold deregulation vs. time-matched controls) was normalized over all samples by dividing the value in a particular sample through the maximum deregulation found for that gene in all samples, resulting in n-fold deregulation values from –1 to 1. Then, the mean of the normalized values was calculated for each single sample from all genes belonging to the toxicological categories of DNA damage response, detoxification response, cell survival/proliferation, and inflammation, as indicated above each diagram. The genes used are as listed in Table 3. This mean thus represents the overall response of the genes of a particular pathway. See the text for further details.
appeared to be strongest for 2-nitrofluorene, which may explain the fact that liver hypertrophy was observed after treatment with 2-nitrofluorene. Except for NNK, this response was maximal at day 3. Overall, the effects on the pathways mentioned above appear to be relatively time independent for 2-nitrofluorene and aflatoxin B1, with easily detectable gene deregulations at all time points analyzed. In the case of dimethylnitrosamine and NNK, the responses were weak yet detectable at day 1 and then increased with time. The results from the different time points suggest that the day 3 or day 7 time point alone might have been enough to reveal the characteristically deregulated genes. In contrast, deregulation of genes involved in inflammation was observed mainly in those samples where inflammation was detected histologically, with the overall extent of deregulation roughly correlating with the severity of pathology and being strong for day 7 and day 14 after dimethylnitrosamine treatment and weaker but present at all time points after treatment with aflatoxin B1.

Thus, a DNA damage response, a detoxification response, and survival/proliferation signaling may reflect the efforts of the cells in the liver to cope with damage inflicted by exposure to genotoxic agents, which may at the same time set the conditions to allow later proliferation of cells with genetic alterations. Overall, this study successfully identified gene expression profiles of the liver after subacute exposure of rats to genotoxic carcinogens, which suggests that a particular combination of mechanisms, both previously known ones and new aspects thereof, characterizes the response to this class of agents early after treatment. Whether the examination of gene expression profiles in short-term studies can reliably predict the development of cancer at much later time points, as suggested with our study, seems worthwhile for further investigation.

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