Cell proliferation and regulation of negative growth factors in mouse liver foci

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Foci of altered hepatocytes are preneoplastic lesions capable of progressing to hepatocellular carcinomas. To characterize the growth of preneoplastic hepatic lesions, size of hepatic foci was analyzed with regard to growth factor regulation and hepatocyte proliferation in focal and non-focal hepatocytes. Twelve-day-old female B6C3F1 mice were initiated with a single dose of the potent mutagen N-nitrosodiethylamine (DEN) (5 mg/kg body weight). Beginning at 6 weeks of age, mice were exposed for 16 weeks to 2038 p.p.m. unleaded gasoline (UG) vapor or 1 p.p.m. ethinyl estradiol (EE) in the diet. Analysis of hepatic foci demonstrated that UG significantly increased, but EE significantly decreased the size of DEN-initiated foci. Hepatic labeling index (LI), as measured by the incorporation of 5-bromo-2'-deoxyuridine, was similar in non-focal hepatocytes at 16 weeks in all groups (0.4–0.8%) and greatly increased in hepatic foci. Hepatocyte LI was significantly increased in DEN/UG foci (29%, n = 41) and significantly decreased in DEN/EE foci (6%, n = 23) relative to DEN/control focal hepatocytes (18%, n = 25). The mean LI of foci correlated with the focal size differences observed in the treatment groups. Immunohistochemical analysis with antibodies directed to the negative growth regulator transforming growth factor-beta1 (TGF-β1) demonstrated a consistent decrease of TGF-β1 in DEN/Ct and DEN/UG hepatic foci relative to non-lesion hepatocytes. Similar results were seen with mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGF-II R), which facilitates activation of latent TGF-β1. In contrast, only 50% of DEN/EE foci had decreased levels of TGF-β1 and M6P/IGF-II R relative to non-focal hepatocytes. These data suggest that proliferative responses observed in hepatic foci may be correlated with foci size. In contrast, chemically induced proliferative responses in non-focal hepatocytes after subchronic exposure cannot necessarily be used to predict proliferative effects in preneoplastic cell populations. Furthermore, these studies suggest that hepatic foci may occur by M6P/IGF-II R enhancing activation of latent TGF-β1 in non-focal hepatocytes but not in the focal hepatocytes, thereby affording focal hepatocytes a selective growth advantage.

Introduction

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*Abbreviations: DEN, N-nitrosodiethylamine; UG, unleaded gasoline; EE, ethinyl estradiol; LI, hepatic labeling index; Ct, control; TGF-β1, transforming growth factor-beta1; M6P/IGF-II R, mannose 6-phosphate/insulin-like growth factor II receptor; G0, non-proliferating phase of the cell cycle; RB, retinoblastoma; PB, phenobarbital; BrdU, 5-bromo-2'-deoxyuridine; H&E, hematoxylin and eosin.

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Animals

the American Association of Laboratory Animal Care. Chemical Industry Institute of Toxicology (CUT). Male C3H/HeNCrlBR mice and female C57BL/6NCrlBR mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). These mice were bred to obtain B6C3F1 mice as previously described (19). All mice were housed in humidity- and temperature-controlled HEPA-filtered facilities accredited by the American Association of Laboratory Animal Care.

Materials and methods

Chemicals

PS-6 blend UG was kindly provided by the American Petroleum Institute (Washington, DC). TGF-β and M6P/IGF-II R antibodies were from Dr Randy Jirtle, Duke University (Durham, NC). Anti-5-bromo-2’-deoxyuridine (BrdU) was purchased from Becton–Dickson (Mountain View, CA). Aminoethyl carbazole and peroxidase-conjugated streptavidin were from Zymed Laboratories, Inc. (San Francisco, CA). Normal goat serum and biotin-conjugated antibody dilution buffer was from Biomedica Corp. (Foster City, CA). Ethynyl estradiol-supplemented diet (1 p.p.m.) was prepared by Dyets, Inc. (Bethlehem, PA). Isoflurane was obtained from Anaquest (Madison, WI). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Animals

All experiments were conducted under NIH guidelines for the care and use of laboratory animals (20) and approved by the Institutional Animal Care and Use Committee. Chemical Industry Institute of Toxicology (CUT). Male C3H/HeNCrlBR mice and female C57BL/6NCrlBR mice were obtained from Charles River Breeding Laboratories (Raleigh, NC). These mice were bred to obtain B6C3F1 mice as previously described (19). All mice were housed in humidity- and temperature-controlled HEPA-filtered facilities accredited by the American Association of Laboratory Animal Care.

Results

As previously reported, UG exhibited hepatic tumor-promoting activity in DEN-initiated female B6C3F1 mice (18). Exposure to UG vapors for 4 months significantly increased the size of foci 2.7-fold and the volume fraction of the liver occupied by hepatic foci 3.9-fold as compared to DEN-initiated controls (Ct). DEN/UG hepatic foci and decreased size of DEN/EE foci as compared with DEN-initiated control (DEN/Ct) mice (18). These data suggest that UG is a tumor promoter. In contrast, ethynyl estradiol (EE) decreased the size of DEN-initiated hepatic foci and volume fraction of the liver occupied by foci as compared with DEN/Ct mice suggesting that EE acts as a chemopreventive (18). Thus exogenous estrogen inhibits and UG promotes hepatic foci development in DEN-initiated mice.

Since UG increases and EE decreases the size of DEN-initiated foci in female mouse liver, focal and non-focal hepatocyte proliferation was assessed in DEN/Ct, DEN/UG and DEN/EE mice to determine if focal size is related to cell proliferation. Little is known about the mechanisms of foci development and growth in mouse liver. Given the importance of growth factors in hepatocyte growth, DEN-initiated hepatic foci were evaluated for alterations in the negative growth regulator TGF-β1. We report that non-focal hepatocyte proliferation was similar in non-initiated controls (Ct/Ct), DEN/Ct, DEN/UG and DEN/EE treatment groups indicating that cell proliferation in normal hepatocytes does not necessarily equate with hepatic foci development. However, a significant increase in cell proliferation was seen in DEN/UG foci and a significant decrease in DEN/EE foci relative to DEN/Ct foci. This pattern of activity correlates with the increased size of DEN/UG hepatic foci and decreased size of DEN/EE foci relative to that of DEN/Ct foci. Increased focal growth was accompanied by growth factor alterations that may afford focal hepatocytes a selective growth advantage relative to the non-focal hepatocytes.

Initiation-promotion experiment

Twelve-day-old female B6C3F1 mice were initiated with a single i.p. injection of N-nitrosodimethylamine (DEN) (5 mg DEN/kg body weight) or saline [initiation controls (Ct)] in accordance with the infant mouse initiation-promotion model system (18,20,21). Beginning at 5–7 weeks of age, groups of 12 DEN-initiated and saline-injected mice were exposed to 0 p.p.m. or 2038 p.p.m. PS-6 UG 6 h/day, 5 days/week for 16 weeks or 1 p.p.m. ethynyl estradiol in the diet as previously described (18). Approximately 20 h after the last exposure administration, mice were anesthetized with isoflurane and euthanized by exsanguination. Sections of the left, right medial, and right anterior lobes of the liver were fixed in 10% buffered formalin. Formalin was replaced 48 h later with 70% ethanol. Liver sections were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and examined microscopically for hepatic foci ≥ 10 cells in size. Foci were identified and classified according to histopathological phenotype using standard criteria (22).

Hepatocyte proliferation

At 3.5 days before euthanasia, at ~2.00 p.m., mice were implanted s.c. with 7-day osmotic pumps (Alzet model 2001, 1.06 μl/h, Alza Corporation, Palo Alto, CA) containing 16 mg/ml BrdU (CAS no. 59-14-3, > 99% pure) in phosphate-buffered saline. The pH of the solution was adjusted with NaOH to 7.2 ± 0.2, and the solution was filtered through a 0.45-μm filter before loading pumps.

Coded paraffin sections of liver were stained for BrdU by established immunohistochemical methods (23). BrdU incorporation in duodenum on the same tissue sections was employed as a positive control for immunohistochemical staining and for systemic delivery of BrdU to the mouse. Computer-generated random fields were scored for BrdU incorporation by light microscopy with the investigator blind as to animal treatment. Hepatic labeling index (LI) was determined by dividing the number of labeled hepatic nuclei as denoted by red pigment over nuclei by the total number of hepatocytes scored and multiplying by 100. For non-focal hepatic nuclei, LI of at least 2000 non-focal hepatocellular nuclei in the left lobe of the liver were counted and scored as labeled or unlabeled. Care was taken to avoid counting focal hepatocytes in calculating the non-focal LI. For focal hepatic LI, serial sections 5 μm in thickness were cut. One liver section was stained with H&E; the next serial section was immunohistochemically stained for BrdU. Foci were located on H&E-stained sections, and the corresponding focus was identified on BrdU-stained slides. All hepatocyte nuclei within the focus were counted and scored as labeled or unlabeled. Labeling index was calculated by dividing the number of labeled nuclei by the total number of hepatocellular nuclei counted and multiplying by 100. Because the hepatic LI data were not normally distributed, they were transformed by arcsin of the square root of LI before testing for statistical significance. Statistical significance of group non-focal LI was determined by the Student–Neuman–Keuls tests, while the significance of the focal LI was determined by Bonferroni t-tests after determining a df difference by ANOVA. A P-value of < 0.05 was considered significant. The mean ± standard deviation from each group of at least eight animals was calculated after tissue-slides were decoded.

TGF-β and M6P/IGF-II R immunohistochemistry

Formalin-fixed liver sample sections of 5 μm thickness on Probe-On-Plus slides were deparaffinized and subjected to an immunohistochemical procedure to visualize the localization of TGF-β1 or M6P/IGF-II R. TGF-β1 is a polyclonal rabbit antibody to residues 78–108 of human mature TGF-β1 (12,24). M6P/IGF-II R is a polyclonal rabbit antibody to purified rat M6P/ IGF-II R (24,25). For TGF-β1 and M6P/IGF-II R the following schedule was used: (1) a total exposure of 6 min to microwaves; (2) 0.3% hydrogen peroxide to block endogenous peroxidases (45 min); (3) goat serum (1:200) to block non-specific protein binding; (4) 10 ng/ml anti-TGF-β1 or anti-M6P/IGF-II R (24,25). For TGF-β1 and M6P/IGF-II R the following schedule was used: (1) a total exposure of 6 min to microwaves; (2) 0.3% hydrogen peroxide to block endogenous peroxidases (45 min); (3) goat serum (1:200) to block non-specific protein binding; (4) 10 μg/ml anti-TGF-β1 or anti-M6P/IGF-II R R at 4°C overnight; (5) goat anti-rabbit IgG diluted 1:200; (6) horseradish peroxidase diluted 1:200; and (7) and horseradish peroxidase substrate, aminoethyl carbazole, for the appropriate color development. Negative controls were performed by replacing the antibody with identical concentrations of non-immune rabbit IgG.

Results

As previously reported, UG exhibited hepatic tumor-promoting activity in DEN-initiated female B6C3F1 mice (18). Exposure to UG vapors for 4 months significantly increased the size of foci 2.7-fold and the volume fraction of the liver occupied by hepatic foci 3.9-fold as compared to DEN-initiated controls. In contrast, treatment with EE for 4 months significantly decreased the mean size of foci 3.9-fold and volume fraction of...
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Table I. Parameters of hepatic foci in DEN-initiated female mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Density (no/Liver)</th>
<th>Mean volume (mm³)</th>
<th>Volume fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN/Ct</td>
<td>353±210</td>
<td>69±63</td>
<td>1.1±0.7</td>
</tr>
<tr>
<td>DEN/UG</td>
<td>533±202</td>
<td>186±129*</td>
<td>4.3±2.5*</td>
</tr>
<tr>
<td>DEN/EE</td>
<td>228±184*</td>
<td>18±12*</td>
<td>0.3±0.3*</td>
</tr>
</tbody>
</table>

Female B6C3F1 mice were injected with DEN (5 mg/kg body weight, 7.1 ml/kg body weight) at exactly 12 days; beginning at 5–7 weeks of age, they were exposed to 0 p.p.m. or 2038 p.p.m. UG vapor for 6 h/day, 5 days/week or 1 p.p.m. EE in the diet for 16 weeks. The area occupied by hepatic foci was used to determine the volume of foci and volume fraction of liver occupied by foci using quantitative stereology. Values are means ± SD of 11–12 mice. Data are from Standeven et al., 1994.

• Significantly different from DEN/Ct (P < 0.05).

the liver occupied by foci 3.7-fold (Table I), indicating that EE inhibits hepatic foci growth.

Hepatic foci phenotype

Microscopic analysis of H&E-stained liver sections indicated that approximately 85% of DEN/Ct foci were basophilic, and over 95% of DEN/UG and DEN/EE foci were basophilic (Figure 1). Remaining foci were of the amphophilic phenotype. Hepatocytes within basophilic foci were generally more vacuolated and smaller than non-focal hepatocytes and exhibited an increased nuclear to cytoplasmic ratio. Non-focal hepatocytes in all treatment groups were similar in H&E-stained sections, although DEN/UG exhibited centrilobular hypertrophy and DEN/EE demonstrated a periporal pattern of intensely stained hepatocytes that was not evident in DEN/Ct mice.

Focal and non-focal hepatocyte proliferation

Hepatic LI was determined to investigate the relationship of foci size to focal and non-focal proliferation. Non-focal hepatic LI was similar in untreated controls (Ct/Ct), DEN/Ct, DEN/UG and DEN/EE mice. Mean non-focal LI was 0.38% ± 0.06% (n = 8) in Ct/Ct, 0.49% ± 0.30% (n = 9) in DEN/Ct, 0.37% ± 0.17% (n = 8) in DEN/UG and 0.83 ± 0.33% (n = 9) in DEN/EE (Figures 1 and 2). These LI are within the range of those previously reported for mouse liver under these experimental conditions (26).

Mean focal LI of each treatment group was calculated from the individual labeling indices of 27 DEN/Ct foci, 41 DEN/UG foci and 23 DEN/EE foci. No significant difference in focal LI was found in basophilic foci as compared with amphophilic foci. In accordance with what others have demonstrated (1), the LI of hepatocytes within foci was greatly increased relative to non-focal hepatocytes (Figure 2). The mean hepatic LI in DEN/UG foci was significantly increased, while the mean hepatic LI in DEN/EE foci was significantly decreased relative to DEN/Ct focal LI (Figure 2). These data support the increased size of DEN/UG foci and decreased size of DEN/EE foci relative to DEN/Ct foci (Table I). A wide range of focal LI was observed in all treatment groups (Figure 3). With DEN/Ct hepatic foci, the LI were dispersed among all LI classes. With DEN/UG foci, 40% of foci had a focal LI in the 31–35% LI class. In contrast, 60% of DEN/EE foci had a focal LI of less than 5% (Figure 3).

Immunohistochemistry

To determine if the alterations in focal growth were linked to the loss of negative growth controls, liver sections were stained immunohistochemically for mature TGF-β1. A slight but consistent increase in TGF-β1 immunoreactivity was seen in UG-exposed non-focal hepatocytes as compared to control non-focal hepatocytes. There was decreased immunoreactivity for mature TGF-β1 in over 95% of DEN/Ct and DEN/UG hepatic foci relative to non-focal hepatocytes (Figure 4). The most intensely positive staining cells were generally observed in non-focal hepatocytes adjacent to focal lesions. This produced a halo effect of TGF-β1 positive non-focal hepatocytes surrounding the foci (Figure 4). In contrast to DEN/Ct and DEN/UG groups where 95% (66/68) of foci examined were TGF-β1-negative, approximately 50% of DEN/EE foci were negative for TGF-β1. Remaining DEN/EE foci were either
positive for TGF-β1 or elicited a staining intensity similar to that of non-focal hepatocytes. There was a slight centrilobular pattern of TGF-β staining in DEN/UG and DEN/EE non-focal hepatocytes.

M6P/IGF-II R

M6P/IGF-II R co-localized with TGF-β in both non-focal and focal hepatocytes (Figure 4). A decrease of M6P/IGF-II R was found in DEN/Ct and DEN/UG foci (66/68) relative to non-focal hepatocytes. The one DEN/Ct focus and one DEN/UG focus that were positive for TGF-β were also positive for M6P/IGF-II R. Similarly, the 50% of DEN/EE liver foci that were negative for TGF-β were also negative for M6P/IGF-II R.

Discussion

In a two-stage initiation–promotion model system, we demonstrated differential effects of chemicals on hepatic foci growth relative to non-focal hepatocytes. Although non-focal hepatocyte proliferative responses were similar in all treatment groups, DEN/UG increased and DEN/EE decreased both the volume fraction of the liver occupied by foci and focal hepatic LI as compared to DEN/Ct. This indicates that the focal LI may be valuable in investigating mechanisms of non-genotoxic carcinogens, while non-focal LI are not necessarily predictive of effects on hepatic foci development.

Although there was a positive correlation between the size of individual foci and their LI (r² = 0.77), a wide range of LI (2–45%) was observed in hepatic foci (Figures 2 and 3). This suggests that individual hepatic lesions exhibit unique growth characteristics and complicates generalizations regarding lesion growth and cancer potential. Foci with a higher LI may have unique genetic alterations, i.e. activation of oncogenes, inactivation of tumor suppressor genes or may progress to hepatocellular carcinomas at a faster rate. However, experimental studies indicate that inhibition of apoptosis is an equally important parameter in the mechanism of some liver tumor promoters (27). In spite of the wide range of LI and overlap among the treatment groups, the LI of focal hepatocytes was greatly increased over non-focal hepatocytes in all cases, and a treatment-related effect could be observed.

There are numerous mechanisms by which chemicals can alter cell proliferative responses. One possible mechanism involves modulation of growth factor signals. We demonstrated that DEN/Ct and DEN/UG hepatic foci have reduced levels of the potent mito-inhibitor TGF-β1 relative to surrounding non-focal hepatocytes. This suggests that focal hepatocytes may not respond to normal mito-inhibitory signals and may escape the proliferative constraints of TGF-β1. A concomitant decrease in the levels of M6P/IGF-II R within these tumors supports this hypothesis (Figure 5). A decrease in focal levels of M6P/IGF-II R suggests that there is a decrease in the activation of latent TGF-β1 to its active form. Thus the focal hepatocytes are exposed to reduced levels of mito-inhibitory signals, potentially giving them a selective growth advantage relative to surrounding non-focal hepatocytes.

The increased size and LI of DEN/UG foci relative to DEN/Ct may be due to the slight increase in M6P/IGF-II R and TGF-β1 immunohistochemically seen in non-focal DEN/UG hepatocytes as compared to non-focal DEN/Ct hepatocytes. However, non-focal LI was not significantly decreased in DEN/UG liver as compared to DEN/Ct liver. The reason for this is unknown at this time but may indicate that a 3.5 day BrdU pump was not sufficient to detect small differences in normal hepatic LI. In this respect, UG may function in a manner similar to that of the rat tumor promoter PB.

Long-term exposure to PB dramatically decreased the growth potential of rat hepatocytes (25,28). A subset population of DEN/PB rat liver foci exhibited decreased levels of M6P/IGF-II R and TGF-β1 (13). PB and UG are both hepatic mitogens, that is, they produce a transient increase in hepatocyte proliferation in the absence of hepatotoxicity (19,29,30). Further work
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Fig. 4. (a) TGF-β immunohistochemical staining of DEN/UG focus. Note decreased TGF-β staining in the focal hepatocytes as compared to non-focal hepatocytes. (b) M6PR/IGF-II R immunohistochemical staining of DEN/UG focus. Note colocalization of M6PR/IGF-II R and TGF-β staining.

Fig. 5. Proposed model for chemically modified hepatocyte proliferation and negative growth factor regulation in hepatic foci. o represents hepatic LI. There was no significant difference in non-focal LI in any treatment groups. However, LI was significantly increased in DEN/UG and significantly decreased in DEN/EE foci relative to DEN/Ct foci. TGF-β1 immunoreactivity, as noted by the shading of liver sections in the model, was similar in Ct/Ct, DEN/Ct and DEN/EE non-focal hepatocytes. A slight, but consistent increase in TGF-β1 immunoreactivity was observed in DEN/UG non-focal hepatocytes. TGF-β1 was decreased in 95% of DEN/Ct and DEN/UG foci relative to non-focal hepatocytes, while TGF-β1 was not decreased in 50% of DEN/EE foci. M6PR/IGF-II R co-localized with TGF-β1.

will be required to determine if the decreases in M6PR/IGF-II R and TGF-β1 in foci are a phenomenon of other hepatic mitogens and tumor promoters. The DEN/UG hepatic foci that had decreased TGF-β1 and M6PR/IGF-II R foci in this study were induced in mouse liver, while in Jirtle’s study the decreased levels of TGF-β1 and M6PR/IGF-II R in DEN/PB liver foci were induced in rats. This suggests that the reduction of TGF-β1 and M6PR/IGF-II R in hepatic lesions may be a general mechanism of altered growth and tumor promotion across species and among different chemicals.

DEN/Ct and DEN/UG hepatic foci were M6PR/IGF-II R- and TGF-β1-negative, but only a small proportion of hepatic foci advance to form hepatocellular adenomas and carcinomas. Therefore, other genetic events or growth factor alterations allow a subpopulation of TGF-β1-negative foci to persist and develop into neoplasms. Dragan et al. have proposed TGF-α, a positive regulator of hepatocyte growth, as a marker in the rat liver of increased genomic instability and progression of non-malignant phenotypes to malignant ones (31). Markers of cancer progression would assist in the identification of lesions that may progress and could also be valuable in investigating the mechanisms of hepatocarcinogenesis.

There are undoubtedly other mechanisms by which hepatic foci develop and grow since 50% of DEN/EE foci, one DEN/Ct focus and one DEN/UG focus, were not negative for M6PR/IGF-II R and TGF-β1. Since the negative growth effects of TGF-β1 are dependent upon the interaction of TGF-β1 with TGF-β receptors, foci that do not have reduced levels of TGF-β1 may still be afforded a growth advantage by decreased TGF-β receptor levels. Decreased levels of TGF-β receptor have been found in some rat liver tumors (32,33).

The mechanism by which EE inhibits hepatic focal cell proliferation and hepatocarcinogenesis relative to DEN/Ct is unknown. Although the presence of M6PR/IGF-II R and TGF-β1 in 50% of DEN/EE foci may contribute to the inhibitory effects of EE on DEN-initiated foci growth, we were not able to establish a correlation between DEN/EE foci that demonstrated M6PR/IGF-II R and TGF-β1 staining and decreased hepatic LI or size. However, cell proliferation is not the only determinant of foci size. Programmed cell death or apoptosis is also an important factor, and TGF-β1 induces apoptosis in primary rat hepatocytes in vivo and in vitro (34,35). Thus in M6PR/IGF-II R and TGF-β1 negative foci, there may be insufficient mature TGF-β1 to induce apoptosis. Conversely, in the 50% of DEN/EE foci that were not negative...
for M6P/IGF-II R, TGF-β1 may be of sufficient concentration to induce or elevate apoptosis. In rats treated with DEN and then exposed to the mono-terpene chemopreventive agent, perillyl alcohol, M6P/IGF-II R levels as well as the apoptotic index were elevated (36). Future studies are needed to evaluate foci for apoptosis and its regulation for a more comprehensive picture of clonal growth.

In summary after subchronic exposure to a tumor promoter or chemopreventive agent, focal hepatocyte proliferation correlated with the increased size of DEN/UG foci and decreased size of DEN/EE foci relative to DEN/Ct foci. Non-focal hepatocyte proliferation was not a predictor of foci development. The differential effects of chemicals on M6P/IGF-II R and TGF-β1 in focal hepatocytes relative to non-focal hepatocytes may provide a selective growth advantage to hepatic foci.

References


