Identification of genes whose expression is altered during mitosuppression in livers of ethinyl estradiol-treated female rats

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SHORT COMMUNICATION

In this study, our goal was to identify genes whose expression in liver is altered in female F-344 rats during mitosuppression induced by 42 days of ethinyl estradiol (EE) treatment (Yager et al., Carcinogenesis, 15, 2117-2123, 1994). Northern analysis demonstrated that the mRNA levels for transforming growth factor-β1 (TGF-β1) and the mannose 6-phosphate/insulin-like growth factor II receptor were significantly increased by EE treatment. Ten cDNA clones representing mRNAs whose expression was increased two- to four-fold in the mitosuppressed livers were identified by differential display. Sequence analysis revealed that one was homologous to the S-24 ribosomal protein and another to mitochondrial ATPase subunit e. The remaining clones showed no homology to known genes in GenBank. However, the expression of clones 15, 16 and 17 was increased in HepG2 cells following treatment with doxorubicin suggesting their induction by oxidative DNA damage. These results suggest that two independent but interrelated signalling pathways, one mediated through transforming growth factor-β and the other through oxidative DNA damage, may contribute to hepatic mitosuppression caused by EE, perhaps through activation of cyclin-dependent kinase inhibitors.

A common effect shared by several hepatic promoters including phenobarbital (PB*), clofibrate and ethinyl estradiol (EE), is an initial, transient stimulation of hyperplastic growth (1-5). Continued exposure to these promoters leads to a subsequent inhibition in basal and/or induced liver growth in vivo (5-7). These studies demonstrate that mitosuppression is a common effect shared by several hepatic promoters and may be a contributing factor to their promoting activities.

While the mechanism(s) underlying hepatic mitosuppression is not clear, Jirtle and co-workers demonstrated increased levels of transforming growth factor-β1 (TGF-β1) protein in PB-induced mitosuppression (8,9). The mRNA and protein levels of the mannose 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor, which facilitates the binding and proteolytic activation of latent TGF-β1 (10), were also increased (8,9). These results provided support for the hypothesis that the reduced proliferative capacity of hepatocytes from PB-treated rats was due, at least in part, to increased levels of activated TGF-β1 (9). Furthermore, a small subset of preneoplastic hepatic lesions promoted by PB treatment contained reduced levels of both TGF-β1 and the M6P/IGF-II receptor compared to surrounding hepatocytes (9). Such changes could provide the preneoplastic cells with selective growth advantages.

Since both EE and PB cause mitosuppression, we carried out Northern blot analyses to determine whether the mRNA levels of TGF-β1 and the M6P/IGF-II receptor were increased during mitosuppression induced by EE (5 μg/day, 42 days). The livers from the same rats used in our previous study (7) were used as the source of RNA which was isolated from homogenates as described (11). Figure 1A shows Northern blots for the mRNAs of TGF-β1, the M6P/IGF-II receptor, c-myc, C/EBP-α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in control (lanes 1-4) and mitosuppressed (lanes 5-8) livers. Figure 1B shows the mean ± SD of the band intensities normalized for GAPDH and expressed as a percentage of control. These analyses revealed a significant 3.5-fold (P < 0.05) and 1.7-fold (P < 0.05) increase in mRNA levels of TGF-β1 and the M6P/IGF-II receptor, respectively, in EE-induced, mitosuppressed livers. In contrast, liver mRNA levels for c-myc and the transcription factor C/EBP-α were similar (P > 0.05) in control and EE-treated rats. This observation is somewhat analogous to the findings of increased TGF-β1 and M6P/IGF-II receptor protein levels in the livers of PB-treated rats made by Jirtle et al. (8,9). However, since our study was limited to the use of Northern blot analysis, it is not possible to compare directly the extent of increase in expression of the genes caused by PB and EE until we investigate protein levels. On the other hand, our finding of increased mRNA levels for these genes is similar to that of Rumsby et al. (12) in rats treated with several peroxisome proliferators. After 7 days they observed 1.5- to 1.8-fold increases in TGF-β1 mRNA and 1.9- to 2.1-fold increases in M6P/IGF-II receptor mRNA. While not determined in their study, this increase likely coincided with cessation of the transient growth that accompanies treatment with these agents.

The increases in mRNA levels of these genes suggests that increased activated TGF-β1 protein levels will be observed. If so, then a TGF-β1-mediated signal transduction pathway leading to activation of specific cyclin-dependent kinase inhibitors (CDKIs) such as p27, p15INK4B and p16INK4A which are induced by TGF-β (13-17) may be involved in mitosuppression. Thus, it should be informative to determine whether these CDKIs or others unique to liver are activated during mitosuppression induced by EE and other liver tumor promoters which have these effects.

We performed differential displays (18-20) to identify genes whose expression was altered in EE-induced, mitosuppressed livers. Forty candidate polymerase chain reaction (PCR) cDNA fragments, which were seen to be differentially expressed in at least three individual control and EE-treated samples, were
Fig. 1. Northern blot analysis of the M6P/IGF-II receptor, TGF-β1, c-myc (from Oncogene Science, Cambridge, MA), C/EBP-α and GAPDH mRNA levels in livers of 42 day control and EE-treated (5 μg/day) female rats used in our previous study (7). (A) Representative autoradiogram from the Northern analysis on one rat/group. Northern blots were performed using standard methods. RNA from four control and four EE-treated livers were denatured at 65°C for 15 min and run on a 1% agarose gel containing 2% formaldehyde. The resolved RNAs were transferred to nitrocellulose membranes and UV-crosslinked. The probes were 32P-labelled using random primers (Boehringer Mannheim, Indianapolis, IN). (B) The figure shows expression as a percent of control of the mRNAs for these genes relative to GAPDH. There were four rats/group and each bar represents the mean±SD. *, significantly greater than control, P < 0.05 (Student’s t-test).

Table I. Properties of the 10 clones identified by differential display and confirmed by Northern analysis to show increased expression in the mitosuppressed livers of EE-treated rats

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Size of homologous mRNA (kb)</th>
<th>Fold induction caused by EE treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.6</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>2.7</td>
<td>1.7</td>
</tr>
<tr>
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<td>1.7</td>
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<tr>
<td>5</td>
<td>1.5</td>
<td>2.8</td>
</tr>
<tr>
<td>8</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>9</td>
<td>1.3</td>
<td>2.1</td>
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<tr>
<td>11</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>15</td>
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<tr>
<td>16</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td>17</td>
<td>0.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

isolated, reamplified and cloned. Northern blots (not shown) identified 10 clones whose expression, relative to GAPDH, was increased 2- to 4-fold over controls in EE-induced mitosuppressed livers (Figure 2).

Fig. 2. Expression levels, relative to controls, of the mRNAs for the 10 cDNA clones identified by differential display as showing increased expression in livers of 42 day EE-treated female rats. The differential displays were done according to the technique of Liang et al. (18,19) using kits from GenHunter, but with some modifications as described by Baurer et al. (20). The amplified cDNAs were cloned directly into a plasmid vector, Bluescript II SK+, using a TA cloning kit (Invitrogen, San Diego, CA) or into the pcr-TRAP cloning vector (version 2.0) using a cloning kit from GenHunter. The expression of each clone was corrected for expression of GAPDH. There were four rats/group and each bar represents the mean±SD. *, significantly greater than control, P < 0.05 (Student’s t-test).
that EE-induced mitosuppression might be a consequence of known and/or novel CDKIs will be explored.

that hepatic mitosuppression induced by EE is mediated by signals stimulated by oxidative DNA damage. The possibility of oxidative DNA damage. Taken together, these results suggest that oxidative DNA damage results from the metabolism of EE to catechols which can cause increased levels of oxidative DNA damage in liver. However, since these cDNAs represent 3' ends of the mRNAs, they were shown to include activation of p53 which in turn also homologous to the S-24 ribosomal protein. Its expression was increased at two times, 1 and 5 days after partial hepatectomy, which represent periods of greatly elevated growth and growth cessation, respectively (22). However, the reason for the increased expression of S-24 during liver growth and its cessation and suppression is not clear and deserves investigation.

The sequences of the remaining eight cDNA clones have no significant homology to the known genes in GenBank. However, since these cDNAs represent 3' ends of the mRNAs, it is possible that at least some may be untranslated sequences not reported in the data base. Screening of the cDNA library prepared from mRNA isolated from the mitosuppressed livers of EE-treated rats should allow the isolation and characterization of these clones.

We (Seacat, O'Gorman, Young, Groopman and Yager, unpublished observations) and others (23) have found that EE can cause increased levels of oxidative DNA damage in liver. In light of this we investigated the effects of doxorubicin, which causes oxidative DNA damage (23), on the expression of three of the clones in HepG2 cells. Table II shows the results from a representative experiment where the cells were treated with the indicated concentrations of doxorubicin for 24 h. The mRNA levels for clones 15, 16 and 17 were increased significantly (P < 0.05) 2- to 5-fold by doxorubicin treatment suggesting the possibility that they may represent oxidative DNA damage response genes. It is possible that such damage results from the metabolism of EE to catechols which then undergo redox cycling leading to oxidative DNA damage (25). Responses to oxidative DNA damage in other cell types have been shown to include activation of p53 which in turn induces the expression of the CDK1 p21/WAF-1/Cip1 (26) which causes cell cycle inhibition. Our results suggest that a similar response may occur upon prolonged EE treatment.

In summary, we found increased expression of TGF-β1 and the M6P/IGF-II receptor as well as 10 clones identified by differential display during hepatic mitosuppression induced by EE. Three of the unidentified clones were also seen to be induced in HepG2 cells treated with doxorubicin which causes oxidative DNA damage. Taken together, these results suggest that EE-induced mitosuppression might be a consequence of signalling through the TGF-β pathway as well as through signals stimulated by oxidative DNA damage. The possibility that hepatic mitosuppression induced by EE is mediated by known and/or novel CDKIs will be explored.

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**References**


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