Prolonged Ornithine Decarboxylase Induction in Regenerating Carcinogen-treated Liver

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ABSTRACT

A cascade of events leading to hypertrophy has been proposed and implicated in growth regulation in a variety of normal and neoplastic cells and tissues. There is a tightly coupled temporal sequence: (a) cyclic adenosine 3':5'-monophosphate-dependent protein kinase (cAPK) activation; (b) ornithine decarboxylase (ODC) induction; and (c) the accumulation of the organic cation, spermidine, resulting in an increased spermidine/spermine ratio characteristic of both normal and neoplastic growth. The specific activation of type I cAPK has been implicated to ODC induction, and the amounts of type I and type II cAPK alter as a function of growth and transformation.

Therefore, we wished to study the alterations in these biochemical parameters as well as that of a putative marker of neoplastic growth. The specific activation of type I cAPK has been implicated to ODC induction, and the amounts of type I and type II cAPK alter as a function of growth and transformation.

INTRODUCTION

The analysis of several recently developed models which result in rapid development of liver carcinomas has substantiated the theory that chemical hepatocarcinogenesis is a sequential process with definable stages (13, 24, 47, 50–53, 64). Prior to the development of these models, it was not possible to experimentally separate the specific stages of liver carcinogenesis for biochemical examination; therefore, the majority of the studies of hepatocarcinogenesis were limited to studies of the resulting hepatomas. Using the model of Farber et al. (24, 64, 65) of discrete initiation and promotion events to generate the rapid formation of liver tumor foci, it is now possible to monitor biochemical events during the preneoplastic stages of tumor progression.

Analyses of the multistep development of liver carcinomas in the Farber model (24, 64, 65) have suggested that initiation and promotion stages occur in liver carcinogenesis that are analogous to the stages in the mouse epidermal carcinogenesis model. Substantial data have been accumulated in the mouse skin system to suggest that the induction of ODC3 is a specific and essential event in the promotion of skin tumors (1). The induction of ODC, the rate-limiting enzyme in the polyamine-biosynthetic pathway, is an early-marked event in all growth systems studied to date (4, 37, 58). Polyamines accumulate during cell growth processes and are now thought to function as the source of organic cations for the cells since their synthesis is sequentially regulated in parallel with nucleic acid synthesis during hypertrophy and hyperplasia (15, 37, 55, 58).

Increased ODC activity and accumulation of polyamines have been demonstrated during the development of experimental animal tumors and human tumors (37, 58). The relationship of ODC expression to hepatocarcinogenesis has been studied only in models which require months to develop, and ODC activity was monitored only at sporadic weekly or monthly intervals. A recent report demonstrated that rats receiving a complete liver carcinogen (4-dimethylaminoazobenzene) continuously in their diet had substantially elevated hepatic ODC activity 4 to 5 months prior to the appearance of visible hepato-cellular carcinomas (62). In another study, DEN was administered in the drinking water for 4 weeks, followed by continuous dietary feeding of phenobarbital (25). ODC activity and polyamine levels were measured in the liver at 1, 11, and 20 weeks after initiation of phenobarbital diets, and no alterations were observed in either ODC activity or polyamine concentrations.

cAPK's have been shown to be present universally in eukaryotic tissues and have been implicated in the regulation of all CAMP-mediated events (40). The rapid activation of cAPK followed by the transcriptional induction of ODC is observed in response to a variety of trophic stimuli (58). This temporal sequence of events has been observed following such diverse trophic stimuli as mitogens (7, 38), drugs that induce enzymes (3, 17, 45), trophic hormones (5, 6, 9, 54, 57), analogs of CAMP and/or phosphodiesterase inhibitors (8, 9, 11), and compensatory growth systems such as regenerating rat liver and the hypertrophic adrenal gland (6, 10). A recent study (39) reports the ability to induce ODC in mouse adrenal tumor cell line Y1 by the addition of cAMP, CAMP analogs, or adrenocorticotropic hormone. Several mutants of the cell line exhibited impaired ability to activate cAPK in response to adrenocorticotropic hormone and attenuation of ODC induction.

Alterations in cAPK isozyme activities and/or CAMP-binding proteins have been observed in numerous transformed cells.

3 The abbreviations used are: ODC, ornithine decarboxylase; DEN, diethylnitrosamine; cAMP, cyclic adenosine 3':5'-monophosphate; 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy; GGT, y-glutamyltranspeptidase.

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lines (28–30, 32, 36, 42, 43, 49), in rapidly growing hepatomas (19), in rat adrenocortical carcinomas (63), in transplantable mouse tumors (33), during cell cycle progression of Chinese hamster ovary cells (18), during embryonic development (31), in X-radiation-induced adenocarcinomas of the rat small bowel (66), in human neuroblastomas (35), and in mammary (23) and renal (26) carcinomas. Several studies have examined cAPK activity during the chemical induction of carcinogenesis. Lung tumors induced in mice by a single i.p. injection of urethan had significantly higher basal and cAMP-stimulated protein kinase activities than did the uninvolved normal lung tissues (44). Mice fed a diet of 2-AAF (500 ppm) for 18 months exhibited significantly increased amounts of the cAPK isozymes (59). Ethionine-fed rats had increased hepatic cAPK activity by 8 weeks of treatment; after 38 weeks of ethionine ingestion, cAPK was activated in the resulting hepatomas (20).

Recently, we have reported (48) that a single carcinogenic dose of DEN rapidly activated hepatic cAPK and induced hepatic ODC, and both remained significantly elevated for at least 7 days. A single noncarcinogenic dose of DEN did not alter either cAPK activity or ODC activity, while serial noncarcinogenic doses resulted in cumulative increases in cAPK and ODC. These results suggest that prolonged elevation in hepatic ODC activity may be specific for hepatocarcinogenesis and hepatoma formation. We report in this paper a consistent sequence of biochemical events. i.e., cAPK activation, ODC induction, spermidine accumulation, and foci formation, in response to PH and carcinogen treatment which produced preneoplastic foci as assessed by the marked elevation of GGT activity in the nodules (12). This trophic response sequence occurs in normal and altered growth states such as preneoplastic processes. However, the appearance of foci is concomitant with elevations of all major measured biochemical events that are prolonged by weeks.

**MATERIALS AND METHODS**

\[ \text{[^yr-32P]ATP (2.0 to 3.5 Ci/mmol) and L-[1-14C]ornithine (60 mCi/mol) were obtained from Amersham/Searle Corporation, Arlington Heights, Ill. Unlabeled ATP and L-ornithine, cAMP, histone H2B, L-γ-glutamyl-p-nitroanilide, glycylglycine (free base), p-nitroanilide, and Triton X-100 were purchased from Sigma Chemical Company, St. Louis, Mo. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Company, Milwaukie, Wis. DEN was from Eastman Kodak Company, Rochester, N. Y. DEAE-cellulose (DE52) and No. 3MM filter paper were from Whatman, Inc., Clifton, N. J. The silica gel-imregnated glass fiber sheets (ITLC) were from Gelman Instrument Company, Ann Arbor, Mich.} \]

**Animals and Carcinogenic Treatment.** The carcinogenic treatment was similar to that of Solt and Farber (64). Male Sprague-Dawley rats (130 to 150 g) from Charles River Breeding Laboratories, Inc., were maintained on a standard basal high-casein diet (Bio-Serv, Frenchtown, N. J.) and water ad libitum for 7 days prior to the initiation of experiments. The animals were housed individually in cages in a room kept at constant temperature with a 7 a.m. to 7 p.m. photoperiod.

Putative premalignant liver foci were produced by the following procedure. Two weeks after a single dose of DEN (200 mg/Kg i.p.), rats were placed on the basal diet containing 0.02% 2-AAF (Bio-Serv) for 14 days. One week after the 2-AAF diet was initiated, a standard two-thirds PH was performed. After 14 days of a diet containing 2-AAF, the animals were returned to the standard basal diet. Multiple foci approximately 1 mm in diameter had developed at this time (28 days after the experiment was initiated with DEN).

All animals were sacrificed by cervical dislocation between 9 a.m. and 12 noon, and tissue samples from whole liver, preneoplastic foci, or nodules were rapidly removed and chilled. Separate samples were frozen in liquid nitrogen and stored at −80° for determination of GGT activity, polyamine analysis, and DEAE-cellulose chromatography to assess cAPK profiles. Determination of cAPK and ODC activity were performed on fresh samples from each individual animal. All enzyme preparations were carried out at 4°. Prior to Day 28 (7 days post-PH), tissue samples were obtained only from whole liver since foci or nodules were not available in sufficient quantity for biochemical analysis. After Day 28, only foci or nodules were analyzed. GGT was used as a marker of preneoplastic hepatocytes as suggested by Cameron et al. (12).

**Enzyme Assay for ODC Activity.** Liver samples were homogenized using a Brinkman Polytron PT-10 homogenizer (full speed, 2 bursts for 5 sec each) in 5 volumes of chilled 50 mM sodium-potassium phosphate buffer containing 40 μM pyridoxal phosphate, 1 mM diithiothreitol, and 100 μM phenylmethylsulfonyl fluoride, pH 7.2. Homogenates were centrifuged for 10 min at 48,000 × g. Fifty to 100 μl of the supernatant fraction were assayed for ODC activity in a final volume of 200 μl in 15-ml tapered centrifuge tubes fitted with rubber stoppers and center wells (Kontes Company, Vineland, N. J.). The buffer used in the assay was the same as the homogenization buffer and included 0.5 mM ornithine. ODC activity was expressed as the amount of 14CO2 released from 0.5 μCi of [L-14C]ornithine at 37° during a 30-min assay (81). The assay was stopped with 0.5 ml of 1 M citric acid, and the 14CO2 released was collected on Whatman No. 3MM filter papers prespotted with 20 μl of NCS (Amersham/Searle). The filter papers were counted in toluene/Omnifluor scintillant. All enzyme activities were corrected for blanks which contained 4-bromo-3-hydroxybenzoxaline dihydrogen phosphate in the reaction mixture. The enzyme activity was linear with respect to incubation time and enzyme concentration.

**DEAE-Cellulose Chromatography of cAPK Isozymes.** Tissues were rapidly excised, frozen by immersion in liquid nitrogen, and stored at −80°. Frozen tissue was homogenized with a Brinkman Polytron PT-10 homogenizer (full speed, 2 bursts for 5 sec each) at 4° in 5 volumes of chilled 5 mM Tris-HCl buffer containing 2 mM EDTA and 3 mM sodium fluoride, pH 7.5. Homogenates were centrifuged for 5 min at 20,000 × g, and the resulting supernatant was diluted with the same buffer to 1/10 (v/v). Five hundred μl of this solution were applied to a DEAE-cellulose column (0.7 x 14 cm) previously equilibrated with the same buffer. Protein concentration was assessed by the method of Bradford (2). Columns were washed with 14 ml buffer, and the cAPK was eluted with a linear gradient of 0 to 0.35 M buffered NaCl (total volume, 30 ml). Fractions of 1 ml were collected, and 50-μl aliquots were assayed for cAPK activity. Tissue preparation in a low-ionic-strength buffer and the slow application of a relatively small sample size on a proportionately large column under low-salt conditions ensured complete reassociation of the regulatory and catalytic subunits. Type I and type II kinase fractions were shown to represent the
holoenzymes by their dependence on exogenous cAMP for maximal activity (e.g., 0.2 versus 1.0 nmol 32P per min per ml, minus and plus cAMP, respectively). Under these low-salt conditions, no free catalytic subunit was detected. Free catalytic subunit elutes at 0.015 to 0.035 M NaCl, whereas type I elutes at 0.060 to 0.012 M NaCl; therefore, there is no overlap in their elution profiles. Recovery rate was consistently 75 to 90% of the supernatant kinase activity applied. Salt inhibition was 39% for type I and 52% for type II. Specific activities of the isozymes were calculated for the total kinase activity (pmol/min) eluted in the respective peak (corrected for salt inhibition) on the basis of the amount of supernatant protein applied to the column.

**Determination of cAPK Activity Ratios.** The conditions for tissue preparation and assay of cAPK were optimized to best preserve the hormonal effects on the enzymes. The procedure was similar to that of Cherrington et al. (14) and Byus et al. (5).

Liver samples were rapidly excised and immediately homogenized at 4°C using a Brinkman Polytron PT-10 homogenizer (full speed, 2 bursts for 5 sec each) in 5 volumes of 10 mM potassium phosphate buffer/1 mM EDTA/5 mM sodium fluoride/5 mM 2-mercaptoethanol/0.5 mM 3-isobutyl-1-methylxanthine/100 mM sodium chloride, pH 6.8. Whole homogenates were centrifuged for 5 min at 10,000 x g, and the resulting supernatant was diluted with the same buffer to 1/133 (v/v). Twenty µl of this solution were assayed for cAPK activity in the presence and absence of saturating amounts of cAMP (10 µM) in a total volume of 75 µl. The final incubation mixture contained 20 mM sodium-potassium phosphate buffer (pH 6.8), 0.5 mM 3-isobutyl-1-methylxanthine, 10 mM magnesium chloride, 50 µg H2B histone, 100 µM ATP, and 1 µCi [γ-32P]ATP, plus or minus 10 µM cAMP. The assay was initiated by the addition of ATP and incubated for 3 min at 30°C. cAPK activity was determined, essentially as described by Huang and Robinson (34), from the amount of 32P incorporated into acid-precipitable material. Utilizing the above conditions, 32P incorporation was linear with respect to both time and kinase concentration. The above procedures preserved the hepatic cAPK activity ratio (−cAMP/4cAMP) present in the tissue.

**Polyamine Determinations.** Liver samples, which had been frozen in liquid nitrogen and stored at −80°C, were homogenized using a Brinkman Polytron PT-10 homogenizer (full speed, 2 bursts for 5 sec each) in 8 volumes of 10% trichloroacetic acid. Homogenates were centrifuged at 40,000 x g for 20 min. The supernatant fraction was diluted 1/5 in 0.1 N HCl, and a 50-µl aliquot of the resulting solution was analyzed for putrescine, spermidine, and spermine concentrations utilizing a Durrum D-500 automatic amino acid analyzer (22).

**Biochemical Assay for GGT Activity.** Tissue preparation and assay of GGT activity was performed according to the method of DeYoung et al. (21). Liver samples which had been frozen in liquid nitrogen and stored at −80°C were homogenized using a Brinkman Polytron PT-10 homogenizer (half-speed, 2 bursts for 5 sec each) in 24 volumes of chilled 5 mM potassium phosphate/10% glycerol/1% Triton X-100, pH 7.0. Homogenates were centrifuged at 10,000 x g for 30 min at 0–4°C. Three hundred µl of the supernatant fraction were assayed for GGT activity in 1.0 ml of assay solution. The assay solution contained 100 mM N,N′-bis(hydroxymethyl)glycine buffer (pH 8.8)/11 mM MgCl2·6H2O/4.4 mM L-γ-glutamyl-p-nitroanilide/40 mM glycylglycine free base. The assay was initiated by the addition of the assay solution and incubated for 30 min at 25°C. The assay was stopped by the addition of 2 ml of 1.5 M acetic acid. The mixture was immediately vortexed and centrifuged at 10,000 x g for 20 min at 0–4°C, and the absorbance was measured at 405 nm. GGT activity was determined from standard curves for p-nitroaniline prepared under the same experimental conditions as those for the tissue samples. Blanks were prepared by boiling the supernatant fraction for 10 min and assayed as above. The enzyme activity was linear with respect to incubation time and enzyme concentration.

Since most of the GGT activity present in tissue is thought to be bound to cell membranes (46), it was necessary to demonstrate that Triton X-100 solubilization produced enzyme activity comparable to that found in membrane fractions not solubilized with detergent. The enzyme kinetics of liver GGT was determined in both membrane preparations (105,000 x g microsomal pellet and 10,000 x g supernatant solution from samples homogenized with Triton X-100). The Triton X-100-solubilized samples were shown to have the same kinetics as did the microsomal pellet preparations (data not shown). Therefore, Triton X-100-solubilized enzyme preparations were routinely analyzed for the assay of liver GGT.

**RESULTS**

**Alterations in cAPK Activity in Regenerating Carcinogen-treated Liver.** The activity ratio (−cAMP/+cAMP) of cAPK was significantly elevated within 24 hr of a single injection of DEN (Table 1). It remained significantly elevated at 2, 3, and 7 days after DEN and then returned toward control value 14 days after the administration of a single dose of DEN. While dietary 2-AAF alone for 14 days increased the activity ratio, this was not due to an increase in −cAMP activity but to an apparent decrease in the total pool of cAPK activity as assessed in the presence of saturating cAMP. DEN plus dietary 2-AAF resulted in an increased activity ratio with no apparent decrease in the amount of kinase activity present. When rats were fed 2-AAF in the diet and cAPK activity was measured at 21 days, again there was a significant increase in the −cAMP activity and the activity ratio, with no detectable alterations in the total amount of kinase activity. After PH in carcinogen-treated liver, there was a rapid and prolonged increase (for 4 days) in the activation of cAPK with a detectable decrease in the amount of activity present 7 days post-PH. After PH alone, it was increased only at 24 hr.

**Alterations in the Amount of Type I and Type II cAPK in Regenerating Carcinogen-treated Liver.** DEAE-cellulose chromatography was utilized to determine the total amount of hepatic type I and type II cAPK activity. Table 2 indicates that, within 24 hr of PH alone, there was a marked and significant decrease in the amount of type I kinase present with no alteration in the amount of type II detectable. Thereafter, the amount of type I appeared to recover and was not significantly different at 4 and 7 days post-PH from that present in control liver. No alterations in the type I-type II isozyme patterns were
detected in any of the regimens except those that involved PH. Dietary 2-AAF plus PH resulted in a significantly decreased ratio of type I and type II, apparently a result of increased type II holoenzyme. Within 1 day of PH in the carcinogen-treated liver, the total type I kinase activity had decreased to approximately 30% of the control value and remained significantly decreased through 7 days post-PH.

Alterations in ODC Activity in Regenerating Carcinogen-treated Liver. A single dose of DEN (200 mg/kg i.p.) resulted in a greater than 13-fold increase in ODC activity within 24 hr (Table 3). The activity remained significantly elevated at 2, 3, and 7 days DEN and returned toward controls 14 days after the administration of DEN alone. Dietary 2-AAF alone for 14 days resulted in no increased ODC activity, nor did DEN plus 2-AAF result in any increase when measured at Days 17 and 20 after the initial DEN administration. When 2-AAF was fed in the diet for 14 days with a PH performed at 7 days after the initiation of the 2-AAF diet, and ODC activity was measured at 21 days, there was a greater than 16-fold elevation of ODC activity. Within 1 day after PH (22 days post-DEN), ODC was 3-fold above the level detectable in rats that had received DEN plus 2-AAF and that were assayed 20 days post-DEN. ODC activity increased to 7-fold of controls 4 days post-PH and remained significantly elevated through 14 days, thereafter returning toward control values at 147 or 168 days after the initial DEN injection. After PH alone, ODC activity was elevated only at 24 hr.

Alterations in Polyamine Concentrations in Regenerating Carcinogen-treated Liver. A single dose of DEN resulted in a near doubling of liver spermidine within 7 days and an increase in the spermidine/spermine ratio from 1.0 to 3.0 (Table 4). At 14 days, spermidine concentration returned toward a control level, and the ratio dropped toward 1.0. No significant alterations in the polyamine pools were detectable with dietary 2-AAF alone or DEN plus 2-AAF when the liver was assayed at 17 and 20 days after the initial DEN administration. However, when 2-AAF was fed in the diet for 14 days and PH was performed 7 days after the initiation of the 2-AAF diet, spermidine concentration was greater than 2-fold as measured 21 days after the initial DEN administration, and the spermidine/spermine ratio was 3.6. Within 1 day after PH in carcinogen-treated liver (22 days post-DEN), spermine concentration was significantly decreased with a spermidine/spermine ratio of 2.0. This can be compared to the DEN plus 2-AAF 20-day data in which the ratio was 1.1. Spermidine concentration was significantly elevated at 4, 7, and 14 days post-PH in those

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Table 1

<table>
<thead>
<tr>
<th>cAPK activity (pmol/min/mg protein)</th>
<th>-cAMP</th>
<th>+cAMP</th>
<th>-cAMP/+cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control liver</strong></td>
<td>227 ± 13</td>
<td>838 ± 27</td>
<td>0.27 (25)</td>
</tr>
<tr>
<td><strong>DEN alone</strong></td>
<td></td>
<td></td>
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<tr>
<td>24 hr</td>
<td>443 ± 56</td>
<td>937 ± 80</td>
<td>0.47 (5)</td>
</tr>
<tr>
<td>48 hr</td>
<td>292 ± 31</td>
<td>975 ± 72</td>
<td>0.30 (5)</td>
</tr>
<tr>
<td>7 days</td>
<td>227 ± 26</td>
<td>910 ± 84</td>
<td>0.25 (5)</td>
</tr>
<tr>
<td><strong>DEN plus 2-AAF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>540 ± 49</td>
<td>972 ± 77</td>
<td>0.56 (4)</td>
</tr>
<tr>
<td>2 days</td>
<td>709 ± 46</td>
<td>1062 ± 116</td>
<td>0.67 (4)</td>
</tr>
<tr>
<td>3 days</td>
<td>923 ± 103</td>
<td>1335 ± 36</td>
<td>0.69 (4)</td>
</tr>
<tr>
<td>7 days</td>
<td>297 ± 13</td>
<td>709 ± 41</td>
<td>0.42 (6)</td>
</tr>
<tr>
<td>14 days</td>
<td>290 ± 13</td>
<td>774 ± 35</td>
<td>0.36 (6)</td>
</tr>
<tr>
<td><strong>Dietary 2-AAF alone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td>227 ± 24</td>
<td>578 ± 34</td>
<td>0.40 (4)</td>
</tr>
<tr>
<td><strong>Dietary 2-AAF plus PH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 days</td>
<td>382 ± 74</td>
<td>879 ± 34</td>
<td>0.43 (3)</td>
</tr>
<tr>
<td><strong>Time after PH in carcinogen-treated liver</strong></td>
<td>401 ± 36</td>
<td>836 ± 51</td>
<td>0.48 (4)</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>459 ± 27</td>
<td>961 ± 78</td>
<td>0.46 (3)</td>
</tr>
<tr>
<td>7 days</td>
<td>309 ± 35</td>
<td>670 ± 23</td>
<td>0.46 (4)</td>
</tr>
<tr>
<td>14 days</td>
<td>331 ± 38</td>
<td>831 ± 23</td>
<td>0.40 (3)</td>
</tr>
<tr>
<td>21 days</td>
<td>255 ± 26</td>
<td>649 ± 21</td>
<td>0.39 (3)</td>
</tr>
<tr>
<td>147 days</td>
<td>260 ± 14</td>
<td>770 ± 70</td>
<td>0.33 (3)</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of triplicate determinations on each rat liver.

** Numbers in parentheses, number of animals in each group.

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Table 2

<table>
<thead>
<tr>
<th>cAPK activity (pmol/min/mg protein)</th>
<th>Type I</th>
<th>Type II</th>
<th>I/II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control liver</strong></td>
<td>623 ± 41</td>
<td>723 ± 41</td>
<td>0.866 (20)</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>390 ± 16</td>
<td>725 ± 65</td>
<td>0.551 (4)</td>
</tr>
<tr>
<td>4 days</td>
<td>496 ± 61</td>
<td>800 ± 20</td>
<td>0.623 (4)</td>
</tr>
<tr>
<td>7 days</td>
<td>553 ± 57</td>
<td>928 ± 36</td>
<td>0.603 (4)</td>
</tr>
<tr>
<td><strong>Dietary 2-AAF alone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td>679 ± 33</td>
<td>741 ± 23</td>
<td>0.922 (4)</td>
</tr>
<tr>
<td><strong>Dietary 2-AAF plus PH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 days</td>
<td>499 ± 30</td>
<td>975 ± 128</td>
<td>0.536 (3)</td>
</tr>
<tr>
<td><strong>Time after PH in carcinogen-treated liver</strong></td>
<td>213 ± 34</td>
<td>586 ± 31</td>
<td>0.369 (4)</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>233 ± 16</td>
<td>770 ± 59</td>
<td>0.309 (3)</td>
</tr>
<tr>
<td>7 days</td>
<td>175 ± 37</td>
<td>626 ± 34</td>
<td>0.274 (3)</td>
</tr>
<tr>
<td>14 days</td>
<td>502 ± 151</td>
<td>990 ± 97</td>
<td>0.517 (3)</td>
</tr>
<tr>
<td>21 days</td>
<td>397 ± 30</td>
<td>828 ± 38</td>
<td>0.485 (3)</td>
</tr>
<tr>
<td>147 days</td>
<td>500 ± 73</td>
<td>793 ± 29</td>
<td>0.628 (3)</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of triplicate determinations on each rat liver.

** Numbers in parentheses, number of animals in each group.

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Footnote:

* Data differ from those of controls (p < 0.05).

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rats that received the complete carcinogen diet. At 147 days post-PH (168 days after the initial DEN administration), the spermidine/spermine ratio was significantly lower than that for controls, having dropped to 0.6. This was due to a decrease in the spermidine concentration and an increase in the spermine concentration, although neither decrease was significant at the 0.05 level. After PH alone, putrescine and spermidine were significantly elevated through 7 days, whereas spermine was decreased significantly at 24 and 48 hr. The spermidine/spermine ratio at 48 hr was 5.4, higher than that observed in any other regimen. Alterations in GGT Activity in Rat Liver during Hepatocarcinogenesis. Rats which received the complete PH-carcinogen treatment had small grayish-white nodules detectable 25 days after the initial DEN administration (4 days post-PH). GGT activity was significantly elevated to 4-fold of controls within 1 day post-PH and was elevated 18-fold 7 days post-PH in carcinogen-treated liver as assayed in the dissected nodules which were up to 1 mm in diameter (Table 5). Within 2 weeks of PH, nodular GGT activity was 40-fold of control, a level of activity maintained for the duration of the experiment (19 weeks). Dietary 2-AAF alone for 14 days did not significantly increase GGT activity, whereas dietary 2-AAF plus PH resulted in a significant elevation of GGT to approximately 9-fold of control as assayed in the small foci detectable at that time. Note that PH alone had no effect on GGT activity.

**DISCUSSION**

The generality of the involvement of cAMP in the transcriptional induction of ODC in a variety of growth systems has been recently reviewed (60). Further, proposed major steps in a trophic response (57) that include cAMP mediation of ODC induction have been elucidated in studies of cell cycle progression (18), thyroid hypertrophy (16), cardiac hypertrophy (3), lymphocytes stimulated to mitogenesis (7, 38), and liver regeneration in response to PH (6). The inhibition of any one of these events inhibits cell growth and the expression of the subsequent events.

Chart 1 summarizes the prolonged activation of cAMP, and the largest continual elevation of ODC reported to date related to both initiation and promotion of the rapid development of hepatocarcinomas in rats according to the model of Farber et al. (24, 64, 65). This is coupled to the generation of sustained spermidine levels and an increased spermidine/spermine ratio characteristic of normal and neoplastic growth (37, 58). Foci

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### Table 3

<table>
<thead>
<tr>
<th>ODC activity (pmol/30 min/mg protein)</th>
<th>Time after PH in carcinogen-treated liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td></td>
</tr>
<tr>
<td>17 ± 2 (22)</td>
<td></td>
</tr>
<tr>
<td>PH alone</td>
<td></td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>DEN alone</td>
<td></td>
</tr>
<tr>
<td>1 day (22 days post-DEN)</td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>Dietary 2-AAF alone</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>DEN plus 2-AAF (d)</td>
<td></td>
</tr>
<tr>
<td>17 days</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td></td>
</tr>
<tr>
<td>Dietary 2-AAF plus PH (d)</td>
<td></td>
</tr>
<tr>
<td>21 days</td>
<td></td>
</tr>
<tr>
<td><strong>Table 4</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polyamine levels</th>
<th>Time after PH in carcinogen-treated liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td><strong>Table 4</strong></td>
<td></td>
</tr>
</tbody>
</table>

147 days                               |                                            |
with detectable GGT activity appear in the complete carcinogenic regimen within 25 days after DEN, with maximal enzyme activity within 14 days of PH. GGT is a useful marker of preneoplastic hepatocytes (24, 52).

Chart 2 summarizes the alterations in the amount of type I and type II cAPK as a function of time and variation of treatment.

### Table 5

<table>
<thead>
<tr>
<th>Time after PH in carcinogen-treated liver</th>
<th>GGT activity (nmol/30 min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td>12 ± 2 (10)</td>
</tr>
<tr>
<td>PH alone</td>
<td>10 ± 2 (3)</td>
</tr>
<tr>
<td>24 hr</td>
<td>12 ± 2 (3)</td>
</tr>
<tr>
<td>7 days</td>
<td>10 ± 2 (3)</td>
</tr>
<tr>
<td>DEN alone</td>
<td>36 ± 6 (3)</td>
</tr>
<tr>
<td>7 days</td>
<td>14 ± 2 (3)</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>Dietary 2-AAF alone</td>
<td>14 ± 2 (3)</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>DEN plus 2-AAF</td>
<td>9 ± 2 (3)</td>
</tr>
<tr>
<td>17 days</td>
<td>15 ± 2 (3)</td>
</tr>
<tr>
<td>20 days</td>
<td></td>
</tr>
<tr>
<td>Dietary 2-AAF plus PH</td>
<td>100 ± 40 (2)</td>
</tr>
<tr>
<td>21 days</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E. of triplicate determinations on each rat liver.

b Numbers in parentheses, number of animals in each group.
c Data differ from those of controls (p < 0.05).
d 2-AAF was fed in the diet for 14 days with PH performed 7 days after initiation of the 2-AAF diet.

Chart 1. Schematic summary of the temporal sequence of biochemical events during progression through the preneoplastic stages of chemical hepatocarcinogenesis.

Chart 2. Diagrammatic summary of alterations of the patterns of cytosolic type I and type II cAPK specific activity as a function of time and variation of treatment.
which included DEN, 2-AAF diet, and PH. The amount of type I isozyme has been shown to decrease in other systems in which increased biosynthesis occurs, and type I has been demonstrated to translocate to the nucleus to alter genetic transcription events (41).

Recent studies suggest that the induction of ODC appears to be selectively regulated by the amount of and degree of activation of type I cAPK in lymphocytes stimulated to divide by concanavalin A (7, 38) in response to isoproterenol-induced cardiac hypertrophy (3) and in the ventral prostate of the castrated rat relative to androgen supplementation (27). In stimulated lymphocytes, the early specific activation of type I cAPK is positive for ODC induction, RNA synthesis, DNA synthesis, and mitosis, whereas the early activation of both type I and type II cAPK which results from treatment with high concanavalin A or with concanavalin A plus dibutyryl cAMP results in inhibition of ODC induction and inhibition of mitogenesis (7, 38). The decrease in type I cAPK specific activity in the carcinogenesis model of Farber et al. (24, 64, 65) coincides with the rapid growth period of the initiated cells.

To our knowledge, this is the first study in which proposed key growth-regulatory enzymes were sequentially monitored during progression through the neoplastic stages of hepatocarcinogenesis. Based on these experiments, it is suggested that prolonged induction of ODC may be a necessary event for the development of neoplastic foci and the eventual formation of hepatomas. The induction of ODC in this system and in several others studied (56, 60) appears to be temporally correlated to the selective decrease in the amount of recoverable cytosolic type I cAPK. The appearance of foci is concomitant with prolonged elevation of parameters of the trophic response and further implicates a chronic elevation of ODC activity in the process of carcinogenesis.

REFERENCES


Prolonged Ornithine Decarboxylase Induction in Regenerating Carcinogen-treated Liver

Jack W. Olson and Diane Haddock Russell