Carcinogenesis vol.18 no.4 pp.731–738, 1997

Choline deficiency selects for resistance to p53-independent apoptosis and causes tumorigenic transformation of rat hepatocytes

Steven H. Zeisel1, Craig D. Albright, Ok-Ho Shin, Mei-Heng Mar, Rudolf L. Salganik and Kerry-Ann da Costa

Department of Nutrition, School of Public Health and School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7400, USA

1To whom correspondence should be addressed

The mechanisms which drive initiated cells to progress to form carcinomas are poorly understood. CWSV-1 rat hepatocytes, in which p53 protein is inactivated by SV40 large T antigen, respond by inducing p53-independent apoptosis when switched to medium containing low choline (16% apoptotic at 48 h in 5 µM choline) as compared with controls (1% apoptotic at 48 h in 70 µM choline). The rate of apoptosis was inversely correlated with cellular phosphatidylcholine content. Choline deficiency (CD)-induced apoptosis is probably mediated by TGFβ1 and reactive oxygen species, since immunoneutralization of TGFβ1 in the medium or treatment with N-acetylcysteine (an antioxidant) or addition of neocuproine (a transition metal chelator) prevented CD-induced apoptosis. CWSV-1 hepatocytes could be gradually adapted to survive in 5 µM choline. CD-adapted cells had increased membrane phosphatidylcholine concentrations (compared with acute CD cells). Adapted cells acquired relative resistance to CD-induced apoptosis (7% of adapted cells compared with 19% of non-adapted cells were apoptotic at 48 h in 5 µM choline). They also became relatively resistant to another p53-independent form of apoptosis (TGFβ1-induced). CD-adapted hepatocytes developed increased capability for anchorage-independent growth and formed tumors when transplanted into nude mice; passage-matched control hepatocytes did not possess these properties. Cell transformation was dependent on exposure to the selective pressure of CD apoptosis, as we observed that when CD apoptosis was inhibited with an antioxidant during adaptation, cells did not become anchorage independent. Acquisition by p53-deficient cells of resistance to p53-independent inducers of apoptosis (CD, TGFβ1 and reactive oxygen species) may leave cells without another important apoptotic defensive barrier and may be responsible for the progression of initiated cells to frank carcinomas.

Introduction

The process of carcinogenesis involves an initiating event which induces genetic damage, followed by survival and progression of selected clones of the mutant cells to form tumors. Little is understood about the mechanisms which drive initiated cells to progress to carcinomas. Something peculiar to the choline-deficient (CD*) environment enhances tumor progression, as rats deprived of dietary choline develop hepatocellular carcinomas despite the absence of any known carcinogens (1–5).

In order to study the underlying mechanisms involved in progression of carcinogenesis after initiation, we developed a cell culture model using immortalized CWSV-1 rat hepatocytes, in which p53 protein is inactivated by SV40 large T antigen. Many cancers are p53 defective (6), suggesting that their precursor (initiated) cells also share this defect. Previously, we showed that CD medium-induced apoptosis in CWSV-1 cells via a p53-independent pathway (7). In normal tissues, apoptosis provides a physiological way to eliminate terminally differentiated, damaged or genetically altered cells, thus facilitating tissue remodeling following cell injury (8). Apoptosis is an important defensive barrier which inhibits carcinogenesis by eliminating initiated cells, usually via p53-dependent mechanisms (9). However, in p53-defective cells, alternative, p53-independent apoptosis pathways may serve as a mechanism(s) for eliminating initiated cells. When both p53-dependent and p53-independent apoptosis are inactivated, an environment is created in which initiated cells may have a high survival rate, significantly enhancing carcinogenesis.

In the following studies, we describe some of the mediators of CD-induced apoptosis [altered membrane phospholipids, transforming growth factor β1 (TGFβ1) and oxidants]. We observed that CWSV-1 cells which are gradually deprived of choline can adapt and become resistant to CD apoptosis. These adapted cells express a tumorigenic phenotype and treatment with an antioxidant during the CD adaptation process can substantially abrogate the acquisition of a transformed phenotype.

Materials and methods

Cell culture techniques

We used an SV40 large T antigen-immortalized CWSV-1 hepatocyte line at passages 25–35 (a gift from Dr Harriet Isom, Pennsylvania State University) which was created from normal male rat (Fischer 344) hepatocytes (10–12). Microscopically, CWSV-1 hepatocytes appear similar to mature, differentiated hepatocytes. They secrete multiple liver-specific proteins (12). CWSV-1 hepatocytes were grown in RPCD defined medium: RPMI-1640 (American Biogenics Inc., Niagara Falls, NY) containing 11.5 µM bovine serum albumin (Sigma Chemicals Co., St Louis, MO), 2 mM L-glutamine (Sigma), 20 µM oleate (Sigma), 7.2 µM linoleate (Sigma), 1.27 µM ethanolamine (Sigma), 10 nM glucagon (Sigma), 10 nM dexamethasone (Sigma), 100 µg/ml penicillin (Gibco-BRL), 100 µg/ml streptomycin (Gibco-BRL) and choline (Sigma). They secrete multiple liver-specific proteins (12). CWSV-1 hepatocytes (passages 20–50) are not tumorigenic in syngeneic newborn rats (12). CWSV-1 hepatocytes were grown in RPMI defined medium: RPMI-1640 (American Biogenics Inc., Niagara Falls, NY) containing 11.5 µM bovine serum albumin (Sigma Chemicals Co., St Louis, MO), 2 mM L-glutamine (Sigma), 20 µM oleate (Sigma), 7.2 µM linoleate (Sigma), 1.27 µM ethanolamine (Sigma), 10 nM glucagon (Sigma), 10 nM dexamethasone (Sigma), 10 nM HEPES, pH 7.4 (Sigma), trace metals (Sigma), 100 µg/ml penicillin (Gibco-BRL, Grand Island, NY), 100 µg/ml streptomycin (Gibco-BRL) and choline (Sigma) as indicated. In some experiments N-acetylcysteine (Sigma) or neocuproine (Sigma) were added as indicated in figure or table legends. Hepatocytes were gradually adapted to choline deficiency (5 µM choline) by stepwise withdrawal of choline from the medium (from 70 to 20 to 15 to 12 to 10 to 8 to 5 µM choline) over a 2 month period (each passage was split ~1:8), allowing cells to aclimatize to each level of choline for ~2 weeks and achieve 70% density before proceeding. Passage-matched control (70 µM choline) or acutely deficient (5 µM choline) hepatocytes were prepared for comparisons with...
CD-adapted hepatocytes by growing them in the indicated medium for 48 h. In other experiments, as noted, this adaptation procedure was carried out in the presence of 300 µM N-acetylcysteine. Cells were cultured on 100 or 35 mm plates (Falcon) or on LabTek® cell culture chamber slides (Nunc Inc., Naperville, IL) as indicated. Experiments were performed on subconfluent cells.

**Growth in soft agar**

Anchoraging dependence of cells was assessed by plating CWSV-1 and control cells (at each phase of adaptation to the stated concentrations of choline in the medium) in soft agar as per Hsieh et al. (13). When cells were CD adapted in the presence of 30 mM N-acetylcysteine, this antioxidant was included in the medium. Anchorage-dependent cells were identified and scored as positive colonies, counted, and measured after 8 weeks of growth in soft agar containing 70 µM choline and colony forming efficiency (CFE) was calculated.

**Tumorigenicity in nude mice**

Tumorigenicity was measured by inoculating s.c. 10^6 CWSV-1 hepatocytes with 0.2 ml medium into 8-week-old nude mice (Charles River Breeding Laboratories, Raleigh, NC). Mice were fed rodent chow (ProLab RMH 3000) and water ad libitum and were housed at the University of North Carolina animal facility, an approved facility supervised by a full time veterinarian. Animals were observed for development of palpable tumors and mice were killed after 8–10 weeks had passed. Tissue specimens for microscopic examination were collected at necropsy.

**Biochemical determinations**

DNA was measured using the fluorometric method of Labarca (14). Protein was measured using a protein-dye binding method (15). Choline, betaine, phosphocholine, glycerophosphocholine and phosphatidylcholine (PtdCho) were separated by HPLC for water-soluble choline compounds or by TLC for PtdCho, samples were hydrolyzed to form free choline and choline content was measured using gas chromatography/mass spectrometry (16). Betaine was measured by forming the 4’-bromophenacyl derivatized, which was then analyzed using HPLC with spectrophotometric detection (17–19). Cellular phospholipid ratios were assayed after lipids were extracted from ~7–10^6 cells (20) and PtdCho (Rf 0.57) and phosphatidylethanolamine (PtdEtn; Rf 0.43) were separated and identified by TLC. Cell methylation of 4¢-methylmethionine, 60:20:5 v/v and quantitated with a phosphate assay using inorganic phosphorus (phosphorus standard solution; Sigma) as a standard (21).

**S-Adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH)** were extracted from 2–4×10^6 cells or from 50 mg liver pulverized in liquid nitrogen, using 100 µl 0.1 M acetate buffer, pH 6.0, and 75 µl 40% TCA solution. The extracts were washed three times with petroleum ether and stored at –80°C until assayed. SAM and SAH were separated by HPLC (22). The initial solvent contained 25 mM NaH2PO4, 10 mM L-histidine-sulfate acid, pH 3.2 and 10% methanol. SAM and SAH eluted from a C18 column (UltraspHERE, 4.6 mm×25 cm; Beckman) using a linear gradient up to 25% methanol. Peak area responses were measured at 254 nm.

**Assessment of apoptosis**

**DNA fragmentation.** DNA was isolated from cells cultivated in each experimental medium, using guanidine/detergent lysing solution (DNA zol™; Molecular Research Center Inc., Cincinnati, OH). Briefly, 8×10^6 cells were homogenized with guanidine/detergent lysing solution by pipetting up and down and incubated for 30 min with agitation at room temperature. The DNA solution was transferred to a new microtube after centrifugation (10,000 rpm, 10 min) using a microcentrifuge (model HSC-10K-115; Savant). DNA was precipitated by 1/2 vol. ethanol, washed twice using a 95% ethanol solution, dried and dissolved in Tris-EDTA buffer, pH 8.0. Aliquots of 5–10 µg DNA were analyzed for fragmentation by electrophoresis on a 1% agarose slab gel containing 1 µg/ml ethidium bromide. A 1 kb DNA ladder was included as a standard. The bands were visualized under UV light and photographed (23).

**In situ detection of apoptotic cells with DNA strand breaks.** Apoptotic cells were detected using a direct immunoperoxidase method (ApopTag™; Oncor Inc., Gaithersburg, MD) to visualize the incorporation of digoxigenin-labeled nucleotide into the 3’-hydroxy ends of DNA fragments (7). Cells, cultured in chamber slides, were fixed by cold 70% ethanol solution and mixed with reaction solutions modifying the 3’-hydroxyl end of DNA. Nuclei showing DNA strand breaks were visualized by diaminobenzidine solution (peroxidase substrate kit; Vector laboratories Inc., CA) (24). Non-apoptotic nuclei were counterstained with hematoxylin solution and counted by light microscopy.

**Morphological detection of apoptotic cells.** End-stage apoptotic bodies were detected after hematoxylin and eosin staining using a light microscope and morphological markers for apoptotic bodies (23, 24). This modification of the TUNEL method, morphology and DNA ladders to establish the presence of apoptosis, as recommended by Schulte-Hermann and co-workers (25). The TUNEL method will also detect strand breaks that occur in necrosis and autolysis due to post mortem DNA changes (26). When DNA degradation occurs in necrosis, it follows morphological changes which include membrane rupture and shut-down of mitochondria and other organelles and thus occurs after a loss of viability. During necrosis, DNA is non-specifically degraded into a conspicuous smear of non-uniform sizes, as opposed to the characteristic DNA ladder pattern often observed in apoptosis. The TUNEL technique consistently provides much higher rates of apoptosis than does counting apoptotic bodies (see Table I). This may be because it detects single-strand as well as double-strand DNA breaks or because the time during which end-stage nuclear degeneration is detected (~24 versus 3 h).

**TGFβ1 treatment and TGFβ1 immunoprecipitation**

CWSV-1 hepatocytes were plated in 70 µM choline and grown for 4 days (~50% confluent) and then placed for an additional 2 days in experimental medium (70 or 5 µM choline) supplemented with 0–1 ng/ml TGFβ1 (R&D Systems, Minneapolis, MN) or with 0–10 µl/ml TGFβ1 neutralizing polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).

\[ \text{H}^3 \text{Methionine or H}^3 \text{Ethanolamine incorporation into phosphatidylcholine} \]

CWSV-1 hepatocytes were cultivated in control medium until 50% confluent and then pulsed with 5 µCi [methyl-\( \text{H} \)]methionine (70 µCi/µmol; Dupont New England Nuclear) or [H]ethanolamine, cells were collected and washed with phosphate-buffered saline. Lipids were extracted from cells and PtdCho was separated by TLC (chloroform/methanol/40% methylamine, 60:20:5 v/v) and quantitated using inorganic phosphorus (phosphorus standard solution; Sigma) as a standard (21).

\[ \text{TGF} \beta 1 \text{neutralizing polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).} \]

**Statistics**

We used analysis of variance and appropriate multiple comparisons procedures to determine statistical significance between the treatment groups (JMP Version 2, SAS, 1989) (26).

**Results**

CD-induced apoptosis was detected as early as 12 h, with most hepatocytes killed within 96 h after switching them from medium containing 70 µM to 0 or 5 µM choline (data for TUNEL, apoptotic bodies and DNA ladders not shown because similar data have been published in our previous report; 7), however, if hepatocytes were gradually adapted to low choline as described above, they grew at a rate that was identical to that of the control cells growing in 70 µM choline (data not shown). Cell population dynamics for CWSV-1 cells growing in 0, 2, 5 and 70 µM choline have been previously reported (7); cell accumulation is negligible in 0 µM choline and is diminished significantly in 5 µM choline compared with 70 µM.

### Table I. Adaptation to choline deficiency is associated with a diminished apoptosis rate in 5 µM choline

<table>
<thead>
<tr>
<th>Condition</th>
<th>Apoptosis (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 µM choline</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Acutely switched</td>
<td>19.3±2.4±6</td>
</tr>
<tr>
<td>to 5 µM choline</td>
<td>6.6±0.8±b</td>
</tr>
<tr>
<td>Gradually adapted</td>
<td>1.9±0.7</td>
</tr>
</tbody>
</table>

CWSV-1 hepatocytes were plated in 70 µM choline-sufficient serum-free medium until 50% confluent and then cultured for an additional 2 days in CD or control medium. Nuclear incorporation of digoxigenin-11-UTP into DNA (TUNEL) and apoptotic body counts were measured as described in Materials and methods. Data are expressed as mean ± SD, n = 6/treatment for TUNEL, n = 3/treatment for apoptotic bodies. 

\[ bp < 0.01 \text{ different from 70 µM choline.} \]

\[ H^3 \text{Methionine or H}^3 \text{Ethanolamine incorporation into phosphatidylcholine} \]

\[ \text{TGF} \beta 1 \text{ treatment and TGF} \beta 1 \text{ immunoprecipitation} \]
Resistance to apoptosis and tumorigenic transformation

Choline (where doubling time was ~36 h). CD-adapted cells had much lower rates of apoptosis (measured by TUNEL and by counting apoptotic bodies) than did hepatocytes acutely switched to equivalent CD medium (Table I).

Treatment with the antioxidant N-acetylcysteine or with the metal chelator neocuproine reduced the rate of apoptosis (measured by DNA strand breaks) in CD CWSV-1 hepatocytes to levels similar to that observed in choline-sufficient cells (Figure 1).

Choline-sufficient CWSV-1 hepatocytes responded to addition of TGFβ1 by undergoing apoptosis (Figure 2). Hepatocytes which were gradually adapted to CD did not respond to added TGFβ1 with increased rates of apoptosis (Figure 2). Neutralization of soluble, endogenous TGFβ1 diminished the apoptotic rate in CD CWSV-1 hepatocytes (Figure 3).

CWSV-1 hepatocytes grown in 5 µM choline (whether acutely deficient or gradually adapted to deficient medium) had lower intracellular concentrations of choline, betaine, phosphocholine, glycerophosphocholine and PtdCho than did hepatocytes grown in 70 µM choline (Table II). The rate of synthesis of PtdCho via the methylation of PtdEtn increased several-fold in adapted cells compared with control cells, with acutely deficient cells having intermediate rates of PtdCho synthesis (Table III). Concentrations of SAM, the methyl donor for this pathway, were the same in control and in acute CD hepatocytes, but were increased ~6-fold in adapted CD hepatocytes (Table III). Concentrations of PtdEtn, another substrate for this pathway, also increased in CD-adapted cells (Table III), changing the PtdCho/PtdEtn concentration ratio in membranes (Table II). In other experiments, we modified cellular PtdCho concentrations and PtdCho/PtdEtn concentration ratios of CWSV-1 cells by varying the choline concentration in the culture medium. The rate of genomic DNA fragmentation in CWSV-1 cells was inversely related to the cellular PtdCho concentration or the PtdCho/PtdEtn concentration ratio of the cells (Figure 4).

CWSV-1 cells which were gradually adapted to CD survived, grew and appeared to be morphologically similar to cells grown constantly in 70 µM choline (data not shown). CWSV-1 hepatocytes (passages 29–35) grown in 70 µM choline formed few colonies when grown in soft agar. However, CFE doubled with each step in reduction of choline from 12 to 8 to 5 µM during the adaptation process (Figure 5). Passage-matched CWSV-1 hepatocytes grown in 70 µM choline were not tumorigenic in nude mice, while seven of eight mice injected with CD-adapted hepatocytes developed tumors (Table IV and Figure 6A). These were histologically undifferentiated to moderately differentiated hepatocellular carcinomas (Figure 6B); only normal tissue was detected at the site of injection of control cells (data not shown). When 30 mM N-acetylcysteine was included in the growth medium throughout the gradual adaptation to CD, the resultant CD-adapted hepatocytes did not exhibit enhanced anchorage-independent growth. CFE in soft agar for N-acetylcysteine-treated cells was only 1.4 ± 0.4%, compared with the 10.6 ± 3.5% observed in hepatocytes that were CD adapted in the same experiment without antioxidant (P < 0.01 by t-test).

Discussion

Choline deficiency caused hepatocytes to undergo apoptosis via a pathway that involved reactive oxygen species (ROS) as...
intermediates. Neocuproine prevents hydroxyl radical formation and was more effective in suppressing CD-induced apoptosis than was N-acetylcysteine, which traps free radicals after they are formed (Figure 1) (27). ROS are involved in several described apoptotic signaling pathways (28). Production of ROS has been previously described in rats fed a CD diet and these ROS caused DNA damage (8-hydroxyguanosine residues) (29).

TGFβ1 is a multifunctional growth factor expressed in hepatocytes undergoing apoptosis (it is not found in quiescent or proliferating hepatocytes nor in hepatocytes undergoing necrosis) (30–32). TGFβ1 can inhibit DNA synthesis (33) and trigger apoptosis in primary hepatocytes (34,35). It induces the sequential expression of the cdk inhibitors p15INK4B and p27Kip1 which effect growth arrest and possibly apoptosis (36). The TGFβ1 apoptotic pathway may not require the p53 (37), Bcl-2 or Bax gene products (38). ROS are mediators of TGFβ1 signaling (39). In animal studies, by 1 week after rats begin a CD diet, TGFβ1 mRNA synthesis in liver increases and this elevation persists for 6 weeks (31). We observed that TGFβ1 is capable of inducing apoptosis in CWSV-1 hepatocytes (Figure 2) and that immunoneutralization of TGFβ1 diminished CD-induced apoptosis (Figure 3). This suggests that TGFβ1 is another of the intermediate messengers in CD-induced apoptosis.

We have previously reported that CD-induced apoptosis occurs because of a deficiency in choline moieties, rather than because of methyl deficiency (40). We report the first direct correlation of cellular PtdCho concentration with apoptosis (Figure 4), but several other investigators have suggested that PtdCho is a critical molecule that is associated with apoptosis (41–44). PtdCho is the major component in eukaryotic membranes and survival of the cells depends on the integrity of the membrane structure (45). PtdCho synthesis is also needed for progression through the cell cycle (46, 47). When choline was withdrawn from cultured fibroblasts they arrested in G1 phase (47), making them susceptible to induction of apoptosis (48).

We found that hepatocytes can be adapted so that they can survive in low choline medium by becoming resistant to CD-induced apoptosis (Table I). Adapted cells did not survive because they corrected intracellular concentrations of choline or water-soluble choline metabolites (Table II). However, adapted cells did slightly increase intracellular PtdCho concentrations compared with acutely deficient cells (Table II). This increase in PtdCho concentration could be the result of enhanced endogenous production of PtdCho via the methylation of PtdEtn (Table III). This observation is consistent with reports that the activity of PtdEtn N-methyltransferase activity is increased in the livers of CD rats (49). We observed that increased PtdEtn N-methyltransferase activity was associated with diminished apoptosis. This differs from the findings of other investigators, who observed that over-expression of a specific isoform of the enzyme was associated with increased cell death (50). CWSV-1 cells were grown in medium con-

![Fig. 3. Immunoneutralization of TGF-β1 protects against apoptosis from choline deficiency.](image)

Table II. Intracellular concentrations of choline and choline metabolites are depleted in choline-deficient CWSV-1 hepatocytes

<table>
<thead>
<tr>
<th>Concentration (pmol/μg DNA)</th>
<th>70 μM Choline</th>
<th>Acutely switched to 5 μM choline</th>
<th>Gradually adapted to 5 μM choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>43±2</td>
<td>1.9±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Betaine</td>
<td>112±29</td>
<td>17±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18±1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphoholchoine</td>
<td>747±52</td>
<td>31±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9±1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycero-phosphocholine</td>
<td>195±31</td>
<td>31±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10±2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>1500±120</td>
<td>800±20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1000±70&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phosphatidylcholine/Phosphatidylethanolamine ratio</td>
<td>1.72±0.02</td>
<td>0.84±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86±0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

CWSV-1 hepatocytes were grown in RPCD defined medium containing 70 μM choline until 50% confluence was attained, they were then switched for 2 days to medium containing 70 μM choline (open bars) or 5 μM choline (hatched bars). TGFβ1 was neutralized using a polyclonal antibody (Collaborative Biomedical) in the amounts indicated. Apoptosis was assessed by counting cells containing apoptotic bodies. Data are expressed as mean percent of cells that were apoptotic ± SD, n = 3/point. This experiment was replicated twice and similar experiments were performed using PC12 and 3T3 cells. *P < 0.05 by 2-way ANOVA and Bonferroni modified statistic.
Resistance to apoptosis and tumorigenic transformation

Table III. CWSV-1 cells adapted to choline deficiency increased synthesis of PtdCho using SAM and PtdEtn as substrates

<table>
<thead>
<tr>
<th>Condition</th>
<th>[3H]methionine</th>
<th>SAM (pmol/10^6 cells)</th>
<th>PtdEtn (nmol/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±7</td>
<td>408±61</td>
<td>0.97±0.02</td>
</tr>
<tr>
<td>Acute 5 µM</td>
<td>301±13</td>
<td>532±38</td>
<td>0.99±0.02</td>
</tr>
<tr>
<td>Adapted 5 µM</td>
<td>491±25</td>
<td>3225±483</td>
<td>1.09±0.05</td>
</tr>
</tbody>
</table>

CWSV-1 cells were treated as described in the legend to Table II. PtdCho synthesis from methionine and ethanolamine after 48 h was measured as described in Materials and methods. SAM and PtdEtn concentrations were assayed as described in Materials and methods. Data are expressed as mean ± SEM (n = 4–6/group for SAM assay; n = 3/group for PtdEtn assay; n = 6–9/group for PtdCho synthesis). Statistical difference was determined by 1-way ANOVA and Dunnett’s test. 100% [3H]methionine labeling was 1300 d.p.m./10^6 cells; 100% [3H]ethanolamine labeling was 1700 d.p.m./10^6 cells.

^aP < 0.01 different from control.

^bP < 0.05 different from control.

Fig. 4. A decrease in cellular phosphatidylycholine concentration or PtdCho/ PtdEtn concentration ratio is associated with DNA fragmentation. CWSV-1 hepatocytes were cultivated in control medium (70 µM choline) until 50% confluent and then switched to experimental medium containing various choline concentrations (1, 0 µM choline; 2, 2.5 µM choline; 3, 5 µM choline; 4, 10 µM choline; 5, 20 µM choline, 6, 70 µM choline) for 40 h. Cellular phosphatidylycholine (PtdCho) concentration and PtdCho/ phosphatidylyethanolamine (PtdCho/PtdEtn) concentration ratios were measured as described in the text. DNA fragmentation was analyzed using a 1% agarose slab gel.

Fig. 5. CFE in soft agar increases as hepatocytes are adapted to low choline. CWSV-1 hepatocytes were gradually adapted to 5 µM choline as described in Materials and methods (hatched bars). Passage-matched controls were carried in 70 µM choline (shaded bars). CFE in soft agar was assessed at several stages of the weaning process (concentrations of choline in medium indicated above data bars; if not indicated concentration was 70 µM choline). Data are expressed as mean ± SEM, n = 6–9 point. **P < 0.01 different from 70 µM choline at same passage by Student’s t-test.

We found that hepatocytes adapted to CD became resistant not only to CD apoptosis, but also to another p53-independent apoptotic pathway (TGFβ1-induced). This observation is consistent with a role for TGFβ1 as a mediator of CD-induced apoptosis and suggests that CD adaptation involves acquisition of a defect in apoptosis that is downstream of TGFβ1.
phenobarbital in chemically initiated liver, where it is postulated that inhibition of apoptotic cell death in preneoplastic foci enhances carcinoma development (57,58). We observed that when N-acetylcysteine was present during adaptation to CD (presumably preventing CD-induced apoptosis), the resulting cells did not develop the capacity for anchorage-independent growth. This is consistent with the hypothesis that ROS-induced mutagenesis and the selective pressure created by CD apoptosis is important for the progression from initiated cell to transformed cell.

We suggest the above hypothesis, but we cannot exclude the possibility that a subset of CWSV-1 cells exists with the propensity for tumorigenicity that eventually comes to dominate the cell population after many passages. Perhaps CD, by increasing the rate of cell turnover, accelerates this transformation process. The CWSV-1 hepatocyte line is not tumorigenic at the passages that we used it, but becomes tumorigenic after passages 52–61 (six of 19 clones formed undifferentiated tumors in nude mice), forming undifferentiated tumors that appear to be different from the ones we describe in CD-selected tumorigenic cells (12). Even if it turns out that CD itself doesn’t create a new mutation and is merely selecting for cells predisposed to carcinogenic transformation, this model is still useful for understanding the effects of CD in rats. It has been estimated that the normal rat liver contains 500–1000 initiated cells at any given time (59) and these cells may be predisposed to carcinogenic transformation.

Humans can become depleted of choline and develop liver damage when fed purified diets (60,61) or when fed parenterally with low choline formulations (62–64). In CD rats there is enhanced hepatocyte cell death (65) and compensatory increases in hepatocyte DNA synthesis and liver regeneration (66). After 6 months on a CD diet, foci of preneoplastic hepatocytes appear in rat liver (67,68) and hepatocellular carcinomas occur at ~12 months, despite the absence of any known carcinogens (1–5). The mechanism for the cancer promoting effect of choline deficiency is not completely elucidated (69–72). Perturbations in important intracellular signaling mechanisms involving hepatic 1,2-sn-diacylglycerol concentration and protein kinase C (PKC) activity occur in choline deficiency (73,74). Prolonged activation of PKC is associated with mitogenesis and cell transformation (75–77).

The chronic cell death and cell proliferation in CD livers, with its associated increased rate of DNA synthesis (65,69) could cause greater sensitivity to chemical carcinogens (78). Alterations in the genome that occur in choline deficiency, such as hypomethylation of DNA (72,79) and DNA damage caused by free radicals (29,80), also probably enhance carcinogenesis. We add to this list of mechanisms the possibility that loss of an apoptotic pathway contributes to carcinogenesis in the liver of CD rats.

Table IV. Hepatocytes gradually adapted to choline deficiency become tumorigenic

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Number of tumors/site</th>
<th>Tumor size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 70 µM</td>
<td>CD-adapted 5 µM</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

CWSV1 hepatocytes which were gradually adapted to 5 µM choline were injected under the right forelimb and passage-matched cells kept in 70 µM choline were injected under the left forelimb of nude mice. Tumors were counted and measured (maximum mean diameter) at 8–10 weeks after inoculation.

We have discovered that CD is a new p53-independent trigger of apoptosis in hepatocytes. CWSV-1 cells which survive CD are resistant to CD-induced p53-independent apoptosis when fed purified diets (60,61) or when fed parenterally with low choline formulations (62–64). In CD rats there is enhanced hepatocyte cell death (65) and compensatory increases in hepatocyte DNA synthesis and liver regeneration (66). After 6 months on a CD diet, foci of preneoplastic hepatocytes appear in rat liver (67,68) and hepatocellular carcinomas occur at ~12 months, despite the absence of any known carcinogens (1–5). The mechanism for the cancer promoting effect of choline deficiency is not completely elucidated (69–72). Perturbations in important intracellular signaling mechanisms involving hepatic 1,2-sn-diacylglycerol concentration and protein kinase C (PKC) activity occur in choline deficiency (73,74). Prolonged activation of PKC is associated with mitogenesis and cell transformation (75–77).

The chronic cell death and cell proliferation in CD livers, with its associated increased rate of DNA synthesis (65,69) could cause greater sensitivity to chemical carcinogens (78). Alterations in the genome that occur in choline deficiency, such as hypomethylation of DNA (72,79) and DNA damage caused by free radicals (29,80), also probably enhance carcinogenesis. We add to this list of mechanisms the possibility that loss of an apoptotic pathway contributes to carcinogenesis in the liver of CD rats.

We have discovered that CD is a new p53-independent trigger of apoptosis in hepatocytes. CWSV-1 cells which survive CD are resistant to CD-induced p53-independent apoptosis when fed purified diets (60,61) or when fed parenterally with low choline formulations (62–64). In CD rats there is enhanced hepatocyte cell death (65) and compensatory increases in hepatocyte DNA synthesis and liver regeneration (66). After 6 months on a CD diet, foci of preneoplastic hepatocytes appear in rat liver (67,68) and hepatocellular carcinomas occur at ~12 months, despite the absence of any known carcinogens (1–5). The mechanism for the cancer promoting effect of choline deficiency is not completely elucidated (69–72). Perturbations in important intracellular signaling mechanisms involving hepatic 1,2-sn-diacylglycerol concentration and protein kinase C (PKC) activity occur in choline deficiency (73,74). Prolonged activation of PKC is associated with mitogenesis and cell transformation (75–77).

The chronic cell death and cell proliferation in CD livers, with its associated increased rate of DNA synthesis (65,69) could cause greater sensitivity to chemical carcinogens (78). Alterations in the genome that occur in choline deficiency, such as hypomethylation of DNA (72,79) and DNA damage caused by free radicals (29,80), also probably enhance carcinogenesis. We add to this list of mechanisms the possibility that loss of an apoptotic pathway contributes to carcinogenesis in the liver of CD rats.
apoptosis as well as to p53-dependent apoptosis. These cells are highly tumorigenic. Acquisition of resistance to the CD form of apoptosis (presumably a back-up pathway) may leave the cells without an apoptotic defensive barrier and may greatly accelerate the process of carcinogenesis. We suggest that, given inactivation of the p53 gene (a common occurrence in human cancers), it is the rare acquisition of resistance to p53-dependent pathways that is crucial to carcinogenesis.

Acknowledgements

We thank Rong Liu for technical assistance. This research was supported by a grant from the American Institute for Cancer Research and from the National Institutes of Health (AG09525) awarded to S.H.Z. An abstract describing a portion of this work was first presented at the Keystone Apoptosis meeting, March 10, 1995, Durango, CO.

References


