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Advances in Brief

In Vivo Administration of Dichloroacetic Acid Suppresses Spontaneous Apoptosis in Murine Hepatocytes[1]

Ronald D. Snyder, Janice Pullman, Julia H. Carter, Harry W. Carter, and Anthony B. DeAngelo[2]

Wood Hudson Cancer Research Laboratory, Newport, Kentucky 41071 [R.D.S., J.P., J.H.C., H.W.C.], and Health Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, North Carolina 27711 [A.B.D.]

Abstract

Spontaneous apoptosis in hepatocytes of male B6C3F1 mice that received dichloroacetic acid (DCA) in their drinking water for 5–30 days (28–58 days of life) was examined as part of ongoing studies to determine the molecular basis of the hepatocarcinogenicity of this nongenotoxic water chlorination by-product. DCA at 0.5 and 5.0 g/liter, significantly reduced apoptosis relative to untreated controls in a dose-dependent fashion. Regression analysis indicated that apoptosis declined over the 30-day period in the livers of control, age-paired animals receiving no drug. Animals receiving low-dose DCA exhibited a similar, although quantitatively depressed, trend line, whereas animals receiving high-dose DCA showed maximal depression of apoptosis at 5 days, which was sustained throughout the course of the 30-day period. These studies suggest that DCA has the ability to down-regulate apoptosis in murine liver. When taken together with previous data demonstrating DCA-dependent decrease in labeling index in these same livers, these data further support the hypothesis that the carcinogenic mechanism of DCA may involve suppression of the ability of the liver to remove initiated cells by apoptosis rather than by induction of selective proliferation of initiated cells.

Introduction

DCA[3] is a water chlorination by-product found in finished drinking water at concentrations ranging from 34 to 160 μg/liter (1, 2). It is a complete hepatocarcinogen in B6C3F1 male mice (3–5); neoplasms were found in 97% of mice given 5 g/liter DCA in their drinking water for 60 weeks (5). The nature of the carcinogenic activity of DCA remains unclear. Most *in vitro* and *in vivo* studies have shown DCA to have little or no genotoxic activity (6–9) although findings to the contrary have been reported recently (10–11). DCA is also known to be a peroxisome proliferator (12, 13). Many peroxisome proliferating agents possess mitogenic and hepatotrophic properties (14) and because increased proliferation is often found to be associated with the carcinogenic process (15), it has been proposed that DCA may act in a similar proliferation inducing fashion. DCA has, in fact, been reported to increase hepatocellular proliferation (4); however, in recent studies, we have demonstrated a clear dose-dependent mitoinhibition in livers of B6C3F1 mice receiving DCA in their drinking water for 5–30 days (16), arguing against a proliferative mechanism of action at least at short exposure times.

An alternative mechanism for the hepatocarcinogenicity of DCA is that DCA might allow outgrowth of initiated cells, not through increasing the proliferation of these cells, but rather through suppression of apoptosis, which would normally remove these cells from the liver. This has been proposed as a general carcinogenic mechanism of tumor promoters and of the hepatocarcinogenicity of certain peroxisome proliferators (17–19). Withdrawal of the liver hyperplastic agents cyproterone acetate and phenobarbital results in massive cell death by apoptosis and reduction in liver mass (20). Readministration of either drug blocks this reduction (21, 22). Nafenopin, another peroxisome proliferator and nongenotoxic hepatocarcinogen, has been shown to inhibit spontaneous apoptosis in preneoplastic liver foci *in vivo* (23) and to partially block transforming growth factor β-induced apoptosis in primary hepatocytes and in the Reuber hepatoma cell line FAO (24). These studies were interpreted as indicating that peroxisome proliferators reversibly suppress apoptosis, and that this suppression could result in outgrowth of initiated cells and subsequent tumor formation.

The present studies were designed to examine the effects of 5–30-day exposures to hepatocarcinogenic concentrations of DCA in the drinking water of male B6C3F1 mice on the frequency of spontaneous apoptosis in liver hepatocytes. It is shown that animals receiving DCA exhibit significant decreases in hepatocyte apoptosis at the earliest time points studied. These results are consistent with the hypothesis that DCA-induced hepatocarcinogenesis may result from enhanced outgrowth of initiated cells due to down-regulation of the apoptotic process.

Materials and Methods

Chemicals. DCA was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI) and was dissolved in distilled water at 0.5 and 5 g/liter. The pH was adjusted to between 6.8 and 7.2 with 10 N NaOH. DCA concentration was verified by using gas chromatographic analyses. TDT was purchased from Boehringer Mannheim (Indianapolis, IN); bdUTP was from Clon-Tech Laboratories (Palo Alto, CA); avidin-biotin detection system and diaminobenzidine substrate kits were from Vector Laboratories, Inc. (Burlingame, CA).

Animals and Treatments. Male B6C3F1 mice, 21 days old, were purchased from Charles River Laboratories (Portage, MI). The animals received standard laboratory chow and water *ad libitum* and were housed in a temperature- (22 ± 2°C) and humidity- (40–60%) controlled environment under a 12-h light/12-h dark cycle. A complete description of the treatment protocol has been reported previously (16). Briefly, 28-day-old animals were distributed randomly into three treatment groups receiving either distilled water or 0.5 or 5.0 g/liter DCA in drinking water for up to 30 days. The study was conducted in two phases: Phase I encompassed 5–15 days, and Phase II encompassed 20–30 days of treatment. Groups of five animals were sacrificed at 5-day intervals by CO_2 asphyxiation.

Histology. Blocks of liver from the left lobes were fixed in neutral-buffered formalin and embedded in paraffin blocks. Histological sections (5 μm) were stained with H&E for pathological analysis. Additional sections were mounted on Probe-On Plus slides (Fisher Scientific, Pittsburg, PA) to facilitate the use of a capillary gap stainer for end-labeling analysis.

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[2] To whom reprint requests should be addressed, at United States Environmental Protection Agency, MD-68, Research Triangle Park, NC 27711.

[3] The abbreviations used are: DCA, dichloroacetic acid; TDT, terminal deoxynucleotidyl transferase; bdUTP, 21-biotinylated dUTP; TUNEL, terminal transferase-mediated nick end-labeling; H & E, hematoxylin and eosin.

Image Analysis. Cellularity of tissues was determined from histological sections by using a Zeiss IBAS 2000 image analysis system and a ×40 objective as described previously (16). Monochromatic light at 623 nm was used to optimize contrast between nuclei and cytoplasm. Hepatocyte number was determined by counting nuclei in 50 fields. Nonparenchymal cells were excluded by a size filter. Images of nuclei were dilated by addition of X and Y coordinates so that adjacent nuclei in multinucleated hepatocytes appeared to touch. This permitted multinucleated cells to be counted as a single cell by the computer.

***In Situ* Nick End-labeling Analysis.** Apoptotic cells were visualized by the *in situ* TDT nick end-labeling assay essentially as described by Gavrielli (25). This assay measures the TDT-catalyzed incorporation of bdUTP into cells containing DNA strand breaks resulting from apoptotic DNA fragmentation and the visualization of these cells by standard avidin-biotin immunohistochemical methods. The assay was conducted with the use of a manually operated capillary gap-staining apparatus that allowed minimal reagent use, assured that all samples were similarly treated, and simplified all handling procedures. Consistent results were obtained with 15 units TDT and 1 nmole bdUTP/2 sandwiched slides in a reaction volume of 127 μl for 1 h at 37°C. Detection was with the Vectastain Elite ABC kit with a 5-min incubation in diaminobenzidine. Tissues were very briefly counterstained with methyl green. Negative controls were run in which TDT was omitted, and positive controls, ventral prostate from 3-day post-castration rats, were run with each set of samples to assure that the assay was working optimally. Sections were examined by light microscopy, and the total number of apoptotic hepatocytes/section was recorded. Multiple fragments in the same immediate vicinity were scored as one event. Cells from extreme tissue margins were excluded from consideration due to occasional artifactual staining of these areas. Care was also taken to exclude positively staining endothelial and bile duct cells. Apoptotic cells took on varied forms depending on their stage in the apoptotic process. These forms were characterized by: (*a*) diffuse staining of cytoplasm with only minimal nuclear condensation; (*b*) dense staining of nuclei retaining normal nuclear structure; (*c*) distinct apoptotic bodies resulting from nuclear disintegration; and (*d*) normal cells (both hepatocytes and Kupffer cells) containing phagocytyzed apoptotic fragments. For the purposes of analyses, all forms were considered equivalent. The inclusion of apoptotic Kupffer cells or rare necrotic cells in our analyses cannot be rigorously discounted. However, sizes and general morphology of the most positively staining cells are consistent with hepatocyte apoptosis.

Statistics. Apoptotic cell determinations were derived from 2–4 different liver sections from each control or treated animal. The average number of apoptotic cells was then determined for each animal in each group of 5 similarly treated animals, and the percentage of apoptotic cells was calculated from measurements of tissue cellularity. The resultant values were subjected to the Student's *t* test. Differences were considered statistically significant when $P < 0.05$.

Results

Histological detection of apoptotic cells in paraffin-embedded tissues has, until recently, relied on the differential morphology of those cells in H&E sections. Despite the fact that this analysis is subjective and has the potential for misidentification of apoptotic events, reasonably accurate measurements of at least late stage apoptotic events have been made for many mammalian tissues. In rat liver, for example, the estimated frequency of spontaneous apoptosis is approximately 0.1% (21–22). Similar estimates have not been reported for mouse liver. The advent of *in situ* methodologies for specific detection of apoptotic cells has made visualization and quantification of apoptosis much easier. We show in the present studies (Fig. 1) that TUNEL analysis reveals a variety of apoptotic forms in murine liver, some of which would not be visualized by H&E staining. All of these cells clearly stain dark brown against the counterstained blue-green background and are, therefore, readily visible. These forms range from round brown nuclei often with internal structure (Fig. 1*A*) to clusters of apoptotic bodies resulting from nuclear fragmentation (data not shown), to cells with normal (nonstaining) nuclei but with overlying light cytoplasmic staining (presumably resulting from leakage of DNA fragments into the cytoplasm). These latter forms are often also seen to contain internalized apoptotic bodies (Fig. 1*B*), indicative of a phagocytic process. Phagocytized apoptotic bodies were seen both in hepatocytes (Fig. 1*B*) and Kupffer cells (Fig. 1*C*). In none of the tissues examined was necrotic foci observed nor was there any indication of lymphocyte or neutrophil infiltration indicative of an inflammatory response. It is unlikely, therefore, that necrotic cells contributed substantially to our analyses.

Fig. 2 demonstrates the effects of DCA administration on the frequency of detectable apoptotic cells or fragments in the liver. Control animals exhibited apoptotic frequencies ranging from 0.04 to 0.09%, and regression analysis indicates a trend toward decreased apoptosis over the 30-day period. Although the biological significance of this trend is unclear, we have observed that, consistent with earlier reports, the livers of these young animals are undergoing rapid changes in size and proliferation (16), and it is not unreasonable that the rate of cell death may also undergo changes. Moreover, this trend

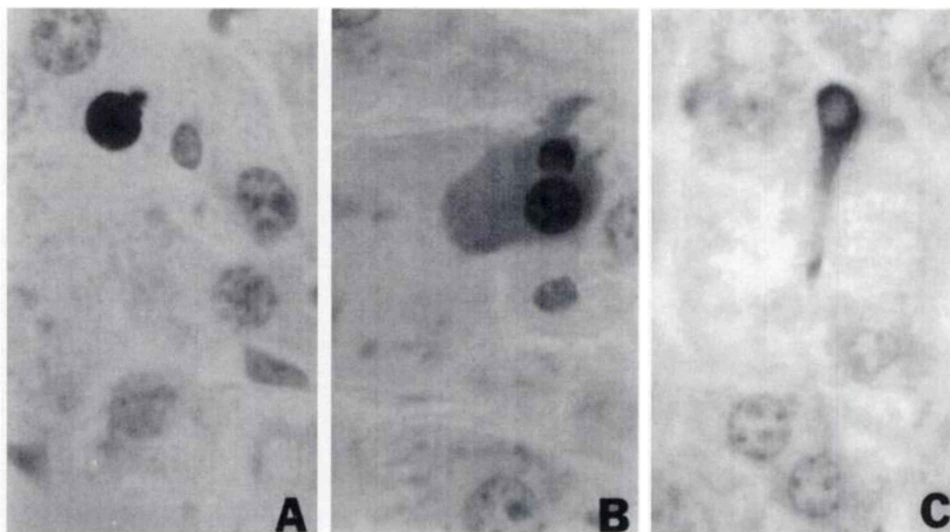

Fig. 1. Examples of apoptotic figures in mouse liver. *A*, intact apoptotic hepatocyte prior to formation of apoptotic bodies and phagocytosis. *B*, apoptotic hepatocyte phagocytyzed by neighboring hepatocyte. Note normal nucleus of intact hepatocyte, internalized apoptotic body, and overlying cytoplasmic staining resulting from apoptotic DNA leakage. This particular photomicrograph is from a liver tumor resulting from long-term DCA exposure (16), but it is representative of forms scored in the present study. *C*, apoptotic cell phagocytyzed by Kupffer cell. Magnification of original micrographs was × 1400.

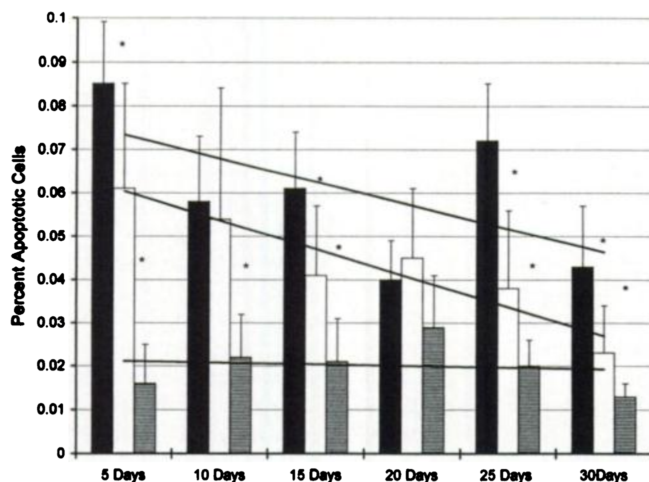

Fig. 2. Effect of 5–30-day exposure to DCA on the frequency of spontaneous apoptosis in mouse liver. Mice were either untreated (■) or received 0.5 g/liter (□) or 5.0 g/liter (▤) DCA in their drinking water for the specified period of time before sacrifice and histological analysis of livers. Percentage of apoptotic cells was based on cellularity measurements on adjacent sections. *, statistical significance (see "Results"). Trend lines were generated by linear regression by using Microsoft Excel software. R^2 values were 0.34, 0.89, and 0.016 for control, low-dose, and high-dose DCA, respectively.

was also observed in animals receiving 0.5 g/liter DCA supporting the possibility that this is a physiological phenomenon. This low dose of DCA also decreased the percentage of apoptotic hepatocytes at the earliest time point studied. Statistically significant suppression was observed at 5, 15, 25, and 30 days ($P < 0.05$, 0.037, 0.004, and 0.033, respectively). Animals receiving the high dose of DCA (5 g/liter) exhibited very significant reductions in apoptosis at the earliest time point studied, and sustained this level of suppression throughout the course of the study. Statistically significant differences were observed on days 5, 10, 15, 25, and 30 ($P < 0.00002$, 0.0017, 0.0005, 0.0001, and 0.012, respectively).

Discussion

Suppression of apoptosis has been proposed as a fundamental mechanism of action of tumor promoters as a class (26). In this paradigm, a certain fraction of cells is either spontaneously or chemically initiated into a preneoplastic state, and these cells are normally recognized and removed by apoptosis. Disruption of apoptosis results in outgrowth of initiated cells and tumor formation. Both phenobarbital and cyproterone acetate have been hypothesized to promote hepatocarcinogenesis in this fashion (23). Nafenopin, a peroxisome proliferator and hepatocarcinogen have also been shown to suppress spontaneous apoptosis in normal liver and in preneoplastic liver foci (23). Moreover, isolated rat hepatocytes die rapidly under normal culture conditions, but continuous treatment with nafenopin stabilizes these cultures for six weeks (24). Subsequent removal of nafenopin results in immediate cell death by apoptosis, suggesting that exposure to nafenopin results in a long-lived but reversible block to apoptosis.

The present studies indicate that another nongenotoxic hepatocarcinogen, DCA, suppresses liver apoptosis suggesting a similar mechanism of hepatocarcinogenesis. Relevant to this hypothesis is the finding that DCA-induced liver tumors in B6C3F1 mice exhibit similar mutational spectra at the H-*ras* and K-*ras* loci (27). These data can be interpreted as indicating that rather than inducing mutation itself, DCA treatment either confers a growth advantage to these initiated cells, or more likely in light of the present studies, suppresses the loss of these cells through apoptosis. Studies are currently under way to examine the effects of long-term (60–100 week) exposures to DCA on the apoptotic rate in hyperplastic foci, adenomas, and carcinomas and to determine if DCA withdrawal results in reversal of the block to apoptosis in this model.

The mechanism by which DCA down-regulates apoptosis is unknown. However, previous studies strongly suggest a critical role for transforming growth factor β in facilitating apoptosis in hepatocytes (reviewed in Ref. 19), and it is possible that DCA causes down-regulation of this growth factor, although this has not yet been investigated. Alternatively, a recent study has demonstrated that seven structurally distinct peroxisome proliferating agents (DCA was not tested) caused increased expression of protein kinase C in cultured hepatocytes (28). Protein kinase C is known to interfere with the apoptotic process (29). Additional studies are required to determine if altered expression of these, or other apoptosis regulatory genes in hepatocytes of DCA-treated animals, contributes to the apparent suppression of apoptosis.

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