The Quantitative Distinction of Hyperplasia from Hypertrophy in Hepatomegaly Induced in the Rat Liver by Phenobarbital

Philip Carthew, Richard E. Edwards, and Barbara M. Nolan

MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, United Kingdom

Received December 4, 1997; accepted April 10, 1998

Cell proliferation is thought to be an important mechanism by which mutations are fixed prior to possible promotion and progression to tumors (Ames and Gold, 1990a,b; Berman, 1988; Butterworth and Goldsworthy, 1991; Butterworth et al., 1991; Cohen and Ellwein, 1990, 1991; Cohen et al., 1991; Clayson et al., 1991, 1993; Preston-Martin et al., 1990). However, the relative contribution of this concept to carcinogenesis is the subject of some controversy (Farber, 1995; Melnick, 1992). One reason is because there have been very few cell proliferation time course studies carried out to validate such a concept, for compounds known to cause cell proliferation (Melnick, 1992). Many compounds cause an initial burst of cell proliferation, which is not subsequently sustained (Carthew et al., 1995). There is also the possibility that cell death may occur during, or because of, treatment, and this may cause further proliferation as is the case with cycloheximide and tamoxifen (Carthew et al., 1996). No studies have attempted to relate the number of cells present in the liver to the relative contributions of cell death and cell proliferation. This is because of the difficulty in determining the relative rates of cell death and proliferation, which can be time dependent during treatment (Carthew et al., 1995, 1996), or in the case of apoptosis, difficult to determine, morphologically, because of the short half-life of apoptotic cells (Bursch et al., 1990). It is relatively easy to measure treatment induced cell proliferation, relative to control tissue using 5-bromo-2'-deoxyuridine (BrdU) incorporation into DNA (Wynford-Thomas and Williams, 1986) or by measuring the proliferating cell nuclear antigen (PCNA) labeling indices (Foley et al., 1993), although the rate of cell proliferation may change with time of treatment (Carthew et al., 1995). Although cell death by apoptosis can be estimated using the standard morphological criteria (Bursch et al., 1991) and cell death in general can be estimated using in situ end labeling techniques (Ansari et al., 1993), the question of whether an organ is actually increasing in size by cell division is still often difficult to determine absolutely. This is especially true for the liver where there is a considerable proportion of binucleate cells and various levels of polyplody (Carriere, 1969). Treatment may change the proportion of these, making the measurement of changes in the numbers of nuclei invalid as a method of estimating cell numbers. A problem commonly associated with estimating hepatocyte cell number is the phenomenon of hypertrophy (increase in cell size) which occurs with enzyme inducers such as phenobarbital, dioxin, and, to a lesser extent, peroxisome proliferators (Carthew et al., 1997). This can also occur due to uptake of water by hepatocytes (hydropic degeneration), an increase in the amount of protein per cell, or due to an increase in the glycogen content of cells, which may be reversible after the
cessation of treatment (Foley et al., 1993). The increase in size of hepatocytes in the zone three region of the liver is often seen to be an obvious morphological result of treatment, which can confound any estimation of whether the increase in liver size is due to cell division (hyperplasia) or an increase in the size of a significant proportion of the hepatocytes (hypertrophy). If there is a sustained hyperplasia of the liver, this would be viewed as possibly indicating that the compound inducing this was more likely to act as a carcinogen in the long term, especially in the case of peroxisome proliferators (Takagi et al., 1992). A recent study which discriminated hyperplasia from hypertrophy in the rat liver, due to treatment with the peroxisome proliferator gemfibrozil did not find a significant increase in overall hepatocyte cell number, even though the liver increased in size and weight by 20% over the treatment period (Carthew et al., 1997). A purely histological method to determine the absolute numbers of hepatocytes in the liver would be particularly useful to indicate whether increases in cell number were occurring upon treatment with compounds that cause hepatomegaly. Using the established technique of morphometry and the "new stereology," by applying the optical dissector principle to the determination of the number of hepatocyte nuclei in a given volume of liver tissue, it was possible to adopt a hierarchical approach to analyzing the liver and determine both the relative changes in hepatocyte cell number with treatment and also the absolute hepatocyte cell number. PB was used as a candidate compound as it is known to cause liver enlargement, hypertrophy of hepatocytes, and an increase in DNA synthesis in hepatocytes, so that the techniques for determining the changes in hepatocyte number, after treatment, as well as the presence of hypertrophy, could be determined.

MATERIALS AND METHODS

Animals and treatment. Thirty-two male F344 rats (150-160 g weight) were fed RM 1 pelleted diet (Special Diet Services, Witham, Essex, UK) ad libitum. Sixteen were given PB in the drinking water (1000 ppm) and the remaining 16 controls were given normal tap water. At the euthanization time of 2, 4, 8, and 12 weeks after the commencement of PB treatment, groups of 4 PB and 4 control animals were euthanized in a rising concentration of carbon dioxide.

Necropsy and tissue processing. At necropsy all livers were weighed and the volume of the liver was measured by the standard fluid displacement principle of weighing the organ suspended in saline (Weibel, 1979). Representative 3-mm slices of the major liver lobes were prepared and the cross sectional areas of the slices were recorded using the NIH Image program (Dr. Wayne Rasband, National Institutes of Health) so that the wet area was known before fixation and processing for histology and the volume of the liver was measured by the standard fluid displacement principle of weighing the organ suspended in saline (Weibel, 1979).

Measurement of hepatocyte nuclear profile density. The calculation of the total number of hepatocytes in each liver was carried out from the estimation of the nuclear profile density measurements. The average of the zone 1 and 3 nuclear profile estimates was used to derive the mean number of nuclear profiles per unit volume for each animal.

Determination of hepatocyte nuclear profile density. This was carried out by capturing two images of an H&E-stained section of liver, separated by 4 μm vertically through the section, using the NIH Image program. The two images were placed in a stack using the same program and viewed consecutively, so as to determine the number of nuclei that were present in the reference but not the look up image using an unbiased counting frame (Gundersen, 1978; Gundersen et al., 1988) overlaid on the image. The principle of the optical dissector is that the determination of the number of such nuclear profiles, which are visible in one image and not the other, determines the number of such profiles in the volume of tissue determined by the area of the frame and its depth (the distance between the two images, 4 μm) (Gundersen, 1978; Sterio, 1984).

The distance between the two captured images must be less than the diameter of the smallest feature being counted. A total of nine zone 1 fields and nine zone 3 fields, per animal, were examined in this way at a magnification of 330. Three fields were selected from zones 1 and 3 of each of the three main liver lobes per rat (the left lateral, the median, and the posterior lobes) and the optical dissector pairs, 4 μm apart were captured. The stacked images were used to determine the number of nuclear profiles in the volume defined by the two fields.

The nuclear profile density per unit volume can be determined using the optical dissector from the standard relationship (Ma et al., 1995)

\[ N_s = \frac{N_o}{V_o} \]

where \( N_s \) is the number of nuclear profiles per unit volume, \( N_o \) is the number of nuclear profiles in the dissector volume of the section, and \( V_o \) is the volume of the dissector. The number of nuclei in the volume of the dissector was determined using an unbiased counting frame and the counting rule outlined by Gundersen to obtain an unbiased estimate of the number of profiles per unit area (Gundersen, 1978; Gundersen et al., 1988). The mean number of nuclear profiles per cubic millimeter per zone was then derived. After individual corrections for tissue shrinkage, the mean nuclear profile density for zones 1 and 3 per unit volume of liver was derived for each treatment group of four animals (Table 1). The number of fields to be examined was determined, initially, by increasing the number of measurements until the coefficient of

<table>
<thead>
<tr>
<th>Treatment and time</th>
<th>Mean, Zones 1 and 3</th>
<th>Zone 1</th>
<th>Zone 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 2 weeks</td>
<td>3.2 ± 0.07</td>
<td>3.2 ± 0.06</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>PB, 2 weeks</td>
<td>2.7 ± 0.17</td>
<td>3.2 ± 0.2</td>
<td>2.2 ± 0.15a</td>
</tr>
<tr>
<td>Control, 12 weeks</td>
<td>3.7 ± 0.2</td>
<td>3.9 ± 0.15</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>PB, 12 weeks</td>
<td>3.1 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Significantly different from the zone 1 value at the 5% level.

a Significantly different from the control 12 weeks value at the 5% level.

Downloaded from https://academic.oup.com/toxsci/article-abstract/44/1/46/1708132 by guest on 09 April 2019
Determination of volume changes in liver tissue due to processing and slide preparation. By also measuring the areas of the H&E-stained sections prepared for nuclear profile density counts, it was possible to determine the relative changes in the size of the liver tissue from its unfixed state (where the total volume and unfixed slice area had been measured), to the section area on which the morphometry measurements of nuclear profile density were made. This allowed a correction for processing shrinkage and slide preparation to be made in the final calculation of cell numbers, assuming the changes in tissue volume to be isotropic, so that area corrections were equivalent to volume corrections. If \( l_s \) and \( l_o \) are characteristic lengths of the shrunken and original tissue block, then the original volume \( V_o \) is determined from the relationship

\[
V_o = V_s l_s^{-3},
\]

where \( V_s \) is the volume after shrinkage (Weibel, 1979). This correction for tissue shrinkage was applied in the determination of the total number of hepatocytes per liver.

Determination of the relative proportion of binucleate hepatocytes. This determination was carried out by examining liver sections stained with H&E using an oil immersion objective and a 550-nm Olympus filter which allows visualization of the hepatocytes plasma membranes, so that the mononuclear and binuclear hepatocyte numbers could be determined (Carthew et al., 1997).

Measurement of the volume fraction of hepatocytes in the liver. The volume fraction of the treatment and control livers was also determined to ensure that there were no significant changes in the percentage of volume of hepatocytes due to edema or selective cell toxicity and proliferation (Carthew et al., 1997).

This was carried out by point counting to derive the area fraction of hepatocytes in the liver and applying the principle of Delesse, which states that for large numbers of sections, the volume fraction and the area fraction are identical (Weibel, 1978). Eighteen fields were examined (6 from each of three lobes) at a magnification of 120, to determine the area fraction (volume fraction) of hepatocytes in each liver.

Calculation of the hepatocyte cell number per unit volume. Once allowance has been made for the changes that can occur in the numbers of binucleated hepatocytes, and estimates were obtained for the mean numbers of nuclear profiles in a measured volume of liver tissue, the calculation of the number of hepatocytes per unit volume was determined from the volume density of nuclear profiles, also corrected for binularity and tissue shrinkage and adjusted for the volume fraction of hepatocytes in the liver. Hence, total liver hepatocyte number = number of nuclei/unit volume X liver volume X \% of hepatocytes in liver volume (corrected for binuclearity) X correction for shrinkage during processing.

Estimation of growth fraction of hepatocytes by PCNA labeling index (LI), as an indicator of hyperplasia. To independently determine whether the increase in liver size was due to cell replication and division, the PCNA labeling indices for all treatment groups and controls were determined as described previously (Carthew et al., 1995).

Paraffin sections (5 μm) from Carnoy’s-fixed liver were rehydrated. For PCNA detection, a monoclonal mouse anti-PCNA antibody was used (1:25 dilution, Novocastra, Newcastle upon Tyne, UK) followed by a rabbit anti-mouse IgG2a peroxidase-conjugated antibody (1:50 dilution, Serotec, Oxford, UK). All procedures were carried out at room temperature. Immunoreaction was visualized using 3,3’-diaminobenzidine/H₂O₂ substrate. Sections were lightly counterstained with hematoxylin. Sections of duodenum, processed at the same time, served as positive controls. At least 2500 hepatocyte nuclei were examined on each section to derive the percentage LI.

Statistical analyses. The results of the quantitation of hypertrophy and hyperplasia were expressed as mean values ± the standard error of the means (group size 4), and analyzed by Student’s t test for significant treatment-related effects at the 5% level.

RESULTS

Indicators of Hepatomegaly

The mean liver weights of rats given PB in the drinking water were increased significantly, compared to the corresponding control at all times examined after the start of treatment, except for the 2-week time point (Fig. 1). The density of liver tissue did not change significantly with treatment or time (Table 2).
TABLE 2
Summary of the Changes in Liver Density, Number of Hepatocytes per Gram of Liver, and Total Number of Hepatocytes per Rat, between 2 and 12 Weeks with Phenobarbital Treatment

<table>
<thead>
<tr>
<th>Treatment and Time</th>
<th>Density (g/cm³)</th>
<th>Mean No. of Hepatocytes/g of Liver $\times 10^8$</th>
<th>Mean Total No. of Hepatocytes/rat $\times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 2 weeks</td>
<td>1.08 ± 0.004</td>
<td>2.1 ± 0.06</td>
<td>20.5 ± 0.5</td>
</tr>
<tr>
<td>PB, 2 weeks</td>
<td>1.06 ± 0.002</td>
<td>1.8 ± 0.14</td>
<td>20.9 ± 2</td>
</tr>
<tr>
<td>Control, 12 weeks</td>
<td>1.08 ± 0.002</td>
<td>2.4 ± 0.14</td>
<td>24.3 ± 1.7</td>
</tr>
<tr>
<td>PB, 12 weeks</td>
<td>1.07 ± 0.007</td>
<td>2.1 ± 0.11</td>
<td>30.8 ± 1.5*</td>
</tr>
</tbody>
</table>

* $P = 0.027$ compared to control mean value at 2 weeks.
* $P = 0.037$ compared to control mean value at 12 weeks.

Indicators of Hypertrophy

To determine whether hypertrophy was occurring to a measurable extent the nuclear profile density volume was determined for zones 1 and 3 of the livers, based on the observation that the hypertrophy associated with PB exposure affects zone 3 preferentially. As zone 1 was unaffected in the PB animals compared to the controls (Table 1) comparing of the nuclear profile density per unit area of zone 3 and zone 1 within a group of PB-treated animals allowed the degree of hypertrophy to be evaluated. Although it was not necessary to correct for tissue shrinkage, this was carried out for the sake of consistency, since it was required for the comparison of the effects between treatment groups. The control groups at 2 and 12 weeks did not show a significant difference between their nuclear profile densities in zones 1 and 3 (Table 1).

PB treatment caused a significant increase in zone 3 hypertrophy at both 2 and 12 weeks of treatment (Table 1), and this was consistent with the observations at the light microscope.

The zone 3 hypertrophy at 12 weeks was great enough to make the overall mean hepatocyte nuclear profile density significantly different to the control value at this time (Table 1).

Indications of Hyperplasia

The calculation of the mean number of hepatocytes per gram of tissue did not demonstrate any significant differences between the PB and control values at either 2 or 12 weeks in the study (Table 2). However, the mean total hepatocyte number, although not significantly different at 2 weeks, was significantly different at 12 weeks after PB exposure (Table 2). The PCNA labeling indices for hepatocytes were also increased at all time points, with respect to the corresponding control values (Fig. 2).

DISCUSSION

The distinction of the relative contribution of hypertrophy and hyperplasia to hepatomegaly has been a perennial problem in rodent toxicology. Previous studies with compounds such as PB, dioxin, and the peroxisome proliferators have
been limited by the qualitative description of hypertrophy, by zone, in the liver. A previous attempt to resolve this was successful in demonstrating that there was an important contribution of both hypertrophy and hyperplasia to the 20% hepatomegaly seen with the peroxisome proliferator gemfibrozil in the rat (Carthew et al., 1997). This method relied on the measurement of the section thickness, for which the confocal microscope was used. As this is not a technique that is available to all experimentalists, the present method was developed. All of the measurement used in the present study can be achieved with standard microscopes and relatively standard image capture and analysis equipment. The reason that this technique, in particular, can be applied is because of the advent of what has been described as the new stereology which uses the principles of unbiased counting (Gundersen, 1978; Gundersen et al., 1988) along with the simple and elegant concept of the optical dissector, to derive profile density measurements simply and reliably (Sterio, 1984; Cruz-Orive and Weibel, 1990).

Unfortunately the method cannot be reliably applied to archival tissues, even where the liver weight was recorded at autopsy. This is because the shrinkage factor between euthanasia and initial fixation will not be known, and this has been found to be the largest and most variable factor that needs to be corrected for (Carthew et al., 1997) in the calculation of the total number of hepatocytes.

For the case of PB-induced hepatomegaly, the results obtained by combining these techniques in the present methodology have given reliable and statistically demonstrable contributions for both hypertrophy and hyperplasia. Thus treatment-induced liver enlargement can be attributed, in part, to an increase in the absolute number of hepatocytes present. The technique will be useful in discriminating whether true hyperplasia occurs with hepatomegaly in rodent studies and contribute a more meaningful endpoint, other than the labeling indices that are commonly used for surrogate markers of cell proliferation, which in fact they are not. The incorporation of BrdU in particular is commonly used as a method of assessing DNA synthesis and hence "DNA at risk" in carcinogenicity studies. However, cells synthesizing such new DNA may not undergo cell division, and therefore the mutation that may have occurred during transcription may not be fixed for subsequent clonal expansion. The value of the technique presented here is that it gives unequivocal results in terms of whether there is a real increase in the number of cells in the liver, which is a true indicator of hyperplasia.

ACKNOWLEDGMENTS

The authors thank Jennifer Edwards and Linda Wilkinson for histological preparations and Dr. Michael Festing for advice on statistical analyses.

REFERENCES


