Cell Cycle Kinetics of Rat Hepatocytes in Early Putative Preneoplastic Lesions in Hepatocarcinogenesis

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ABSTRACT

This set of experiments is the first of a series designed to explore facets of cell proliferation of hepatocytes during the carcinogenic process induced in liver by chemical carcinogens. A rat model for hepatocarcinogenesis, the resistant hepatocyte model, was used. A major advantage of this model is the unusual degree of synchrony in the early steps. Carcinogenesis was initiated by the administration of a necrogenic dose of diethylnitrosamine. Resistant hepatocytes so induced were stimulated rapidly to proliferate by partial hepatectomy in the presence of a brief exposure to dietary 2-acetylamino fluorene sufficient to inhibit the proliferation of the majority of hepatocytes, the nonresistant population. Cell cycle parameters were measured in the early carcinogen-altered resistant hepatocyte populations and in regenerating hepatocytes. Growth fraction and doubling time were experimentally determined in the altered hepatocytes. The mean cell cycle length of the resistant cells was 38.6 hr, somewhat longer than that of regenerating hepatocytes, which was 33.6 hr. Most of the increase was due to a prolonged S phase which was 13.8 hr in the altered cell population as compared to 7.0 hr in hepatocytes in regenerating control liver. The hepatocytes in normal regenerating liver had a mean duration of 21.6 hr for G1, as compared to 20.4 hr for the altered hepatocytes and a G2 of 3.4 hr as compared to 3.0 hr for carcinogen-altered hepatocytes. M was assumed to be 1.6 hr in both populations. The growth fraction in the altered cell population was determined to be a minimum of 0.83, and the doubling time was about 45 hr. Thus, the resistant hepatocytes which represent an early putative preneoplastic population show, in general, a prolongation of the cell cycle, mostly due to a prolonged S phase.

INTRODUCTION

Alterations in the expression and control of cell proliferation are among the most prominent phenotypic manifestations of hepatocytes during the development of hepatocellular carcinoma (1, 4, 9, 10, 13, 15, 17, 23, 24, 27, 29, 30, 35, 43, 44). Involvement of cell proliferation in overall cancer induction with several carcinogens (4, 24, 29, 43) as well as an intimate involvement in the initiation process (3, 5, 14, 21, 32, 37, 41, 45–47) and in promotion (16, 20, 21, 24, 30, 34, 36, 37) have been documented. However, cell cycle kinetics, despite its obvious importance in carcinogenesis, has been the subject of relatively little study (1, 13, 15, 16, 23, 27, 35). Because of the availability of newly developed models for liver carcinogenesis (5, 8, 9, 21, 44), it is now possible to analyze the presumptive precursor populations at the different stages of carcinogenesis with respect to parameters of cell cycle kinetics.

The resistant hepatocyte model was chosen for this study because it provided analyzable cell populations that are homogeneous with respect to their stage in the hepatocarcinogenic sequence (8, 36–38, 41). In this experimental model, initiation is achieved by a necrogenic dose of one of several hepatocarcinogens, such as DENA (37, 38) or by a nonnecrogenic dose of one of many carcinogens coupled with PH (2, 41, 45). This procedure induces rare resistant hepatocytes which can be selectively stimulated to proliferate by providing a mitogenic stimulus, such as PH, in the presence of a level of dietary 2-AAF sufficient to inhibit the proliferation of the uninitiated nonresistant hepatocytes. This regimen leads to the vigorous proliferation of the rare initiated resistant hepatocytes to form visible hepatocyte nodules within 10 days. Thus, it becomes possible to identify and study the early foci of altered hepatocytes that were induced during initiation. Many of these nodules will remodel to form normal-appearing liver, and a few will persist (6, 40). It is important to note that this brief selective stimulation of altered hepatocytes to form nodules has a strong promoting effect on the development of hepatocellular carcinoma when DENA, 1,2-dimethylhydrazine, or benzo(a)pyrene are used as initiating carcinogens (36). Also, hepatocellular carcinoma (38) and other less advanced new cell populations have been found within the confines of persistent nodules (8). Thus, a continuity has been seen between resistant hepatocytes, hepatocyte nodules, persistent hepatocyte nodules, and hepatocellular carcinoma (8, 22, 40), justifying the designation of foci and nodules of resistant hepatocytes, as a population, as early putative preneoplastic lesions.

Parameters of cell cycle kinetics, such as duration of cell cycle phases, growth fraction, and doubling time of hepatocytes in these early preneoplastic lesions are compared with those of regenerating hepatocytes. The results form the subject matter of this communication.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (Charles River Breeding Laboratories, Kingston, NY) initially weighing 150 to 180 g were used. The rats were maintained on a chow diet (Purina Laboratory Chow 5001).

Experimental Designs. All the experiments follow an experimental regimen which is based on the model described by Solt and Farber (37). Rats were given a single dose of DENA (200 mg/kg i.p.) (Eastman-Kodak Co., Rochester, NY). Two weeks later, the animals were fed 2-AAF (0.02% in semisynthetic Diet 121; Dyets, Inc., Bethlehem, PA) for 2

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Determination of Cell Cycle Length and Duration of Cell Cycle Phases. These parameters were determined by using a modified RFLM method (26, 28). The protocol is outlined in Chart 1. One hundred and twenty rats were randomly separated into 2 groups of 60 each. One group was placed on the experimental regimen and the other on a control regimen. These were on the same experimental regimen as outlined in Chart 1. On Day 4 after PH, all rats received an i.p. implant of an osmotic minipump (α). At 1, 4, 12, 24, 48, and 72 hr after implantation, groups of animals were killed and their livers examined as described in the text. dThd, thymidine.

The livers were removed, and samples were fixed in formalin for autoradiography. Autoradiography was performed by the dipping method, using Kodak NTB2 emulsion and exposure of the slides for 1 week at 4°C. After development, the slides were stained with hematoxylin and eosin for microscopic examination. About 100 mitotic figures from the focal populations were counted from each sample. These were scored as labeled when the number of silver grains was more than 3-fold higher than that of the equivalent background.

RFLM curves were constructed and analyzed with the use of the computer program of Macdonald et al. (11, 18, 19). In this program, the phases of the cell cycle were assumed to have a γ-distribution. In addition, the curves were evaluated by the use of the Quastler and Sherman method (25).

Growth Fraction. The fraction of proliferating cells in a population is called the growth fraction. This parameter was determined in the foci of proliferating hepatocytes by a continuous-labeling method. The animals were on the same experimental regimen as outlined in Chart 1. Four days after PH, all rats received an i.p. implant of an osmotic minipump [Model 2001 Alzet (Alza Corp., Palo Alto, CA)] filled with 1.25 mCi in 200 μl of [methyl-3H]thymidine (specific activity, 77.2 Ci/mmol). The osmotic minipump has a capacity to deliver 6.25 μCi/μl/hr for 7 days. At 1, 4, 12, 24, 48, and 72 hr after implantation of the osmotic minipump, groups of animals were sacrificed. The procedure is outlined in Chart 2. The livers were excised, and samples were prepared for autoradiography, as in the previous experiment. The labeling index of the focal population was determined by counting at least 1000 focal hepatocytes in each animal, and the growth fraction was calculated according to the method of Steel (39).

Doubling Time. Rats on the experimental regimen (Chart 1) were sacrificed at various times after partial hepatectomy, as indicated in Chart 5. The area of the 3 largest γ-GT-positive foci in each section was measured with the use of an image analyzer (Hewlett-Packard Digitizer 9874A).

RESULTS

The RFLM curves are plotted in Chart 3. From these curves, the values for the various cell cycle parameters were calculated by 2 methods, that of Macdonald et al. (11, 18, 19) with a computer program and that of Quastler and Sherman (25). As
Experimental Time (hours) Charts. Top, RFLM curve of developing nodule population; bottom, RFLM curve of regenerating hepatocyte population. Both curves were constructed and analyzed by the computer program of Macdonald et al. (11, 18, 19). Each point, RFLM value from one animal derived from about 100 mitotic figures.

Table 1

<table>
<thead>
<tr>
<th>Population</th>
<th>Phase of cell cycle</th>
<th>Macdonald et al. method (11, 18, 19)</th>
<th>Quastler and Sherman method (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developing nodule</td>
<td>G₁</td>
<td>20.4 ± 3.7a</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>13.6 ± 8.5</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>G₂</td>
<td>3.0 ± 0.9</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1.6f</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;c&lt;/sub&gt;</td>
<td>38.5 ± 9.3</td>
<td>41.2</td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>G₁</td>
<td>21.6 ± 20.0</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>7.0 ± 2.6</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>G₂</td>
<td>3.4 ± 0.9</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1.6</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;c&lt;/sub&gt;</td>
<td>33.8 ± 20.2</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- Mean ± S.D., derived from measurements of 10 to 60 foci at each time point.
- a-S-phase of developing nodule vs. S-phase of regenerating liver, p < 0.0001.
- f Fixed value.
- NA, not available. This was used when phase duration and T<sub>c</sub> could not be derived.

The second peak of the fraction-labeled mitoses curve is much more ill-defined under these circumstances.

The great majority of the hepatocytes in the developing nodules are in a proliferative phase, as judged by the calculation of the growth fraction (Chart 4). The shape of the continuous-labeling curve seems reasonable, assuming the durations of the phases of the cell cycle are correct and that there is no significant cell loss. The curve approximates a plateau at a time about equal to the combined durations of G<sub>2</sub>, M, and G₁. This result is consistent with the assumption that the last cell to be labeled would require this period of time in order to be labeled.

The calculation of doubling time for the developing hepatocyte nodules was made by converting the values for area into volumes. This was based on the assumption that the nodules were spheres. As an approximation, this was reasonable because foci, in this model, are nearly circular. Also, in this study as in previous studies with the resistant hepatocyte model (e.g., Refs. 20 and 38), the foci enlarge in a remarkably synchronous fashion such that the developing nodules vary little in diameter. Despite this reservation, the observed and calculated values agree remarkably well (Chart 5). The number of cells per lesion volume was calculated by dividing the volume of a focus by the volume of a single cell (Table 2). The theoretical plot started from an origin derived from the observed points. The subsequent points were calculated on the basis that every 38.6 hr (the cell cycle time), the population increased by 83%, the growth fraction. There was no significant difference between the theoretical and the observed plots. It can be concluded then that the experimentally derived values for the cell cycle length and growth fraction do

![Chart 3. Top, RFLM curve of developing nodule population; bottom, RFLM curve of regenerating hepatocyte population.](image)

![Chart 4. Labeling index at various times after implantation of osmotic minipump.](image)

![Chart 5. Observed nodule volume and cell number (○) are compared to theoretical values (O) with time after PH.](image)
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Table 2
Increase in developing nodule size with time

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Area (sq mm)</th>
<th>Volume (cu mm)</th>
<th>Number of cells ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.06 ± 0.021*</td>
<td>8.58 ± 2.15b</td>
<td>780 ± 195c</td>
</tr>
<tr>
<td>38.6</td>
<td>9.97 ± 0.047</td>
<td>23.7 ± 7.25</td>
<td>2155 ± 659</td>
</tr>
<tr>
<td>77.2</td>
<td>NA±</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>115.8</td>
<td>16.6 ± 0.09</td>
<td>50.7 ± 20.6</td>
<td>4605 ± 1873</td>
</tr>
<tr>
<td>154.4</td>
<td>30.8 ± 0.11</td>
<td>129.0 ± 28.7</td>
<td>11685 ± 2609</td>
</tr>
</tbody>
</table>

* Mean ± S.D. x 10².
b Mean ± S.D. x 10⁻⁴.
± Mean ± S.D.
± NA, not available.

The times in the tables are multiples of the cell cycle time of the altered hepatocytes (38.6 hr). The values for areas are the mean of the 3 largest focal areas in each section from every animal killed at that time point. These values were directly measured from γ-GT-stained liver sections, with the use of an image analyzer. The volumes were calculated from the areas, by assuming that the lesions were circles (πr²), and the volumes were spheres (4/3πr³). The number of cells observed per volume was derived by dividing the volume of a nodule by the volume of a single cell (1.1 x 10⁻⁵/cu mm). The theoretical values were derived by extrapolating the observed data from each observed point back to zero time, then averaging these values. The averaged value was then used with the cell cycle time and the growth fraction to construct the theoretical curve.

DISCUSSION

The results demonstrate that the mean duration of the cell cycle of the hepatocytes in the carcinogen-induced foci, during the promotion phase of this model, is longer than that of the normal regenerating hepatocytes. This increase is due to a prolongation of the S phase, since G₁, G₂, and M in the 2 hepatocyte populations do not differ greatly.

PH is associated with partial synchronization of the cell population, an effect that is known to influence the shape of the curves in the “fraction-labeled mitoses” method for measurement of cell cycle parameters using the labeling index. This complication is minimized by the use of the RFLM method (28) since it allows a choice of time of sacrifice in which the synchronization of cell cycle events is diminished (28).

The computer program used in this study for the analysis of the RFLM curves has been compared with other programs, and the values for the lengths of the phases of the cell cycle for standard cell systems are quite similar (11). This is consistent with the results of this study, in which the derived mean values for the duration of the phases of the cycle for regenerating liver are similar to those in the literature for animals of comparable age (28).

The reasons for the lengthened S phase in the nodule hepatocytes are not known. Some of the possibilities are: (a) this is a basic property acquired by the carcinogen-induced resistant hepatocytes during initiation or selection. Consistent with this possibility is the observation that the S phase is prolonged in at least one hepatoma induced by a carcinogen (23); (b) the dietary 2-AAF could conceivably be interacting with the DNA (7) or other cell constituents to a degree that prolongs the cell cycle but still allows cell proliferation in response to the mitogenic stimulus provided by PH. 2-AAF and its active metabolite, N-hydroxy-2-AAF, are known to prolong DNA replication time in the liver (48); or (c) a selective increase in DNA, such as by gene amplification, could conceivably prolong the S phase. Gene amplification is a known mechanism for the development of at least one type of resistance, that to methotrexate (33). This type of genomic modification has been suggested as possibly playing some role in the carcinogenic process (42).

To our knowledge, the only other study on cell kinetics of carcinogen-induced altered hepatocytes during liver carcinogenesis was by Rabes and Szymkowiak (27). The model that they used was that of continuous exposure to DENA in the drinking water. At 20 days after the start of the exposure to DENA, the lengths of G₁, S phase, and G₂ were 55.6, 9.6, and 3.9 hr, respectively. At 118 days, the corresponding values were 19.4, 11.6, and 3.8 hr. Thus, with the continuous-DENA model, the G₁ period early in carcinogenesis is very much more prolonged than it is with the resistant-hepatocyte model. Since, in both studies, the measurements were made at times of exposure to an exogenous factor (DENA or 2-AAF), it is conceivable that a major basis for the differences resides in different effects of 2-AAF or DENA on hepatocytes as related to their cycling. In addition, in the present study, the early nodule population is still under the influence of an intense proliferative stimulus, namely PH.

Another aspect of considerable importance to the growth of foci and nodules is cell loss. The failure to find a major difference between the observed and theoretical doubling times suggests that cell loss is not a large quantitative factor during the time interval studied. This conclusion seems at variance with that of Schulte-Hermann et al. (34) using a quite different model. They found no progressive growth of early foci of γ-GT-positive hepatocytes, even though the labeling indices with [³H]thymidine were as high as 30%. Since, in their model, the foci generally show only very slow and unpredictable development into nodules in contrast to the rapid development of nodules in the resistant hepatocyte model, it is conceivable that the biological history of the altered hepatocytes in the foci in the 2 systems may be quite different, at least in some respects.

The relationship of the findings in this study to the ultimate development of cancer is not clear at this time. In this model, as in many others of liver and elsewhere (9), the early consequences of exposure to an initiating dose of a carcinogen and the expansion of the initiated cells is the development of many focal proliferations (nodules, papillomas, polyps, etc.). In the rat liver, as many as 10³ such small nodules (islands or foci) per liver have been observed frequently (21, 32). In the resistant-hepatocyte model, a very tiny subset, no more than 1 to 2% of such nodules, persist (6, 40) to act as precursors for subsequent steps leading to the appearance of hepatocellular carcinoma. Even though a reasonable sampling of foci and nodules was made in this study, given the low probability of persistence, very few, if any, would probably be of the persistent disposition. It is, therefore, conceivable that what has been studied in these experiments does not relate to the ultimate cancer but only to the early steps in the carcinogenic process. The examination of the persistent nodule and the comparison between the results of experiments with such nodules and those examined in the present study may obviously throw new light upon the linkage between early and later steps in the carcinogenic process as they relate to alterations in the expression and control of liver cell proliferation.

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