Cell Proliferation and Tumor Growth in Hepatomas 3924A


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SUMMARY

Attempts have been made to improve upon current methods used for determination of growth rates in this large series of hepatomas. The sum of the length plus width is now used. Change in tumor volume in 3924A with time has been determined by assuming that the tumors had a hemiellipsoidal configuration. A second-order polynomial, obtained by the method of least squares fit by the computer, was a satisfactory way to express changes of tumor volume with time. The volume-doubling time for 3924A was 5.5 days.

The mean cell cycle time for 3924A was calculated to be 28.2 hr on the basis of this method. The mean values for the different phases of the cell cycle were: \( T_G^1 \), 15 hr; \( T_G^2 \), 3.4 hr; \( T_S \), 9.4 hr; and \( T_M \), 0.4 hr. These median values are given also in that they may be a more realistic appraisal of the different phases of the cell cycle time than mean values. The potential doubling time was calculated to be 42.8 hr, the actual doubling time was 132 hr, the cell loss factor was 0.67, and the growth fraction was 66.3%.

The relative amounts of neoplastic to nonneoplastic tissue also are needed for a better quantitative understanding of tumor growth in this large series of hepatomas. The preliminary results of 9 sections from 5 different 3924A tumors, varying in weight between 1.6 and 12.4 g, have been obtained. The tumor tissue comprised \(68 \pm 3\%\) of the hepatoma; necrotic tissue was calculated to be \(13 \pm 2\%\); connective tissue was \(18 \pm 2\%\); and blood, was \(0.5 \pm 0.1\%\).

INTRODUCTION

Morris hepatomas have been used extensively by many institutions in this country, as well as in foreign countries, in basic studies of neoplastic transformation (16). In spite of the extensive biochemical, genetic, enzymatic, metabolic, and morphological research, no systematic study of the quantitative relationship between the rates of increasing tumor volume, tumor weight, cell proliferation, and cell loss has been made. In addition, no systematic study has been made of the quantitative relationships between neoplastic cells and fibrous connective tissue, necrotic tissue, and blood. These studies have been initiated to obtain more precise quantitative information about these parameters in this large series of hepatomas.

Information obtained from these studies may provide: (a) additional information about the mechanisms involved in the general problem of neoplastic growth; (b) quantitative information that may assist in the interpretation of the extensive biological, genetic, metabolic, enzymatic, and morphological studies; and (c) results that can provide the necessary control information for subsequent studies on the perturbation of these processes by irradiation, alone or in combination with chemotherapeutic agents.

The net growth rate of a self-reproducing cell population such as a tumor is determined by the interactions of several variables. One of the most prominent variables is the time necessary for a somatic cell to reproduce itself to make 2 daughter cells. This time interval has been defined as the cell cycle. A second important variable is the fraction of the total cell population that is actively reproducing itself; this has been defined as the growth fraction (7). Third, the net growth of a tumor cell population is affected both by the loss of individual cells by migration and by cell death; this has been defined as the cell loss factor (12). Finally, the overall change of tumor volume with time is dependent upon not only the relative amounts of different types of individual cells but also the relative amounts of structural constituents. Most of these growth parameters have been determined for the rapidly growing Morris Hepatoma 3924A.

MATERIALS AND METHODS

Animals and Labeled Thymidine Administration. Female ACI rats were inoculated bilaterally in the back with 3924A by Dr. Harold Morris in Washington, D. C., and then shipped to this laboratory. The rats were maintained under standard laboratory conditions. Measurements of each tumor were made 3 times weekly over the period of the study. On Day 17 after inoculation, 50 \(\mu\)Ci of thymidine-5-methyl\(^{3}\)H (3 \(\mu\)Ci/mM), at a concentration of 0.017 \(\mu\)M/ml in 0.9% NaCl...
solution were given to each rat by i.p. injection. All rats were given injections between 8:00 and 9:00 a.m. in order to avoid the introduction of error due to the daily oscillations in thymidine metabolism which have been reported by Potter (9). Two animals with bilateral tumors were used to establish each experimental point. Sacrifices were made every hr for the first 8 hr after the administration of thymidine, every 2 hr between 8 and 26 hr after injection, and every 4 hr between 26 and 60 hr after injection.

Autoradiographic Techniques. Tumor sections were fixed in 10% formalin for 72 hr and then stored in 70% alcohol. The sections were later embedded in paraffin, cut at 4 μ, and stained with hematoxylin and eosin. Kodak AR-10 stripping film was applied, and the slides were stored in black boxes at 4°C until developing, at which time they were developed in Kodak D-10 developer and fixed in a 20% solution of sodium thiosulfate. A 14-day exposure was necessary for the autoradiographs of the 4- through 16-hr tumors, and the time had to be increased to 28 days of exposure for the 30- through 34-hr points and to 42 days of exposure for the 40- through 70-hr points.

Three slides were counted for each tumor; approximately 750 cells/slide were counted to determine percent labeled cells; 50 mitoses/slide were used to determine percent labeled mitoses. This results in a total of approximately 2250 cells counted per tumor and 150 mitoses/tumor.

The growth rate for Hepatoma 3924A has been determined by the serial measurements of the length, width, and height of the tumors, made 3 times weekly over a 3-week period with vernier calipers. Measurements of length, width, and height of each tumor were also made immediately before and after sacrifice to determine the accuracy of the method of measurement of the tumor under the skin with measurements of the excised tumors. The tumors were then weighed in order to correlate the measurements of tumor dimension with the actual weights and volumes of the tumors.

Three different methods have been used to express the change in the dimensions of 3924A with time: (a) the sum of the length and width, as originally used by Morris and Wagner (8), to compare the growth rates of the different hepatomas; (b) the product of length X width of the tumors, which gives the change in the rectangular area enclosing the tumor with time (13); (c) volume calculated on the assumption that the tumors were hemiellipsoids according to the method of Dethlefsen et al. (4), where volume = [(4/3) x (1/2) x (w/2) x h]/2.

Determination of Various Cell Types in Several Different Morris Hepatomas. Sections of 3924A were stained according to the Masson (6) trichrome procedure. This staining regimen results in purple nuclei and tumor cell cytoplasm, green connective tissue, and red erythrocytes. Necrotic tissue is easily recognized because necrotic nuclei are pycnotic, stain very darkly, and are irregular in shape. The estimates were done by counting the number of squares in the eye piece grid that were occupied by a particular cell type. From 12 to 20 fields were evaluated in each section, and at least 9 sections of each tumor type were analyzed. If these same estimates had been made by counting each individual cell, a total of at least 3000 cells would have been counted for each tumor. The method used gives the percentage of the area or volume of the tumor which is occupied by a particular cell type rather than the number of each cell type.

Methods for Analysis of Percent Labeled Mitotic Curves. The mathematical analyses of the data were carried out in previous studies of Hepatoma H-35 tc, and the current studies of 3924A. Estimates of the different phases of the cell cycle have been analyzed by 5 different methods: fitting of data points individually to polynomials by the method of least squares (5); measurement of the time between the midpeaks of the 1st and 2nd cycles; averaging of the 50% intercepts of the 2 ascending limbs and the 50% intercepts of the 2 descending limbs (3); determination of the phase deviations, assuming a log normal distribution for the deviations of the phases (1); and determination of the area of the percent labeled mitotic curves to the first trough (15).

RESULTS

The changes of the sums of the lengths and widths of 32 tumors showed a linear increase. This linear function was extrapolated to 30 days in order to determine the rate of change of the sum of the length and width of the tumor in cm/month. This extrapolated value was determined to be 7 cm for 1 month of growth.

A linear fit by the computer with the least squares method was unsatisfactory for the product of length X width, as well as for volume changes in which the tumor was assumed to be a hemiellipsoid. Estimates of the tumor volume, based on the assumption that the tumor is a hemiellipsoid, were compared with measurements based on the product of length X width; the mean values of the 32 tumors were used in all determinations of changes of tumor volume with time. The changes in the product of length X width of tumor 3924A were plotted by means of the least squares fit by the computer.

A 2nd-order polynomial was also used to describe the changes in the tumor volume with time when a hemiellipsoid was used the equation for these changes if shown in Chart 1.
Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor weight (g)</th>
<th>Tumor tissue (%)</th>
<th>Necrotic tissue (%)</th>
<th>Connective tissue (%)</th>
<th>Blood (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>556 D</td>
<td>1.6</td>
<td>59</td>
<td>18</td>
<td>23</td>
<td>0.5</td>
</tr>
<tr>
<td>556 D</td>
<td>1.6</td>
<td>70</td>
<td>13</td>
<td>16</td>
<td>0.3</td>
</tr>
<tr>
<td>556 E</td>
<td>2.4</td>
<td>67</td>
<td>9</td>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td>556 E</td>
<td>2.4</td>
<td>57</td>
<td>14</td>
<td>30</td>
<td>0.08</td>
</tr>
<tr>
<td>556 A</td>
<td>5.5</td>
<td>71</td>
<td>4</td>
<td>24</td>
<td>0.2</td>
</tr>
<tr>
<td>556 C</td>
<td>8.2</td>
<td>74</td>
<td>11</td>
<td>16</td>
<td>0.4</td>
</tr>
<tr>
<td>556 C</td>
<td>8.2</td>
<td>62</td>
<td>21</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>556 B</td>
<td>12.4</td>
<td>75</td>
<td>18</td>
<td>7</td>
<td>0.3</td>
</tr>
<tr>
<td>556 F</td>
<td>12.4</td>
<td>78</td>
<td>11</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>Mean value</td>
<td>68±3</td>
<td>13±2</td>
<td>18±2</td>
<td>0.5±0.1</td>
<td></td>
</tr>
</tbody>
</table>

The relative amounts of tumor tissue, necrotic tissue, connective tissue, and blood were determined in 9 sections from 5 different tumors. They were arranged in ascending order of weight to see whether any changes in the relative amounts of the different tissues occurred. The preliminary results from 5 different 3924A tumors, varying in weight between 1.6 and 12.4 g, are shown in Table 1. Tumor tissue comprised 68% of the tumor, and necrotic tissue was 13%; the remainder of the tumor was connective tissue (18%) and blood (0.5%).

The lack of a distinct 2nd wave of mitoses for Hepatoma 3924A made graphic analysis unsatisfactory because of the inability to determine accurately either the peak of the 2nd wave or the characteristics of the 2nd wave with any degree of precision. A model of the mitotic cycle, with independent probability density functions for the durations of phases $T_{G2}$, $T_{S}$, and $T_{G1}$, proposed by Barrett (1) has been utilized for 3924A. The analysis of these data by this method is shown in Chart 2.

The mean cell cycle time for 3924A was calculated to be 28.2 hr on the basis of this method. The mean values for the different phases of the cell cycle were: $T_{G1}$, 15 hr; $T_{S}$, 9.4 hr; $T_{G2}$, 3.4 hr; and $T_{M}$, 0.4 hr. The median values were: $T_{G1}$, 12.7 hr; $T_{G2}$, 2.4 hr; $T_{S}$, 7.9 hr; and $T_{M}$, 0.4 hr.

**DISCUSSION**

**Tumor Measurements and Methods for the Correlation of Size, Weight, and Volume**

The rate of change of tumor value with time is one of the basic measurements for the study of tumor growth. The analysis of growth curves provides important theoretical and practical information for clinical and experimental oncology. Mathematical functions with 2 constants are used most frequently for such analyses. These functions include the 1st-order exponentials in which log volume increases linearly with time and an equation in which the diameter or cube root of volume is linear with time. More complex functions with 3 constants have been used also. Also, interest has revived recently in the Gompertz function by demonstration of its applicability to various experimental tumors.

Previous methods by Morris have used the time between tumor transfers as an index for the differences in growth rates. These ranged between 0.6 month for 3924A and 13 months for the slowest growing tumor, 7794B. When the increased size is computed from increases in the tumor dimensions, it is calculated to be 8.4 cm/month, and, for the slower growing 7794B, 0.5 cm/month. This estimate of 8.4 cm/month for 3924A is similar to the estimate of 7 cm/month obtained in this study.

These determinations are based only on the sum of changes of length and width of the tumor with time. They are, therefore, inadequate to describe changes in tumor volume with time. No systematic study has been made concerning the rates of increase in tumor volume and tumor weight in this large series of hepatomas, in spite of the general utilization of these tumors by many investigators. Obviously, these determinations are important in the overall problem of relating cell proliferation with tumor growth rate.

Methods similar to those used by Steel et al. (13) have been used to determine the closeness of correlation between the tumor weight obtained at sacrifice with the area enclosed by the tumor (product of length X width). The correlation coefficient between product of length X width with weight is very good. A correlation coefficient of 0.94 between weight and tumor surface was found for 129 tumors; the standard error was estimated to be ±1.19.
Experimentally Determined Volume-doubling Time

The actual volume-doubling time for 3924A was determined from the 2nd-order polynomial for the changes in tumor volume with time (Chart 1). The tumor volume, rather than tumor area, was used because it approximates more closely the actual changes of tumor volume with time. A cell kinetics study was carried out on Day 17 after inoculation; therefore, \( t = 17 \) days in the following equations:

\[
V = 16t^2 - 131t + 325
\]

where \( V \) = volume in cu mm and \( t \) = time in days.

\[
V = 16(17)^2 - 131(17) + 325
V = 2722 \text{ cu mm on Day 17}
\]

By substitution into a quadratic equation and solving for \( t \), it was found that the tumor would double in size by 22.5 days; therefore, the tumor-doubling time at 17 days is 22.5 - 17, or 5.5 days.

The instantaneous volume-doubling time for Day 17 was determined graphically by extending the slope of the tangent to the curve on Day 17. The instantaneous volume-doubling time was also determined to be 5.5 days.

Plots of the changing tumor volumes with time were made on semilogarithmic charts. Exponential growth was not demonstrated, since there was a gradual reduction of the slope of the curve with time.

Mathematical Analysis of the Percent Labeled Mitotic Curves

Several means have been used to overcome the inherent difficulties of the original approach by Quastler and Sherman (10), the model of the mitotic cycle with independent probability density functions for the durations of the phases \( T_{G_1}, T_s, \) and \( T_{G_2} \) was proposed by Barrett (1). A Monte Carlo program for the computation of percent labeled mitoses in accordance with the model has been devised for a log-normal distribution of the phase durations (Chart 2).

Mitotic Time, Potential Doubling Time, Cell Loss Factor, and Growth Fraction for 3924A

Mitotic Time \( (T_M) \). The duration of mitoses can be calculated by

\[
\frac{T_M}{T_S} = \frac{M.I.}{L.I.}
\]

where \( T_M = \) duration of mitoses; \( T_S = \) duration of DNA synthesis; \( M.I. = \) mitotic index; \( L.I. = \) labeling index; \( T_M = \frac{0.7 \times 9.4}{17.6} = 0.4 \text{ hr} \).

Mean Cell Cycle Time \( (T_C) \).

\[
T_C = T_{G_2} + T_S + T_{G_1} + T_M
T_C = 3.4 + 9.4 + 15 + 0.4
T_C = 28.2 \text{ hr}
\]

Potential Doubling Time \( ([T]) \). Steel and Bensted (14) estimated the potential doubling times of tumors from the experimental data found on the labeling indices of various tumors and the time for DNA synthesis. A single equation is utilized to express this relationship based on the assumption that normal liver growth and the distribution of cell cycle times are invariant. The equation for the potential doubling time \([T]\) is:

\[
[T] = \frac{T_s}{L.I.}
\]

where \( T_s = \) time for DNA synthesis in hr and \( L.I. = 1\text{-hr labeling index} \).

For cell populations in other than linear growth, one must take into account the fact that the probability of finding a cell in different parts of the cell cycle is not constant. In the extreme case of exponential growth, for instance, the phase distribution diagram is an exponential function. The effect of this on the equation is to introduce a constant of proportionality \(\lambda\)

\[
[T] = \lambda \frac{T_s}{L.I.}
\]

where \(\lambda\) must be found from the shape of the phase distribution diagram. In the case of tumors, accurate values for \(\lambda\) are generally not known, but for a wide range of tumor-doubling times, an assumed value of 0.80 appears to be sufficiently accurate (within 10%). The estimated potential doubling time for 3924A, based on this equation and with the 0.80 value for \(\lambda\), is:

\[
[T] = \frac{(0.8 \times 9.4)}{17.6} = 42.8 \text{ hr}
\]

Actual Doubling Time. This value, taken from analysis of growth curves in the first part of the discussion, was 132 hr.

Cell Loss Factor \( \phi \). The ratio of cell loss to the rate of cell production gives the cell loss factor (12).

\[
\phi = 1 - \frac{T}{T_d}
\]

where \( T = \) potential doubling time and \( T_d = \) actual volume-doubling time.

\[
\phi = 1 - (42.8/132) = 1 - 0.33 = 0.67.
\]

Growth Fraction. Estimates of the proportion of proliferating cells in the tumor, which Mendelsohn (7) has called the growth fraction, can be made by determining the ratio of the percent labeled cells and the ratio of the experimentally determined 1-hr thymidine-labeling index to the predicted 1-hr thymidine-labeling index, in the following manner (12):

\[
\text{Predicted labeling index} = \lambda \frac{T_s}{T_C}
\]

where \( T_s = \) duration of DNA synthesis, \( T_C = \) duration of cell cycle, and \( \lambda = \) the constant of proportionality. The predicted labeling index for 3924A is:

\[
(0.8 \times 9.4)/28.3 = 26.6 \text{ T}
\]

Growth fraction = 1 hr thymidine-\(^3\)H labeling index/predicted labeling index

The growth fraction for Hepatoma 3924A is:

\[
\text{Growth fraction} = 17.6/26.6 = 66.3\%
\]
The following values were obtained for Hepatoma H-35 tc2, assuming a log-normal distribution for phase duration: \( T_{G_2} \), 4.2 hr; \( T_g \), 6.6 hr; \( T_{G_1} \), 11 hr; and \( T_M \), 0.4 hr. The total cell cycle time was calculated to be 22.2 hr, the growth fraction time was 53%, and the potential doubling time was 43 hr.

Sasaki et al. (11) have made similar studies on other hepatomas. The length of the cell cycle (\( T_C \)) and its phases and the growth fraction were studied in vivo in 3 transplantable rat hepatomas: a fast-growing one (5123 tc), one of intermediate growth rate (7794A), and a slow-growing one (7793). The cell cycle times and growth fractions for each of these tumors were as follow: 27 hr and 0.8 for 5123 tc; 49 hr and 0.42 for 7794A; and 234 hr and 0.4 for 7793. However, the values for 7793 are open to a considerable degree of uncertainty; all tumors showed marked variations in the cell cycles of individual tumor cells, especially in the \( T_{G_2} \) period. For instance, the length of \( T_{G_2} \) in Hepatoma 7793 varied from 5 to 40 hr. Determination of the percentage of neoplastic cells, as well as the percentage of fibrous connective tissue, necrotic tissue, and blood, was done. The relative amounts of neoplastic to nonneoplastic tissue in this large series of hepatomas is another basic measurement with regard to a better quantitative understanding of tumor growth. The actual measurement used in many instances is the tumor volume-doubling time, and it is sometimes used synonymously with cell population-doubling time. Different biological processes can operate to modify this assumption equating tumor volume with cell volume. The cell size distribution could change with time, or there could be an accumulation of intercellular connective tissue, blood, or cystic fluid. This would obviously make the tumor volume increase faster than the cell population, so the use of the volume-doubling time would underestimate cell loss.

In view of this lack of correlation between the different components of the tumor with increased weight, little can be said about the changing of these components with tumor growth. Other investigators (2) have shown that increased cell loss does occur in certain experiments in tumors with increased tumor size.

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