Cellular Kinetics of Invasive Squamous Carcinoma of the Human Cervix¹,²

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SUMMARY

The cell kinetics of two solid human tumors, invasive squamous carcinomas of the cervix, were studied by parenteral administration of tritiated thymidine followed by serial biopsy of the tumors at intervals for 24 hours, and subsequent radioautographic analysis for mean grain counts and percent labeled mitoses at these times.

In these two tumors, the majority of labeled cells were at the periphery of the tumor nodules (growth fraction of 41–53 percent). Tumor nodules appeared to grow by expansion from within, as newly formed cells migrated toward the centers of nodules. A significant proportion of interior cells (growth fraction of 17–20%) retained the capacity to divide, a function lost in the differentiation of normal epithelial cells, and thus were able to contribute to the total proliferative pool.

Cell cycle times were 15.5 and 14.3 hours, and DNA synthesis times were 11.8 and 9 hours. These times are not unlike those of normal mammalian cells, adding to the evidence that rapid tumor growth is not necessarily dependent upon rapid cell proliferation.

The observations that in these tumors: (a) the majority of dividing cells were at the periphery of nodules, (b) the growth fraction was reduced, i.e., maximum of 53 rather than 100 percent of basal cells were capable of dividing, and (c) periods of DNA synthesis were fairly long, points up the potential errors in estimating the cell cycle time from in vitro labeling studies.

The possibility that cells in the interior of tumor nodules may be arrested in mitosis is described, a factor which may prevent even faster growth than is already observed.

INTRODUCTION

By the use of radioactive precursors of deoxyribonucleic acid and high resolution radioautography, it has been demonstrated that the rate of cell proliferation in several rapidly growing tumors of experimental animals is no greater than the rates of cell proliferation in the corresponding normal tissues of the same animals (3, 17).

The purpose of this investigation was to study by similar technics the patterns of cellular kinetics in a rapidly growing human carcinoma, invasive squamous carcinoma of the cervix.

MATERIALS AND METHODS

Carcinoma of the cervix was chosen for study because this tumor is readily accessible for biopsy and subsequent treatment of the patient precludes further reproduction. Studies were done with in vivo labeling of the tumors because, in our experience (unpublished results), the growth of normal cervical epithelium in tissue culture is too inconsistent to obtain reliable results from analysis of endocervical tissue incubated in tissue culture fluid and sampled periodically after labeling.

Patients accepted as volunteers had been judged to have a shortened life expectancy on the basis of extensive tumor invasion, but were fully informed of the nature of the experiment. Sterile, pyrogen-free, tritiated thymidine (Nuclear Research Chemicals, Inc., Orlando, Florida) with an initial specific activity of 5 c/micromole was administered intravenously (0.1 microcurie per gram body weight in 125 ml of 5 percent dextrose and water). Biopsies were taken within 30 to 45 minutes after administration of thymidine-³H and then every three hours for 24 hours. The biopsy specimens were fixed in neutral buffered formalin, embedded in paraffin, and cut at 4 microns. Radioautographs were prepared using Kodak NTB 3 photographic emulsion and were poststained with hematoxylin and eosin.

The greatest number of labeled mitoses at each time interval and the majority of labeled nonmitotic cells were found in the first two rows of cells at the periphery of invasive tumor nodules. These cells are subsequently designated as basal cells; all other tumor cells within each tumor nodule are designated as interior cells. Tumor nodules are defined as the smallest, discrete subdivisions of the tumor formed as insular or peninsular structures surrounded by stroma. At each time interval, 6000 basal cells and 6000 interior cells were counted to obtain at least 200 mitoses for analysis in each category of cells.

RESULTS

In sections of normal cervix, which were included with the biopsy taken at 30 minutes from Patient 1, labeling in ectocervical cells was concentrated in the first two rows of cells adjacent to the underlying stroma (Fig. 1). Cells above these...
layers had lost the ability to incorporate thymidine-³H (synthesize DNA) and showed the usual signs of normal cervical epithelial cell differentiation, i.e., an increase in the amount of cytoplasm, a reduction in the size of the nucleus, a more rounded to polygonal cell shape, and a horizontal rather than perpendicular axis as compared to cells in the basal layer. Nuclei in columnar cells of normal endocervix included with these sections showed no labeling. In areas of in situ carcinoma, labeling was primarily concentrated in the basal cells, but labeled cells were also evident well above the basal layer, indicating that some cells which had left the basal layer had retained the capacity to synthesize DNA (Fig. 2). Other authors have reported similar findings based on in vitro labeling of in situ cervical carcinoma with thymidine-³H (10, 16, 40).

In foci of invasive carcinoma, labeling was present in basal and interior cells (Fig. 3). The percentage of basal cells and interior cells of tumor nodules synthesizing DNA in Patients 1 and 2 (labeling index) is shown in Table 1.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Basal cells</th>
<th>Interior cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient #1</td>
<td>Patient #2</td>
<td>Patient #1</td>
</tr>
<tr>
<td>Growth fraction</td>
<td>0.53</td>
<td>0.41</td>
</tr>
<tr>
<td>Labeling index</td>
<td>0.40</td>
<td>0.26</td>
</tr>
<tr>
<td>Mitotic index</td>
<td>0.045</td>
<td>0.03</td>
</tr>
<tr>
<td>$t_C$</td>
<td>$15.5 \pm 1.5 \text{ hr}$</td>
<td>$14.3 \pm 1.5 \text{ hr}$</td>
</tr>
<tr>
<td>$t_{G_1}$</td>
<td>1.3 hr</td>
<td>2.5 hr</td>
</tr>
<tr>
<td>$t_M$</td>
<td>1.3 hr</td>
<td>1.0 hr</td>
</tr>
<tr>
<td>$t_S$</td>
<td>11.8 hr</td>
<td>9.3 hr</td>
</tr>
<tr>
<td>$t_G$</td>
<td>1.1 hr</td>
<td>1.5 hr</td>
</tr>
</tbody>
</table>

Indices and times of cell-cycle components in invasive carcinomas of the cervix.

The length of the cell cycle ($t_C$) in the invasive carcinoma of both patients was estimated by plotting the percentage of labeled mitoses for basal cells against the time of biopsy and subsequently measuring the time interval between corresponding points on the first and second cycles. These plots are shown in Charts 1 and 2. Once estimates had been made for these cell cycle times (Patient 1, 15.5 hr; Patient 2, 14.3 hr), the extent of grain count dilution in mitoses for a similar time interval (15 hr) was determined. The 15-hour interval chosen was from 6 hours, when the majority of mitoses were labeled, to 21 hours. In both patients the grain count was approximately halved during this interval (Table 2). This finding lends support to our estimates of $t_C$ based on the plots of labeled mitoses.

An alternative method for approximating the cell cycle time ($t_C$) is by the use of the following equation:

$$t_C = \frac{t_S \times N}{nS}$$

where $t_S$ is the DNA synthesis time (the time interval between the 50% point on the ascending and descending limb of the first wave of plotted mitoses; Table 2), and $nS$ is the percentage of cells in DNA synthesis (percentage of labeled cells among all cells counted before the appearance of the first labeled mitosis; Table 1).

In using this formula, one assumes that the cells counted are all capable of cell division, i.e., $N = 100\%$. This assumption would have resulted in values 29.3 and 34.6 hours for the two cell cycle times, values nearly twice as long as obtained from plotting waves of labeled mitoses and from evaluating grain count dilution. The longer cell cycle times arrived at by the use of this formula are due to incorrectly assuming that 100% of cells counted were capable of proliferating. By rearranging the formula to solve for $N$,

$$N = \frac{t_C \times nS}{t_S}$$

we can obtain the actual percentage of cells capable of proliferating, i.e., the growth fraction (28), for basal and interior cells of both tumors (Table 1).

The other components of the cell cycle were obtained as follows: (a) Mitotic time ($t_M$), by the formula

$$t_M = \frac{\text{Mitotic index} \times t_C}{\text{Growth fraction}}$$

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Mean grain count reduction for basal mitoses, interior mitoses, and interior nonmitotic cells during an interval equivalent to one cell cycle.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean grain count</td>
<td>Basal mitoses</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient #1</th>
<th>6 hr</th>
<th>12 hr</th>
<th>17.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% reduction</td>
<td>47%</td>
<td>29%</td>
<td>44%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patient #2</th>
<th>6 hr</th>
<th>14.2</th>
<th>17.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% reduction</td>
<td>48%</td>
<td>23%</td>
<td>47%</td>
</tr>
</tbody>
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(b) Premitotic gap ($tG_2$), by measuring the time interval from the injection of thymidine-3H to the point where 50% of mitoses are labeled on the ascending limb of the first wave of plotted mitoses (equals $tG_2 + \frac{1}{2}tM$) and subtracting $\frac{1}{2}$ of the value of $tM$ obtained in the previous step. (c) Postmitotic gap ($tG_1$), by the formula

$$tG_1 = tC - (tG_2 + tS + tM)$$

The times for these components of the cell cycle for both tumors are given in Table 1. Because the peak of the second wave of mitoses cannot be fixed precisely, due to the 3-hour interval between biopsies, the values for $tC$ are given as ±1.5 hours.

In the tumor nodules of both patients, the maximum frequency of labeled mitoses for basal and interior cells was at 9 hours. The waves of labeled mitoses among basal and interior cells were synchronous within the same tumor (Charts 1 and 2), and while over 90% of the mitoses in basal cells and less than 45% of mitoses in interior cells were labeled at 9 hours, the plots of mitoses of basal and interior cells of the same tumor would be nearly superimposable if the curve for interior cells were scaled up to that for basal cells.

Among the unlabeled mitoses in interior cells at 9 hours, many were intact; others were clearly degenerating. Cells surrounding the unlabeled mitoses were generally intact, and many were adequately labeled. At the periphery of tumor nodules at this time, there were very few unlabeled basal cell mitoses, and unlabeled mitotic figures were intact.

**DISCUSSION**

This in vivo study provides information on the mechanisms by which invasive squamous carcinoma of the cervix grows. Conventionally, one thinks of a carcinoma invading into adjacent tissues by outward infiltration of newly proliferated cells. Indeed, certain rapidly growing sarcomas and carcinomas appear to behave in this fashion and have been described by Hamperl (13) as being of the invasive-destructive type. In the tumors reported here, the majority of malignant cells capable of dividing were in the first two rows of cells at the periphery of tumor nodules – the basal layer. However, instead of infiltrating centrifugally into the surrounding stroma, newly formed cells arising from the basal layer migrated toward the center of the tumor nodule causing the nodule to expand by pressure from within. Hamperl (13) also recognizes this type of growth in epithelial tumors and refers to it as expansive.

The conclusion that cells actually migrate toward the center of the tumor nodule is based on the following observations: (a) No unlabeled malignant cells were observed external to the labeled basal cells at the periphery of nodule. (b) Many telophase mitotic figures in the basal layer appeared to be forcing one-half of the labeled, dividing cell into the predominately unlabeled parabasal layer, but none were directed toward the exterior of the basal layer. (c) In most tumor nodules there was a progression of tumor-cell maturation from the periphery to the center of the tumor nodule, reminiscent of that seen in normal ectocervical epithelium. This pattern of growth has been described in the sarcomas of rats (14, 25) and in nonneoplastic tissue in hepatic lobules of partially hepatectomized rats (9).

In most normal epithelia studied to date (4, 16, 24, 33, 40), the predominance of labeling has been in the basal layer with only insignificant labeling in the parabasal and intermediate layers. In vitro labeling of cervical biopsy specimens has indi-
icated that as one moves along the histologic spectrum from dysplasia of the cervix to carcinoma in situ (10, 16, 40), there is an increased tendency for cells above the basal layer to incorporate thymidine-3H. They, therefore, represent potential proliferating cells above the basal layer. The fraction of labeled basal cells (labeling index) also has been reported to be significantly greater in dysplasia and carcinoma in situ of the cervix than in normal ectocervix (10, 16).

In the columnar cells of the normal endocervix, one rarely finds mitotic figures (21), and in vitro labeling studies have failed to reveal any significant degree of incorporation of thymidine-3H into the columnar cells (16, 39). The failure to find labeled nuclei in endocervical cells may be interpreted as indicating that these cells have extremely long G1 or G2 periods and require periodic stimulation to recommence proliferation. However, the negligible number of mitotic figures found in columnar cells of normal and pregnant women and lack of labeling in squamous metaplasia of the endocervix (16, 40) suggest that the columnar cell is completely differentiated, unable to divide, and is derived from an inconspicuous precursor cell not easily distinguished from adjacent stromal cells.

In the tumors reported here, the frequency of labeling of basal cells in foci of in situ carcinoma and in the adjacent areas of invasive carcinoma was greater than that found in the occasional fragments of normal ectocervix included with the biopsies. However, there was not sufficient in situ carcinoma or normal ectocervix to compare the labeling index of basal cells in in situ carcinoma with those of invasive carcinoma or to permit comparison of labeled interior cells in the areas of in situ carcinoma with those of invasive carcinoma. The fact that occasional cells in the interior of tumour nodules of invasive carcinoma are capable of division and that they divide synchronously with basal cells indicates that some regulatory mechanism maintains a similar rate of cell division throughout the tumor in the nodules of various sizes. The presence of labeled mitotic figures in interior cells also suggests a mechanism by which tumors grow rapidly.

The surface/volume ratio of a sphere decreases with an increase in its radius. Therefore, the contribution to the total proliferating pool of a small but constant percentage of dividing tumor interior cells increases relative to the number of basal cells on the surface (at the periphery) of a tumor nodule as the nodule grows.

As an example, a spherical tumor nodule 100 microns in diameter composed of cells 10 microns in diameter would contain 1,002 cells, 513 cells (51 percent) in the interior and 489 cells (49 percent) on the surface. A tumor nodule of twice this diameter (200 microns) would contain approximately eight times as many (8,017) cells with 5,845 cells (73 percent) in the interior and 2,172 cells (27 percent) on the surface.

While the growth rate of such a tumor nodule would be increased by virtue of the contribution of dividing interior cells, the combined effect of basal cells providing the greater proportion of dividing cells in early stages and interior cells providing the greater proportion of dividing cells in later stages of the growth of the tumor nodule could produce the effect of exponential retardation of the growth rate. Mitotic arrest in interior cells, as was observed in the tumors, would further enhance such retardation.

Exponential retardation of growth produces a Gompertzian growth curve (19). While we have no evidence that the growth of these tumors was Gompertzian, several studies have indicated that the growth rates of tumors of experimental animals are (5, 19, 26, 31, 42, 44), and in this sense this simple mathematical model suggested by our observations on these human epithelial tumors is consistent with what has been found in animal tumors.

Slowing of the growth rate of tumors generally has been ascribed to a reduction in the growth fraction (14, 29, 42) or a slowing of the rates of cell division (increase in the cell cycle time) as a result of the tumor outgrowing its blood or nutritional supply (12, 14, 20, 26). However, demonstrations of a lengthening of the cell cycle time in tumors have been based on studies of the Ehrlich ascites tumor (12, 20), a system which may not be at all analogous to solid tumors.

If the mathematical model for growth of epithelial tumors proposed here is applicable, it may be used to explain the slowing of growth without implicating a change in cell cycle time. That is, even if the cell cycle time and its components are fairly well fixed for an individual tumor, retardation of growth may be brought about by: (a) reduction in the growth fraction of basal and/or interior cells, (b) arrest of division of dividing basal and/or interior cells, and (c) changes in the average size that tumor nodules attain before budding or branching to form new tumor nodules.

Only occasional (approximately 5%) unlabeled basal cell mitotic figures were found at the time when the maximum number of labeled mitoses were present (9 hours), but many (approximately 55%) unlabeled interior cell mitotic figures were found at this time in tumor nodules. The majority of unlabeled interior cell mitoses were adjacent to adequately labeled cells, and, therefore, the lack of labeling was presumably due to the cells not being in DNA synthesis at the time thymidine-3H was administered, rather than to thymidine-3H not being available in the interior of tumor nodules.

The alternative possibility that unlabeled interior cell mitoses can be accounted for by over half of the cells in the growth fraction of the interior of the tumor nodules having extended G2 periods seems unlikely. If this were the case, the two populations of cells comprising the growth fraction of interior cells would result in great variability in the rate of passage of cells through the cell cycle. Since we did find a second wave of mitoses in the interior of tumor nodules in both tumors, and did observe that the grain count of interior nonmitotic cells was approximately halved in the length of one cell cycle (Table 2), it may be concluded that the unlabeled interior mitoses do not result from an extended G2 period.

Arrest of cells in mitosis is further supported by the findings that: (a) many of the unlabeled interior cell mitotic figures showed some evidence of degeneration, (b) the number of labeled mitoses did not exceed 50% in the interior of either tumor, and (c) while the grain count of basal cell mitoses halved in the time of one cell cycle, it was reduced by only 24–28% in interior cell mitoses, consistent with one-half of the interior cell mitoses being arrested in M for periods up to 1 cell cycle in length. In tissue culture studies using colcemid to produce mitotic arrest, cells blocked in mitoses for longer than 5–6 hours failed to complete mitosis (18).
The finding of cells arrested in mitosis in carcinoma of the cervix adds another phase to the cell cycle where the process of cell division may be arrested. Other investigators have previously reported arrest of cells in G$_2$ (1, 6, 8, 27, 28, 32, 41), G$_1$ (6, 8, 11, 30, 34, 37), and in certain cell lines, S (25, 45). It is now becoming generally accepted that the rapid growth of neoplasms is not due merely to the rapid proliferation of neoplastic cells (2, 3, 15, 17, 35, 36). Indeed, the cell cycle times reported here are not unlike those of a host of mammalian tissues, normal and neoplastic (2). If anything, there is a tendency for tumors to have a somewhat longer generation time than normal tissues.

Reports have also been made of human tumors with exceptionally long generation times, the majority of which are summarized in a review by Baserga (2). These studies employing the Quastler and Sherman (38) formula for estimating $t_C$ and based on in vitro labeling of the tumor contain at least two major sources of error. First, there is no way to determine the growth fraction in such material, and if the growth fraction were significantly reduced, as was observed in the tumors reported here and previously by others (5, 19, 26, 31, 44, 42), the generation time would be proportionally increased beyond its actual value. Second, one must use an estimate of $t_S$ to carry out this calculation in in vitro studies, and it has now been well established that $t_S$ is quite variable in neoplastic (7) as well as normal human cells (22, 43) so that any value chosen for $t_S$ could easily be off by several orders of magnitude.

While the late effects in humans of doses of thymidine-$^3$H sufficient for generalized labeling have not been fully discounted (31), it does appear that our knowledge of tumor cell kinetics for various human tumors will be greatly hampered until results of more in vivo studies are available. The possibility that alterations in the cell kinetics of a neoplasm following irradiation or chemotherapeutic treatment may aid in prognosis (16), or guide future therapy, makes such studies even more imperative.

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