Cell Kinetics of Head and Neck Cancers¹

Valery M. Kotelnikov,² John S. Coon IV, Abdul Haleem, Samuel Taylor IV, James Hutchinson, William Panje, David D. Caldarelli, Katherine Griem, and Harvey D. Preisler


ABSTRACT

We measured the tumor cell proliferative rate in 26 patients with head and neck cancer, 22 of which were squamous cell carcinomas (SCCs). Patients received sequential infusions of idodeoxyuridine and bromodeoxyuridine, after which the tumor was biopsied and studied. The percentage of labeled cells [labeling index (LI)] in well-differentiated SCCs was 20.4 ± 2.7% (mean ± SE) and 23.8 ± 2.1% in moderately differentiated SCCs (P = 0.135). The LIs of two poorly differentiated SCCs were 39.4 and 55.9%. The LI was 2.5% in a high-grade lymphoepithelioma and 24.8% in a malignant lymphoma. In one well-differentiated and one poorly differentiated mucoepidermoid tumor, the LIs were 3.0% and 29.1%, respectively. S-phase duration time measurements ranged from 5.1–21.5 h (12.8 ± 1.5). The calculated potential doubling times ranged from 18.8–84.5 h (47.3 ± 6.7). The duration of G₂ was between 90 and 180 min. To track the fate of labeled cells, in four patients a repeat biopsy was obtained 7–14 days after the idodeoxyuridine/bromodeoxyuridine infusion. These patients did not receive treatment between the biopsies. Due to the dilution of the label, most labeled cells in the second biopsy had undiluted label, suggesting that these cells had not divided after incorporating idodeoxyuridine/bromodeoxyuridine. On Day 7 labeled cells migrated to keratinized parts of tumors and to necrotic foci. Thus, the arrest of cell cycle transition, tumor cell differentiation, and cell death may be major routes of tumor cell loss from the proliferative compartment. This may explain the difference between very short potential doubling times and the actual rate of tumor growth.

INTRODUCTION

The proliferative rate of head and neck cancers plays an important role in determining the course of the disease and the outcome of treatment. Chauvel et al. (1) reported an inverse relationship between percentage of S-phase cells and patient survival. The preliminary results of two recent clinical trials (2, 3) have suggested that knowledge of proliferative rate prior to the initiation of radiotherapy may be used as a major parameter for selecting radiotherapy regimens and for predicting treatment outcome. In addition to the importance of the pretherapy proliferative rate, evidence has been presented that the proliferative rate of these tumors may accelerate during radiotherapy, permitting the neoplastic cells to rapidly regrow during treatment (4, 5). This regrowth may be responsible for a high proportion of treatment failures.

Earlier studies of head and neck tumor cell kinetics have utilized either in vitro DNA labeling with [³H]thymidine or have used FCM³ (6–9). In general, these methods underestimate the proportion of S-phase cells (10, 11). Begg et al. (12) introduced a method for measuring cell cycle parameters using an in vitro injection of BrdUrd with subsequent FCM of disaggregated tumor cells. During the last decade this method was widely applied to study cell kinetics in a variety of human tumors (2, 3, 11, 13–15). In spite of its popularity this methodology is not optimal as indicated by recent studies (11, 16, 17). Begg (18) pointed out two major problems. The first one is the admixture of nontumor cells in the disaggregated tumor biopsy, resulting in inaccurate determination of the proportion of tumor BrdUrd-labeled cells (LI). This problem can be avoided if an immunohistochemical detection of BrdUrd-labeled cells in a tumor biopsy section is used instead of FCM to measure LI. Bennett et al. (11) demonstrated that for diploid tumors FCM LI is nearly four times lower than LI measured in a biopsy section. The second problem concerns the accuracy of the Tₛ measurement which depends on the mathematical method applied (16, 17). The validity of Tₛ measurements only can be confirmed by using an alternative methodology (18).

The tumor cell kinetic studies in humans mentioned above led to a calculated potential doubling time which is shorter than the actual tumor volume doubling time. The difference is due to the cell loss factor. This factor in human head and neck SCCs was estimated to be between 85 and 93% (19, 20). Cell loss pathways have been well documented for experimental tumors (20), but yet have not been directly studied in humans.

In the study described here we used the method of in vivo labeling of cells with IdUrd and BrdUrd and subsequent immunohistochemical visualization of labeled cells, a method which

Received 8/16/94; accepted 1/20/95.
¹ Supported by funds from Rush Cancer Institute.
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³ The abbreviations used are: FCM, flow cytometry; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; LI, labeling index; SCC, squamous cell carcinoma; NCI, National Cancer Institute; FDA, Food and Drug Administration; Tₛ, S-phase duration time; Tₚ₀, potential doubling time; T₀, cell cycle time; T₂₀₂, duration of G₂.

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Table I
Summary of clinical and histopathological data of patients given IdUrd and BrdUrd for cell kinetics studies

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Histology</th>
<th>Grade</th>
<th>Localization</th>
<th>Stage</th>
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<tr>
<td>HN-4</td>
<td>SCC</td>
<td>Moderate</td>
<td>Maxillary sinus</td>
<td>Recurrent</td>
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<td>High</td>
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has been successfully used to study cell proliferation in human leukemias and solid tumors in our laboratory (21) and in that of Hoshino et al. (22). This technique not only provides precise cell cycle parameter determinations, but also gives valuable information regarding the morphological aspects of tumor growth and provides a unique opportunity to track the fate of labeled tumor cells if serial biopsies are obtained. The latter may help to understand the routes of cell loss from a tumor.

The results of this study confirm that a high proportion of head and neck tumors grow rapidly and that maintenance of the integrity of the tumor tissue is essential to the analysis of the proliferative characteristics of these tumors.

MATERIALS AND METHODS

The IdUrd/BrdUrd protocol (H&N 1392) utilized in this study was reviewed and approved by the Institutional Review Board of the Rush University, the NCI, and the FDA. IdUrd and BrdUrd were supplied by the NCI. Informed consent required by the local Institutional Review Board, NCI, and FDA was obtained from every patient before the administration of IdUrd and BrdUrd.

Patients

All patients who participated in this study were diagnosed and treated either in Rush-Presbyterian-St. Luke's Medical Center (Chicago, IL) or West Suburban Hospital (Oak Park, IL). Both newly diagnosed (16 patients) and locally recurrent (10 patients) patients were eligible for study. Table 1 provides the clinical and histological information regarding the patients and tumors which were studied. In all cases patients were diagnosed as having a malignant neoplasm before study. Nineteen patients received both IdUrd and BrdUrd on the same day. When IdUrd and BrdUrd were infused on the same day, each was given i.v. over 30 min with a 0.5-h interval between the infusions. At the end of the last infusion the tumor was surgically removed and a portion was fixed in Bouin's solution for 3 h, dehydrated in methanol, and finally embedded in glycol methacrylate. Two-mm-thin sections placed on Alcian blue coverslips were used for immunohistochemical processing. Seven patients received the infusion of IdUrd only followed by the biopsy. In 10 cases paraffin sections obtained from the Department of Pathology were also stained with the same immunohistochemical methods. In four patients a second biopsy was obtained 7–14 days after the first one. No therapy was given to these patients between the two biopsies.

Immunohistochemistry

Immunohistochemical procedures for single and double labeling have been described in detail earlier (23). In short they are as follows.

**Single-labeling Procedure.** The tissue specimens are first rehydrated in distilled water for 10 min. They are then incubated with 3% H$_2$O$_2$ for 30 min, then treated with Pronase (1 mg/ml) for 45 min and 4 N HCl for 20 min to expose the binding sites for the anti-IdUrd/BrdUrd antibodies. The mouse mAb 3D9 (a gift from Dr. G. L. Mayers, Roswell Park Memorial Institute, Buffalo, NY; 1:200), which is specific for both IdUrd and BrdUrd, is applied for 30 min followed by biotinylated antimouse IgG (1:200, 30 min), followed by the ABC reagent (a mixture of avidin-DH solution, 1:50, and biotinylated enzyme 1:50). These reagents were supplied in the Vectastatin Elite ABC Kit (Vector, Burlingame, CA). At the final steps
specimens are stained with 0.025% 3,3'-diaminobenzidine tetrahydrochloride in 0.003% H2O2 and counterstained with Harris' hematoxylin solution. Paraffin sections are treated in the same way except that the HCl application lasts for 40 min. In 14 specimens we used a slightly different labeling procedure. Rat anti-Brdu antibody and rat ABC kit (see below) instead of 3D9 antibody were used to demonstrate cells labeled with Brdu only. As a negative control for rat anti-Brdu antibody, we used sections from several tumor samples labeled with Idud only. There was no cross-reactivity of the anti-Brdu antibody with Idud.

Double-labeling Procedure. To calculate the temporal parameters of the cell cycle, we apply double immunohistochemical staining which allows us to distinguish between Idud- and Brdu-labeled cells. In the first step, tumor sections are stained according to the procedure described above using rat anti-Brdu antibody (Sera Labs, Crawley Down, United Kingdom) diluted 1:200 and a Vectastatin Elite rat ABC kit. 3D9 antibody (1:20) in PBS containing 0.5% Tween 20 and 1.5% normal horse serum is applied as the second label. This is followed by sequential treatment of the sections with rabbit anti-mouse immunoglobulin and APAAP mouse mAb (both from DAKO Corporation, Copenhagen, Denmark). The sections are treated then with fast blue substrate for final staining. As a result, three types of cells can be identified in the section: those labeled with Idud only (blue nuclei), with Brdu only (gold nuclei), and those labeled with Idud and Brdu (nuclei stained with both gold and blue).

Labeled Cells Counting

All stained sections are first examined to determine the proportion of viable tumor tissue, interstitial tissue, necrotic areas, and normal non-neoplastic tissue. The distribution of the labeled nuclei is then assessed. The LI (percentage of labeled tumor cells) is determined using a 10 x 10 square eyepiece graticule in selected fields of the section containing viable tumor cells. From 500-1000 tumor cells are counted in every field. For four to six fields (2000-5000 tumor cells) representing areas with high, low, and intermediate proportions of labeled cells are studied to calculate minimum, maximum, and average values of the LI. Necrotic fields without viable tumor cells and interstitial tissue are excluded from the assessment of the tumor proliferative rate. In tumor islands with keratin pearls only basal layers of nonkeratinized cells are counted.

Calculation of Cell Cycle Time

For these studies double-stained sections were used. Three hundred to 500 cells labeled with Idud only (blue nuclei) and with Brdu Idud and Brdu/Idud (golden and golden/blue nuclei) are counted. The Tc is calculated using the formula:

\[ Tc = N_{double} + Brdu/Idud \times \Delta T/Idud \]  (A)

where \( N_{double} + Brdu/Idud \) is the sum of double-labeled cells and cells labeled with Brdu only, \( N_{Idud} \) is the number of cells labeled with Idud only, and \( \Delta T \) is the interval between the start points of two injections. \( T_{per} \) of the tumor was calculated from the equation:

\[ T_{per} = \lambda \times Tc/LI \]  (B)

where \( \lambda \) is a correction factor for the nonlinear age distribution of cells through the cell cycle. It was assumed to be 0.8 (11, 13-15, 20).

Immunohistochemistry of Cytokeratin K10

Frozen sections are fixed sequentially in cold acetone and absolute cold methanol. The staining is performed using DAKO LSAB Kit (DAKO Corporation) and monoclonal mouse anti-human cytokeratin 10 antibody (DAKO-CK10, DE-K10) with diaminobenzidine as the chromogen. The counterstain is ethyl green.

RESULTS

Distribution of DNA-synthesizing Cells in Tumors

DNA-synthesizing cells were found to be distributed evenly or in clusters in tumor parenchyma showing various labeling patterns. In some SCCs labeled cells were located predominantly in basal layers of tumor islands while the rest of the island consisted of keratinizing structures (Fig. 1). The central parts of these tumor islands were strongly positive for keratin K10 (Fig. 2). In other tumors all regions of the tumor island contained labeled S-phase cells (Fig. 3). In these cases the tumor island contained few K10-positive cells which were scattered within the island. In high-grade SCCs which had lost their organ-specific structure, the labeling was chaotic and did not correlate to any apparent anatomical pattern (Fig. 4). In some regions of high-grade tumors labeled cells were grouped around blood vessels (Fig. 5). Labeled endothelial cells of blood vessels were common within both tumor parenchyma and tumor stroma (Figs. 5 and 6). Lymphoid cells infiltrating interstitial tissue between tumor islands demonstrated different degrees of labeling (Fig. 7).

In samples HN-5, HN-6, HN-8, HN-18, and HN-29, there was considerable heterogeneity in the proportion of cells labeled in adjacent fields. Fig. 8 illustrates this sort of variation in the tumor tissue sample HN-6. In this specimen the LI ranged from 22.4-80%. In the other 21 tumors, however, the proportion of labeled cells in different areas was practically the same. Fig. 9 shows the correlation between the LIs determined in two dif-
different areas of the same tumor: one sample of the tumor was embedded in plastic in our laboratory while the other specimen was originally sent to the Department of Pathology and was embedded in paraffin.

LIs

Average as well as minimum and maximum values of LIs for tumors labeled with both IdUrd and BrdUrd are summarized in Table 2. Data for LIs of cells labeled with IdUrd or BrdUrd only are also in Table 2. Cells labeled with IdUrd only represent cells which ended DNA synthesis during the IdUrd infusion or during the interval before the BrdUrd infusion. Those labeled with BrdUrd only entered S-phase either during the interval or during the BrdUrd infusion. Hence, \( L_{\text{IdUrd}} + L_{\text{BrdUrd}} \) represents effectively an infusion time of 90 min of label.

On average the difference between the 90-min and 30-min label was minimal, \( 23.6 \pm 3.6\% \) versus \( 21.6 \pm 3.3\% \) (mean \( \pm \) SE, \( P = 0.66 \)), indicating that during the succeeding 1 h after the first label the LI increases by only 2%.

The proliferative rate of morphologically well-differentiated SCCs was high, \( 20.4 \pm 2.7\% \) (range, 14.5–27.6). Moderately differentiated SCCs had similar LIs of \( 23.8 \pm 2.1\% \) (range, 16.2–35.1). There is no significant difference between these LIs (\( P = 0.135 \)). The LIs of two poorly differentiated SCCs were higher, 39.4% and 55.9%, respectively. The average LI of 22 SCCs was \( 23.2 \pm 2.0\% \).

The LI of a high-grade lymphoepithelioma was 2.5%, and in two mucoepidermoid tumors the LIs appeared to correlate with the morphological grade (3.0% and 39.4% for well-differentiated and poorly differentiated tumors, respectively). The LI of a malignant lymphoma was 24.8%. LI values did not correlate with either clinical stage or site of the tumors. There was no statistically significant difference between primary (24.4 \( \pm \) 2.2%) and relapsed (25.2 \( \pm \) 5.2%) SCC LIs (\( P = 0.31 \)).

In 10 samples a high level of infiltration by mononuclear cells of the interstitial tissue, which separates tumor cell islands, was detected. The LI of these cells ranged from 0–13.2% (mean \( \pm \) SE, 4.1 \( \pm \) 1.3%).
Duration of S-Phase and Potential Doubling Time

In 12 patients with SCC, full cell cycle parameters were calculated (in others either only IdUrd was infused or viable tumor tissue was present only in paraffin sections in which double labeling cannot be done). The data are presented in Table 3. \( T_s \) durations ranged from 5.1–21.5 h, with a median value of 12.1 h (mean ± SE, 12.8 ± 1.5). The calculated potential doubling times ranged from 18.8–84.5 h, with a median of 43.2 h (mean ± SE, 47.2 ± 6.7). There was no correlation between the LI and \( T_{pot} \) values. Labeled mitotic figures were not detected in any specimen. This suggests that the minimum duration of \( G_2 (T_{G2}) \) is longer than 90 min (the time interval between the beginning of IdUrd infusion and the biopsy). In three patients (not included in Table 3) the biopsy was performed 85–100 min after the infusion of BrdUrd; in other words, approximately 3 h after the start of the infusion of the first DNA label (IdUrd). In these biopsies some mitotic figures were labeled with IdUrd but not BrdUrd. Hence, \( T_{G2} \) in some cells of these tumors must be less than 3 h.

Fate of Proliferating Tumor Cells in the Absence of Treatment

In the second biopsy of four tumors, taken 7–14 days after the infusion of IdUrd and BrdUrd, the following phenomena were observed.

Dilution of Label Resulting from Repeated Cell Divisions. Most of the labeled nuclei in the second biopsy demonstrated a fragmented pattern, indicating that the initially labeled cells had undergone one or more divisions between the first and second biopsies (Fig. 10). In contrast, in some cells the whole nucleus remained stained. The pattern of the label in these nuclei was indistinguishable from the appearance of the label in Day 0 biopsy which suggests that these cells had not divided after being initially labeled or underwent fewer divisions than the cells with fragmented label (Fig. 10b). In patient HN-12 (Table 4) the LI in the first biopsy was 25.9% and 29.1% in the second biopsy. Of the labeled cells, 73.5% in the second biopsy had fragmented label and in 26.5% the label was undilated. In patient HN-25, LI2 was higher (39.4%) than LI1 (21.7%), but the proportion of cells with undiluted label (23.8%) was essentially the same as that in patient HN-12. In contrast, 93.8 and 99.6% of the labeled cells in the second biopsy of patients HN-15 and HN-33 had fragmented label. In patient HN-15, Day 0 LI was 19.9% and Day 9 LI was 12.9%. In patient HN-33, on Day 13 after the infusion, the LI was 9.3% versus 19.4% in the first biopsy.

Within the first 2 weeks after the infusion of IdUrd/BrdUrd, in two of the tumors studied, almost all of the cells passed through at least one division cycle, while in the other two tumors approximately 25% of cells appeared not to have divided.

Migration of Label from the Basal Layer of the Tumor Island to Its Central Cornifying Structures (Pearls). Two of the tumors (Patients HN-25 and HN-33) contained pearl-like structures. In the biopsy obtained immediately after IdUrd infusion and the biopsy. In three patients (not included in Table 3) the biopsy was performed 85–100 min after the infusion of BrdUrd; in other words, approximately 3 h after the start of the infusion of the first DNA label (IdUrd). In these biopsies some mitotic figures were labeled with IdUrd but not BrdUrd. Hence, \( T_{G2} \) in some cells of these tumors must be less than 3 h.

Labeled Debris of Cell Nuclei in Necrotic Foci. In the second biopsy of one patient (Patient HN-12), areas of necrosis were present. In the necrotic area, labeled fragmented and pyknotic cell nuclei were noted (Fig. 12).

Labeled Nontumor Cells. Labeled mononuclear cells and labeled polymorphonuclear leukocytes were present in all four second biopsies.

DISCUSSION

Tumor cell growth kinetics has been recognized as one of the most important determinants of curability of human tumors in general (24) and of head and neck tumors in particular (25). In their comprehensive review Sasaki et al. (6) reported cell cycle parameters for 198 SCCs based mainly on the measurement of \(^{[3}H\)thymidine LI in vitro and FCM. Later, the introduction of BrdUrd and other halogenated nucleotides stimulated a new generation of cell kinetics studies in humans (12, 26, 27). To facilitate the discussion of our results, we summarize the data of cell kinetics in SCC in Table 5.
Proportion of DNA-synthesizing Cells (LIs). The fraction of S-phase cells in our study is higher than that in most other studies (Table 5). This may be partly due to a naturally occurring wide range of proliferative rates in SCC. On the other hand, there may be technical reasons for our observations.

First, FCM underestimates the proportion of S-phase cells because of the admixture of stromal and other nonmalignant cells. In this study we found that tumor tissue not only contains varying proportions of mononuclear cells, but that the percentage of these cells in S-phase varies widely among the tumors studied (LI range, 0–13.2%). It is evident that FCM cannot discriminate between these cells and tumor cells unless tumor cells are tetraploid. The proportion of tetraploid (and higher ploidy) tumors is only 4% of all head and neck SCCs (18). That is why the application of FCM results in an inevitable underestimation of LI values when disaggregated tumor cell suspensions are studied (11, 18).

Second, the use of in vitro labeling results in LI estimates which are lower than those obtained from in vivo studies (7, 10, 14). This may be the case in Hemmer’s (7) study as well (Table 5), in which S fractions were significantly lower in comparison to the data of other groups, with the median percentage of S-phase cells only 2.6% (Table 5). Conversely, LIs reported by
groups which used in vivo labeling procedures (10, 11, 19) are significantly higher.

LI values may be affected by the duration of exposure of S-phase cells to the DNA label in vivo, which in our case was 0.5 h when one of the halogenated nucleotides was injected and 1.5 h when both IdUrd and BrdUrd were administered. In fact, the LIs of cells stained with mAb 3D9 (for both IdUrd and BrdUrd) is on average 2.0% higher than that of cells stained with BrdUrd-specific antibody (Table 2). These observations demonstrate that prolonged infusions of halogenated nucleotides may seriously affect the results of LI determinations.

It is important to obtain the tumor specimen immediately after the administration of a DNA label. The longer the interval between the end of the infusion and the biopsy, the less accurate the LI will be because of the movement of labeled cells from S into G2-M and later because of the division of labeled cells. In previous studies (2, 3, 11, 13, 16) biopsies were taken at least 4–6 h after BrdUrd injection. Many initially labeled cells divide during this period of time, producing two labeled daughter cells resulting in an overestimation of the proportion of S-phase cells. Wilson et al. (13) proposed therefore to make special corrections to obtain more realistic LI values when there is a lag between the labeling of the tumor and acquisition of the specimen for study.

The heterogeneity within individual tumors with respect to cell kinetics may be another potential source of error. However, when we compared LIs of different regions of the same tumor embedded in plastic (our laboratory) and in paraffin (Department of Pathology), we found that LIs were highly correlated (Fig. 9). Similarly, Wilson et al. (13) also did not find significant differences in the cell kinetic parameters measured in different samples from the same tumor.

**Cell Cycle Parameters.** The duration of S-phase in our series range between 5.1 and 21.5 h, with a median of 12.1 h (Table 5). This is the first attempt to assess the duration of S-phase in SCC of the head and neck using double-labeling methodology. An indirect approach to approximate the duration of the S-phase is to use the difference between 30- and 90-min LIs (Table 2). This difference is 2.0%. In other words, every hour 2% of the tumor cell population enters the S-phase. Given a constant rate of cell cycle transition by tumor cells, the average duration of S-phase would be 1 h × 21.6/2.0 = 10.8 h (see Equation 1 in "Materials and Methods"). This is very close to the results obtained by us using double-labeling methodology and by Begg et al. (2) and Bennett et al. (Ref. 11; Table 5).

$T_s$ measurements made by other authors using different methods suggested shorter S-phase duration in SCC in comparison to other tumors (see, for example, Ref. 18). The duration of S-phase in SCCs reported here is also shorter than the $T_s$ determined by the same double-labeling method in human leukemias and other solid tumors (21–23).

While there is an excellent correlation of $T_s$ values obtained by us and reported by authors (2, 3, 11, 15) who used the methodology of Begg et al. (12), the $T_{pot}$ in our study (about 2 days) is shorter than those reported by these authors (4–5 days). This discrepancy may be explained by higher LI values measured in biopsy sections as compared to the values obtained by FCM (see above). Nevertheless, both methods demonstrate very short $T_{pot}$ versus actual tumor doubling times which for SCC of the head and neck were assumed to be 1–6 months and longer (20).

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*This time is slightly longer if one counts postinfusional availability of the drug which in humans is 10–20 min (28).*
There are few reports concerning the duration of $G_2$ in human SCC. Using the labeled mitoses method, Bresciani et al. (19) reported the average $T_{G_2}$ to be as long as 8.1 h. Since we did not find labeled mitotic figures in samples obtained 90 min after the beginning of the IdUrd infusion, but detected labeled mitoses in biopsies removed approximately 3e h after the start of the infusion, the minimum $T_{G_2}$ in these tumors is between 1.5 and 3 h.

Fate of Proliferating Tumor Cells. As mentioned above, there is a large difference between the $T_{pot}$ of a tumor and the actual tumor volume doubling time. The discordance between $T_{pot}$ and actual tumor doubling time is evident if one considers tumor HN-12 (Table 4). The cell cycle time of the tumor was 45.7 h. Hence, in a single week the labeled cells should go through three divisions with a tripling in the volume of the tumor. Similarly, based on the $T_{pot}$, the volume of the tumor HN-25 should increase 9-fold in a 9-day period. To explain this difference Steel (20) introduced the concept of a spontaneous cell loss factor. This factor in human head and neck SCCs was estimated to be between 85 and 93% (19, 20). Our studies performed in serial samples provide direct experimental evidence for these theoretical calculations. The studies described here demonstrate three phenomena which contribute to the difference between $T_{pot}$ and tumor doubling time. One phenomenon is the egress of cells from the proliferating pool. For example, in tumors HN-12 and HN-33 (Table 4) approximately 25% of the cells appeared to have stopped proliferating after completing DNA synthesis. This was suggested by the fact that the initial DNA label remained undiluted 7-9 days after the infusion of IdUrd/BrdUrd, indicating that these cells did not undergo mitosis.

A second mode of egress is via differentiation. In well-differentiated keratinizing SCCs proliferating cells were located only on the margin of a tumor island (Fig. 11a). One week later labeled cells had migrated into the pearls (Fig. 11b). The phenomenon of migration of cells from undifferentiated regions of a tumor to pearl structures was previously reported in rats bearing transplantable SCC (29).

### Table 4  Labeling patterns in serial samples from patients who did not receive therapy between two biopsies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histology</th>
<th>Infusions</th>
<th>$T_{pot}$ (h)</th>
<th>L11</th>
<th>L12</th>
<th>Days between biopsies</th>
<th>Cells with fragmented/unfragmented label (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN-12</td>
<td>SCC,M</td>
<td>IdUrd/BrdUrd</td>
<td>45.7</td>
<td>25.9</td>
<td>29.1</td>
<td>7</td>
<td>73.5/26.5</td>
</tr>
<tr>
<td>HN-15</td>
<td>SCC,M</td>
<td>IdUrd/BrdUrd</td>
<td>19.9</td>
<td>12.9</td>
<td>9</td>
<td>9</td>
<td>93.8/6.2</td>
</tr>
<tr>
<td>HN-25</td>
<td>SCC,M</td>
<td>IdUrd/BrdUrd</td>
<td>22.9</td>
<td>21.7</td>
<td>39.4</td>
<td>9</td>
<td>76.8/23.8</td>
</tr>
<tr>
<td>HN-33</td>
<td>SCC,W</td>
<td>IdUrd/BrdUrd</td>
<td>84.5</td>
<td>19.4</td>
<td>9.3</td>
<td>13</td>
<td>99.6/0.4</td>
</tr>
</tbody>
</table>

Fig. 11  Migration of the label to cornified pearls. a, Day 0 biopsy; labeled cells are limited to the periphery of the tumor island; b, Day 7 biopsy: peripheral cells are unlabeled, the pearl contains compressed remnants of labeled cell nuclei. × 400.

Fig. 12  Labeled fragments of cell nuclei in the focus of necrosis in Day 9 biopsy. V, viable tumor tissue; N, necrosis. × 400.
Table 5  Cell kinetics of SCC of head and neck (literature)

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Methods</th>
<th>S-phase cells, %, mean (range)</th>
<th>(T/T_{pot}), days, mean, (range)</th>
<th>(T_c), h, mean, (range)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>[^{3}H]thymidine in vivo, labeled mitoses curve</td>
<td>17.5 (11-36)</td>
<td>2.5 (2.1-3.6)</td>
<td>23.5 (18-34)</td>
<td>19</td>
</tr>
<tr>
<td>16</td>
<td>[^{3}H]thymidine in vivo</td>
<td>17.0 (2.0-30.0)</td>
<td>1.9</td>
<td>16.1</td>
<td>6</td>
</tr>
<tr>
<td>198 (pooled data)</td>
<td>[^{3}H]thymidine in vivo</td>
<td>12.1 ± 3.9</td>
<td>11.2 (2.3-22.4)</td>
<td>23.5 (18-34)</td>
<td>1</td>
</tr>
<tr>
<td>88</td>
<td>Static cytometry</td>
<td>18.0 (2-48)</td>
<td>40.4 (primary)</td>
<td>24.5 (metastatic)</td>
<td>9</td>
</tr>
<tr>
<td>37</td>
<td>[^{3}H]thymidine in vitro</td>
<td>11.2</td>
<td>28 FCM</td>
<td>17.2</td>
<td>8</td>
</tr>
<tr>
<td>28</td>
<td>FCM</td>
<td>40.4</td>
<td>68 FCM</td>
<td>17.2</td>
<td>7</td>
</tr>
<tr>
<td>68</td>
<td>FCM</td>
<td>2.6</td>
<td>0-23.2</td>
<td>17.2</td>
<td>8</td>
</tr>
<tr>
<td>33</td>
<td>FCM</td>
<td>2.6</td>
<td>0-23.2</td>
<td>17.2</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>Xenograft in nude mice</td>
<td>22-25</td>
<td>3.1</td>
<td>17-19</td>
<td>33</td>
</tr>
<tr>
<td>68</td>
<td>BrdUrd in vitro, FCM</td>
<td>12.7</td>
<td>4.7</td>
<td>11.2</td>
<td>2</td>
</tr>
<tr>
<td>82</td>
<td>BrdUrd in vivo, FCM</td>
<td>(2.4-30.5)</td>
<td>(1.4-16.9)</td>
<td>(4.8-33.2)</td>
<td>15</td>
</tr>
<tr>
<td>123</td>
<td>BrdUrd in vivo, biopsy</td>
<td>(1.2-30.0)</td>
<td>(1.2-40.9)</td>
<td>(7.3-37.5)</td>
<td>11</td>
</tr>
<tr>
<td>31</td>
<td>BrdUrd in vivo, biopsy</td>
<td>14.9 (FCM)</td>
<td>5.7</td>
<td>9.9</td>
<td>11</td>
</tr>
<tr>
<td>22</td>
<td>IdUrd and BrdUrd in vivo, biopsy</td>
<td>9.2 (2.1-28.0)</td>
<td>5.2</td>
<td>9.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.1-15.0)</td>
<td>(6.4-18.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All of these phenomena are presented graphically in Fig. 13. It is important to stress that cell death and differentiation have long been recognized as mechanisms responsible for the cell loss from a tumor’s proliferative pool (20). Disturbances of cell cycle transition demonstrated in our study add to this list and provide a further basis for the difference between \(T_{pot}\) and actual doubling time of the tumor.

Some Methodological Aspects of Cell Proliferation Studies Using In Vivo Infusions of IdUrd and BrdUrd. There are two methodological issues which require clarification. One is time interval between the IdUrd and BrdUrd infusions and biopsy of the tumor. Studies using the methodology of Begg et al. (12) recommend a prolonged interval (4-6 h) between the BrdUrd infusion and the tumor biopsy (12, 13, 16). According to this method the interval must be long enough to permit a significant fraction of BrdUrd-labeled cells to move to G2-M (12, 16). In contrast, the method used in our study is a modification of the \[^{3}H\]- and \[^{14}C\]-thymidine double-labeling technique originally described by Wimber and Quastler (30). For calculation of \(T_c\), we use the proportion of cells labeled with IdUrd only and the cells labeled with BrdUrd (including both single- and double-labeled cells). Cells, labeled with IdUrd only, are the cells which leave S-phase during the period of time between the starting points of the two infusions, which is 1 h (30 min of IdUrd infusion plus 30-min interval). Once being labeled with IdUrd, the proportion of cells leaving S-phase during 1 h would be the same independent of the duration of the infusion and of the availability of the circulating label. The second infusion would label all cells which were in S-phase at the starting point of the infusion (true LI) + cells which enter the cell cycle during the infusion period (30 min), after which the tumor is excised. To provide better precision for the \(T_c\) calculation, it is important that \(\Delta T\) not be longer than the duration of G2 (30).

Fig. 13  Four types of labeled cells that were present in the second biopsy obtained 7-14 days after the infusion of IdUrd/BrdUrd. I, cells whose label had the same intensity as the label in the first biopsy (Day 0), these cells presumably did not divide (arrested in G2); II, cells with diluted (fragmented) label, which divided one or more times; III, labeled nuclei of necrotic cells; IV, labeled compressed nuclei within the cornified pearls. IUdR, iododeoxyuridine; BrdU, bromodeoxyuridine.

A third mode of egress is via cell death. For example, in the second biopsy of the patient HN-12 many labeled nuclei were found in necrotic foci, indicating that the labeled cells had become necrotic (Fig. 12). Additional studies to be published elsewhere indicate that some tumors contain apoptotic cells and that the number of these cells increases markedly during cytotoxic therapy.5

According to the data provided above in some SCCs, $T_{G2}$ is between 1.5 and 3 h. Therefore, we would not recommend the use of a $\Delta T$ longer than 1.5 h.

The second methodological question is the principle of selection of microscopic fields to enumerate labeled cells. In our studies we selected several fields representing viable tumor areas with high, intermediate, and low frequency of labeled cells. In this way we are able to provide an accurate estimate of the mean values as well as the ranges. In addition to the exclusion of necrotic areas, we excluded also keratinized structures. This is supported by the results of cytokeratin studies which demonstrated that in tumors in which cells are undergoing keratinization (exhibiting high levels of expression of cytokeratin 10 and pearl formation), labeled cells are limited to the marginal layers of the tumor island. In normal epithelium K10 is a marker of an early stage of keratinocyte differentiation (31). In other words, K10-positive cells consist of differentiating cells which will be lost from the tumor (20). Additionally, cells from keratinized structures of SCC cannot induce tumors after retransplantation in experimental animals (29). By definition these cells should not be counted when the potential doubling time of the tumor is determined.

The results of this study support the opinion that head and neck cancers are fast-growing tumors that require intensive treatment schedules. The data described above also suggest that the use of the in vivo IdUrd/BrdUrd double-labeling technique provides valuable cell kinetics data, allows study of the distribution of DNA-synthesizing cells within the tumor, and may be used to assess labeled cells in serial biopsies.

ACKNOWLEDGMENTS

We thank Erzsebet Horvath and Jennifer Hartman for skillful technical assistance.

REFERENCES


Cell kinetics of head and neck cancers.


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