Measurement of Potential Doubling Time for Human Tumor Xenografts Using the Cytokinesis-Block Method

Lynn Hlatky, Mariusz Olesiak, and Philip Hahnfeldt

Joint Center for Radiation Therapy, Department of Radiation Oncology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Estimates of the potential doubling time (T\textsubscript{pot}) of seven human tumor xenografts were made using a cytokinesis-block method. This method is currently being investigated as an alternative to flow cytometric assays using the administration of a thymidine analog in the measurement of T\textsubscript{pot}. If perfected, the cytokinesis-block method of measuring T\textsubscript{rpm} would be advantageous as a predictive assay, in that no label is administered to the tumors in situ. Xenografts were grown in nude mice, and following tumor excision and disaggregation, tumor cells were cultured with the cytokinesis-blocking agent cytochalasin B. The flux of cells through mitosis was marked by the accumulation of multinucleate cells. By counting cytokinesis-blocking agent cytochalasin B. The flux of cells through mitosis was marked by the accumulation of multinucleate cells. By counting the total number of nuclei as a function of time, the effective population growth was observed. T\textsubscript{pot} values were obtained by fitting suitable exponential least squares curves to the data, with the doubling time indicated by the fitted functions. For the seven tumors studied, a significant spread in growth rates was observed. T\textsubscript{pot} values generated by this method ranged from ~2 days for a rapidly growing squamous cell carcinoma of the pharynx, FaDu, to ~7.5 days for the slower growing glioblastoma multiforme U251-MG. These values are compared with standard 5-iododeoxyuridine T\textsubscript{rpm} measures and volume doubling times obtained by Perez et al. (Cancer Res., 55: 392-398, 1995) for the same tumor xenografts. Although individual T\textsubscript{rpm} values varied between these methods, the ranking of the seven tumors in order of T\textsubscript{pot} times was the same regardless of method. To estimate T\textsubscript{pot} for each of the tumors, the fraction of clonogenically dead cells that was microscopically apparent, including apoptotic cells and cells expressing micronuclei, was determined as a function of time in culture. Tracking this in vitro cell loss rate provides information on the adjustment of these primary tumor cells to in vitro culture, a factor that needs to be addressed when determining how in vitro measurements of T\textsubscript{pot} can be effectively related to in vivo measurements.

INTRODUCTION

Certain tumors exhibit rapid cellular proliferation, which may lead to reduced local control under standard therapeutic regimens. If rapidly proliferating tumors were identifiable prior to treatment, alternative treatment protocols, such as accelerated fractionation schemes, could be considered. Thus, much effort has been invested in characterizing the kinetic behavior of individual tumors in an attempt to predict their therapeutic responsiveness (1-10). The T\textsubscript{pot} of a tumor is a first-order estimate of the tumor growth rate and is considered a better indicator of the tumor repopulation rate during radiotherapy than is the pretreatment volume doubling time. The T\textsubscript{pot} measure characterizes tumor growth in terms of cell cycle time and proliferation fraction. It ignores tumor cell loss, cell cycle diversity, and time-dependent changes in the growth fraction. For particular histologies, e.g., tumors of the head and neck, individual tumors that exhibit short T\textsubscript{pot} values are considered candidates for accelerated fractionation (12). Presently, the predictive value of T\textsubscript{pot} measures are being assessed in clinical trials using accelerated and conventional radiotherapy schemes (4, 5, 13, 14).

T\textsubscript{pot} is conventionally estimated by a single in situ labeling of the tumor with a thymidine analog such as BrdUrd or IdUrd, followed after a lag time by a tumor biopsy and flow cytometric analysis (15-21). Because T\textsubscript{pot} seems to be a clinically useful parameter, alternative noninvasive methods to measure T\textsubscript{rpm} are being investigated (22, 23). Shibamoto and Streffer (23) demonstrated the potential validity of a cytokinesis-block method for estimating T\textsubscript{pot} values of human tumor xenografts. Here, we further investigate this method by examining the kinetics of seven human tumor lines using the cytokinesis-block method, estimating T\textsubscript{pot} values, and further considering the potential of this method for investigating the fine structure of tumor population kinetics. T\textsubscript{pot} values obtained by the cytokinesis-block method are compared with published IdUrd T\textsubscript{pot} values for the same tumor xenografts (24).

MATERIALS AND METHODS

Cell Culture. In vitro estimates of T\textsubscript{pot} were made for the following human tumor cells grown as xenografts: U87, a glioblastoma multiforme (ATCC); HGL9, a glioblastoma multiforme (MGH); U251-MG, a glioblastoma multiforme (Dr. Bigner, Duke University); FaDu a SCC of the pharynx (ATCC); HCT15, a colon adenocarcinoma (ATCC); STS25T, a schwannoma, soft tissue sarcoma (Dr. Little, Harvard University); and SCC21, a head-and-neck SCC (MGH).

Xenografts of each of these human tumor lines were grown in nude mice in the laboratory of Dr. Herman Suit (MGH). The method of tumor transplantation has been described previously (25, 26). Tissue samples were excised and disaggregated to single cells using a digestion mixture consisting of 0.1 N HCl with 0.4 mg/ml pepsin (Sigma Chemical Co.) for a treatment time of 1 h at 37°C. Following this, 3.3 \times 10\textsuperscript{5} cells/slide were plated on CellTak-coated slides (Collaborative Biomedical Products) in DMEM containing 20% FCS, 0.2 mg/ml gentamycin sulfate, and 2.0 \mu g/ml cytochalasin B (previously aliquoted in DMSO at 1 mg/ml). Coating slides with CellTak ensures that the vast majority of the cell population adheres to the slides, thereby considerably increasing plating efficiencies. Cytochalasin B is a cytokinesis-blocking agent widely used in the assaying of micronuclei following clastogenic damage (27-29) and more recently in the determination of T\textsubscript{rpm} values (23). At low concentrations (<3.0 \mu g/ml), as used in this study, it is considered minimally toxic. Cytochalasin B blocks cell cytokinesis following mitosis but does not block mitosis itself. The result is that cells that have traversed mitosis since the addition of the drug are clearly detectable by the presence of two separate nuclei within a single cytoplasm.

At various times after plating, slides were fixed in 3:1 ethanol:acetic acid and rinsed in 70% ethanol. Slides were stained using the Feulgen technique, staining only the DNA (27). At each time point, the fraction of multinucleated cells in the population was counted. Between 500 and 3000 cells were counted per time point. The percentages of multinucleated cells plotted as a function of culture duration provide the means of estimating the time-dependent fraction of divided cells in the populations. In addition, for these tumors, the fraction of clonogenically dead cells that was microscopically apparent, including apoptotic cells and cells expressing micronuclei, was determined as a function of time in culture.

Theory. A determination of T\textsubscript{pot} from an in vitro assay must take into account the special circumstances of in vitro growth if it is to preserve its essential clinical meaning. In place of flow cytometry, growth in culture was assessed with a minimum of perturbation to the cell population by using...
cytochalasin B to block cytokinesis while permitting karyokinesis. The number of divisions each cell would have undergone is thus readily ascertained by scoring nuclei per cell. A plating efficiency correction was also introduced, because this is a known feature bearing on ultimate culture growth. The “Appendix” details how these two parameters are finally introduced into a slightly modified formalism for finding T\text{pot}.

### RESULTS

Following disaggregation into single cells, the xenograft populations were plated on CellTak-coated slides and incubated in the presence of cytochalasin B. At intervals of ~24 h after plating, cells were fixed, and the fraction of multinucleated cells was determined at each time point. The rate of cell division for each xenograft population was visualized by charting increases in the number of nuclei (over a fixed cell number, e.g., 1000) as a function of time in culture. Three replicate experiments were scored for each tumor type. Reproducible kinetic data for the xenograft populations studied was obtained by this method, as is shown in Fig. 1 for cells from the glioblastoma U87. Data for all the human tumor xenografts, with replicate experiments pooled, are shown in Fig. 2. These curves were fit with exponentials to generate T\text{pot} values. A significant spread in growth rates was observed for the seven tumors studied.

For the purpose of T\text{pot} calculation, initial data points of the growth curves were fitted by least squares to exponential functions (Fig. 2).

![Fig. 1. Total number of nuclei normalized to 1000 cells shown as a function of time in culture for three U87 replicate experiments. Between 500 and 3000 cells were counted per time point.](image)

![Fig. 2. Number of nuclei/1000 cells versus time for all cell lines. These are essentially in vitro "growth curves" for the seven human tumor xenografts. The curves reflect the pooled counts from replicate experiments for each tumor line. These plots were fit with exponentials to generate T\text{pot} values.](image)

### Table 1

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Origin</th>
<th>Cytokinesis-block T\text{pot} (days)</th>
<th>Divided fraction (%)</th>
<th>IdUrd T\text{pot} (days)</th>
<th>Volume-doubling time (T\text{vol}) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FaDu</td>
<td>SCC</td>
<td>2.0 ± 0.2</td>
<td>79.3</td>
<td>1.3 ± 0.1</td>
<td>4.5</td>
</tr>
<tr>
<td>HCT15</td>
<td>Human colon adenocarcinoma</td>
<td>2.5 ± 0.1</td>
<td>73.05</td>
<td>2.2 ± 0.1</td>
<td>4.7</td>
</tr>
<tr>
<td>STS26T</td>
<td>Schwannoma (soft tissue sarcoma)</td>
<td>2.5 ± 0.2</td>
<td>75.78</td>
<td>2.4 ± 0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>U87</td>
<td>Glioblastoma multiforme</td>
<td>3.9 ± 0.3</td>
<td>46.46</td>
<td>2.5 ± 0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>SCC21</td>
<td>SCC</td>
<td>5.9 ± 0.3</td>
<td>45.55</td>
<td>2.9\textsuperscript a</td>
<td></td>
</tr>
<tr>
<td>HGL9</td>
<td>Glioblastoma multiforme</td>
<td>6.2 ± 0.3</td>
<td>26.39</td>
<td>3.9 ± 0.3</td>
<td>4.7</td>
</tr>
<tr>
<td>U251-MG</td>
<td>Glioblastoma multiforme</td>
<td>7.5 ± 0.5</td>
<td>67.10</td>
<td>10.5 ± 1.4\textsuperscript b</td>
<td>8.3</td>
</tr>
</tbody>
</table>

\textsuperscript a L. Perez, unpublished data.

\textsuperscript b This value has since been modified downward, to ~4.2 (L. Perez, unpublished data).

Points after curve rollover were ignored, as described in “Appendix.” The time at which the fitted function indicated a doubling of the population was taken to be the T\text{pot} for the associated cell line. This method is similar to that of Shibamoto and Streffer (23), except that the fits here allow for a fixed, nonproliferating component (see “Appendix”). The form is number of nuclei = A + B*exp(Ct). Numerical values of T\text{pot} determined by the cytokinesis-block method are compiled in Table 1. T\text{pot} values ranged from ~2 days for a rapidly growing SCC of the pharynx, FaDu, to ~7.5 days for the more slowly growing glioblastoma U251-MG. Also listed in Table 1 are the volume-doubling times and T\text{pot} values determined in situ by Perez et al. (24) for the same xenografts using standard IdUrd techniques. It is noted that for this set of xenografts, the cytokinesis-block method ranked the T\text{pot} values in the same order as did the IdUrd method. The T\text{pot} values obtained here by the cytokinesis-block method are all longer than those detected by Perez et al. (24) for these xenografts using the IdUrd method (taking into account the updated estimate by Perez et al. of a shorter T\text{pot} value for the U251-MG).

The divided-fraction graph for the U87 populations demonstrates a bimodal kinetic behavior. This is indicative of subpopulations of cells within the tumor, which either have different cycling rates or resume cycling in distinct cohorts after being put into culture (e.g., as would be expected in mixtures of oxygenated cycling and hypoxic quiescent subpopulations). Such information on cycle heterogeneity is not available from standard T\text{pot} assays.

In scoring data for estimating T\text{pot}, cells in the population were counted and classified as single nucleate, multinucleate, or lethally damaged. Cells that were visibly lethally damaged (including cells with visible apoptotic bodies, micronucleated cells, and cells with detectable lethal chromosome aberrations, e.g., dicentric bridges or nuclear blebbing) were counted, and the fraction of lethally damaged cells was scored as a function of time in culture. Fig. 3. Thus, besides tracking cell division, one can also track a portion of the cell death in culture. Tracking this \textit{in vitro} cell loss rate provides data on the adjustment of primary tumor cells to \textit{in vitro} culture and provides information for judging how well \textit{in vitro} clonogenic assays can be made that reflect the total cell populations for these specific tumors. The colon adenocarcinoma xenograft populations HCT15 were observed to undergo a significant degree of apoptosis (as determined by the presence of apoptotic bodies) on transition from the \textit{in vivo} to the \textit{in vitro} situation. This was not seen for the cells from the other xenografts.
DISCUSSION

The cytokinesis-block method has been suggested as an alternative to thymidine analogue labeling for the measurement of $T_{\text{pot}}$. In this study, as in the studies of Shibamoto and Streffer (23), estimates of $T_{\text{pot}}$ values were obtained for human tumor lines grown as xenografts and evaluated in vitro after the addition of cytochalasin B. These $T_{\text{pot}}$ measures were then compared with $T_{\text{pot}}$ values for the same xenografts determined in the standard way, via in vivo labeling with a thymidine analogue. If perfected as a predictive assay for human tumor kinetics, the cytokinesis-block technique would offer the distinct advantage that no label is administered to the tumor in vivo, alleviating the problems associated with thymidine analog injection. Only a microscope is required for analysis; therefore, the method is accessible to all laboratories, and normal cells such as stroma or macrophages infiltrating the tumor sample could be excluded by visual inspection. In addition, information on individual tumor radiosensitivity, via micronuclei detection, could be obtained simultaneously when assessing cytokinesis-blocked cells (27-29, 31). If useful information for estimation of $T_{\text{pot}}$ can be obtained from the observation of tumor cells in vitro, disadvantages of the method are that it is labor intensive, and, unlike flow cytometric $T_{\text{pot}}$ measurements, data are not rapidly available, because the cells must be cultured on the order of 100 h after removal from the tumor. Even so, in vitro, culture periods of much longer duration are tolerated in the predictive assay measurements of radiosensitivity via clonogenic assays such as SF2._

When possible, $T_{\text{pot}}$ values were obtained directly from the data plots by noting at what times the population size $N = 2000$ was achieved. Otherwise, the times at which $N = 2000$ in curves fitted to initial points were used. In these cases, the nuclei/cell versus time graphs were fitted by least squares to exponential functions. The individual $T_{\text{pot}}$ times generated via this method were both significantly different and longer than the $T_{\text{pot}}$ times derived by IdUrd labeling (24). Yet, when the seven human xenografts were ranked in order of $T_{\text{pot}}$, both methods yielded the same ranking. (Note that for HCT15 and STS26T, there was essentially no difference in rank.) In the data of Shibamoto and Streffer (23) the $T_{\text{pot}}$ values obtained for eight tumor xenografts (human and murine) by the cytochalasin B method did not vary significantly (with the exception of one murine xenograft, HP melanoma in BALB/c mice) and were not systematically larger or smaller than $T_{\text{pot}}$ values obtained using BrdUrd labeling. For these same xenografts, excluding HP, the ranking based on $T_{\text{pot}}$ values between the two methods agreed.

By counting multinucleate versus mononucleate cells in vitro, divided cell fractions are obtained for the populations. Information on divided fraction versus time amplitudes that gained from the growth curve, _i.e._, total nuclei in the population versus time. In this study, particular tumor lines, _e.g._, U87, demonstrate nonhomogeneous transitional behavior in their divided-fraction curves, which show inflection points separating concave-up and concave-down behaviors. These results are indicative of subpopulations of cells within the xenografts, which either have different cycling rates or resume cycling in distinct cohorts after transfer to _in vitro_ culture. It is worth noting that both of these conditions, different cycling rates and distinct cell cohorts (nonconstant growth fraction), are inconsistent with the assumptions of $T_{\text{pot}}$ as standardly defined. By use of the divided-fraction curves, diversity in the cell proliferation rate could be disentangled from the resumption of cycling of distinct cohorts. This cytochalasin-block assay potentially has the ability to detect heterogeneous cellular behavior and to distinguish among asynchronous and synchronous cycling patterns, uncovering diversity in cellular kinetics not revealed by flow cytometric studies. In theory, by taking sufficient data points, mixtures of oxygenated cycling cells and hypoxic quiescent subpopulations within tumors should be discernible due to the "resumption kinetics" of the hypoxic tumor subpopulations when disaggregated and exposed to oxygen _in vitro_ (32). Although assays, such as the cytokinesis-block assay, that follow proliferation over time offer a means of measuring certain aspects of intratumor cycle diversity, the problematic nature of tumor proliferation heterogeneity and the time-dependent alterations in the proliferating fraction cannot be fully addressed _in vitro_.

There are not enough tumors examined in this study to see whether characteristic kinetic fingerprints exist for the different tumor histologies. However, this assay potentially provides detailed information on the adjustment of tumor populations to culture and could thereby reveal characteristic kinetic structures of specific histologies (_e.g._, the existence of quiescent subpopulations). The glioblastomas, U87, HGL9, and U251-MG, seem relatively slow growing and exhibit bimodal growth curves. Besides its value as an alternative method for estimating $T_{\text{pot}}$ values, fuller development and exploration of this cytokinesis-block method should provide an enhanced understanding of: (a) how individual primary tumor populations grow in culture; and (b) the extent to which the proliferative heterogeneities _in vitro_ indicate analogous diversities _in vivo_. The documentation of intertumor cell growth differentials may be a useful augment to the concept of $T_{\text{pot}}$ in the prediction of tumor growth and therapeutic response.

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APPENDIX

To determine $T_{\text{pot}}$ values, the numbers of nuclei/1000 cytokinesis-blocked cells were plotted as a function of time. Best exponential projections of population growth (nuclear proliferation) were then made on the basis of these data. The exponential fits include certain classic kinetic assumptions of tumor growth used in standard $T_{\text{pot}}$ derivations (e.g., Steel (11)). There is the observation _in vivo_ that there is a fraction of the population that is nonproliferating, but which nonetheless increases in size to maintain a constant proportion with the proliferating population (fixed-growth fraction). This nonproliferating subpopulation increases in size by receiving an influx of cells that are nonproliferating progeny of dividing cells. The effect of the presence of such a nonproliferating fraction is to extend the $T_{\text{pot}}$ from $T_c$ to $T/f$, where $T_c$ is the...
cell cycle time for the proliferating cells, and $f \equiv \ln(1 + GF)/\ln(2)$, where $GF$ is the growth fraction. We do not expect equivalent behavior in vitro but note that any such effect will be absorbed into the solution for a time coefficient, which appears in the in vitro kinetics statement to follow.

Beyond in vivo tumor cell kinetics, an additional consideration is evidenced by observations of the acclimation of tumor cells to culture conditions. It seems that a small subpopulation of the original plated population is incapable of further division or is so delayed in the resumption of division that it may be considered dormant for the sake of describing the initial kinetics. (Correction for this dormancy is analogous in principle to the plating efficiency adjustment routine in colony assay studies.) This subpopulation is of fixed size, distinct from the variable-size, nongrowing fraction seen in vivo.

An extension of standard tumor kinetic theory more suitable for capturing both in vitro kinetic considerations just discussed is to now consider the $T_{pot}$ to be the time at which the population has “grown” to an average of 2000 nuclei/1000 cytokinesis-blocked cells, where $N$ is the population size (average nuclear count/1000 cells) at time $t$. $A$ is the nongrowing portion of the population, $B$ is the coefficient of exponential growth, and $C$ is a coefficient introducing the proper time scaling and/or net growth retardation:

$$N = A + B \exp(Ct)$$

where, specifically

$$2000 = A + B \exp(C T_{pot})$$

For fitting the data, $A$, $B$, and $C$ are solved for by using least squares methods. The fits are actually made to $\log(N - A) = \log(B) + Ct$, inserting trial values for $A$ and in each instance solving for $B$ and $C$ by linear regression fits to the resulting linear equation in $t$. The values of $A$, $B$, and $C$ that gave the least squares fit to this equation overall were ultimately determined. Portions of the curves after “rollover” (negative curvature) are attributed to mitotic growth inhibition in the population, a dependence not applicable in the usual meaning of $T_{pot}$, and, therefore, were not considered in the fits. The origins were also excluded from the fits, except for the case of STS26T cells, for which the origin was included to give the minimum three points necessary to accomplish the fit.

References

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