Quantitative Analysis of Biological Marker Synthesis in Tumor Cell Cycle

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A quantitative relationship between tumor cell number and biologic marker concentration has been investigated and characterized by the previously developed discrete-time kinetic model for the study of cell kinetics. Here, this model is further expanded to cope with both cell cycle kinetics and biologic marker synthesis. A synchronized tumor-cell population is examined to determine the time course of the synthesis of markers in relation to cell cycle. The DNA content distributions, measured by flow microfluorometry, are analyzed by use of the model, and the cell age distribution is extracted. The average marker content per cell in cell cycle is measured for each time sequence in synchronized cell populations. The model, incorporating the cell age distribution and the average marker content per cell in cell cycle, enables one to generate the marker content distribution from which the cell number is estimated from the marker concentration. The model performance is further evaluated with Chinese hamster ovary cells, and their polyamine content and the total polyamine concentration during synchronization is calculated and related to the total cell number.

Additional Keyphrases: tumor–marker interactions, cell cycle kinetics, cancer, DNA, discrete-time kinetic model, polyamines

In the clinical management of patients with malignancy, quantitative and specific methods for assessing the effectiveness of therapeutic modalities and determining disease status are needed. Many types of tumor cells have identifiable biological markers, the kinetic patterns for which are indicative of the presence of a malignancy and may reflect the total number of neoplastic cells involved and their changes due to therapy. The tumor–marker kinetic approach is designed to integrate the growth kinetics of tumor cells with their synthesis of biological markers in experimental animal models and humans and to establish a quantitative relationship between tumor cell burden and biological marker content. Studies on biological marker production in patients with malignancy by use of the tumor–marker kinetic relationship represent a new approach to the evaluation of patient status and response to treatment. The assessment of marker concentrations reflecting total tumor burden and various aspects of cell proliferation and regression could provide the clinician with information of importance in decisions concerning therapy for particular patients. Also, kinetic information related to marker content and excretion in body fluids of these patients will aid in predicting and monitoring their clinical course during treatment.

A kinetic approach has been developed to measure immunoglobulin synthesis and the growth kinetics of multiple myeloma as well as to categorize myeloma patients according to the stage of their disease and assess their response to chemotherapy (1–3). An animal model of myeloma has been investigated for correlation with the clinical model of myeloma cell kinetics (4, 5). The metabolism of murine myeloma IgM was studied in vivo, to determine tumor cell number in mice with localized MOPC 104E tumor (6–8). Rates of secretion in vitro of human chorionic gonadotropin and of its subunits α and β by established clonal cell lines of a bronchogenic carcinoma and choriocarcinoma were quantitatively compared with growth curves of these cell lines in terms of cell number and cell protein (9). Polyamines were measured in the tumor, liver, and serum of rats with spontaneously regressing MTW9 carcinoma (10). This animal model suggested that intracellular spermidine, which increased during tumor growth, was diminished by excretion during regression, and, further, that spermidine concentrations in the serum or urine reflected tumor cell death. Because changes in polyamines in physiological fluids reflect tumor cell kinetics, Russell (11) reviewed evidence of their efficacy as biochemical markers of cancer and suggested their possible usefulness to clinicians in rapidly assessing tumor response to chemotherapy or to multimodality therapy. A high correlation was observed between the rates of cell proliferation and polyamine synthesis during the in vitro growth cycle of a tumor cell line, which indicates that the polyamines may be functionally involved in the cell's preparation for division (12).

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polyamines putrescine, spermidine, and spermine in relation to the cell cycle have been examined in Chinese hamster ovary cells synchronized by selective detachment of mitotic cells (13, 14). The polyamine concentrations of AKR leukemic cells from the mouse thymus were assayed in relation to the cell cycle (15).

Here, we present a general approach to integrating growth kinetics with synthesis of biological marker in tumor cells; with it, one can characterize the synthesis of biological markers in relation to tumor growth and cell cycle. The tumor–marker relationship, when established, will be used to develop a quantitative framework necessary for comparing biological markers to total tumor cell burden in individual animals and patients. The discrete-time kinetic (DTK) model previously developed (16, 17) is capable of generating the cell age distribution and predicting distributions of cell size and DNA content during the proliferation of various experimental tumors. This model is used in the present study to relate tumor cell number to polyamine concentration. Chinese hamster ovary cells synchronized by mitotic selection are considered, and the total polyamine concentration during synchronization is calculated and related to total cell number.

**Analytical Methods**

The discrete-time kinetic model. The DTK model (17) will be briefly described and will then be extended to encompass the synthesis of markers in cell cycle. In the DTK model analysis of cell cycle kinetics, the total cell population consists of two groups, namely proliferating and nonproliferating cells, for which the state vectors are defined. The nonproliferating group consists of non-cycling cells, which either retain their proliferative potential or are incapable of replication. During any specified interval, the nonproliferating cells may die or re-enter the proliferating cycle compartment. The model is based on the assumption that the cell cycle may be subdivided into a finite number of time segments such that each of these segments constitutes a cell cycle compartment. The mean generation time of the cell, denoted by $T_0$, is divided into $n$ equal intervals $\Delta T_0$, called the “biological unit time.” The biological age of cells is denoted by $T$ and is defined as $T = k \Delta T_0$, where $\Delta T_0$ is a function of real time $t$ if $T_0$ is time dependent, and $k$ is the discrete time. Each of these intervals constitutes a “cell age compartment.” All cells within the interval of one specific age compartment are considered as one subpopulation and are characterized by the mean cell age of that compartment. The dynamics of proliferating cells $x$ and nonproliferating cells $q$ is then represented in the form of state equations as follows:

\[
x(k + 1) = \Phi_{pp}(k + 1, k)x(k) + \Phi_{pm}(k + 1, k)q(k)
\]

(1)

\[
q(k + 1) = \Phi_{mp}(k + 1, k)x(k) + \Phi_{mm}(k + 1, k)q(k)
\]

(2)

where $x(k)$ is the $n$-dimensional proliferating cell age state vector at time $k$, and $q(k)$ is the $m$-dimensional nonproliferating cell age state vector at time $k$. In $x(k)$, the $j$th element denotes the number of cells in the $j$th cell age compartment at time $k$. The state vector $q(k)$ contains the two elements $q_d(k)$ and $q_o(k)$, where $q_d(k)$ is the number of dead cells and $q_o(k)$ the number of cells arrested in the $G_0$ phase if it exists. The state transition matrices $\Phi_{pp}$, $\Phi_{pm}$, $\Phi_{mp}$, and $\Phi_{mm}$ represent the relation between the state vectors at the time instants $k$ and $k + 1$. For example, in equation 1, $\Phi_{pm}(k + 1, k)q(k)$ denotes the subset of cells that at time $k$ were nonproliferating but at time $k + 1$ have regained their ability to replicate and have returned to the proliferating group. $\Phi_{mp}(k + 1, k)x(k)$ in equation 2 denotes those cells that at time $k$ were proliferating but at time $k + 1$ have transferred from the proliferating group to the nonproliferating group. Each of the transition matrices expresses the cell interactions between the cell age compartments and contains the elements expressed in terms of cell kinetic parameters, namely, the cell-loss rate, the cell-cycle time, and the growth fraction, whose changes may bring about a change in the rate of tumor growth.

The distribution of cell age described by equations 1 and 2 may be related to the distribution of DNA content by knowledge of: (a) the DNA content curve in cell cycle, as characterized by the DNA content per cell with $2n$ for the $G_1$ phase and $4n$ for the $G_2 + M$ phase and (b) a gaussian form of the DNA distribution of cells at any particular age with a known coefficient of variation. Given the cell age vectors $x(k)$ and $q(k)$, one can obtain the DNA content vector $c(k)$ (16). An analytical method has recently been developed for extracting the DNA content curve from DNA content distribution by the DTK model with a variational method of the parameter optimization.

**Marker content distribution.** The marker content distribution represents the distribution of cells according to the cellular content of marker, and provides the relationship between the total tumor cell number and the total marker concentration of a population of cells. In determining the marker content distribution for a cell population, the cellular marker content is defined in the same manner as the cell DNA state was defined. The marker content vector $m(k)$ has the elements which form the cell distribution according to marker content. At any discrete time $k$, a relationship between the cell age vector and the cell marker vector exists,

\[
m(k) = Vx(k) + V_mq(k)
\]

(3)

where $V$ and $V_m$ are the linear transformation matrices. The elements of $V$ are obtained from the knowledge of marker synthesis in the cell cycle. Also, the elements of $V_m$ for nonproliferating cells are determined from the marker content at which cells are transferred from the proliferating to the nonproliferating group. Each of these elements is expressed by a gaussian form with the average marker content for any specified age compartment in the cell cycle.

A method of determining the average marker content
per cell in cell cycle is presented, utilizing both experimental techniques and quantitative approaches. The time series of a synchronized cell population is obtained by either mitotic selection or chemical agents, and its marker content is measured. The corresponding series of flow microfluorometric measurements of DNA content distributions is analyzed by the DTK model, and the fraction of cells in each phase of the cell cycle is determined as a function of time. From these DNA content distributions, the cell age distributions are extracted and predicted. Because the degree of cell synchronization is somewhat limited, the synchronized cell population not only contains the cells in any specific cycle phase with a large fraction but also a mixture of cells in other phases as well. Therefore, the determination directly from the synchronized cell population of the average marker content per cell in specific stage of the cell cycle may not be accurate. An iterative scheme for the parameter optimization has been developed to improve the estimation of the average marker content per cell. An initial estimate of the average marker content in the cell cycle is based on the experimental determination of a marker for a time sequence of the synchronized cell population. With this estimate of the average marker content and the predicted cell age distribution, the model simulates a series of marker content distributions for the synchronized cell population. The marker content distribution determines the total marker content in each cell sample and the corresponding cell number. The simulated total marker content is then compared with the experimental measurement of the marker, and any difference between the simulated content and the experimental measurement becomes a basis for further adjustment of the estimated average marker content in cell cycle. This iteration continues until the best-fit of the simulated content to the measured content is obtained. During the iteration the cell number remains the same. The best estimate of the average marker content determines the characteristics of the transition matrix $V$ in equation 3. With this, one can establish the relationship between the total cell number and the total marker content and calculate the total marker during the growth and regression of cell populations. For a cell population perturbed by chemical agents, the perturbed average marker content per cell in cell cycle should be established by similar procedure discussed above, which results in a perturbed form of the transition matrix.

**Experimental Data for Analysis**

The polyamines putrescine, spermidine, and spermine constitute a unidirectional biosynthetic pathway whose biosynthetic enzymes and accumulation patterns appear to play important roles in the regulation of growth processes. Polyamines appear to have clinical utility for monitoring cancer therapy. There are studies of polyamine biosynthesis throughout the various phases of the mammalian cell cycle. In synchronously growing Don C cells, the activity of ornithine decarboxylase (EC 4.1.1.17), the first enzyme in the biosynthetic pathway to the polyamines, displays three peaks of activity during the cell cycle: one during mitosis, one at the boundary of $G_1$ and $S$ phases, and the third at late $S$ phase (21). Polyamine concentrations have been assessed in AKR lymphoma cells from the mouse thymus that represent various phases of the cell cycle (15). Putrescine increased at the boundary of $G_1$ and $S$ phases, and spermidine and spermine concentrations in late $S$.

Synchronized cells have been used to investigate the quantitative relationship of the changes in cellular polyamine content during the cell cycle. The polyamines in relation to the growth of synchronous Chinese hamster ovary cells (22), studied by using the mitotic selection method (23), were then cold-accumulated to obtain a large number of cells at the beginning of each experiment (24). Heby et al. (13) have examined the time of synthesis of the polyamines putrescine, spermidine, and spermine in relation to the cell cycle of Chinese hamster ovary cells synchronized by mitotic selection. Cell cycle analysis of the Chinese hamster ovary cells was accomplished by flow-microfluorometry of the DNA content distributions (25, 26) coupled with quantitative computer modeling (27).

In the present analysis, Chinese hamster ovary cells and polyamine interactions, experimentally determined (13), have been evaluated by a scheme discussed above. A time-sequence cell sample was collected after mitotic cells were plated into culture vessels, and each sample was analyzed to determine the DNA distribution and the polyamine content. The cellular DNA content was monitored throughout one complete cell cycle, the cell population doubling time being about 15.0 h. The cells at the time of the experiment were in the middle of their exponential growth phase, and about $3 \times 10^7$ cells, essentially all of them in metaphase, were collected. Flow-microfluorometric data for an exponential culture of Chinese hamster ovary cells, grown in suspension culture in F-10 medium supplemented with 15 ml of newborn calf serum per deciliter, were provided by Tobey (unpublished data) with a generation time of 15.0 h, subdivided into a pre-DNA synthetic period $G_1$ of 7.9 h, a DNA synthetic period $S$ of 4.4 h, a post-DNA synthetic period $G_2$ of 2.2 h, and mitosis lasting 0.5 h. These values are comparable to those estimated and used in the computer analysis of flow-microfluorometric data by Heby et al. (13): $T_0 = 14.5$ h with $T_{G_1} = 8.5$ h, $T_s = 3.2$ h, and $T_{G_2+M} = 2.8$ h. The cell age distribution for exponential growth phase in vitro was constructed as shown in Figure 1a, in which the shaded area represents cells in $S$. The flow-microfluorometric DNA content distribution was compared with the simulated DNA content distribution (Figure 1c). The closed circles represent experimental data points, the solid line represents the simulated result of total cells, and the shaded area inside indicates cells in $S$. The predicted average DNA content per cell in cell cycle used for DNA simulation was shown with the DNA increasing from $2n$ in $G_1$ to $4n$ in $G_2 + M$ (Figure 1b).
The time sequence of DNA distributions was obtained by flow-microfluorometry every 2 h after plating (Figure 2), and the intracellular polyamine content in the Chinese hamster ovary cells was determined with a Durrum D-500 amino acid analyzer (13) (Figure 5).

**Results: The Tumor Cell and Marker Content Relationship**

To determine the marker content distribution by using equation 3, the cell age distribution at time \( k \) must be known. The total marker content \( m \) may be obtained experimentally for a given cell population. Figure 2 shows the time-course of DNA content distributions; the solid lines represent the simulated total cell distribution, the shaded area indicates cells in \( S \), and the closed circles represent the flow-microfluorometric measurements of DNA distributions. From hours 2 through 6, essentially all cells possessed a 2n DNA content, i.e., they were progressing through the \( G_1 \) phase of the cell cycle. No cells were engaged in DNA synthesis for about 6 h after the start of synchronization. After this time, however, virtually the entire population started to produce DNA; 8 h after synchronization, 70% of the cells had entered the \( S \) phase, and by 10 h 80% of the cells were in the \( S \) phase, 10% were in the \( G_1 \) phase, and another 10% had entered the \( G_2 + M \) phase. At 12 h, most of the cells were progressing through the \( G_2 \) and \( M \) phases of the cell cycle. About 15 h after synchronization, most of the cells had completed one cycle and 75% of the cells possessed the DNA content of \( G_1 \) cells. The corresponding sequence of cell age distribution during synchronization, as obtained by the DTK model, was illustrated in Figure 3.

As shown in Figures 2 and 3, each cell population collected during synchronization contains a mixture of cells in each cycle phase; thus, the experimental determination of the cellular polyamine content does not accurately reflect the content in any specific stage of cell cycle. The mean polyamine content per cell in relation to specific stages of cell cycle was predicted by the model, as illustrated in Figure 4. As expected, when the collected mitotic cells divided, the cellular spermidine content decreased to a value that was half of that at the mitotic phase. In the late-\( G_1 \) phase the spermidine began to accumulate and in the beginning of the \( S \) phase they sharply decreased to the minimum value. Spermidine increased significantly in the mid-\( S \), but as the cells started to enter the \( G_2 + M \) phase, they began to accumulate spermidine again. On the other hand, the intracellular spermidine content in the Chinese hamster ovary cells, measured at various times after synchronization, is shown in Figure 5. The closed circles represent the experimental data points (13); the solid curve rep-
represents the simulated results. The putrescine content was about 10-fold lower than the spermidine and spermine content at all times. However, the patterns of change for putrescine, spermidine, and spermine were very similar throughout the cell cycle (13).

The relationship between the total number of cells and the total concentration of polyamines is readily analyzed from the polyamine content distribution with time. This relationship is demonstrated in Figure 6. The solid line represents the total concentration of spermidine and the dashed lines represent the number of cells during the one-cycle period of synchronization. Non-linearity is a characteristic of the relationship. The tumor cell number remains about the same during the

first 11-h period, then sharply increases up to twofold. On the other hand, the spermidine content varies considerably during the first 7 h, then gradually increases up to about twofold the initial content, indicating that the polyamine content largely depends on cell age distribution as well as cell number.

Discussion

A quantitative approach was developed to establish the kinetic relationship between the tumor cell burden and the cellular marker content. The previously developed DTK model for cell kinetic analysis (16, 17) was further expanded to encompass the synthesis of biological markers in relation to the cell cycle, and computer simulation demonstrated the usefulness of the model approach for relating the kinetic behavior of Chinese hamster ovary cells to their synthesis of polyamines during synchronization.

Information on the cell cycle kinetics of Chinese hamster ovary cells in vitro was obtained by the flowmicrofluorometric analysis of DNA distribution and autoradiography. The cell age distribution for exponentially growing cells was then estimated, but it may be extracted from the DNA content distribution. The DTK model simulated the mechanism of cell synchronization by mitotic selection, and its performance was examined with respect to the time series of flowmicrofluorometric DNA distributions for Chinese hamster ovary cells synchronized by selection, revealing the successive progression of mitotic cells throughout the cell cycle (Figure 2) and predicting the corresponding series of cell age distributions (Figure 3). The cell age distribution extracted was of particular value, because it became baseline information for constructing the marker content distribution. The model also showed that the growth pattern of synchronized Chinese hamster ovary cells was similar to one of HeLa cells synchronized by selection (23) and one of Chinese hamster ovary cells synchronized by hydroxyurea (28).
In the present study, the polyamine (spermidine) content distribution for synchronized Chinese hamster ovary cells was generated and subsequently analyzed in order to relate quantitatively Chinese hamster ovary cells to polyamine content (Figure 6). A nonlinear relationship was dominant throughout the cell cycle, whereas the marker content was related in terms of cell number by computer simulation. Cellular marker content is mainly characterized by two factors: (a) the distribution pattern of cell age at any specific time and (b) the shape of the curve for average marker content per cell in cell cycle.

The average marker content per cell in cell cycle (Figure 4) was determined by using both marker measurements and simulated marker contents. The polyamine concentrations in relation to the cell cycle have been studied on cells perturbed by chemical agents (14, 21), separated by sedimentation (15), or collected by the mitotic selection method (13, 24). These studies of perturbed systems or mitotic selection are limited by the dispersion of cell cycle and the degree of synchronization, which results in a mixture of cells in different cycle phase; therefore, those changes in polyamine synthesis cannot be accurately correlated with the cell cycle. The average polyamine content predicted by the DTK model enables the accurate estimation of polyamine synthesis in relation to specific stages of the cell cycle. In the present study of synchronized Chinese hamster ovary cells, all the polyamines showed the same pattern of fluctuation during the cell cycle (13); as a result, the marker content distribution for spermidine alone was considered and analyzed in detail. With the average spermidine content in cell cycle, the model simulated the time-course of spermidine content in exponentially growing Chinese hamster ovary cells, as shown in Figure 7. In this case, a linear relation appears to prevail; however, we could not correlate spermidine synthesis with specific stages of the cell cycle. Information on cycle-stage-specific spermidine synthesis would provide better insight into its relation to the kinetic behavior of Chinese hamster ovary cells and allow more accurate estimate of the cell number by the cellular content of spermidine. With the evidence that polyamine synthesis is cell-cycle dependent (10, 14, 15, 20), a hypothesis was proposed that spermidine can serve as a marker of tumor-cell kill, whereas putrescine reflects the proliferation of the tumor (11, 18); however, the Chinese hamster ovary cells and polyamine interactions appear not to support the hypothesis, although further investigation is required.

The marker content distribution presents an analytical insight into the quantitative relationship between tumor cell number and cellular marker content. Experimental determination of the marker content distribution with plasminogen activator in Chinese hamster ovary cells has been presented in this symposium, by use of the flow-metrofluorometric technique (29). The analytical method for in vitro studies could be further extended to in vivo studies as well, and could provide a framework of a scheme for estimating the total tumor burden based on the body fluid content of markers. The latter requires developing a marker content distribution for body fluid content. The relationship between the body fluid marker content distribution and the marker content distribution in vitro or in vivo, or both, must then be established in order to relate body fluid marker content to total tumor burden.

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References


