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Possible Implication of Long-lived Non-dividing Metaphase Cells in the Fraction of Labeled Mitoses Method

— A New Foundation of Cell Cycle Analysis —

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INTRODUCTION

Ever since the introduction of the flow cytometric technique for the measurement of DNA content in individual cells, a number of investigators have endeavored for its application to the diagnosis and prognosis of neoplastic diseases as well as the monitoring of cancer treatment. It is because the technique has provided a simple means to know how cells are distributed in the cell cycle. Certainly, when there are few cells in S- and G_2M-phases, the cell population may be considered as non-proliferative. However, the data do not give us any information as to the rate of cell proliferation. In principle, cell proliferation rate cannot be estimated without making reference to a clock in some way or another. It means that the measurement must be made at least twice at a known time interval as in the double labeling method for the measurement of S-phase length. For more detailed analysis, many more measurements are required.

The cell cycle analysis using the "fraction of labeled mitoses" curve (FLM curve), called previously as "percent labeled mitoses" curve (PLM curve) by Quastler and Sherman (1959), continues to be the most powerful method. Despite the method is laborious in its experimental procedure and despite it suffers from a considerable statistical errors involved in the analytic result, it is the only method available for estimating the mean and the standard deviation of phase transit time. The FLM curve rises and falls in the form of a trapezoid when a cohort of pulse-labeled cells goes through M-phase. In the actual experimental data, the trapezoid is degenerate due to variable transit times (Quastler and Sherman, 1959; Wimber, 1960; Takahashi, 1966; 1968). There are several computer programs with which to calculate cell cycle parameters by fitting a stochastic model to the experimental data (Steel and Hanes, 1971; Takahashi et al., 1971).

According to the models currently available including mine (Takahashi, 1968; Takahashi et al., 1971), the slopes of the FLM curve become less and less steep with advance of time. This characteristic is consistent with most of the experimental FLM curves but there are some exceptions (e.g. Hamilton and Dobbin, 1994) in which the first wave rises slowly and falls precipitously in a way that cannot be simulated by such models. Common to all the models proposed so far is that M-phase was assumed to be of short duration and little attention was paid to the distribution (of the duration). If it is assumed that some fraction of cells take a long time in M-phase whereas the remaining fraction divide quickly, the resulting FLM curve will be much different from traditional ones. As will be described below, such a biphasic (or bimodal) distribution of M-

phase transit time seems to be the case with malignant tumor cells whose DNA content in G_2M phase is greatly variable. This paper describes a new method for the analysis of the FLM curve that can be applied to such malignant tumor cell populations.

BACKGROUND OF RECONSIDERATION

The background for a new method of FLM analysis is that there are dividing and non-dividing metaphase cells. As will easily be understood, the shape of the FLM curve reflects not only the distribution of S-phase length but also the distribution of metaphase transit time. A low mitotic index does not necessarily mean that all of the mitotic cells quickly pass through M-phase.

In a proliferating cell population, more than half of the cells entering into mitosis divide to generate daughter cells. It is possible, however, that a sizable fraction of mitotic cells are non-dividing and it may exceed the fraction of dividing mitotics. It may sound strange or paradoxical but it is acceptable if we consider the situation in which the dividing metaphase cells leave quickly from the scene whereas non-dividing metaphases stay much longer in the population. We have to admit the possibility that many of the metaphase cells which we see in the population are destined to lysis but cell proliferation is sustained by a rapid renewal of so small a fraction of mitotics that may even escape recognition.

M-PHASE AS A CHECKPOINT OF DNA CONTENT

To the astonishment of investigators in the early days of flow cytometric study of tumor cells, G_1 DNA content is constant in a variety of tumor cell strains in spite of a marked variation of chromosome number (Kraemer et al., 1971, 1972). As long as we think that many of the mitotic cells divide, a constancy of G_1 DNA content remains an enigma.

To evade from this difficulty, most flow cytometrists considered that the histogram of G_2M peak is merely representing the variation of fluorescence intensity due to variable degrees of chromosome condensation. However, for those who have ever observed metaphase chromosomes microscopically which are so variable in shape and in number, it is hard to believe that G_2M DNA content is constant unless positively supported by reliable experiments. This discrepancy is resolved only if we accept an idea that mitotic cells with variable DNA content are generated continuously, although at a relatively low rate (i.e. below 50% at highest), in malignant tumors and eliminated before proceeding to G_1-phase. There must be a cytogenetic mechanism to check an abnormal DNA content in mitotic cells.

A NEW METHOD OF FLM ANALYSIS

A negatively skewed FLM wave is usually interpreted to result from the addition of variable S-phase length. The method of estimating variable transit time through phases (Wimber, 1960) was supported by the results of simulation using a mathematical model that assumes gamma-distributed phase transit times (Takahashi, 1966, 1968) although there is no experimental evidence to support this assumption directly. The situation is much different if there are cells that stay long in metaphase and consequently the slopes in the FLM curve may be accounted for without assuming variation of transit time.

For the sake of simplicity, we will first consider the cell populations in which phase lengths are constant (Fig. 1). Transit times through dividing and non-dividing metaphase will be denoted as T_M and T_M^* and the relative numbers of cells in these phases as F_M and F_M^*, respectively (Fig. 1). Hence, $F_M + F_M^* = 1$. If the numbers of dividing and non-dividing metaphase cells are M and M^*,

$$F_M = M/(M+M^*) \text{ and } F_M^* = M^*/(M+M^*)$$

T_M will be short but T_M^* may be quite long ($T_M << T_M^*$). If a reproductive coefficient, α, is defined as the probability with which a G_2- cell enters into a dividing metaphase, the following relationship holds:

$$F_M : F_M^* = \alpha T_M : (1-\alpha) T_M^*$$

Hence,

$$T_M^*/T_M = \beta(F_M^*/F_M) = \beta(1/F_M - 1)$$

where

$$\beta = \alpha/(1-\alpha)$$

(see Fig.1)

In order that the tumor grows, the reproductive coefficient should be greater than 1/2:

$$1/2 < \alpha \leq 1$$

A mean transit time through metaphase, T_{MM}^*, that takes T_M^* into consideration is given by

$$T_{MM}^* = \alpha T_M + (1-\alpha) T_M^*$$

On the other hand, mitotic index, MI, increases in proportion to T_{MM}^* (under the condition of a fixed cell cycle time). Therefore, when MI is small as is actually so even in rapidly growing cell population, T_{MM}^* should also be small. Then, a question of whether T_M^* can be long or not will naturally arise. Given with T_{MM}^* and $\alpha(1/2 < \alpha < 1)$, the maximum length of T_M^* that can be attained is

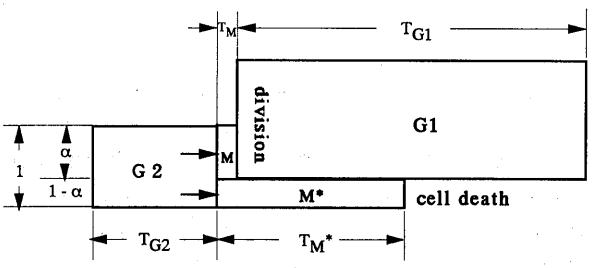

Fig. 1 Structure of mitotic phase : G_2-phase cells enter into dividing and non-dividing metaphase (i.e. M- and M*-phases) at relative rates of α and $1-\alpha$, and they stay in these phases for T_M and T_M^*, respectively. The numbers of cells in M- and M*-phases are written as M and M^* whereas F_M and F_M^* are the relative numbers of cells in M- and M*-phases. Note that M^* may exceed M even in proliferating cell populations. In this figure, mitotic cells after division are included in G_1.

$$T_M^* = (1-\alpha)^{-1} T_{MM}^*$$

and this indicates that T_M^* can be very long even when T_{MM}^* is short and hence MI is small.

AN EXAMPLE

Given with the cell cycle parameters, a FLM curve can be constructed using this model (Fig. 3). For a sample calculation, it will be assumed that (i) the cells pulse-labeled in S-phase enter into mitosis at $t_0 =$

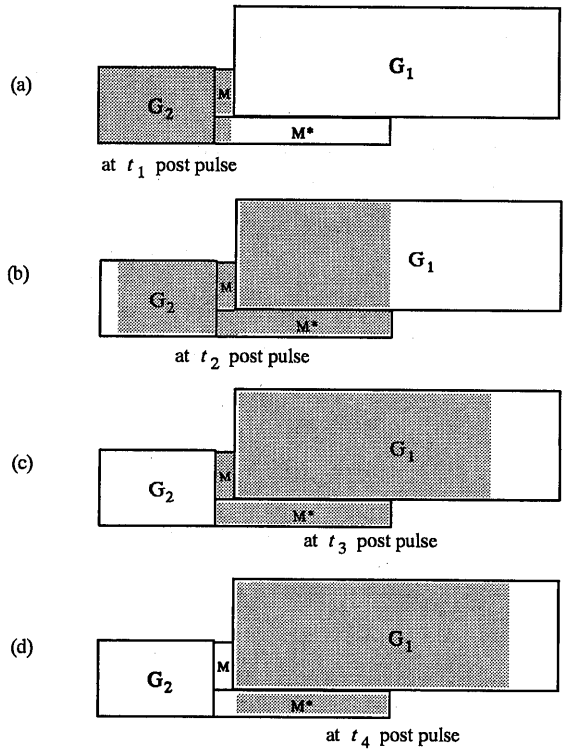

Fig. 2. Passage of labeled cells through metaphase: A labeled cell cohort is indicated by a shaded area. A fraction of labeled metaphase cells (FLM) is the fraction of shaded area in $M + M^*$. (a) At $t_1 = T_{G2} + T_M$, labeled cells occupy M completely but occupy only a small fraction of M^*. (b) and (c) During the period from $t_2 = T_{G2} + T_M$ to $t_3 = T_{G2} + T_S$. M and M^* are occupied by labeled cells. Therefore, FLM stays at the level of unity. (d) At $t_4 = T_{G2} + T_S + T_M$, none of M are labeled but most of M^* consists of labeled cells. Although not shown, FLM drops to zero level by $t_4 = T_{G2} + T_S + T_M$

T_{G2} (T_{G2} =3 hr), (ii) the fraction of cells that enter into dividing metaphase compartment is α (= 75%), (iii) they divide after a period of T_M (= 0.5 hr) at $t_1 = t_0 + T_M$ (t_1 = 3.5 hr) (iv) the fraction of cells that enter into non-dividing metaphase compartment is 1 - α (= 25%), (v) they stay in this compartment for a period of T_M^* (= 4.5 hr) before being disintegrated at $t_2 = t_1 + T_M^*$ (t_2 = 8 hr). Then, it follows that

$$F_M / F_M^* = 1/3$$

and it means that F_M = 1/4 and F_M^* = 3/4.

At $t_0 = T_{G2}$ (3 hr), labeled cells begin to enter into mitosis and the FLM curve begins to rise from zero, When a period of T_M (0.5 hr) has elapsed after entry into mitosis (at $t_1 = t_0 + T_M$ = 3.5 hr), dividing metaphase compartment is filled completely with labeled cells but in non-dividing metaphase compartment the fraction of labeled cells is T_M / T_M^* (=1/9). Therefore, in a period of (t_0, t_1), the FLM value increases linearly from

$$\text{FLM } (t_0) = 0$$

to

$$\text{FLM } (t_1) = F_M + (T_M/T_M^*)\, F_M^* \ (=1/3)$$

During a succeeding period from t_1 to t_2 (= $t_1 + T_M^*$) it increases to reach a value of unity (provided that $T_M^* < T_S$) and then remains at this plateau level for a period of $T_S - T_M^*$(=2.5 hr) until $t_3 = t_2 + T_S$ (=15 hr)

$$\text{FLM}(t_2)=1 \quad \text{and} \quad \text{FLM}(t_3)=1$$

Since t_3 is the time when the tail of a labeled cohort begins to leave from G_2/M boundary, the FLM begins to decrease and at $t_4 = t_3 + T_M$ (= 15.5 hr) it reaches to a value of

$$\text{FLM } (t_4) = (1 - T_M/T_M^*) F_M^* \ (= 2/3)$$

Then, it takes another 4 hr (i.e. $T_M^* - T_M$) before the FLM curve returns to zero. Therefore, at $t_5 = t_3 + T_M^*$ (= 19.5 hr)

$$\text{FLM } (t_5) = 0$$

This example clearly indicates that the ascending and descending slopes of the first FLM wave depend so much upon the transit times of dividing and non-dividing cells through metaphase that the slopes cannot be attributed directly to variable S-phase transit times. As a matter of course, in a special case in which dividing and non-dividing cells have the same transit time through metaphase, the new model makes no difference from the traditional one.

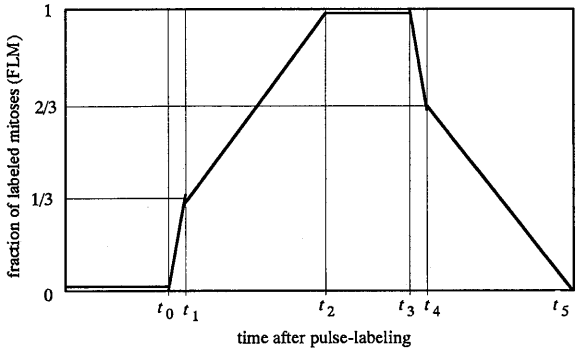

Fig. 3 A FLM curve for a sample cell population: (i) Up to $t_0 = T_{G2}$ (3 hr), FLM = 0. (ii) Then it rises sharply until $t_1 = t_0 + T_M$ (3.5 hr) (iii) The slope is less steep to $t_2 = t_1 + T_M^*$ (8 hr) (iv) From t_2 to $t_3 = t_2 + T_S$ (15 hr), FLM stays at the level of unity. (v) From t_3 to $t_4 = t_3 + T_M$ (15.5 hr), FLM drops sharply and (vi) From t_4 to $t_5 = t_3 + T_M^*$ (19.5 hr), FLM drops slowly again.

Anyway, this new model provides a means to analyze such FLM curves that will never be fitted well by the traditional methods, for example, the data as reported by Hamilton and Dobbin (1983). In addition, it draws our attention to the possibilities that have never been seriously considered, i.e. S-phase transit times in individual cells may not be so variable as has been estimated from the skewness of the first FLM wave by the traditional methods.

CELLS WITH VARIABLE TRANSIT TIMES

By generalizing the method described above, a stochastic model of the FLM curve can be constructed, which can be used for the simulation of the cell populations with dis-

tributed phase transit times.

In principle, a mathematical model for the generation of a theoretical FLM curve utilizes a multiple phase birth process (Kendall, 1948) which consists of a series of Poisson processes, as described previously (Takahashi, 1968, Takahashi et al., 1971). A cell changes its state multiple times to go through a phase of the cell cycle. Only different in this new version is to calculate the numbers of both dividing and non-dividing metaphase cells separately.

Because of the assumption that the state changes according to the law of a Poisson process, a sojourn time of a cell in i-th state obeys an exponential distribution.

$$p_i(t) = \exp(-\lambda_i t)$$

where $p_i(t)$ is the probability density that a sojourn time in i-th state is t and λ_i is a transition probability density (i.e. the probability with which a cell in i-th state shifts to the next state in unit time). If it is assumed that a cell changes its state k times according to a Poisson process before it goes through a phase and that the transition probability is unchanged within a phase

$$\lambda_1 = \lambda_2 = \quad = \lambda_k = \lambda$$

then it follows that a phase transit time obeys a gamma distribution:

$$P(t) = \Gamma(k)^{-1} \lambda^k t^{k-1} \exp(-\lambda t),$$

and the mean, $T(\lambda, k)$, and the coefficient of variation, $CV(k)$, of phase transit time are

$$T(k, \lambda) = k/\lambda$$

and

$$CV(k) = k^{-1/2}$$

The number of cells in each state can be described by a differential equation (i.e. compartment equation):

$$dn_i(t)/dt = \lambda_{i-1} n_{i-1}(t) - \lambda_i n_i(t) \qquad (i = 2, 3, \cdots, k)$$

where $n_i(t)$ is the mean number of cells in i-th state (or compartment) at time t. Note that the equation begins with $k = 2$ (as for the equation with $k = 1$ see below). The first and the second terms in the right hand side of the equation represent influx into i-th compartment and efflux from it, respectively. For ease of mathematical treatment, all the transition probability densities within each phase are assumed to be equal:

$$\lambda_i = \lambda_{G1} \qquad (i = 1, \cdots\cdots, g_1)$$
$$\lambda_i = \lambda_S \qquad (i = g_1+1, \cdots, s)$$
$$\lambda_i = \lambda_{G2} \qquad (i = s+1, \cdots, g_2)$$

and

$$\lambda_i = \lambda_M \qquad (i = g_2+1, \cdots, k)$$

The cells in the first compartment is special and the mean cell number, $n_1(t)$, is described by

$$dn_1(t)/dt = 2\alpha \lambda_k n_k(t) - \lambda_1 n_1(t)$$

because only a fraction of the cells in the last (i.e. k-th) compartment divide to yield $n_1(t)$ The remaining fraction, $(1 - \alpha)\lambda_k n_k(t)$, do not divide and stay in the population as non-dividing mitotics for a variable time that obeys a gamma (or exponential) distribution.

If there are k^* compartments in non-dividing mitotic phase, a mean number of cells in these compartments are described by

$$dn^*_1(t)/dt = \lambda_k n_k(t) - \lambda^* n^*_1(t)$$

and

$$dn^*_i(t)/dt = \lambda^* n^*_{i-1}(t) - \lambda^* n^*_i(t) \qquad (i = 2, 3, \cdots, k^*)$$

Solving the simultaneous differential equations (compartment equations) for $n_i(t)$ $(i = 1, 2, \cdots, k)$ and $n^*_i(t)$ $(i = 1, 2, \cdots, k^*)$ under an appropriate initial condition, the distribution of cells in these compartments can be calculated. Relative cell number in each compartment in an asynchronous population, r_i, is that corresponds to $n_i(\infty)$. In order to calculate the number of labeled metaphase cells (dividing or non-dividing), $M(t)$, the compartment equations must be solved under the initial condition that

$$n_i(0) = r_i$$

and $M(t)$ is given by

$$M(t) = \sum_{i=G2}^{k} n_i(t) + \sum_{i=G2}^{k^*} n^*_i(t)$$

The FLM curve synthesized in this way can be fitted to the experimental data by changing the parameters iteratively as described previously (Takahashi et al., 1971), using a function minimization technique whereby the function to be minimized is the sum of the square deviations of the FLM curve from the data

DISCUSSION

The above consideration has demonstrated that the first FLM wave gives us information about the distribution pattern of metaphae transit time because the shape changes remarkably depending on the existence of a cell group whose sojourn time in metaphase is long as expected to be the case in many malignant tumor cells. For the reasons described above, they are the cells which do not divide but disappear from the population by lysis after long sojourn times in metaphase. They correspond to the metaphase cells which have variegated chromosome complements with variegated DNA contents and cannot yield G_1 cells with a constant DNA content. When a fraction of non-dividing metaphase cells is reduced to zero (i.e. $1-\alpha = 0$), this new version becomes identical to the traditional method. By contrast, the traditional method, when applied to such tumor cells, does not give us right answer. We have to admit the possibility that we might have misinterpreted the first FLM wave with a long tail. This will be important especially in evaluating the FLM curves of some tumors (e.g. McCarter and Quastler, 1962; Post and Hoffman, 1964; Reiskin and Mendelsohn, 1964). In addition, a revised version of FLM method will allow to estimate the fraction of cells that enter into non-dividing metaphase.

An idea that the tail of the first FLM wave reflects passage of cells through non-dividing metaphase has its basis on the consideration that the cells in G_2M phase with abnormal DNA contents are eliminated in metaphase. In this respect, it is important to make a direct cinemicrographical observation of mitotic process in order to see if there is a difference of transit time between dividing and non-dividing mitotics. According to Ebe et al. (personal communication), HeLa cells divide soon after they round up on the bottom of culture bottles but a considerable fraction of cells which stay long in mitosis do not divide and ultimately disappear by cell lysis. It has a profound biological implication because it means that the mechanism to terminate S-phase is distroyed in some malignant tumor cells but metaphase serves as a checkpoint of DNA content to preserve constancy of G_1 DNA content. The checking mechanism is unknown but it can be expected that any cell which stays long in metaphase will certainly die due to suppressed gene expression. Only when an abnormal cell acquires a new mechanism to go through the barrier of a checkpoint, a new tumor cell line develops.

In order to examine whether or not a synthetic FLM curve fits well to data in its fine details of curvature, statistical data noise must be reduced to as small values as possible by increasing the number of cells scored. However, the traditional method of scoring labeled and unlabeled mitotic cells under microscope is so tedious that it was practically impossible to count more than several hundreds per scoring time. Fortunately, a flow cytometric technique allows quick measurement of both DNA content and the amount of bromodeoxyuridine (thymidine-analogue) incorporated at the time of pulse-labeling. Therefore, if the FLM method is modified in such a way that the fraction of labeled G_2M cells is used instead of the fraction of labeled mitoses, the cell cycle analysis will be greatly facilitated by the application of flow cytometric technique.

REFERENCES

Quastler, H. & Sherman, F. G. (1959). Cell proliferation kinetics in the intestinal epithelium of the mouse. *Exp. Cell Res.* **17**, 420-438.

Wimber, D. E. (1960). Duration of the nuclear cycle in Tradescantia paludosa root tips as measured with H^3-thymidine. *Am. J. Bot.* **47**, 828-834.

Takahashi, M. (1966). Theoretical basis for cell cycle analysis. I. Labelled mitosis wave method. *J. Theor. Biol.* **13**, 202-211.

Takahashi, M. (1968). Theoretical basis for cell cycle analysis. II. Further studies on labelled mitosis wave method. *J. Theor. Biol.* **18**, 195-209.

Takahashi, M., Hogg, J. D. & Mendelsohn, M. L. (1971). Automatic analysis of FLM curves. *Cell Tissue Kinet.* **4**, 505-518.

Steel, G. G. & Hanes, S. (1971). The technique of labelled mitoses: analysis by automatic curve-fitting. *Cell Tissue Kinet.*, **4**, 93-105.

Hamilton, E. & Dobbin, J. (1983). The percentage labelled mitoses technique shows the mean cell cycle time to be half its true value in Carcinoma NT. *Cell Tissue Kinet.* **16**, 483-492.

Kraemer, P. M., Deaven, L. L., Crissman, H. A. & Van Dilla, M. A. (1972). DNA constancy despite variability in chromosome number. *Adv. Cell Mol. Biol.* **2**, 47-108.

Kraemer, P. M., Petersen, D. F. & Van Dilla, M. A. (1971). DNA constancy in heteroploidy and the stem line theory of tumors, *Science* **174**, 714-717.

Kendall, D. G. (1948). On the role of variable generation time in the development of a stochastic birth process. *Biometrika* **35**, 316-330.

McCarter & Quastler, H. (1962). Effect of dimethylbenzanthracene on the cellular proliferation cycle. *Nature* (*Lond.*) **194**, 873-875.

Post, J. & Hoffman, J. (1964). The replication time and pattern of carcinogen-induced hepatoma cells. *J. Cell Biol.* **22**, 341-350.

Reiskin, A. B. & Mendelsohn, M. L. (1964). A comparison of the cell cycle in induced carcinomas and their normal counterpart. *Cancer Res.* **24**, 1131-1136.