Cell Cycle Kinetics and Mitotically Linked Chemotherapy\textsuperscript{1}

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Summary

Recent developments in the kinetics of solid and ascites tumors are discussed in terms of models of tumor growth and treatment with mitotically linked chemotherapeutic agents. Evidence for and the significance of the following phenomena are summarized: feedback control of cell proliferation in solid tumors, nonproliferating tumor cells, the reversion of tumor cells from nonproliferating to proliferating status, tumor cell death, clonogenic tumor cells, and changes of tumor kinetics in response to therapeutic perturbation.

Introduction

In the past ten years the kinetic analysis of tumor growth has undergone a rapid and impressive development. Increasingly complex and realistic tumor models are being dealt with, and for the first time the methods are being extended to tumors in the midst of the perturbations of therapy. Such details of tumor growth are a vital concern to the cancer chemotherapist, since he looks to kinetic data for the clues that will further improve his therapy. But much like the parallel situation in radiotherapy \textsuperscript{(13, 24)}, the empirical practice of chemotherapy (both in the laboratory and the clinic) manages to stay ahead of all attempts at understanding the underlying mechanisms. The best that kinetics can do at the present is to suggest some vaguely rational framework which may give a sense of order to the selection of a few of the infinitely possible schedules that can be used.

General Models of Tumor Therapy

A fully developed kinetic model of chemotherapy would apply to both tumor and the relevant dose-limiting normal tissues. It would incorporate the characteristics of the known types of agents, including the mitotic linkage if any, dose-response curves, variations in distribution of agent, and secondary perturbations caused by the agent. And finally it would have to embrace some presently mysterious aspects of the problem: the characteristics of the most resistant cells in the populations, the dynamic responses to initial perturbation, and the definition of cure, toxicity, or other end-points. Such models are still over the horizon, but meanwhile it is worth reviewing the current status of what is known about the cellular basis of this approach.

The earliest tumor models contained a single compartment of proliferating, exponentially growing tumor cells. In 1932 Mayneord formalized the role of central necrosis \textsuperscript{(14)}, and in 1962 and 1966 the ideas of growth fraction \textsuperscript{(15)} and cell death \textsuperscript{(21)} were added. The technics of kinetic analysis and the available data on tumor kinetics were reviewed thoroughly by Baserga in 1965 \textsuperscript{(1)}, and in 1967 we described a sequence of increasingly complex models of tumor growth \textsuperscript{(17)}. The most complex of these models is the stepping off point for the discussion of recent developments that follows. This model contains clonogenic cells in the proliferating and nonproliferating state, and a nonclonogenic pool containing proliferating, G\textsubscript{O}, sterile and dying tumor cells.

Proliferating Cells

The driving force for tumor growth and the key to mitotically linked chemotherapy is the cell cycle. It can be characterized by two parameters: the cell cycle time, \(T_c\), and the coefficient of variation of cell cycle time, \(CV_c\). Both parameters can be estimated in any population of asynchronous cells by pulse labeling with a radioactive precursor of DNA, fixing samples periodically, and constructing a PLM (percent labeled mitoses) curve from autoradiographs. When \(CV_c\) is zero, the labeled cells will cycle at identical velocities, and this results in a PLM curve consisting of a series of trapezoids recurring at \(T_c\) intervals. When \(CV_c\) is greater than zero, the sides of the trapezoids become sigmoidal, and the series degenerates into a progressively damped sinusoid. The period of the oscillations gives an approximation of the average cell cycle time, \(T_c\), but does not equal it precisely, and the damping gives an estimate of \(CV_c\).

The average cycle times of tumors that have been measured range over a factor of 10 and include values on the order of half a day for L1210 (25) and Sarcoma 180 (19) and \(T_c\)'s of several days for human ascitic and solid tumors (4, 7). Longer values probably exist since the nature of the PLM curve method tends to bias the sampling toward the shorter \(T_c\)'s. Some examples of \(CV_c\)'s are: 40 to 50 percent for spontaneous and transplantable C3H mammary tumors (16, 18) and 25 and 64 percent for two transplantable rat tumors studied by Steel et al. \textsuperscript{(21)}.

One of the most intriguing aspects of tumor kinetics is the relationship between \(T_c\) and \(T_e\). Remember \(T_e\) is a cellular characteristic, and its variability gives rise to \(CV_c\) and the

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damping of the PLM curve. \( T_c \) is a population characteristic, and an example of variability here would be the behavior of \( T_c \) in a series of experimental tumors of the same type. But operationally the only way to get a PLM curve from small experimental tumors is to assemble it from data on 50 or 100 tumors. Thus the characteristics extracted from such a PLM curve are a composite of intercellular and intertumor effects. In this situation, an indirect expression of intertumor variability can be gotten by comparing the PLM values for experimental tumors is to assemble it from data on 50 or 100 operations across the entire curve. Given a \( CV_c \) of 50 percent, this is precisely what would be expected from the random sampling error of 100 or so cells, and hence the error for tumors per se approaches zero. It must be emphasized that these tumors arise independently and are widely divergent in growth and morphologic characteristics even though they have a common etiology and tissue of origin.

Tannock (23) and Frindel et al. (6, 8) have recently compared the kinetic behavior of tumor lines that grow both in the ascitic and the solid form. Both groups find that when the tumors are ascitic the generation time of the tumor cells increases dramatically with the age of the tumor. For example, at 2, 6, and 10 days after implantation respectively, Tannock found the \( T_c \) (and \( CV_c \)) were 12 hours (11.6 percent), 44 hours (12.2 percent), and approximately 83 hours (52 percent). However, Tannock's solid tumor has a \( T_c \) of 17 hours, and a \( CV_c \) of 27.6 percent; and Frindel et al. have shown that the \( T_c \) of their solid tumor is largely independent of tumor volume. Clearly the ascitic cells respond to aging, crowding, or some similar consequence of growth in an across-the-board lengthening of their cycle. One could argue that the cells in solid form have a similar response as a function of distance from vessels or some other local but not general property of the tumor. This would explain the independence of \( T_c \) and volume, as well as the intermediate value of the solid \( T_c \). But some other factor must be operating as well because the \( CV_c \) of the solid tumors is smaller than the range of variability seen during the life history of the ascitic form. Thus no matter how good the local conditions, individual cells in solid form divide less rapidly than they do in a young ascites tumor, and no matter how poor the local conditions, individual cells in solid form do not lengthen their cycle to the same extent as cells in an old ascites tumor.

Although the effect is small, cells in solid tumors do respond somewhat to their environment. Hermens and Barendson (9, 10) have described differences in labeling index and \( T_c \) between the outermost and the innermost parts of a rat rhabdomyosarcoma. Similarly, Tannock has shown a fall-off of labeling index with distance from the vessel in a tumor that grows as perivascular cylinders only several cells thick (22).

Thus it appears that in a solid tumor \( T_c \) is stable with age, responds only slightly to altered conditions of growth, and is somewhat longer than the \( T_c \) the same tumor cells can achieve in an early ascitic form. The individual cellular variability in \( T_c \) is quite large, but it is not as great as the range of behavior found in ascites tumors of different ages. And finally, the very fact that reasonable PLM curves can be obtained from populations of tumors of similar type or origin means that the \( T_c \), \( CV_c \), and other population attributes of the cycle must be relatively invariant within such tumor classes. These observations strongly support the hypothesis that tumor cell proliferation is a controlled or negative feedback process in aging asces and in all solid tumors. In the solid form, the control appears to be loose at the level of individual cells and tight at the tissue or regional level. The nature of the tight control of \( T_c \) remains obscure. The negative feedback loop could take various forms including a specific growth inhibitor driven by cell concentration, a depletion of nutrients or of growth stimulants, a control involving the growth of blood vessels or other aspects of blood supply, and a control involving differentiation of the tumor cells to conform to architectural or functional specifications that interfere with further proliferation.

This feedback model of tumor cell proliferation has several important implications to the therapist and the cancer biologist. To the therapist, the relative invariance of the control with tumor type may make \( T_c \) and other cycle characteristics predictable from one patient to another. On the negative side, however, a reversible inhibition of the cell cycle can be expected to render some tumor cells resistant to mitotically linked therapy. In addition, the model predicts that the destruction of some tumor cells could temporarily release the remaining cells from feedback inhibition. This means that during the recovery phase after treatment, the surviving tumor cells would shorten their cell cycle or recruit cells into the cell cycle until the accelerated growth brought them back under feedback control. To the biologist, the model emphasizes the need to study the nature of growth controls, and it nurtures the hope that with better understanding one may someday be able to restore homeostasis to the malignant population.

Nonproliferating Cells

The presence of nonproliferating cells seems to be universal in solid tumors. Such cells are inferred from labeling statistics, from attempts to saturate a population with chronically administered label, and from the disparity of \( T_c \) and the doubling times of tumors (5, 12, 15, 16, 21). In many tumors the nonproliferating cells are the majority, that is the growth fraction (the ratio of proliferating to total cells) is less than 0.5. Nonproliferation may be an aberration of disordered growth, but normal tissues are an excellent example of mixed proliferating and nonproliferating cells, and some remnant of this complex phenomenon may explain the similar behavior of tumors (3). Nonproliferating cells have a particular significance to chemotherapy since they are immune to mitotically linked agents. The details of reversibility of the nonproliferative state are crucial to this problem. In spite of many efforts there is still no decisive evidence on the extent, the rate, the control, or even the existence of conversion from the nonproliferating to the proliferating state in solid tumors. In anticipation of what is suggestive, but as yet unproven, the best we can do is to divide the nonproliferating cells into a \( G_0 \) (reversible) and a
sterile (irreversible) compartment and await further developments.

Cell Loss

Several recent studies have convincingly demonstrated the importance of cell loss in tumor growth (7, 18, 20, 21, 23). In some slowly growing tumors the rate of loss is so large and so similar to rate of cell production that these systems approximate the steady state renewal condition of normal tissues (3). Migration of cells is one possible cause of loss, but cell death is the predominant factor. Death involves both proliferating and nonproliferating cells, and it may also occur on a regional basis.

From the chemotherapeutic point of view, cell death has many ramifications which are still poorly understood. It means that cell proliferation and net growth are loosely coupled, and hence that net growth may be a poor indication of the vulnerability of a tumor to mitotically linked agents. It also means that tumor shrinkage may be uncoupled from therapeutic effect. For example, a slowly growing tumor may have its rate of proliferation decimated and yet not show a loss of mass because of a parallel cessation of cell loss. And finally, cell death raises some interesting questions about G0 cells.

The turnover time of G0 cells is a good general descriptor of their accessibility to mitotically linked agents. In the absence of cell death, the only source of turnover is the reversion of G0 cells to proliferative status. Unless the reversion rate is high enough to give complete turnover during the course of treatment, there will inevitably be a failure to kill some G0 cells. Any built-in mechanism of death of G0 cells would increase the turnover of the G0 compartment and would reduce the fraction of G0 survivors after therapy. An even more striking effect could occur if the built-in mechanism of G0 death was nonrandom. Two hypothetical modalities of such nonrandomness are death due to a predefined age distribution of G0 cells and death caused by a fixed size of the G0 population with the cells furthest from the proliferating centers dying first. We have been studying this problem in a line of mouse mammary tumors in which the growth rate is slow and the death rate is high. The nonproliferating fraction in these tumors is unexpectedly easy to label with chronically administered tritiated thymidine, and the only interpretation we have that fits this phenomenon is nonrandom death of nonproliferating cells with the oldest cells dying first.

Death due to aging of cells would probably continue independently of therapy. On the other hand death secondary to the size of the nonproliferating pool would faithfully follow the rate of influx of cells into the pool. In the latter case reaching G0 cells by turning the compartment over through growth might have some advantages over killing proliferating cells and having the G0 cells revert. Mitotically linked radiation sensitizers or some other method that reaches the cells but does not interfere with growth until a later time would then be a reasonable approach to treatment. The brominated pyrimidines are examples of radiation sensitizers that could be used in this manner (11). These agents are incorporated into dividing cells without significantly interfering with growth. Subsequent radiotherapy elicits the sensitization, but a point that bears checking is whether the sensitization extends to cells that take up the analog and then go into the G0 state. With any method that attempts to reach G0 cells by encouraging growth, one must accept progression of the tumor during the course of treatment. In the absence of much cell death, this could involve several doublings of the tumor and hence would be prohibitive. But a high spontaneous rate of cell death reduces tumor growth and at the same time promotes turnover of the G0 cells, and under these conditions the G0 cells could be completely sensitized in less than a doubling.

We know nothing at present about the way a tumor changes its feedback settings as it undergoes progressive shrinkage during therapy. However, if there is any tendency to reduce the size of the G0 pool in keeping with the reduction in volume, then the recruitment and the displacement mechanism can be going on side by side. For this to work, the mitotically linked agent must kill proliferating cells by destroying some and converting the others to sterile but intact forms. The sterile cells would gradually fill the G0 pool and displace unaffected cells; meanwhile those G0 cells that revert would in turn be killed by the agent. This, of course, is completely speculative, and it might well turn out that such treatment approaches affect host toxicity more than tumor regression.

Clonogenic Cells

In solid tumors it is the rare cell that has the capability of growing a new tumor; or more precisely, the probability of producing a tumor from a single cell is generally very small, and thousands of cells are usually required to produce a reasonable incidence of tumor take. From evidence such as this it has been suggested that most tumors are like the hematopoietic system in that proliferation goes on at many different levels, but only a few proliferating cells are fully clonogenic.

Bergsagel and Valeriote recently provided an interesting kinetic insight into this phenomenon by studying colony formation of a mouse plasma cell tumor Adj PC-5 (2). The gross tumor has a doubling time of 36 hours, and the doubling time of the 4.4 percent of cells that are clonogenic is 25 hours. A ten-hour treatment of vinblastine reduces the colony-forming cells to zero, suggesting both that the underlying cycle time of these cells is no greater than ten hours and that there is no significant G0 component of clonogenic cells. The Tc of this tumor has not been studied by the PLM method, but unless cell death is a major factor, the doubling times indicate that the nonclonogenic tumor cells will have a longer average cycle time than the clonogenic cells.

If clonogenicity of tumor cells continues to be a valid concept, then the therapist will have to broaden his orientation to include the kinetics of clonogenic cells. The situation will be further compounded if it turns out that clonogenic cells can exist in a G0 state. Eventually, a dichotomy may arise between treatments for immediate relief of symptoms and treatments for cure or long-term palliation. The former would be aimed at the cells in general and the latter at the clonogenic cells in particular. When the clonogenic cells are in the minority, their kinetics cannot be studied by the PLM method. Methods involving clonogenicity per se must be used, and, as in the example just described, these methods turn out to...
involve mitotically linked chemotherapeutic agents with well-understood effects.

**Perturbed Tumors**

A brisk perturbation makes it difficult to carry out conventional kinetic analysis. For example, transient mitotic inhibition prevents the scoring of PLM values, partial synchronization invalidates the analysis, and the kinetic behavior of dying cells may obscure the analysis of survivors. Nevertheless if one is planning to use fractionated or continuously administered therapy, it is critical to know how the tumor cells are behaving in the midst of therapy.

Hermens and Barendsen (10) have circumvented these problems of conventional analysis by developing a tumor system in which cell growth can be followed either in solid tumors *in vivo* or as single-cell clones *in vitro*. Thus PLM curves can be supplemented by surviving fractions and by direct estimates of the regrowth of clonogenic cells as a function of time after a therapeutic perturbation of the solid tumor. In the unperturbed state, their rhabdomyosarcoma has a doubling time of 144 hours, a \( T_C \) of 21 hours, and a plating efficiency of 35 percent. The tumor was perturbed with a single dose of 2,000 rads of 300 kv X-rays, a dose that gives a surviving fraction of 0.008. Between Days 4 and 9 postirradiation, the number of clonogenic cells increased by a factor of 100 at an average doubling time of 18 hours. A PLM curve beginning on Day 4 gave a \( T_C \) of about 12 hours. There can be no doubt that this tumor underwent a prompt and effective repopulation as a result of accelerated kinetics in the posttreatment period.

These studies confirm the prediction of the feedback model that a sudden loss of tumor cells may result in a shortening of \( T_C \) and a rapid repopulation. In general this type of response increases the vulnerability of surviving tumor cells to mitotically linked agents. But the rapid regrowth of tumor minimizes palliation and works against the therapist who uses spaced treatments in order to avoid host toxicity.

**Overview**

There is some satisfaction in the new-found knowledge that tumor growth is far from the simple one-compartment models that were expounded up to several years ago. But with each new complication, the hope for a simple universal strategy of tumor therapy becomes increasingly remote. Nevertheless, the problem is important enough to warrant serious attention, and until a radically different approach to cancer therapy is found, the best strategy for chemotherapy will probably be intimately involved in the kinetic factors of tumor growth. We simply must learn how to recruit or otherwise destroy G₀ cells, how the cell cycle behaves in perturbed and unperturbed tumors, and how best to characterize and take advantage of the behavior of those tumor cells that are primarily responsible for the continued propagation of tumor masses. Above all we need new and better methods of analysis if the day is ever to come when kinetics can dictate the course of tumor therapy.

**REFERENCES**

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