2

Oncology for surgeons—cell kinetics

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INTRODUCTION

Radical surgery alone is insufficient to guarantee cure for many patients with solid tumours, and the search continues for better prognostic indices and adjuvant therapies. Cell kinetic studies describe cell proliferation in both normal and malignant tissues, and in particular the timing and duration of proliferative events. Owing to the limitations of earlier research tools, it has been surprisingly difficult to learn about the dynamic behaviour of living cells in vivo. Static indices such as the thymidine labelling index, or flow cytometric S phase fraction (SPF) have been widely studied under the guise of cell kinetics, but these give no indication of the rate of cell production. For example, a tumour with a high SPF but a long cell cycle time may be proliferating more slowly than a tumour with the converse characteristics.

Developments in flow cytometry, monoclonal antibodies and cell markers have led to considerable recent progress in cell kinetic studies. As the understanding of the integration of cell processes improves, so the science of kinetics merges with research into many other regulatory and enzymatic processes within living tissues. Proliferation markers have been extensively reviewed in recent years (Hall & Levison 1990; Quinn & Wright 1991), and it is the purpose of this chapter to provide a general review of the principles and applications without recourse to mathematics, for which many excellent guides are available (Steel 1977; Wright & Alison 1984).

Before it can be of use in prognosis or therapy, a kinetic measurement (Table 2.1) must fulfil three criteria. It must be easily applied in normal clinical practice, results must be rapidly obtainable so that appropriate therapy can be implemented, and the measurements must be standardized and readily performed in hospital laboratories.

The cell cycle

All tissues, organs and tumours are composed of a complex mixture of cells of different functions, origins and fates. Each subpopulation consists of cells which are terminally differentiated (such as neurons), non prolifer-

Table 2.1 Kinetic indices of clinical utility

Static indices (with no time component)
Mitotic count or index (MI)
Labelling index (LI) (of any named marker or antigen)
S phase fraction (SPF)
Growth fraction (GF)
Dynamic indices (with a time component)
S phase duration (Ts)
Cell cycle time (Tc)
Potential doubling time (T_{pot})
Volume doubling time (Td)
Effective doubling time (T_{eff})
Crypt turnover time (CTT) [of mucosa]
Crypt cell production rate (CCPR) [of mucosa]

ating or quiescent but still able to proliferate in response to appropriate stimuli, proliferating to produce daughter cells, or destined to die or undergo programmed cell death (PCD).

Proliferating cells pass through a series of discrete phases in their life cycle. In 1951 Howard and Pelc at the Hammersmith Hospital described the concept of the cell cycle, composed of five phases (Fig. 2.1). From the G1 (gap 1) phase, cells enter either the G0 (resting) phase, or the S (DNA synthesis) phase. From the S phase they enter the G2 (gap 2) phase, from which they pass into the M (mitosis) phase, and then back into G1. The duration of the S, G2 and M phases is believed to be relatively constant for any one cell type, maximum variation being found in the duration of the G1 phase. All cell populations contain cells in each phase, the majority of cells always being in G1. Rapidly proliferating populations contain a higher

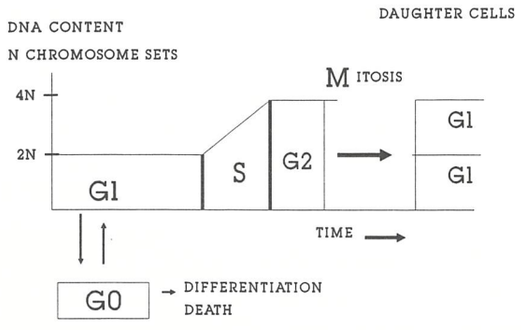

Fig. 2.1 A diagram of the cell cycle which emphasizes the change in DNA content of the cells (y axis) with time (x axis).

fraction of cells in the S phase. Chromosomes are duplicated during the S phase, when cycling cells will take up thymidine analogues, which include tritiated thymidine and the halogenated deoxyuridines.

Concepts in cell and tissue kinetics

The cell cycle model makes complex populations of cells amenable to experimental and mathematical analysis. There are a number of concepts which are important to the understanding of the kinetics of proliferating cell populations.

The *growth fraction* (*GF*) is the fraction of proliferating cells in the total population. In most cell populations it is much less than 100% or unity, because of the large number of quiescent, differentiated and dying cells, even within rapidly proliferating tumours. The growth fraction may approach 100% in the proliferation zone of intestinal mucosa and in cell cultures in exponential growth.

The cell cycle time (*Tc*) is the time for one cell to pass through the entire cell cycle. If all cells in the population are identical and are proliferating (growth fraction is 100%), the time taken for the population to double its numbers will be the cell cycle time.

The potential doubling time (T_{pot}) or apparent cell cycle time is a useful concept. It is the product of the cell cycle time Tc divided by the growth fraction (GF). It is the time taken for cell numbers to double in a population which contains non-proliferating cells, which is the normal biological state. It is only equal to the cell cycle time at the limit where GF = 100%. It takes no account of cell loss.

The effective doubling time (T_{eff}) is a term used in radiotherapy to describe the proliferation of surviving cells after treatment fractions.

The volume doubling time (*Td*) or specific growth rate of a tissue or tumour is the consequence of the balance of all processes of cell production and cell loss (Table 2.2). The Td can sometimes be estimated by serial imaging. For example, serial measurements can be made of hepatic metastases using ultrasonography or computerized tomography. Steel (1977) calculated the mean TD of 56 lung metastases from colorectal cancer to be 95 days. Bolin (1983) estimated the mean Td of 27 colorectal cancers to be 195 days (range 79–2355 days).

Table 2.2 Factors determining tumour volume growth

TUMOUR CELL PRODUCTION
The growth fraction
The duration of the cell cycle
Genetic switches of the cell cycle
Local intercellular signals (e.g. growth factors)
Distant intercellular signals (e.g. hormones)
Adequate local nutrition and vascularity
TUMOUR CELL LOSS
Apoptosis
Exfoliation into blood or gut
Altered cell adhesion
Host response, inflammation and cytokines
Ischaemia and tumour necrosis

The growth curve of tumours

The true pattern of growth of tumours from single abnormal cells to advanced disease is not known. In some experimental models, growth is initially exponential but decays with time (Laird 1964). The growth rate of human tumours probably varies with time according to the interaction of tumour and host factors such as the inflammatory response, nutrition and vascularity (Denekamp 1986). A measurement taken at any point in the life of the tumour is unlikely to be predictive either of the past or future growth characteristics of the tumour.

Cell loss

The rate of growth of a tissue or tumour is determined by the balance between cell production and cell loss. Cell kinetic studies only describe cell production. Cell loss is a critical concept. The *cell loss factor* (θ) is calculated from the ratio of the potential to the actual doubling time.

$$\text{Cell loss factor } (\theta) = 1-(T_{pot}/Td)$$

Cell loss is caused by a number of processes, including cell exfoliation into the bloodstream, lymphatics, body cavities or gastrointestinal tract, cell migration or metastasis, natural cell death (ageing and apoptosis), and tissue necrosis due to immunological response, drug or radiation effects, or hypoxia.

Apoptosis and programmed cell death

The selective elimination of cells which have served their purpose is an important process in the life of tissues and organs. Apoptosis is rapid and

cell fragments are soon redistributed to other cells and macrophages. The programmed death of single cells (PCD) or apoptosis is less easily discerned than tissue necrosis, and does not imply that the dying cell is defective. Indeed, the switches and intrinsic controls of apoptosis may be as complex and important as those regulating cell proliferation. Paradoxically, cells with defective apoptosis mechanisms may survive rather than die, contributing to abnormal growth of the tissue or tumour. Apoptosis is the subject of much current research and is well characterized (Dive & Hickman 1991). For example, specific nuclease-induced DNA fragmentation is recognized on gel electrophoresis as a 'DNA ladder'. The gene *bcl*-2 may specifically inhibit apoptosis and thus promote oncogenesis (Williams 1991).

Cell loss is extremely difficult to measure directly. The cell loss factor of a tumour can be estimated indirectly from the T_{pot} and the Td. This is much easier in experimental tumours than in clinical practice. Estimates of the T_{pot} of colorectal tumours (Rew et al 1991a) and the Td (100 days or more) indicate that the cell loss factor for intestinal tumours may well exceed 90% of total cell production. This need not be surprising, as in a proliferating tissue in the steady state such as intestinal mucosa, cell production balances cell loss, and the cell loss factor is 100 per cent. A relative reduction in cell loss, such as may be due to increased cell adhesiveness or decreased apoptosis, may be as important as an increased rate of cell production in determining tumour or tissue growth.

Regulation of the cell cycle and proliferation associated proteins

Cell proliferation is a cleverly choreographed interaction of molecular processes. It requires control of initiation and termination, and control mechanisms for this complex process have been extensively conserved through evolution. In normal tissues, proliferation and differentiation are related and tightly regulated by positive and negative feedback from both intra- and extra-cellular mechanisms. Many quiescent cells can still proliferate in response to appropriate signals, and the behaviour of tumour cells may be influenced by adjacent stromal cells such as fibroblasts.

Extracellular signals include growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF) and transforming growth factor alpha (TGF-α). Evidence for a control function also exists for genes such as p53, *erb* B_2, *ras*, *fos*, *jun* and *myc* (Pardee 1989).

Some proteins are known to have cycle-dependent expression and defined functions. They include nucleohistones; enzymes of nucleotide and polyamine metabolism such as dihydrofolate reductase and thymidine kinase; protein p105 which is selectively expressed in G0 and M phases; the topoisomerase family of DNA conformational and repair enzymes, and initiators of proliferation such as the proteins p34- *cdc*2 kinase (*cdc* = cell division cycle) and cyclin (Marx 1989).

MEASUREMENT OF CELL KINETICS

Static and dynamic cell kinetic indices can be estimated by a number of methods, each of which have its advantages and limitations (Table 2.3). Mitotic counting, radioisotope labelling techniques and stathmokinetics are documented briefly because they are well covered in earlier texts (Steel 1977).

Static indices

Indices based on the counting of labelled cells in tissue sections are laborious and are subject to observer bias, fatigue and variation. Critically,

Table 2.3 Indices of proliferation: advantages and limitations. The comments apply to clinical applications

(a) Static indices	Advantages	Limitations
Mitotic count	Conventional Histology	Mitoses are rare M phase is short
Labelling indices —tritiated thymidine	Proven method for in vitro studies	Laborious Radioactivity Delay with data
—bromodeoxyuridine —iododeoxyuridine	For in vivo or in vitro studies	Has to be injected preoperatively Fresh tissue only
—Ki-67	Intrinsic marker	Labile Function unknown
—Proliferating cell nuclear antigen (PCNA)	Intrinsic to cell Archival or fresh tissue Histology or FCM	Labile
—AgNORs (Nuclear organizing regions)	Intrinsic marker	Laborious to count Value uncertain
S phase fraction (flow cytometry)	Large numbers of nuclei can be counted	Artefacts common Loss of tissue architecture
(b) Dynamic indices	**Advantages**	**Limitations**
Stathmokinetics (metaphase arrest) —tritiated thymidine	Proven clinical method Historical data for comparisons	Cytotoxic drugs Laborious to count Radioisotope now unacceptable in vivo
Bromodeoxyuridine Iododeoxyuridine	No acute toxicity (low dose) Well preserved in range of fixatives Protease resistant	Prospective studies Consent needed
—histochemistry	Reliable uptake Easily stained	Laborious to count
—flow cytometry	High productivity Dynamic data from a single biopsy	Preparation artefacts Loss of tissue architecture Reproducibility? Expense and training

they give no indication of the dynamic state and rate of tumour proliferation.

The mitotic index

This is the proportion of mitoses within the total population. The mitotic index is a crude assessment of proliferation, because the M phase is short relative to the S phase duration and the cell cycle time (Potten et al 1992).

In vitro tritiated thymidine studies

Tritiated thymidine (3H-TdR) was for many years the mainstay of cell kinetic research. 3H-TdR allows both static and dynamic experiments in animal models. Ethical considerations restricted the study of human tumours to in vitro incubation of freshly excised tumour biopsies with 3H-TdR.

Counting of labelled cells by autoradiography yields the labelling index (LI). Drawbacks of the technique include the problems with handling radioisotopes, the time delay during autoradiographic development, and the need to count labelled cells manually.

In vitro bromodeoxyuridine studies

In vitro incubation of fresh tissue sections with bromodeoxyuridine yields labelling data equivalent to 3H-TdR (Chwalinski et al 1988). Shimosato et al (1989) reported the study of BrdUrd labelling indices in human lung carcinomas by this technique.

Flow cytometric static proliferation indices

In flow cytometry (FCM), coherent light of a known wavelength, usually from a laser source, illuminates a coaxial stream of cells or nuclei which are labelled with fluorescent dyes. Photon emissions are detected at predetermined wavelengths by photomultiplier tubes (PMTs). Computerized data collection allows the simultaneous measurement of six or more cell parameters, including DNA content. The intensity of fluorescence allows quantitation of the marker under study in each cell or nucleus (Watson 1987a,b). Counting of fluorescent events in each of thousands of cells or nuclei is rapid, and the sorting and collection of subsets of cells for further analysis is also possible.

DNA analysis by single parameter flow cytometry

Nuclear suspensions can be obtained from fresh or archival blocks of tissue and analysed by FCM. Dyes such as propidium iodide and ethidium

bromide bind directly to DNA, and fluorescence in direct proportion (stoichiometrically) to the quantity of DNA present. A data plot of nuclear numbers against fluorescence intensity yields a DNA histogram. Normal tissues are diploid and have single G1 and G2 peaks.

Abnormal DNA content, or aneuploidy, reflects gain or loss of sets or fragments or chromosomes. The DNA index can be calculated from the DNA histogram, as the ratio of the mean DNA content of the aneuploid to the diploid peak. Ploidy is not in itself a kinetic marker, but it has considerable bearing on the measurement of cell kinetics by flow cytometry. In most series, 50% or more of tumours are found to be aneuploid (Barlogie et al 1983; Hedley 1989).

The aneuploid population is also made up of cells in G0/G1, S and G2. The aneuploid population usually overlaps the diploid population within the DNA histogram, and may introduce considerable difficulties to the mathematical analysis (deconvolution) of the histogram.

The flow cytometric S phase fraction (FCM SPF)

The DNA histogram yields the relative proportions of nuclei in G0/G1, S and G2 and hence the fraction of nuclei in the S phase (SPF). The SPF does not indicate the rate of proliferation. The relative ease of generation of S phase fraction (SPF) data from archival material using flow cytometry has encouraged the belief that each tumour can be described by a single meaningful proliferative value. The clinical value of FCM SPF measurements is uncertain for a number of reasons. There are problems of variable extraction and preparation of nuclei from archival blocks which render up to 40% of specimens unsatisfactory for analysis (Hedley 1989). Even with standardization of controls and analytical algorithms there is considerable interlaboratory variation (Wheeless et al 1991). Archival studies lack control or assessment of the degree of heterogeneity in the original tumour, and better quality control can be achieved with fresh specimens and fine needle aspirates. The accurate analysis of aneuploidy is confounded by overlapping diploid and aneuploid populations and by the presence of artefact, which may also cause diploid tumours to be misinterpreted as aneuploid. Unfortunately, the tumour population is not distinguishable from stromal cells in diploid histograms.

Dynamic indices of cell proliferation

The following techniques enable time-dependent or dynamic indices to be estimated.

Stathmokinetics

This is the study of the rate of accumulation of arrested cells in vivo. Colchicine, vincristine and vinblastine arrest dividing cells in metaphase.

The method allows the rate of entry of cells into mitosis, the rate of cell birth, the potential doubling time and the duration of mitosis to be calculated (Camplejohn 1982).

Tritiated thymidine

The cell cycle time can be estimated using a 3H-TdR if multiple, serial biopsies are taken after in vivo pulse labelling. This is rarely feasible in clinical practice, but some human tumour kinetic data has been obtained from radiolabelling in vivo (Chavaudra et al 1979).

Fraction of labelled mitoses (FLM)

Cells may be pulse labelled with 3H-TdR in vitro or in vivo and allowed to proceed through the cell cycle. Serial sampling allows estimation of the cell cycle and phase times from the peak numbers of cells in mitosis as the first and second cell cycles are reached. Precise definition of the graphical peaks is rarely possible. Variations on this technique include *Pulse* and *Double labelling* methods.

Grain count halving experiments

As 3H-TdR labelled DNA divides at mitosis, so the grain count falls. The time elapsed from pulse labelling to the first mean halving is a measure of time (Ts + TG2 + TM). Tannock (1978) reviewed 97 clinical studies which had used this techique.

Halogenated pyrimidines and in vivo studies

Bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IUdR) are synthetic, non-radioactive DNA base precursor analogues. They each have similar biological characteristics for cell kinetic studies (Begg 1989). Gratzner (1982) described the first of a series of monoclonal antibodies specific for these agents within denatured DNA.

BrdUrd was developed as a tumour radiosensitizer in the 1950s. Subclinical doses (200–500 mg) are incorporated in measurable quantities into animal and human tumours and tissues (Begg et al 1985; Begg 1989; Wilson 1988). Following intravenous injection, BrdUrd and IUdR are rapidly incorporated into DNA by S phase cells in proliferating tissues throughout the body. It is advised that the use of these agents is restricted to post-reproductive adults with proven malignancy (Rew & Wilson 1991c). Two variations on the in vivo labelling technique can be used to obtain proliferation data.

a. Continuous infusion labelling studies. These agents are given by continuous infusion or pulse labelling immediately before surgery or

biopsy, and labelling (but not time-dependent parameters) is measured by histochemistry (Hoshino et al 1985) or flow cytometry (see below).

b. Pulse labelling studies. An intravenous bolus of BrdUrd or IUdR is given between 3 and 10 h before surgery or biopsy, this being within the normal range of the S phase duration. In this method, both static and dynamic indices can be calculated from single biopsies.

Multiparameter flow cytometry and dynamic studies

Quantitative cell cycle changes in the expression of markers such as BrdUrd or IUdR and regulatory proteins and oncogene products such as c-*myc* (Rew et al 1991b), p53, p21, Ki67 and PCNA can be measured by flow cytometry. The simultaneous analysis of cell surface markers, nuclear antigens and DNA content is even possible (Houck & Loken 1985). The antigen under study is identified by a monoclonal antibody which may be bonded directly to a fluorochrome, or it may be detected indirectly with a second anti-Ig antibody bound to a dye such as fluorescein (FITC). The absorption and emission spectra of the available dyes rather than engineering limitations determine the number of antigens which can be measured simultaneously. The principles of the method are illustrated in Figure 2.2.

In 1985, Begg and colleagues described how cell nuclei pulse labelled in vivo with BrdUrd could be followed through their cell cycle by measuring DNA content and BrdUrd content simultaneously on a flow cytometer (Begg et al 1985, 1989; Wilson et al 1988). This was a revolutionary advance

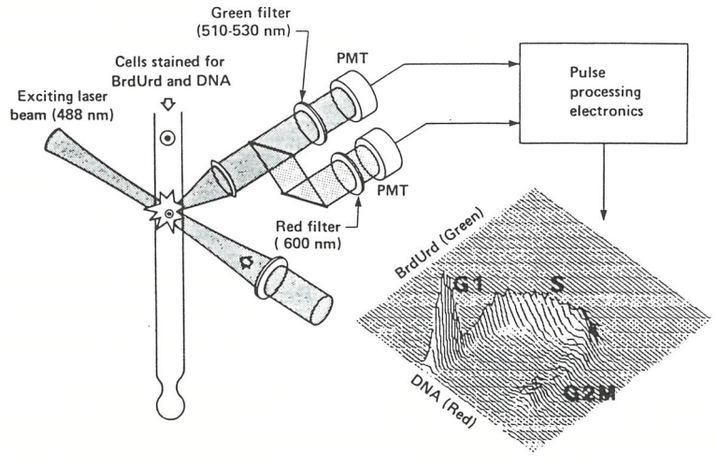

Fig. 2.2 This diagram illustrates the principle behind the multiparameter analysis of bromodeoxyuridine (BrdUrd) content in relation to the G1, S and G2M phases of the cell cycle by flow cytometry (see text). (By courtesy of Dr J W Gray.)

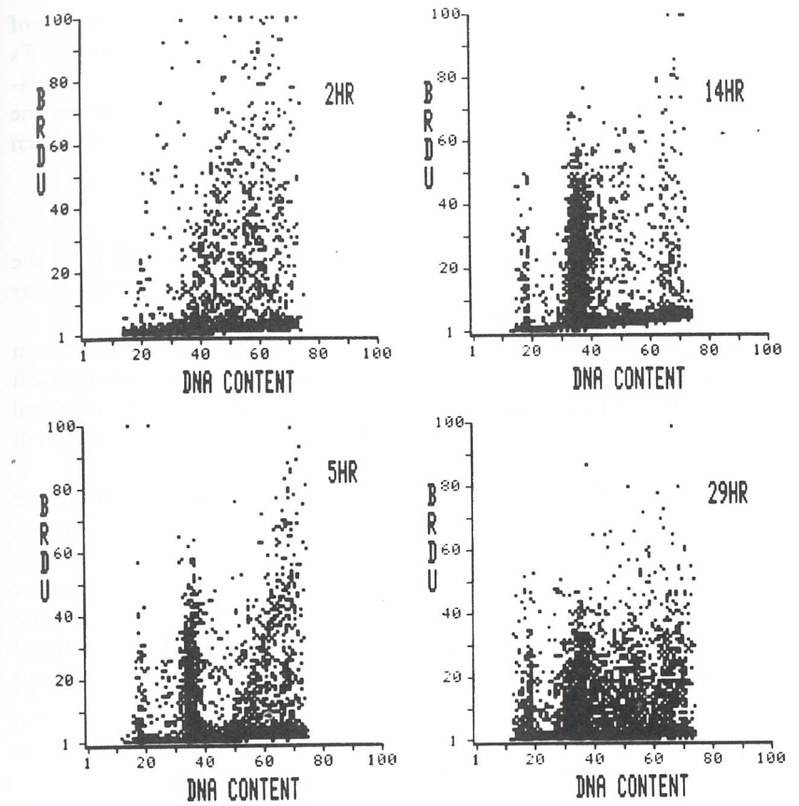

Fig. 2.3 These histograms illustrate the cell cycle progression in an experimental tumour. A series of aneuploid MC28 rat sarcomas were grown subcutaneously in nude mice and sampled at the time intervals shown after pulse labelling with bromodeoxyuridine. Two hours after labelling, most labelled nuclei are distributed in the aneuploid S phase, between channels 36 (G1) and 72 (G2). With time, the nuclei pass through G2M and into the G1 phase of the daughter cell cycle (see channel 36 at 14 hours). By 29 hours, labelled cells have re-entered the S phase. The cell cycle time of this tumour was calculated to be 30 hours. There is very little labelling in the diploid population (G1 at channel 19, G2 at channel 38).

for clinical studies. Figure 2.3 illustrates the way by which flow cytometry can be used to demonstrate cell cycle progression in an animal model. The BrdUrd labelling index, the S phase duration (Ts) and the potential doubling time (T_{pot}) of a human tumour could be estimated from a single biopsy within one day.

The model assumes that at the time of labelling, BrdUrd is evenly taken up by cells throughout the S phase, that all cells in the labelled population are proliferating, that the cell population is in a steady state and homogeneous and that DNA synthesis proceeds uniformly during the S phase. The pulse-labelled population will pass through and clear the S phase in a

time Ts. If the time between labelling and biopsy is known, the change of mean DNA content of the labelled population with time and hence the Ts can be calculated. The precise time interval is not critical to the calculations. This is a practical clinical advantage of the technique, given the difficulties in precise timing of operations and biopsies. The T_{pot} can then be calculated from the formula

$$[T_{pot} = \lambda.Ts/LI]$$

Where LI is the labelling index, calculated by computer 'gating' from the histogram, and lambda (λ) is a correction factor for aging and non-linear distribution of cells through the cycle (Steel 1977).

The method has further refinements. By appropriate gating, studies can be focused on aneuploid populations where present, as these exclude much of the stromal cell population. In animal models, samples from identical series of tumours can be used to measure all cell cycle phase durations in detail.

BrdUrd and IUdR have become established as the gold standard for proliferation studies because they are much safer to handle than radioisotopes; biopsy specimens can be preserved in 70% ethanol or methanol, adding to the clinical convenience of the method; BrdUrd and IUdR can be detected in formalin-fixed histological sections by immunochemistry using the same primary monoclonal antibodies as used for FCM; they are rugged markers which survives formalin, pepsin extraction and other fixation and preparative conditions which destroy labile proteins.

Fig. 2.4 An incidental villous adenoma of the rectum labelled in vivo with bromodeoxyuridine. The proliferating cells are clearly marked with the darker stain. (Magnification × 25.)

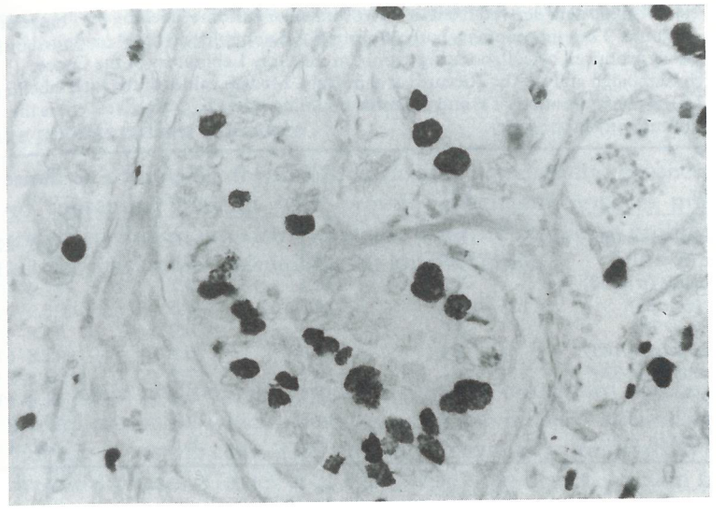

Fig. 2.5 A primary rectal carcinoma labelled in vivo with bromodeoxyuridine. The proliferating cells are clearly distinguishable as in Fig. 2.4. (Magnification × 40.)

Combined flow cytometric and histochemical studies

Flow cytometry consistently underestimates the BrdUrd or IUdR labelling index in diploid tumours when compared with histochemical counting, because the former also counts unlabelled stromal nuclei. The latter method frequently identifies areas of tumour where local labelling may reach 50%. Examples of BrdUrd-labelled biopsies are shown in Figures 2.4 and 2.5.

Very few stromal cells are found to take up BrdUrd in any of the series of tumours yet studied, and it may be valid to combine the flow cytometric Ts data for the tumour cells with the histochemical (manually counted) LI values to calculate a T_{pot} for the foci of rapid proliferation. Such foci often display a T_{pot} of less than two days, and this may be more significant for the rate of tumour proliferation than is the mean labelling amortised over larger areas (Bennett et al 1992).

In vivo multiparameter BrdUrd studies: clinical data

BrdUrd and IUdR are useful in the study of many classes of human haematogenous and solid tumours (Table 2.4) . Because the LI and Ts are independent variables, T_{pot} is a better index of the rate of proliferation than either Ts or LI alone. The data emphasize that a rapid potential doubling time is common to many tumours, and suggests that variable cell loss rather than cell production may be the principal determinant of the clinical growth rate of many tumours.

Table 2.4 Kinetic data derived from in vivo bromodeoxyuridine labelling on a range of human tumours. The table contains both published and unpublished data* (supplied by Dr G D Wilson) produced by collaborating groups at the Gray Laboratory of the Cancer Research Campaign and (+) by Riccardi et al in Italy. Median values of the total labelling index (LI), S phase duration (Ts) and potential doubling time (T_{pot}) are given. (n) is the number of tumours in the series.

Tumour	LI%	TS (h)	T_{pot} (days)	Range of T_{pot}
Colorectal (n = 100)	9.0	13.1	3.9	(0.7–22.2)
Gastric (n = 21)+	10.7	14.4	8.4	(6.8–13.5)
Oesophagus (n = 50)	7.8	12.4	5.2	(1.6–107.1)
Cervix (n = 22)	11.6	15.8	4.5	(2.9–15.8)
Breast (n = 75)	4.2	8.7	8.2	(1.8–47.5)
Head/neck (n = 165)	4.9	9.9	6.4	(1.8–67.8)
Melanoma (n = 52)*	4.2	10.7	7.2	(2.3–33.0)
Lung (n = 38)*	8.0	15.1	7.3	(1.4–132.0)
Gliomas (n = 20)+	6.4	14.8	12.1	(6.0–26.8)
Leukaemia (n = 50)+	7.8	12.7	8.8	(2.8–16.7)

Limitations of flow cytometry in solid tumour kinetics

Multiparameter flow cytometry is not the answer to all problems in cell kinetic research. Solid tumour biopsies must be disaggregated into single cells or nuclei before analysis. This may be done by mechanical grating or fine needle aspiration, by the use of collagenase on fresh tumours, and by pepsin digestion of ethanol- or formalin-fixed material, but this inevitably yields mixtures of subcellular fragments and cell aggregates in addition to single cells. Flow cytometry is not a substitute for conventional histology or immunohistochemistry. The disaggregation of solid tumours damages many antigens and destroys histological structure, while cytoplasmic and cell membrane antigens are particularly difficult to preserve. Stromal, inflammatory and vascular cells cannot yet be distinguished from diploid tumour cells in DNA histograms, although it may prove possible to distinguish stromal from tumour cells using markers such as cytokeratin.

Computer-assisted planar image cytometry (Kamentsky & Kamentsky 1991) allows the measurement of DNA content in propidium iodide stained histological sections. Though still in the development phase, such machines may in time combine the best features of flow and image cytometers, and overcome the need to disaggregate solid tumours.

Gastrointestinal mucosa as a cell kinetic model

Intestinal mucosa is a valuable model for clinical research. It is a highly structured tissue in which the distribution of proliferating cells is well documented (Chwalinski et al 1988). In vitro and in vivo BrdUrd labelling has allowed the detailed description of the proliferating compartment in human colorectal mucosa, including dynamic parameters such as S phase

duration of crypt cells (Potten et al 1992). This will facilitate histochemical studies in mucosa of other proliferation associated proteins.

Other proliferation markers

The need to administer thymidine analogues parenterally, prospectively and to consenting patients with proven malignancy imposes practical constraints on clinical research. A robust, 'intrinsic' marker of proliferation would offer considerable advantages for kinetic studies.

Ki67

This is a labile proliferation associated protein recognized by the Ki67 monoclonal antibody, which is expressed in the nucleus of proliferating cells but not in resting cells. The antigen is maximally expressed in late S and the G2M phases (Gerdes et al 1984). Ki67 can be measured in frozen sections by histochemistry and in fresh preparations by flow cytometry. The biological function of the antigen remains uncertain (Sasaki et al 1988).

Proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta, a 36 000 MW acidic protein which increases in the late S phase of the cell cycle. It is intimately associated with the replication and repair of DNA (Laskey et al 1989). PCNA exists in a nucleoplasmic (loosely bound) form during G1, S and G2 and nuclear chromatin associated (strongly bound) form during the S phase. Fixation and extraction considerably affect the percentage of cells which express the antigen. When only the tightly bound nuclear antigen is preserved, a PCNA S phase fraction (SPF) is measured. When both loosely and strongly bound forms are preserved, an estimate of the growth fraction is obtained. When studied by flow cytometry, levels of whole cell PCNA immunofluorescence increase in late G1 and early S phase of the cell cycle, peaking in mid-S and declining through G2/M (Kurki et al 1988).

The measurement of PCNA is possible by histochemistry using a variety of monoclonal antibodies (MoAb). Histochemical labelling measured using the PC10 MoAb correlates with BrdUrd labelling. Anti-PCNA monoclonal antibody 19A2 (Ogata et al 1987) can also be used on formalin fixed, deparaffinized sections of human tumours. However, the protein is somewhat labile under many preparation conditions, and great care will need to be taken in comparing PCNA labelling data between series and centres.

PCNA may have a role as a proliferation marker in clinical practice, and is now being evaluated at a number of centres. For example, Jain et al (1991) reported PCNA labelling indices and a semiquantitative PCNA

grading system in 93 archival gastric carcinomas in relation to survival. The median PCNA labelling index was 41%. In this study, neither PCNA index or grade correlated with conventional prognostic indices, such as tumour stage, but low PCNA grade correlated with longer patient survival.

AgNORs

Nucleolar organizer regions (NORs) occur within the nucleolus, where they are foci for ribosome production. They can be visualized on interphase chromosomes by electron microscopy and histochemically on condensed, premitotic chromosomes by a silver reduction technique, hence AgNORs. Although they have been extensively studied as proliferation and mitotic markers, their appearance and numbers are in practice very variable (Egan & Crocker 1992). The counting of AgNORs in tissue sections is time consuming and prone to preparation errors. In general, AgNOR studies have been found to contribute little to the conventional histological study of tumours, and they remain a specialist tool.

Sources of experimental kinetic data variation

Tumour heterogeneity confounds the interpretation of kinetic data in several ways. The cells of solid tumours may be monoclonal (derived from a single mutated stem cell line) or polyclonal. The existence and location of separate clones in malignant tissues is difficult to prove, but may lead to the presence of 'tumours within tumours' with different kinetic characteristics. Intratumour heterogeneity affects macroscopic and microscopic architecture, ploidy, stromal content and thymidine analogue labelling (Fig. 2.6) among other indices. Other important sources of variation include inter-assay and interbatch differences in preparation, protocol, reagents and methods; single observer and interobserver counting variation; inter-institutional variation, and interseries description and selection criteria.

In the study of archival material it is rarely possible to assess the representative nature of the available block as compared with the original specimen, or to be certain of the fixation parameters.

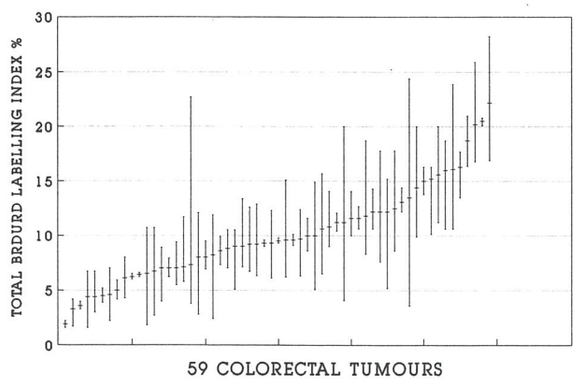

Fig. 2.6 This illustrates the range of random site-to-site variation (heterogeneity) in the flow cytometric BrdUrd labelling index of 59 primary colorectal tumours. Up to 6 sites were sampled per tumour. (Reproduced from Rew et al 1991a, The British Journal of Surgery 78: 1080–1083, by permission of the publishers, Butterworth–Heinemann Ltd.)

KINETIC DATA AS PROGNOSTIC MARKERS

Many authors have reported the relationship between static proliferation indices and prognosis or outcome in various classes of tumour. Tubiana & Courdi (1989) reviewed more than 100 papers reporting SPF and thymidine analogue labelling index (LI) measurements in relation to relapse-free survival or outcome of a range of human solid tumours. In each class of tumours there was a wide range of values of SPF or LI. In 10 of 11 series of breast carcinomas of all stages, there was a significant relationship between static indices and relapse free survival. In 6 out of 7 multivariate studies of breast tumours, LI or SPF had independent prognostic significance. There were similar findings in series of head and neck tumours, ovarian tumours and non-Hodgkin lymphomas.

Measurements of in vivo BrdUrd labelling studied by histochemistry have been shown to correlate with other indices of biological aggressiveness, as in gastric carcinomas (Yonemura et al 1988) and urological tumours (Nemoto et al 1988). In a local study of 100 BrdUrd-labelled colorectal tumours, we have not found a relationship between proliferation parameters and survival, with follow-up to 3 years after surgery (Rew et al, unpublished data). Indeed, given the complexity of proliferation and the wide range of other factors which determine the biological aggressiveness of tumours, it would be surprising if any single kinetic index or marker were able to give a reliable prediction of future tumour behaviour and clinical outcome in all cases.

CELL KINETIC DATA APPLIED TO THERAPY

Dynamic kinetic indices may have a role in the selection of appropriate adjuvant therapy, and kinetic data will be most valuable if they can be put to therapeutic use. Much work has been published on the theory and application of cell cycle kinetic data to chemotherapy and radiotherapy regimes, but there is no conclusive evidence of a survival benefit to be derived from the use of kinetic data to plan treatment. Fractionated scheduling of treatments to take advantage of differences in the proliferative rate of tumour and normal tissues is one approach to cancer therapy.

Chemotherapy

A given dose of drug kills a constant fraction of cells, and there is an inverse relationship between cell number and curability (Skipper et al 1964). Cytotoxic agents may act selectively in the cell cycle, and against rapidly proliferating rather than slowly cycling and quiescent cells.

Tumour cells in vivo are randomly distributed in cell cycle stage (asynchrony). Population synchrony can be induced by treatment with cytotoxic drugs, which leaves surviving cell populations more susceptible to subsequent drug doses. Cytotoxic drug dosage schedules have often been chosen empirically. Many factors may contribute to the failure of tumour chemotherapy, but the timing of the drug doses to achieve optimal synchrony may be a function of the cell cycle phase durations (Steel 1977; Tannock 1978; Mauro et al 1986).

Radiotherapy

Kinetic theory may be important in radiotherapy. The clinical effectiveness of radiotherapy depends upon achieving a maximal tumoricidal effect while minimizing damage to normal tissues. Cells repair, redistribute, reoxygenate and repopulate after radiation damage. Cells tolerate and overcome sublethal radiation doses. The division of a standard course of radiotherapy into smaller fractions, less separated in time, may improve the therapeutic ratio (Denekamp 1986), but the effect of radiation on normal tissues is a limiting factor in therapy.

Cell depletion may be a potent stimulus to bring G0 cells back into the cycling population so that repopulation and redistribution of the proportions of resting to cycling cells of a tumour during treatment will decrease the effectiveness of treatment. Rapidly proliferating tissues will repopulate rapidly, and the rate of repopulation (T_{eff}) approximates to the T_{pot} rather than to the pretreatment volume doubling time (Kummermehr & Trott 1982). Repair and repopulation tend to increase the total dose of radiation needed to achieve a given degree of damage.

Because rapidly proliferating tumour cells which survive initial radiotherapy doses may also repopulate rapidly, frequent low dose bursts of treatment may be more effective in killing large fractions of cells than are traditional, prolonged regimes. Continuous, hyperfractionated, accelerated radiotherapy (CHART) regimes use small but frequent doses to achieve maximum tumour cell kill within the limits imposed by normal tissue tolerance (Dische & Saunders 1989). Such a CHART regime may involve treatments of three fractions daily for 12 successive days to a total radiation dosage equivalent to conventional regimes lasting several weeks. Early data suggest that significant improvements in tumour regression may be achieved over conventional radiotherapy for head and neck carcinomas in

those tumours assessed to be rapidly proliferating by BrdUrd or IUdR labelling (Wilson G D, personal communication), but complete trial data are not yet available.

AN OVERVIEW AND CONCLUSIONS

No one method of measuring the cell kinetics is ideal. Although many authors have reported correlations between static indices and prognosis, the data must be approached with caution because experimental cell kinetic methods all have practical limitations. Even if a technique were available which measured proliferation in biopsies with complete and reproducible accuracy to a defined standard, we would still have problems with data interpretation and application for a number of reasons.

The true pattern of growth of tumours from single abnormal cells to advanced disease is not known, and clinical proliferation parameters are inadequate because they describe the kinetics of a tumour only at one point on the growth curve. Measurement of proliferation at one time does not predict past or future behaviour. It is often the behaviour of the metastases rather than of the primary tumour which kills the patient. The kinetics of micrometastases may be very different to those of the primary tumour. Tumour heterogeneity may produce misleading data from sampling variability.

The case for the regular measurement in clinical practice of dynamic kinetic data in solid tumours is not yet established, although the tools are widely available. The clinical research of recent years has focused attention on the capacity of human tumours for rapid cell production, and much is now known about the architecture of proliferating tissues and tumours. Many intriguing routes remain to be travelled in kinetic research.

KEY POINTS FOR CLINICAL PRACTICE

- Proliferation is a complex process. Single kinetic indices do not adequately describe cell proliferation.
- Cell loss is important in the life of all tumours.
- Proliferation markers may have prognostic utility for some groups of tumours, such as breast carcinomas and lymphomas.
- The halogenated pyrimidines BrdUrd and IUdR have major advantages as proliferation markers.
- Cell kinetic data may improve the scheduling of adjuvant radiotherapy for individual patients.
- Cell kinetics is a rapidly expanding field of study. Many new markers require full evaluation.
- Tumour heterogeneity and assay variability have an important bearing on published data.

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