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Cell kinetics of human solid tumours

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The study of human tumour cell proliferation using *in vivo* administration of bromodeoxyuridine (BrdU) and flow cytometry (FCM) has been studied at the Gray Laboratory and Mount Vernon Hospital since 1985 (Begg et al, 1985; Wilson et al, 1985, 1988; McNally & Wilson, 1989; McNally, 1989; Wilson, 1991; Wilson & McNally, 1992). During this period over 570 patients have been studied, with a variety of solid neoplasms including head and neck, oesophagus, lung, rectal, cervix, uterus, testicular and breast carcinomas and sarcomas, lymphomas and melanomas. These studies have shed new light on the proliferative characteristics of human tumours both by the numbers that have been studied *in vivo* and the cell kinetic parameters that can be generated. Until this technique was developed, studies on human tumours were restricted mainly to *in vitro* investigations in which only the labelling index (LI) could be assessed. The aim of these studies is to provide new biological information and to apply the measurements as a predictive test for therapy. New insight can also be gained on the relationship between proliferation and gross DNA abnormalities as the information is generated simultaneously with total DNA content.

Table I summarizes the basic cell kinetic data obtained from four of the major groups of tumours studied at Mount Vernon Hospital. The information to be assimilated from these data is that (a) there are many tumours from sites suitable for radiotherapy which may possess proliferative characteristics suitable for shorter overall treatment times as judged by the median T_{pot} values; (b) both LI and T_s are important proliferation variables in determining the T_{pot}; and (c) there is tremendous interpatient heterogeneity in proliferation, as judged by the coefficient of variation (CV), reinforcing the need for individual measurements.

The application of BrdU/FCM to study human tumour proliferation has attracted many biologists and clinicians to the laboratory and these collaborations have proven fruitful and rewarding. Two of these collaborations form the basis of this section. These are studies on colorectal cancer by David Rew, which formed the basis of a Master of Surgery thesis, and on melanoma, which will also be submitted for a higher degree by Hamish Laing.

Melanoma

The clinical course and biological behaviour of human malignant melanoma continue to pose many unanswered questions that have been highlighted by the sharply rising incidence in most countries. Attempts to rationalize management and optimize the prognosis for patients whilst minimizing morbidity have been confounded by the substantial number of patients whose disease behaves at variance with that predicted by existing knowledge and prognostic criteria.

The value of parameters of cell proliferation as indicators of disease behaviour has been shown in other solid human tumours, but such studies have previously been difficult to perform in malignant melanoma because of methodological limitations. Most primary cutaneous melanomas are of small volume and after routine histo-

Table I. Summary of cell kinetic parameters of Mount Vernon tumours

Tumour group	Number	Parameter	Median	Range	CV
Head and neck	188/165	LI (%)	4.9	0.9–20.7	76
		T_s (h)	9.9	5.4–27.3	45
		T_{pot} (days)	6.4	1.8–67.8	101
Lung	86/38	LI (%)	8.0	0.7–28.2	70
		T_s (h)	15.1	5.5–37.8	45
		T_{pot} (days)	7.3	1.4–132.0	167
Oesophagus	87/50	LI (%)	7.8	0.7–27.2	64
		T_s (h)	12.4	7.1–28.1	46
		T_{pot} (days)	5.2	1.6–107.1	156
Cervix	25/22	LI (%)	11.6	3.8–23.8	52
		T_s (h)	15.8	10.8–30.4	35
		T_{pot} (days)	4.5	2.9–15.8	61

The number column refers to the number of observations and the number of patients, as in some patients several specimens were studied. CV represents the coefficient of variation.

Dedicated to N. J. McNally

pathological analysis often leave insufficient material for cell cycle analysis using *in vitro* techniques.

Until recently, therefore, research has concentrated on the prognostic value of DNA aneuploidy measured using FCM with only limited studies of cell proliferation using intralesional or *in vitro* tritiated thymidine and autoradiography. The presence of aneuploid cell clones may be detected in malignant melanoma tumours using FCM with either fresh or paraffin-embedded material, allowing both retrospective and prospective analysis. Although there has been variable reporting of aneuploidy rates in melanoma, many studies have shown a strong correlation between the presence of aneuploid cells and poor outcome (Sondergaard et al, 1983; Kheir et al, 1988; Bartkowiak et al, 1991). Early workers using tritiated thymidine *in vivo* (Terz et al, 1971) were able to compare calculated and measured tumour doubling times in a number of human tumours, including melanoma. With intralesional or *in vitro* administration, however, the thymidine LI alone may be derived. Small series of patients with melanoma suggest that the LI may be of prognostic significance (Costa et al, 1987, 1990), as it is in many other solid human tumours. The development of dynamic studies using *in vivo* labelling with BrdU have permitted detailed cell proliferation data to be obtained for the first time.

Current prospective study

Patients and methods

Since 1990, we have studied 110 lesions. Of these, complete FCM analysis has been achieved in 22 primary cutaneous melanomas and 60 metastases. There have been 28 failures owing to mainly small amounts of material. All patients received 200 mg BrdU as an intravenous bolus injection, between 3 and 12 h prior to surgical excision of the lesion. No adverse effects were seen.

Representative samples were taken from the tumours after inspection by a consultant histopathologist and fixed in 70% ethanol for at least 24 h. The samples were then processed for multiparametric flow cytometry using the methods previously described (Begg et al, 1985; Wilson et al, 1988). Data were derived for the DNA index, LI, duration of S-phase and potential doubling time (T_{pot}). Clinical and histopathological data collected included all known prognostic variables. Immunoperoxidase localization of BrdU and of PCNA expression has been carried out on all specimens.

Results

As may be seen from Table II, whilst 32% of primary melanomas studied demonstrated the presence of aneuploid cell populations, a significantly greater proportion (56%) of metastatic lesions were aneuploid ($\chi^2 = 0.05$). Indeed, this rises to 60% if nodal metastases, which characteristically have large diploid lymphocyte populations, are excluded.

The median LI for primaries was 4.1% and metastases 5.4%, and the median T_s 10.2 and 11.6 h, respectively. No significant difference exists between the two groups for these parameters.

Calculation of the T_{pot} for the two groups shows a wide range of proliferation potential as seen in other solid tumours: these melanomas include some of the shortest and longest potential doubling times of all human tumours in the Mount Vernon series. In general, melanomas are less rapidly proliferating than the other groups of tumours studied at Mount Vernon.

Comparison of the T_{pot} with the Breslow thickness (Fig. 1) shows the thinner primary lesions (<1.5 mm maximum thickness) to be proliferating more slowly than the thicker lesions (median T_{pot} 18.3 *versus* 7.9 days: $p = 0.05$).

In those patients in whom we have studied both a primary and a metastatic lesion, there is a strong correlation between the two lesions for the DNA index, LI and T_{pot} (Table III).

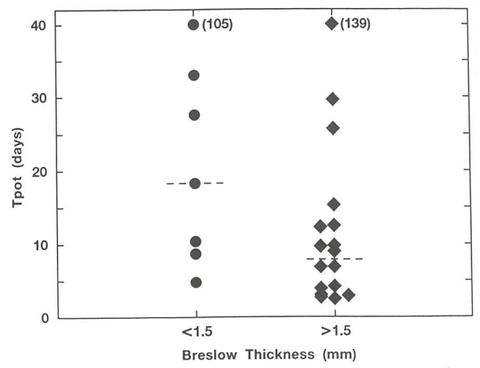

Figure 1. The relationship between Breslow thickness and T_{pot} in primary cutaneous melanoma. Comparison is made between lesions greater and less than 1.5 mm maximum thickness. The dashed line represents the median value.

Table II. Summary of cell kinetic data from primary and metastatic melanoma

	n	% aneuploid	LI (%)	T_s (h)	T_{pot} (days)
Primaries	22	32	4.1 (0.24–9.9)	10.2 (4.4–23.6)	9.4 (2.5–105)
Metastases	61	56*	5.4 (0.6–16.8)	11.6 (6.1–26.2)	7.2 (2.3–139)

The numbers for each proliferation parameter represent the median and below that the range.
*$p < 0.05$ using χ^2-squared analysis.

Discussion

This study has shown that dynamic cell proliferation parameters may be measured in human malignant melanoma, even in some thin primary lesions. Some difficulty has been experienced in obtaining adequate cell yields from some lentigo maligna melanomata.

The finding of larger numbers of aneuploid populations within metastases than in primaries supports the contention that malignant potential is enhanced in cell clones with abnormal DNA content. It is also of interest in this context that the few patients in whom we were able to study primary and metastatic disease did not show any evidence of transformation from a diploid primary to an aneuploid secondary.

The measurement of T_{pot} for the first time in a large prospective study has confirmed that some of these tumours may be rapidly proliferating and that this predominates in thicker primary lesions which, as a clinical group, have a worse prognosis. The outcome of patients whose tumours fall outside this trend will be followed with interest.

Where it has been possible to study, intertumour heterogeneity has been much less than in other tumours. This may be explained by their small volume, histological uniformity and relative lack of necrosis.

The measurement of cell proliferation parameters in malignant melanoma provides an important opportunity to gain a better understanding of the behaviour of this disease. It may allow the better and earlier identification of patients who would benefit from more aggressive therapy.

Colorectal cancer

Intestinal malignancy is an important source of morbidity and mortality. Current prognostic indices of colorectal cancer, in practice the Dukes staging and the histological grade of the tumour, are unable accurately to discriminate patients at risk of subsequent recurrence. Adjuvant treatment has not, as yet, been demonstrated to produce a consistent improvement in survival. Until recently, *in vitro* radioisotope studies and single parameter FCM to measure the DNA content and S-phase fraction have been the mainstay of cell kinetic research in colorectal cancer. The use of BrdU for *in vitro* and *in vivo* single parameter LI studies of human colorectal tumour cell kinetics has been reported (Khan et al, 1988), but little was known of the dynamic behaviour of intestinal tumour cells.

Methods and results: tumours

Between 1988 and 1990, we studied the cell proliferation kinetics of 100 primary previously untreated tumours, 49 colonic and 51 rectal adenocarcinomas, at the time of their definitive conventional surgery. We used *in vivo* BrdU pulse labelling and multiparameter FCM to measure the DNA index, the total and aneuploid LI, the S-phase duration (T_s) and the potential doubling time (T_{pot}) (Rew et al, 1991a).

97 patients, three with synchronous tumours, consented to receive a single bolus dose of 250 mg between 2.4 and 16 h prior to curative or palliative surgery. There were 49 diploid tumours and 51 aneuploid tumours. The median total LI of 100 tumours was 9.0% (range 0.7–22.2%). The median aneuploid LI was 12.0% (range 2.0–25.5%). Both LI and T_s were variables. The intertumour range of the T_s varied from 4.0 to 28.6 h. The median T_s was 13.1 h. The median T_{pot} was 3.9 days with a range of 1.75–21.4 days. No correlation was found between any kinetic parameters and the Dukes stage or histological grade, or with clinical outcome at 2 years follow-up.

The study highlighted other features of colorectal tumour biology. We observed considerable intratumour heterogeneity between widely separated sites within tumours. Of 58 tumours, 42 had a range of variation of T_{pot} of less than 5 days, but seven tumours had a range of variation greater than 10 days. Intratumour heterogeneity is a feature of colorectal tumour growth. We would urge caution on any attempt to classify colorectal tumours by proliferative criteria unless heterogeneity has been properly assessed.

The clinical volume doubling time (T_d) of human colorectal tumours has been commonly estimated to exceed 100 days, compared with our median T_{pot} value

Table III. Comparison of cell kinetic parameters in primary and secondary disease in the same patient

Code	Tumour	DNA index	LI	T_s	T_{pot}	Time between 1° and 2°
HUBU515.1	1°	1.7	5.3	14.3	9.0	Synchronous
HUBU515.2	2° node	1.7	5.9	17.0	9.5	
HUBU314	1°	1.0	3.0	13.8	15.3	12 months
HUBU490	2° node	1.0	3.7	12.4	11.2	
HUBU438	1°	1.8	8.2	10.2	4.2	4 months
HUBU486	2° node	1.7	6.5	11.5	5.9	
HUBU386	1°	1.0	9.5	7.4	2.6	3 months
HUBU440	2° node	1.0	5.6	10.3	6.2	

The time interval between 1° and 2° is the time between the two surgical procedures.

of 3.9 days. Colorectal tumours thus appear to have a high cell loss factor, probably due in combination to exfoliation, apoptosis and necrosis.

Colorectal mucosa studies

Altered cell proliferation may also be important in diseases of intestinal mucosa, particularly in ulcerative colitis, familial polyposis coli (FPC) and in the development of pre-malignant adenomas (polyps). This hypothesis has been difficult to test. The facility to study BrdU labelling in wax-embedded tissue sections using peroxidase immunohistochemistry allowed us to undertake a large descriptive and fully quantitative study of *in vivo* labelled mucosa using normal biopsies from the tumour resection specimens. Among other features, we described considerable uniformity of proliferation throughout the colorectum, and a subtle shift in the proliferative pattern in FPC mucosa (Potten et al, 1992a,b; Kellett et al, 1992). The combination of FCM-derived T_s data with static histochemistry parameters allowed us to calculate the crypt cell cycle time (30 h) from single biopsies.

Conclusions

While the FCM/BrdU assay represents a significant advance for clinical research (Rew & Wilson, 1991), there remain problems with it as applied to colorectal cancer. In particular is the inability to distinguish stromal from tumour cell nuclei in the diploid peak. Preliminary histochemical studies have shown that labelling within maximally proliferative regions of tumour may exceed 50%. It may be that the proliferation of these subpopulations will more accurately reflect the clinical behaviour and therapeutic responsiveness of these tumours, and the more laborious task of manual counting of labelled cells may yet have an important role in such studies.

In vivo BrdU labelling of colorectal tumours in itself offers considerable opportunity for further research into their biology, as it lays down a reliable proliferation marker against which other antigens can be compared. We have studied expression of both p62cmyc (Rew et al, 1991c) and PCNA in this material.

A better understanding of the proliferation of solid tumours may allow a better selection and use of adjuvant therapy along the lines of CHART theory. Advanced rectal cancers and those in unfit or elderly patients may be good candidates for such therapy, as are some bladder and breast tumours, series of which we have also studied by the BrdU/FCM technique (Rew et al, 1991b, 1992). We can have considerable optimism that cell kinetic research may have a real bearing on clinical and surgical practice.

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