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In vitro modelling of human tumour behaviour in drug discovery programmes

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Abstract

Human tumour cell lines have played an important part in our understanding of cancer and have been used extensively in the discovery and characterisation of new chemotherapeutic drugs. A potential weakness of such cell lines is that they may have lost important properties originally possessed *in vivo,* including potential targets for therapy. This review discusses how possible differences between tumour cells in cancer patients and cell lines might be identified by the use of short-term cultures of human tumour cells taken directly from cancer tissue, termed here primary cultures. Cell-cycle time is one important difference between tumours and cell lines and it is known that the cell-cycle times of primary cultures cover the same wide range as estimated *in vivo* cell-cycle times. Because tumour cells have at least two pathways to cell death, one from interphase and one from mitosis, changes in cell-cycle length can modify the balance of such pathways. Responses of primary cultures to DNA-damaging drugs and inhibitors of growth factor receptors also differ from those of cell lines, suggesting that the process of developing a cell line can result in the loss of important cellular responses. Without an appreciation of these changes our ability to discover new targets for the development of improved cancer therapy may be jeopardised. The identification of cell lines that preserve potential targets is an important goal in cancer biology and research using primary cultures will help in this identification.

Keywords: Cell cycle; Mitotic cell death; Apoptosis; Primary culture; Cytotoxic; Epidermal growth factor receptor

1. Introduction

Since the discovery in the 1950s that some human tumour cells could be propagated indefinitely in culture in the presence of the appropriate nutrients and growth factors, human tumour cell lines have played a major part in anticancer drug discovery. However, the main classes of cytotoxic drugs in current clinical use, including DNA-reactive compounds (nitrogen mustard, cyclophosphamide, melphalan, cisplatin), antimetabolites (methotrexate, thioguanine, 5-fluorouracil, cytosine arabinoside), mitotic poisons (vinblastine, vincristine, paclitaxel) and topoisomerase poisons (doxorubicin, etoposide, camptothecin) were discovered using transplantable mouse tumours rather than with cell lines [1]. In the late 1970s, the first human tumour cell line, CX-1 or HT-29 (growing as a subcutaneous xenograft in nude mice) was incorporated into the drug-discovery programme of the US National Cancer Institute (NCI). Interestingly, this xenograft appeared to be unresponsive to all of the clinical drugs developed previously [1]. By the mid-1980s, a sufficiently large number of human tumour cell lines had been developed to allow the initiation of an NCI programme to use a panel of such lines for drug discovery. The rationale was that human tumours might possess tissue-specific targets not exhibited in murine-based tumour models and that these could be identified in a mass-screening programme. Technical methods had been developed to allow multiple cultures of such cells in 96-well culture dishes [2] and these were applied to large-scale screening [3]. More than 70 000 compounds have been tested, and compounds identified as having novel patterns of activity have been tested against human tumour cell lines growing as xenografts. The programme has allowed the acquisition of a large amount of genetic and other data

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on these cell lines and has been reviewed elsewhere in this volume [4].

Over the last decade, attention has turned to the use of rational approaches to drug discovery, where protein targets identified empirically by their involvement in cancer growth control have been isolated, in many cases crystallised, and used for the design and synthesis of specific inhibitors [5]. However, an almost obligatory step in the progress to clinical trial of new anticancer agents is the demonstration of the ability to inhibit the growth of cultured human tumour lines *in vitro* and of xenografted human tumour cell lines *in vivo*. The underlying assumption of this approach, as it is with the NCI cell-line panel, is that such cell lines adequately model the behaviour of human cancer. In this review, we examine how tumour cell lines differ from human tumour cells growing *in vivo*. We have drawn on extensive data obtained by culturing tumour cells directly from human cancer tissue, here termed primary culture, and on the conversion of these to cell lines. We also discuss possible approaches to the use of primary cultures and early-passage cell lines in drug discovery.

2. Differences in cytokinetics of cell lines and tumours *in vivo*

Early *in vivo* studies provided estimates of cell-cycle kinetics from the mitotic index (MI) or S-phase fraction (SPF) by employing the assumption that the transit times for each of these phases did not vary among different tumours. The cycle time could be calculated, with an appropriate correction factor for exponential growth, from the transit time for mitosis divided by MI, or from the transit time for S-phase divided by SPF. The advent of flow cytometry enabled estimation of the *in vivo* S-phase transit time [6]. Cancer patients were treated with a single dose of bromodeoxyuridine to label S-phase tumour cells within the tumour, and a biopsy sample was removed about 6 h later, at which time a proportion of the original S-phase population had left S-phase. The S-phase transit time, calculated from this proportion, was found to vary among different tumours from 6 to 30 h. When divided by the S-phase fraction and multiplied by an appropriate correction factor, estimates of cell time (potential doubling time or T_{pot}) were obtained. A feature of these results was the degree of variation from one individual's tumour to another, with values ranging from 2 days to more than 2 weeks [7]. In Fig. 1 we have compared some of these published values with corresponding cell-cycle times calculated for a series of early-passage (<20 passages) human tumour cell lines developed in this laboratory [8]. While the S-phase transit times of the cell lines, as determined by flow cytometry, cover a similar range to those of tumours determined *in vivo*, cycle times are markedly different. Since the proportion of cells in G_2-phase and mitosis is small, both *in vivo* and *in vitro*, relative to that of cells in G_1-phase, the results suggest that one of the main cytokinetic differences between tumours *in vivo* and tumour cell lines *in vitro* is the length of time that cells spend in G_1.

The entry of G_1-phase cells into S-phase requires the activation of cyclin-dependent kinase 2 (cdk2) by binding to cyclin E, dephosphorylation by cdc25A phosphatase and degradation of inhibitors such as $p27^{KIP1}$ [9]. Entry is an all-or-nothing response probably controlled by positive-feedback loops, for instance by the loop in which cdk2 activates cdc25A phosphatase, which further activates cdk2, and cdk2 facilitates $p27^{KIP1}$ degradation, which further activates cdk2. Thus, the difference between the *in vivo* and *in vitro* G_1-phase transit times could be related to intrinsic factors such as the rate of cellular accumulation of cyclin E. An alternative explanation is that the *in vivo* G_1-phase transit times are long because of extrinsic effects of the tumour microenvironment. For instance, a lack of nutrients or growth factors caused by a poor vascular supply could lead to the arrest of cells in G_1-phase through accumulation of $p27^{KIP1}$.

We investigated the initial rate of *in vitro* cell proliferation of samples of tumour material removed at surgery from cancer patients [10], using primary cultures grown over a 7-day culture. We used a number of specific conditions such as a low (5%) oxygen concentration and the presence of appropriate supplements in the culture medium to ensure adequate survival of these cultures [11]. Measurement of growth from changes in protein or MTT staining was found to be unsuitable because a significant proportion (generally 50–90%) of the sample comprised stromal cells and some of these died during the culture period. Likewise, measurement

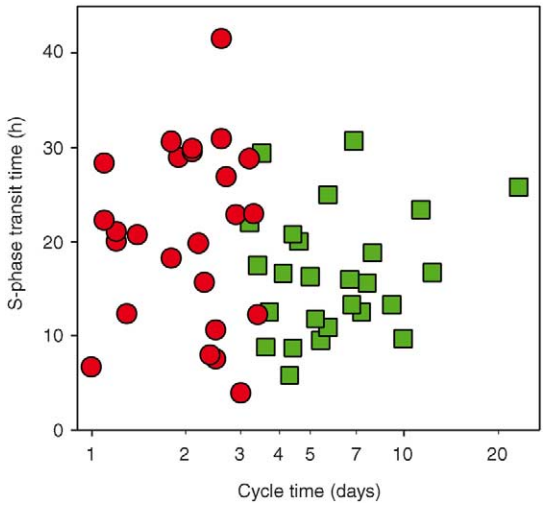

Fig. 1. Relation between S-phase transit time and cell-cycle time for human tumours *in vivo* (■) and for early-passage cell lines *in vitro* (●).

of growth from changes in [^{3}H]thymidine incorporation was also unsuitable because some of the original cells may die during culture. We developed a modified [^{3}H]thymidine incorporation method for the estimation of the cell-cycle time [12]. In brief, we assumed that cells enter S-phase randomly from G_1-phase, so that the rate of entry into the S-phase population reflects the population of G_1-phase cells. This behaviour is consistent with the results of flow cytometry [13]. When a mitotic poison was added to arrest cell division, the number of G_1-phase cells decreased exponentially with time and, after a slight delay, the number of S-phase cells decreased exponentially with time. In contrast, the numbers of G_1- and S-phase cells in control cultures increased exponentially with time. The decrease with time of [^{3}H]thymidine incorporation by S-phase cells following mitotic arrest would therefore be expected to be related directly to cell-cycle time and this has been demonstrated experimentally for a number of cell lines [11].

The distribution of cycle times of primary cultures of carcinoma specimens, as determined by this method, is compared with that of a number of early-passage cell lines in Fig. 2. The cycle times for early-passage cell lines were significantly lower than those for primary cultures from ovarian cancer ($P < 0.001$; Mann–Whitney test), lung cancer ($P < 0.001$) and samples from other carcinoma types including cervical and endometrial ($P < 0.001$).

In a number of cases the cell-cycle time of the cell line was found to be lower than that of the primary culture from which it was derived, raising the question of the mechanism for this change. One possible explanation is that subculture selects for more rapidly growing variants in an original heterogeneous population. Alternatively, culture conditions could be responsible; primary cultures are grown as small three-dimensional multicellular aggregates on agarose [11] while cell lines are generally cultured as a monolayer in a tissue-culture flask. To distinguish these two possibilities we carried out experiments of the type shown in Fig. 3. A primary culture was made of an endometrial cancer and its cell-cycle time was determined as 4.2 days. Over the course of several months, a cell line designated NZEN1 was developed from this tumour and its cycle time was determined as 2.5 days. Tumour cells from the cell line were then implanted subcutaneously in athymic mice and grown as a xenograft in an effort to reconstitute cell-cell and cell-matrix interactions. After reaching a diameter of 8–10 mm, tumours were removed, partially disaggregated and cultured under the same conditions as used for the initial primary culture. The cycle times of these cultures were found to be around 2.0 days, which resembled that of the cell line rather than that of the original primary culture. The relative content of G_1-, S- and G_2/M-phase cells growing *in vivo* as a xenograft was also measured by flow cytometry and found to be similar to that shown by the cell line. This result, together with those from similar experiments with melanoma cell lines (data not shown), suggests that the observed differences between the *in vivo* growth rates of human tumours and the *in vitro* growth rates of derived cell lines is likely to be the result of selection pressures during the derivation of a cell line. Such derivation, requiring between 3 and 12 months, may well allow selection of variants.

Are the cycle times of the primary cultures similar to those of tumour cells growing *in vivo*? While we have not compared growth rates of individual cultures with *in vivo* determinations, it is of interest that the range of

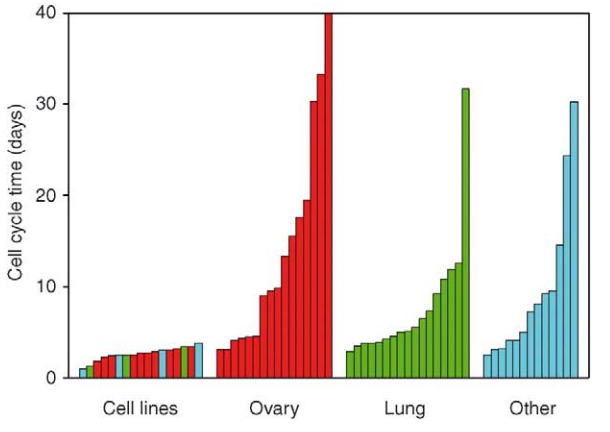

Fig. 2. Measured cell-cycle times of a number of early-passage carcinoma cell lines and of primary cultures derived from patients with ovarian carcinoma, non-small cell lung carcinoma and other carcinomas.

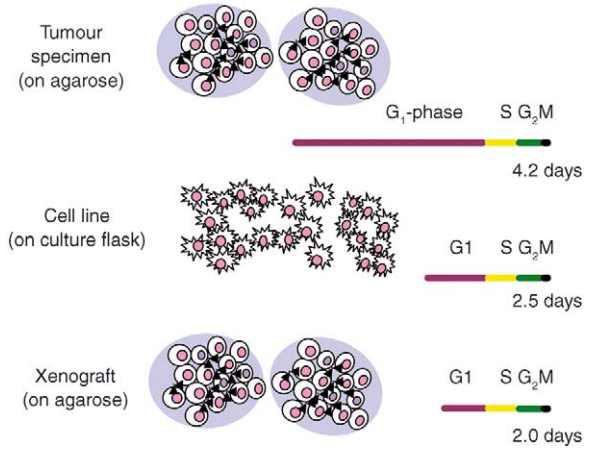

Fig. 3. Comparison of cycle times of a primary tumour and a derived cell line. A primary culture (endometrial cancer) was grown on agarose and found to have a cycle time of 4.2 days. A cell line (NZEN1) was developed from this tumour and had a cycle time of 2.5 days. The cell line was grown as a xenograft, the resulting tumour removed and cultured on agarose. The cell-cycle time of the cells cultured from the xenograft (2.0 days) was more like that of the cell line from which it grew than of the original tumour. Animal experiments were carried in accordance with guidelines of the University of Auckland Animal Ethical Committee.

cycle times for each of the categories in Fig. 1 (from 3 to 40 days) is very similar to that for reported T_{pot}. This similarity supports the hypothesis that the *in vivo* cell-cycle time is initially preserved, to a large extent, when tumour material is subjected to primary culture.

3. Responses to carboplatin

The results described in the previous section suggest that the rates of proliferation of primary cultures are slower than those of cell lines. Does the degree of cell death induced by a cytotoxic drug similarly change during the development of a cell line? Clonogenic assays are traditionally used to quantitate drug-induced cell death in cell lines, and the use of clonogenic or 'stem cell' assays has been extensively studied for the analysis of human tumour samples [14–16]. However, 'stem cell' assays require a long time frame and also necessitate the separation of individual cells from each other and from the extracellular matrix, a process that could perturb their response to drug-induced DNA damage. Because of the large differences in incubation time, it is difficult to use clonogenic assays to answer the question of whether drug sensitivity changes during the development of a cell line.

We have devised a method for estimation of drug-induced cell loss that is applicable to the small multicellular aggregates used for the determination of cell proliferation rates [8,11] and review here its application to the anticancer drug carboplatin (we have also obtained qualitatively similar results with cisplatin). The measurement of such cell loss is complicated by the existence of at least two pathways to cell death. The 'classical' pathway to apoptosis involves activation of a caspase cascade leading to internucleosomal cleavage of DNA, nuclear fragmentation, blebbing of cytoplasm and other changes [17]. The time course is generally short, such that at any given point of time only some of the cells that are destined to die are observable by appropriate staining. A second type of death, typified by the response of cultured cells to mitotic poisons such as paclitaxel, is termed mitotic cell death or mitotic catastrophe and occurs subsequent to mitosis [18]. Cells enter mitotic arrest but the chromatids subsequently decondense, giving the appearance of nuclear fragmentation [19]. We have used flow cytometry, cell counting and mathematical modelling to estimate the time course of cell death following exposure of a cell line to the mitotic poison paclitaxel and have found this process to be comparatively slow, with cellular DNA being progressively degraded over a time period of approximately 70 h [20]. Other pathways, including senescence in whch cells enter an extended non-growing phase before dying, and endoreduplication, in which a proportion of G_2/M-phase cells enter a further S-phase [21], also exist. Each pathway will have a certain degree of selectivity for tumour cells rather than host cells, and the pathways used by a particular drug will influence its overall efficacy.

Our technique measures drug-induced cell death only in interphase (strictly speaking, only in G_1- and S-phase) and the principle is shown in Fig. 4. If cell division is blocked by paclitaxel-induced mitotic arrest, or by DNA damage-induced G_2-phase arrest, the number of G_1-phase cells decreases at an approximately exponential rate [11,13]. The simultaneous induction of G_1- or S-phase cell death will cause a more rapid decrease in these cells with time, in comparison with that of G_2-phase or mitotic arrest. Rather than measuring G_1-phase cells by flow cytometry, which is difficult with clinical tumour samples, we have estimated S-phase cell content by [^{3}H]thymidine incorporation. The method is complicated by the potential of carboplatin to induce G_1- and S-phase cell-cycle arrest, but we have found using flow cytometry that G_1- and S-phase arrest in response to DNA damage generally reverses within 2 days.

We used this method to compare the ability of carboplatin to induce interphase cell death in carcinoma cell lines and primary cultures. We utilised a drug concentration (40 μM) approximating to that obtained clinically following administration [22]. The *in vitro* responses of 10 ovarian cell lines, and of primary cultures from ovarian cancer, lung cancer, and other tumour types, are shown are shown in Fig. 5; they show wide variation in the degree of induced interphase

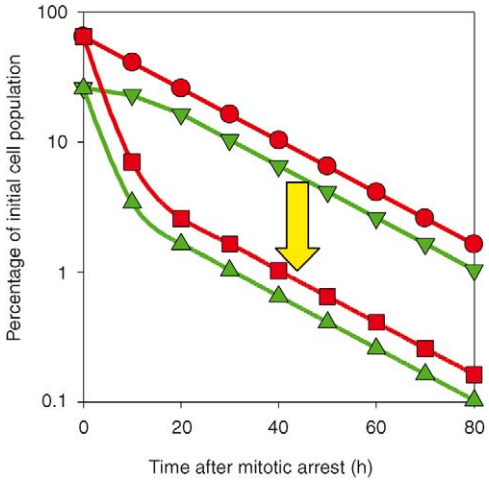

Fig. 4. Strategy for the estimation of drug-induced interphase cell death. If cell division is halted completely by exposure to a mitotic poison, both the G_1-phase (●) and S-phase (▼) compartments will decrease with time because of cell movement through the cycle. If, alternatively, cell division is completely inhibited by DNA-damage responses (i.e. by G_2-phase arrest and mitotic arrest), the G_1-phase (■) and S-phase compartments (▲) will decrease as a consequence of both cell movement through the cycle and induced interphase death. The difference between the two effects gives an estimate of drug-induced cell death before mitosis.

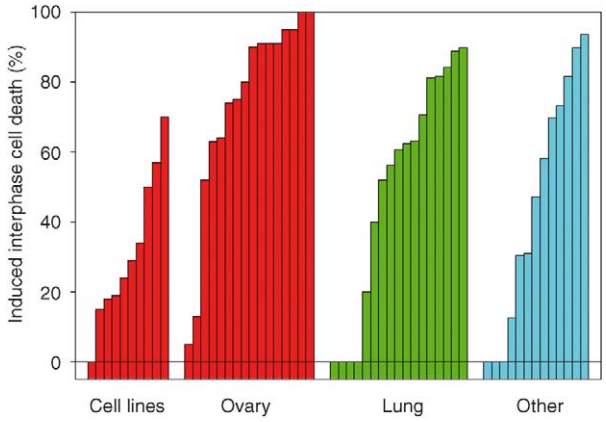

Fig. 5. Estimated values for carboplatin-induced interphase cell death for early-passage ovarian carcinoma cell lines and for primary cultures derived from patients with ovarian carcinoma, non-small cell lung carcinoma and other carcinomas. The mitotic poison used to block cell division was paclitaxel (up to 100 nM) and the exposure time was 7 days (5 days for cell lines).

death. It is of interest that cell lines that showed the largest responses with the [^{3}H]thymidine assay also showed evidence of carboplatin-induced interphase cell death by flow cytometry. The *in vitro* responses of the ovarian cell lines are significantly lower than those of the primary cultures (Mann–Whitney test; $P = 0.002$). In two cases the response of an ovarian cell line could be compared to the primary culture from which it was derived and was found to be decreased. It is also of interest that the *in vitro* responses to carboplatin of the primary cultures of ovarian cancer samples were significantly higher ($P = 0.04$) than those of primary cultures of lung cancer samples.

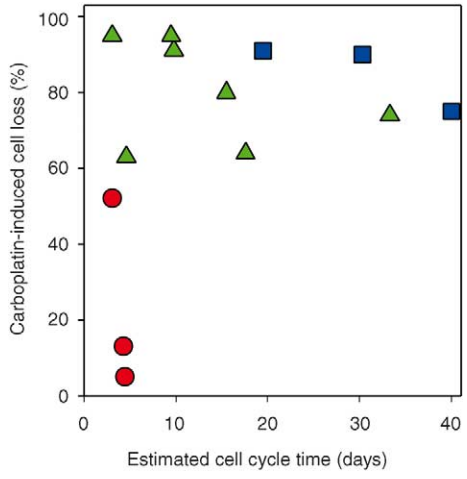

Fig. 6. Relation between *in vitro* variables (cell-cycle time and carboplatin-induced cell loss) and clinically evaluable ovarian cancer patients treated with carboplatin and having no response (●), a partial response (▲) or a complete response (■). Data are from a published study [23] and the figure is reproduced from the Proceedings of the 9th Biennial IGCS Meeting, Seoul, Korea, with kind permission of Monduzzi Editore.

In order to determine whether the results with primary cultures are related to *in vivo* response to carboplatin, we have commenced a study in patients with ovarian cancer who have been treated with carboplatin, generally as a single agent. The results of a preliminary study, involving 13 patients who were treated with carboplatin, have been published [23] and the data are reproduced in Fig. 6. The results suggest that outcome is related to a combination of two *in vitro* varaiables, cell death induced in interphase and cell-cycle time. Of particular interest is the observation that primary cultures taken from patients who subsequently obtained a complete remission had long cell-cycle times. One possible conclusion from these results is that both the degree of carboplatin-induced cell death and the rate of repopulation of surviving tumour cells contribute to the overall response.

4. Responses to inhibitors of signal transduction

Inhibitors of signal-transduction pathways in cancer tissue comprise one of the most critical areas of new anticancer drug discovery, and inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase [5] have increasing clinical application in human cancer [24]. Response to the latter class of drugs requires not only the presence of EGFR but also of the appropriate underlying signalling pathways. As in the case of DNA damage, blocking of EGFR can cause cell-cycle arrest as well as cell loss. Blockade of signalling pathways downstream from the receptor can block the G_1- to S-phase transition, and EGFR can also modulate the effect of epidermal growth factor as a survival factor, leading to apoptosis [25].

We used a quinazoline-based inhibitor of the EGFR tyrosine kinase (SN25531) to compare the responses of cell lines and of primary cultures to EGFR inhibition [26]. This drug, an early member of the series that resulted in canertinib [27], is structurally related to the clinical drug gefitinib (Fig. 7). Since the concentration of SN25531 required for 50% inhibition of EGFR kinase is 0.89 nM [28], we have compared here the effect on [^{3}H]thymidine incorporation of an inhibitor concentration (10 nM) that would be expected to inhibit EGFR kinase by 90%. The results are summarised in Fig. 8 and show that while this drug concentration failed to inhibit the growth of most carcinoma cell lines, it was effective in a high proportion of primary cultures. The degree of inhibition for the lung and ovarian cancer primary cultures were significantly higher (Mann–Whitney test) than those of the cell lines. In the case of one sample of ovarian cancer, we were able to show that sensitivity to SN25531 was maintained during the development of an early-passage cell line. However, it was of interest that the only two cell lines that showed a

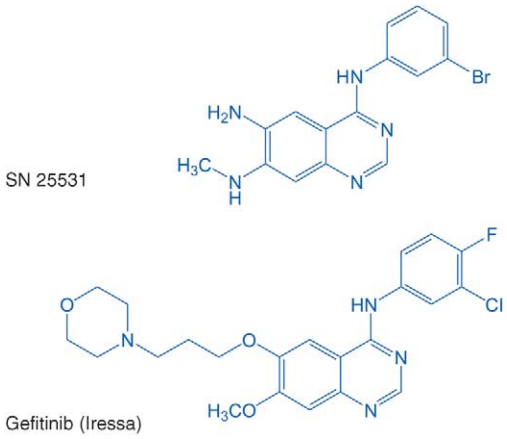

Fig. 7. Comparison of the chemical structures of SN25531, the drug used in the described study, and gefitinib, a clinical inhibitor of the epidermal growth factor-receptor kinase.

good response to the inhibitor were also early passage, one from a lung carcinoma (NZL1) and one from an ovarian carcinoma (NZOV2). We had previously shown that the non-responsive carcinoma cell lines still expressed EGFR, as measured by flow cytometry [26].

Considerable variation in the responses of primary cultures to the inhibitor is evident from Fig. 8. Some were strongly inhibited, consistent with the expected 90% inhibition. Some showed no inhibition and two even appeared to stimulate [^{3}H]thymidine incorporation. This phenomenon needs further investigation, but has previously been observed with a melanoma line, which, unlike all others tested, expressed EGFR [26]. Some cultures responded partially to the drug (Fig. 8): two possible interpretations are that they are heterogeneous (i.e. some cells are inhibited and some not) or that the observed inhibition represents growth arrest rather than

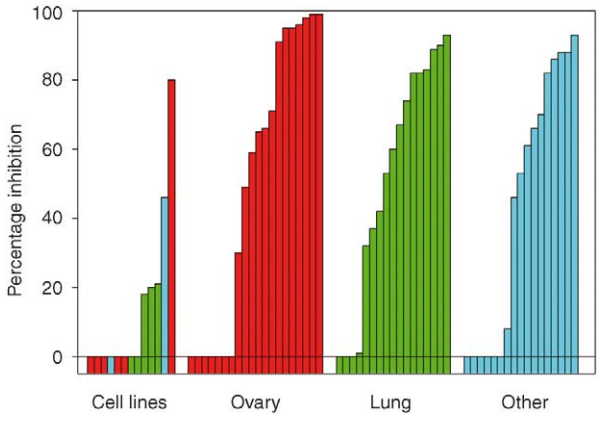

Fig. 8. Comparison of responses of a number of early-passage carcinoma cell lines with those of primary cultures derived from patients with ovarian carcinoma, non-small cell lung carcinoma and other carcinomas to the epidermal growth factor-receptor kinase inhibitor SN25531 at an added concentration of 10 nM [26]. Values show the percentage inhibition of [^{3}H]thymidine incorporation at the end of exposure (7 days for primary tumours and 5 days for cell lines).

the induction of apoptosis. We have not yet determined whether the observed effects are due to growth inhibition or to apoptosis induction. The pronounced inhibition observed with some of the primary cultures raises the question of whether the original tumours from these patients might have been sensitive to the drug. The hypothesis that primary cultures can predict outcome could be addressed by an appropriately designed clinical trial.

5. Perspective

We have reviewed here a comparison of human tumour cell lines and of primary cultures of tumour material taken directly at surgery from cancer patients. We have investigated three different properties: cell-cycle time, sensitivity to the cytotoxic drug carboplatin and sensitivity to an inhibitor of the EGFR tyrosine kinase. Even though the cell lines and primary cultures showed considerable heterogeneity in these properties, there was in each case a significant shift in the cell lines towards higher growth rate and reduced drug response. This is consistent with the results of work using RNA-expression analysis [29] and other techniques that have led to an increasing awareness that human tumour cell lines differ in a number of respects from the tumours from which they were derived.

It might at first seem surprising that human tumour tissue, which develops over a number of years *in vivo*, changes so quickly during the development of a cell line. This is particularly so with regard to cell-cycle time (Fig. 1). A key issue here is kinetics: tumour cells *in vitro* generally grow exponentially while *in vivo* growth is dominated by turnover. Tumour tissue generally comprises islands of malignant cells embedded in an extracellular matrix containing collagen, other proteins and macromolecules, and a variety of host cells including fibroblasts, smooth muscle cells, blood and lymphatic vessels, and immune cells. Fibroblasts (or myofibroblasts) are thought to be primarily responsible for the generation of the matrix and of basement membrane components, but may also produce factors necessary for the survival of the tumour cells. However, they also physically contain the tumour cell population so that the net growth rate of the tumour tissue is only a small fraction of the potential growth rate based on the cell cycle of the individual cells [30]. When tumour tissue is used to develop a cell line, the restriction on exponential growth is relaxed and the observed decrease in cycle time in the cell line might be the inevitable consequence of selecting more rapidly growing variants. This raises the interesting question of why human tumours are not similarly selected for short cycle times *in vivo*. The answer may be related to the regulation of cell turnover within tumour tissue. If more rapidly growing variants

in the population have a correspondingly higher rate of apoptosis, they would have no selective growth advantage. The *in vivo* state might be altered during repopulation following cytoreductive therapy and perhaps in endstage disease where failure of containment of tumour cells by stromal tissue leads to growth at an almost exponential rate.

The selection for increasing resistance to a cytotoxic drug such as carboplatin (Fig. 5) and to inhibition of signal transduction (Fig. 8) may be a consequence both of the selection of more rapidly growing variants and the culture conditions used for the development of cell lines. The conditions of tissue culture may also produce higher degrees of DNA damage and consequent selection of cells that are resistant to such damage. Similarly, exposure of cancer cells to a different set of growth and survival factors, as provided by foetal bovine serum, might select for variants that express different membrane receptors. It is this area that is of most potential importance to the use of cell lines in drug discovery. The development of cell lines may lead to loss of important potential targets. While it is possible to modify cell lines genetically, replacing a specified target such as EGFR, this will not help us to discover as yet unknown targets. Primary cultures of tumour material, by maintaining many of the properties of the original tumour cells, represent a unique resource that may allow identification of new targets, but cannot be used in drug screening programmes. We are currently exploring the hypothesis that some early-passage tumour cell lines may retain some of these targets and thus form the basis for novel research. A disadvantage of such lines is that they are heterogeneous and their properties can change with passage number. Future work with such lines also requires the development of new endpoints that reflect the unique biology of early-passage cell lines.

Acknowledgements

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