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Potentials and Possible Pitfalls of Human Stem Cell Analysis

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Summary. *A clonogenic cell assay for malignant brain tumors that permits the evaluation of tumor cell sensitivity to BCNU and that correlates with patient response to BCNU has been developed. The potential for a stem cell analysis of human tumors has been demonstrated by studies of the reasons for clinical drug failure, tumor heterogeneity, and age-response relationships.*

The basic requirements of a stem cell assay include the ability to dissociate representative single cells from solid tumors, to optimize culture conditions, and to characterize the growth of colonies. Exposure of cells to a drug in vitro must be comparable to the in situ situation; possible significant differences between short-term and 'continuous' treatment methods are emphasized. Also discussed are criteria for in vitro sensitivity of cells, problems inherent in the 'early' evaluation of cultures (at the cell 'cluster' stage), and the effects of system errors, which if overcome should lead to the development of analytic methods with a maximum sensitivity and predictive value.

Introduction

Precise and reproducible systems for determining the effects of oncolytic agents at the cellular level will aid in the rational planning of therapeutic regimens. Changes in tumor size that can be measured by any available method do not provide sufficient information. Recently, emphasis has been focused on clonogenic tumor cells that have the potential for unlimited proliferation; continuous growth of a malignant tumor reflects multiplication of these cells, and, conceivably, a therapy that could destroy all clonogenic cells should produce a cure. It is assumed that a better understanding of tumor biology and the effectiveness of treatment will come from an increased understanding of this clonogenic population.

We have developed in vitro methods to evaluate various factors that affect the growth of clonogenic cells in malignant brain tumors. For tumors that have a similar histological appearance, the number and types of clonogenic cells, their growth kinetics, and their differential sensitivity to cytotoxic agents probably account for variations in tumor growth and variations in the response to chemotherapeutic agents.

The basic requirements for an in vitro clonogenic cell assay include: (1) the ability to disaggregate tumor specimens to obtain a representative single cell suspension; (2) the optimization of culture conditions; and (3) characterization of colonies with regard to the origin of cells and their malignant potential. A cell can be considered a stem cell only if self-renewal can be demonstrated. This paper presents the results of preliminary investigations with a clonogenic cell assay for human brain tumors. Our assay demonstrates both the potential benefits of the in vitro stem cell assay and some possible pitfalls.

Studies of Malignant Brain Tumors

Recently, we developed an in vitro clonogenic cell assay for the investigation of human brain tumors [18]. Briefly, single cells are disaggregated from tumor biopsy specimens and cultured for 4 weeks under conditions optimized for in vitro colony growth. Culture conditions include incubation with heavily χ-irradiated 9L rat brain tumor 'feeder cells' in complete medium, supplemented with 30% fetal calf serum, at 37° C in a 5% CO_2-95% air atmo-

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sphere. Colonies have been grown from the majority of tumors analyzed. In some instances, transplantation of cells harvested from colonies produced tumors in nude mice. In some colonies, cells that contain glial fibrillary acidic protein (GFAP) have been found, and cultured cells from colonies were recognized by two hybridoma monoclonal antibodies (GE2 and BF7) that were developed from and react only with glioma cells [23].

To date, biopsy specimens from 12 glioblastomas have been analyzed for in vitro cell sensitivity to BCNU and comparted to the response of the tumor to the same agent in situ. Cell survival curves (Fig. 1) demonstrated a maximum of 90% cell kill within the range of the clinically achievable dose. Determination of the amount of in vitro cell kill that could reflect in situ tumor response requires a comparison of cell and patient results, with selection of a discrimination point and later confirmation of that relationship. It appears that cell kills of 5%, 20%, and 40% discriminate between tumor response and progression for 10%, 40%, and 100% of the clinically achievable dose, respectively (Fig. 2). The correlation between culture and patient response is better for cell resistance than for cell sensitivity. All seven patients whose cells were resistant in vitro failed clinically, but three of five patients whose cells were sensitive in culture responded clinically. This predictive capability is similar to the soft agar stem cell assay developed for a variety of tumors [22, 26] and to the estrogen receptor assay developed for breast carcinoma [14]. This relationship between in vitro and in situ results implies that cells disaggregated from a tumor biopsy specimen are representative of the clonogenic cells within the solid tumor, at least with regard to chemosensitivity. More data to establish the validity of the assay are necessary.

Clonogenic cell assays may provide information that will help explain the failure of brain tumor chemotherapy. Analysis of cell survival for up to ten times the clinically achievable BCNU dose demonstrated two distinct patterns of cellular response (Table 1): (1) Essentially no cell kill was noted clinically-resistant after treatment with up to ten times the clinically achievable doses of BCNU of cells cultured biopsy specimens from six patients; and (2) cells obtained from biopsy specimens of six other patients showed modest or no cell kill when treated with clinically achievable doses of BCNU, though a marked increase in cytotoxicity was apparent at higher doses; three of the latter patients were clinically resistant. This observation implies that patient failure is caused by inherent tumor cell resistance to drugs in some patients and by inadequate drug delivery in others.

One patient responded to BCNU both in culture and in situ (biopsy 126, Table 1). She had a second operation after her tumor recurred and clinical resistance to BCNU had been documented. The recurrent tumor was partially necrotic, and the assay

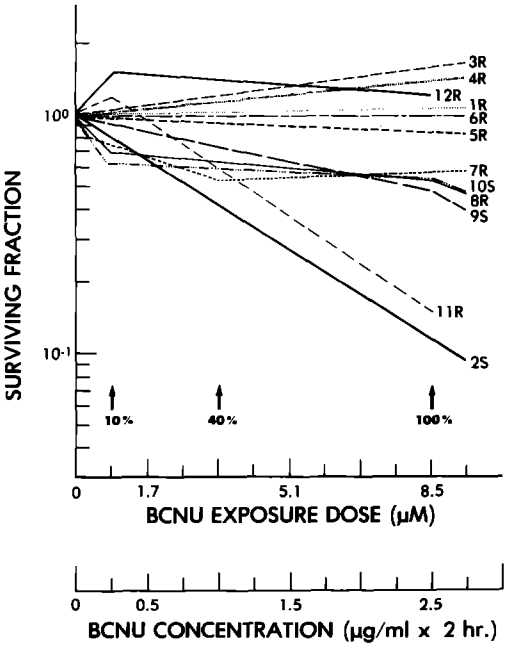

Fig. 1. Cell survival curves for tumor cells disaggregated from biopsies of 12 human malignant gliomas and treated in vitro with various doses of BCNU. Drug dose is expressed as medium concentration (μm/ml), cell exposure dose (μ*M*), and percentage of mean maximal clinically achievable dose (*"inserted values* and *arrows*). Tumor response in situ is represented by R (resistant) or S (sensitive) adjacent to their tumor cell curves. Tumor response in situ S = sensiltive; R = resistant

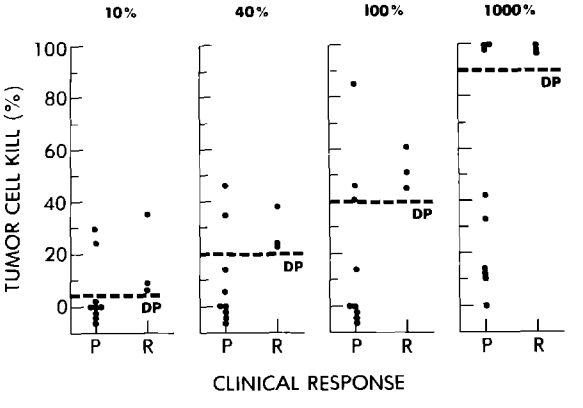

Fig. 2. Relationship between percentage tumor cell kill and clinical response for in vitro doses representing 10%, 40%, 100%, and 1,000% of mean maximal clinically achievable dose. The discrimination point (DP) that apperars to represent in vitro sensitivity is different for each dose

showed persistent, marked cell sensitivity to BCNU (biopsy 167, Table 1). This observation suggests that in individual patients the recurrence of tumor may reflect insufficient drug delivery rather than drug-induced cell resistance, as has been assumed to be the case.

Human brain tumor heterogeneity has been demonstrated histologically [20], by autoradiographic studies of cell kinetics [6], by flow cytometric analysis of cell DNA content [7], and by the in vitro clonogenic capacity from multiple specimens of individual tumors [17, 18]. We have evaluated the feasibility of isolating tumor cell clones by using early culture passage cells from a glioblastoma biopsy specimen. Preliminary studies suggest that different clones may have different sensitivities to BCNU. Clonal heterogeneity might play an important role in determining tumor response to drugs.

Finally, clonogenic cell assays may help explain certain clinical observations. Several centers treating malignant glioma patients with nitrosoureas have observed better clinical results for younger patients [11, 27]. Except for the assumption that older patients tolerate less drug [11], there has been no acceptable explanation for this observation. To investigate the relationship of patient age to cell sensitivity, we grouped all sensitive and all resistant malignant gliomas and compared their median and mean ages. Preliminary evaluation suggests that tumors harbored by younger patients are more sensitive to BCNU. This apparent age dependency is being investigated.

Possible Pitfalls

Tumor Disaggregation

Mechanical methods of dissociating solid tumors invariably produce cell clumps as well as single cells; cell clumps are more likely to grow into colonies than single cells. Unless a 'pure' single cell suspension is plated, the resulting colonies cannot be considered to represent single clonogenic stem cells that were present in the original tumor.

We have recently evaluated several different enzymatic methods for obtaining single cell suspensions from rat and human brain tumors [19]. Rat 9L brain tumors were labeled in situ with a single dose of tritiated thymidine 30–60 min before sacrifice. Tumors were minced and cell suspensions were obtained by mechanical methods alone and with subsequent treatment with several enzyme preparations. Furthermore, an additional aliquot of the tumor mince was used to obtain a preparation of nuclei by a method reported to retrieve 70%–80% of all nuclei in a tissue specimen [8, 9]. The disaggregation methods were evaluated according to the

Table 1. Comparison of cultured cell and patient response to BCNU

Biopsy no.	Culture treatment		Cell response[a]	Patient response[a]
	Percentage cell kill			
	2.5 μg/ml × 2 h	10 μg/ml × 2 h		
Group 1[b]				
125	0	33	R	R
127	0	0	R	R
128	0	10	R	R
131	14	0	R	R
134	0	21	R	R
150	40	14	R	R
Group 2[c]				
126	61	99.3	S	S
159	46	96	S	R
160	51	–	S	S
163	45	90	S	S
167	85	97	S	R
168	0	89	R	R

[a] S, sensitive (in vitro = > 40% cell kill at maximum clinically achievable dose (2.5 μg/ml × 2 h); in situ, significant tumor response); R, resistant

[b] Essentially no cell kill, probable inherent drug resistance

[c] Modest or no cell kill at clinically achievable doses and greater cell kill at larger doses; possible drug delivery problem

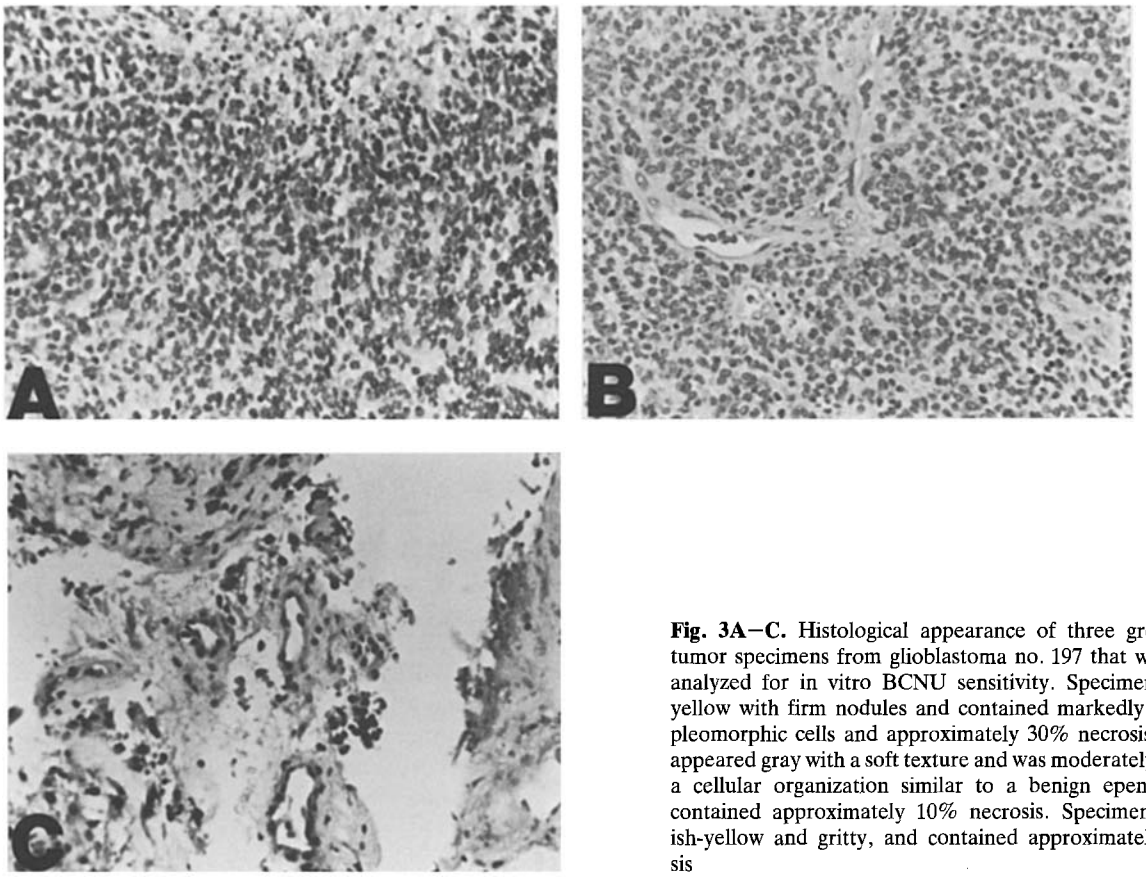

Fig. 3A–C. Histological appearance of three grossly different tumor specimens from glioblastoma no. 197 that were separately analyzed for in vitro BCNU sensitivity. Specimen **A** appeared yellow with firm nodules and contained markedly hypercellular, pleomorphic cells and approximately 30% necrosis. Specimen **B** appeared gray with a soft texture and was moderately cellular, with a cellular organization similar to a benign ependymoma, and contained approximately 10% necrosis. Specimen **C** was whitish-yellow and gritty, and contained approximately 95% necrosis

purity of the single cell suspension obtained, and the labeling index (LI) of the cells and nuclei were compared. Dissociation by means of an enzyme cocktail consisting of pronase (0.05% of 45 PUK/mg, B grade, Cal Biochem, La Jolla, CA), collagenase (0.02% of 125 units/mg, U.S. Biochemical Corporation, Cleveland, OH), and DNAse (0.02% of 7×10^4 dornase units/mg, B grade, Cal Biochem, La Jolla, CA) for 30 min at 37° C produced in a purer suspension of single cells than either trypsin (0.25% × 10 min, GIBCO), neutral protease (1 mg/ml × 60 min, Dispase, Sigma Corp.), or mechanical methods. A human brain tumor (a medulloblastoma) was dissociated by the same method, with similar results. The LI for single cells obtained with the enzyme cocktail was essentially identical with the LI of the cells obtained without the use of enzymes; comparison of methods used to obtain nuclei gave results that were similar. This suggests that the enzyme cocktail produces a cell suspension that is representative of the cells present in the tumor biopsy.

In any assay, the sensitivity of a tumor to a drug usually is determined from analysis of a relatively small biopsy sample. It is conceivable that culture of specimens taken from different areas of an histologically heterogeneous tumor will produce clonogenic cells with different characteristics. If cell sensitivity is markedly different among various areas in a tumor, analysis of a single biopsy specimen may not reflect the sensitivity of the whole tumor, and the use of the stem cell assay to predict chemotherapeutic response for individual patients may be invalid. We found that the CFE of untreated cells from different areas of nine malignant gliomas demonstrated a coefficient of variation of 33% [17]. Recently, we analyzed the in vitro response to BCNU in three grossly distinct specimens of a mixed malignant glioma/ependymoma (Fig. 3). The dose-response curves of all three specimens demonstrated remarkable similarity (Fig. 4). If our assay is to be used for individualizing patient treatment, we must confirm this finding with other tumor samples.

Culture Conditions

Culture conditions must be optimized for the growth of colonies in agar and monolayer systems to most accurately reflect the growth of cells that are clonogenic in situ.

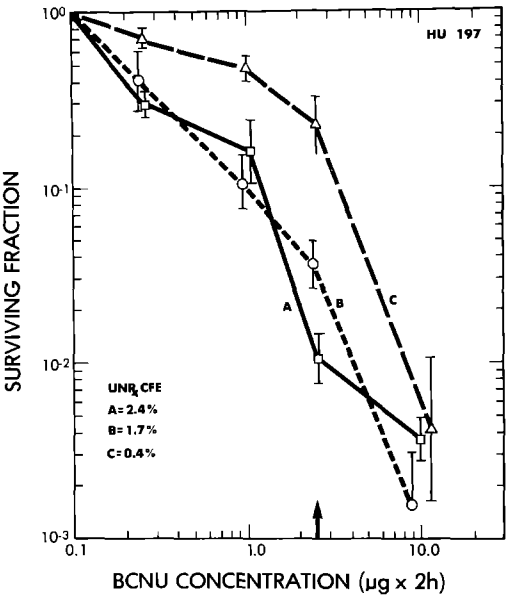

Fig. 4. Survival of cells disaggregated from three separate specimens of glioblastoma no. 197 treated in vitro with various doses of BCNU. *Error bars* represent 95% confidence limits based solely on the variability in colony counts in treated cultures. Cells from specimen C grew more slowly in culture and demonstrated lower untreated CFE than cells from specimens A and B. The three *curves* are remarkably similar; the cells from all three biopsy specimens are considered sensitive to BCNU

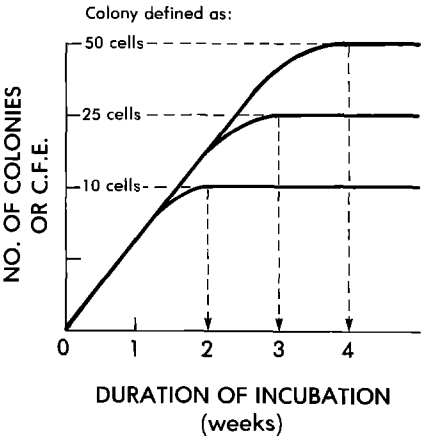

Fig. 5. Hypothetical curves represent changes in number of colonies or CFE with increasing duration of in vitro incubation for each colony definition. Colonies are defined as containing 10, 25, and 50 cells. The minimum incubation period for each colony size is represented by the initial part of the *plateau* on its *curve*

The definition of a colony is frequently arbitrary, varying from an accumulation of 10–100 cells derived from a single cell; culture conditions are not usually considered in determining the threshold for colony size. The number of cells in a colony should differentiate between unperturbed clonogenic and doomed cells. Furthermore, because cells treated with oncolytic agents or χ-rays may not die until four cell divisions have been completed [5], the minimum colony cell number needed to evaluate therapeutic efficacy should be 16 cells. The precise number of cells required in a colony is arbitrary above the minimum limit. Figure 5 shows hypothetical curves used to determine the proper incubation period for colonies of three different sizes. As long as the plateau has been reached on a plot of the number of colonies versus duration of incubation, any group containing more than 16 cells might be considered a colony.

Agents may perturb cell division without causing cell death. For example, treatment of 9L gliosarcoma with dianhydrogalactitol (DAG) produces a delay in colony formation at high doses [13]; unless treated cells are incubated for 16 days instead of the usual 12 days, the activity of DAG would be overestimated. To avoid such a misinterpretation, an additional group of plates should be incubated longer than usual for each new drug evaluated, at least for the high dose(s).

Optimum conditions may be obtained by the addition of feeder cells or by using conditioned medium to make colony growth independent of the number of tumor cells plated. The absence of a cell number dependency should be determined for each tumor analyzed by plating different numbers of cells and demonstrating the same CFE for each group.

Finally, because the CFE of primary tumor cell suspensions is usually very low, in the order of 0.01%–2% [3, 18, 21], drug analyses will be most valid if the CFE is optimized. In our system, we have recently demonstrated that a few passages in culture produce a marked increase in CFE (to 10%–50%) for untreated cells [17]. BCNU dose-response curves were repeated after three to four culture passages for cells derived from four glioblastoma biopsy samples and compared with the original cell survival analysis. The curves were essentially identical (Fig. 6) [29], which implies that the original cell survival studies are valid despite very low CFEs and that, at least for a cell cycle-nonspecific agent such as BCNU, the result of studies of early passage glioma cells are the same as those obtained from cell suspension originally derived from the tumor biopsy.

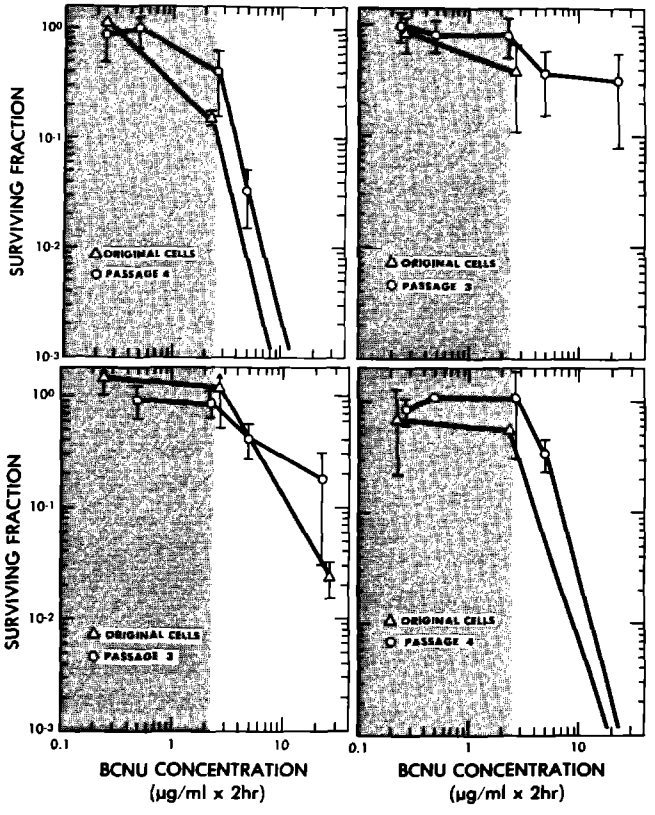

Fig. 6. Comparison of BCNU dose response curves for malignant glioma cells originally disaggregated from 4 biopsy specimens and for cells from the same tumors after three or four passages in culture. The *curves* for original and passaged cells demonstrate remarkable similarity. The shaded areas represent the clinically-achievable dose

Characterization of Colonies

The identity of cell types that grow into colonies must be known for a tumor stem cell assay to be valid. Colonies and cells may be characterized by the production of morphologically distinct products (e.g., melanin production in melanomas [15]), secretions (e.g., human chorionic gonadotropin for choriocarcinomas [1]), standard and immunohistochemical stains (e.g., GFAP for astrocytic tumors [2]), and cell surface characteristics (e.g., monoclonal antibody recognition [23]). The characteristics of normal host cells that may be present in or near the tumor that may grow into colonies in both monolayer (e.g., fibroblasts [4]) and agar (e.g., macrophages [24]) systems must be recognized and eliminated from the final calculation of clonogenic tumor cell survival.

When an infiltrative tumor is biopsied at its site of origin, the malignant nature of the clonogenic cells should be determined. Malignant gliomas exemplify this problem: Because colonies can develop from normal brain tissue [19], biopsies taken at the leading edge of a brain tumor may produce colonies that contain both normal and malignant astrocytes. Because morpohological characteristics alone will not distinguish between the two cell types [19], subsequent cloning and analyses of growth either in culture or in immunodeprived mice may help determine the nature of the malignant cell. It may be possible that studies in which monoclonal antibodies developed against a malignant brain tumor cell culture are used will differentiate between malignant and nonmalignant cells (R. Ceriani, 1980, personal communication).

Pharmacological Considerations

Whenever possible, the treatment of cells in vitro should parallel the in situ exposure of cells within the solid tumor. Dosages should be calculated according to cell exposure dose and expressed in moles/liter if a comparison is to be made between the sensitivity to different agents [30]. The drug half-life ($t_{1/2}$) in situ will be affected by chemical degredation, conjugation, and elimination by several routes (especially kidney and liver), and tumor exposure can be calculated from plasma levels [12]. The condition of in vitro cell exposure should be adjusted to provide the same $t_{1/2}$ values as found in situ. Doses significantly smaller than the maximum clinically achievable dose should be evaluated, because regions within a tumor are usually poorly perfused and would not be expected to accumulate large amounts of drug. A dose larger than the amount usually achievable may be administered for studies of tumor resistance. Furthermore, under certain circumstances, a larger than usual drug dose may be delivered to the tumor by altering the method of administration of the agent. Intraperitoneal administration of adriamycin [16] and megadose BCNU with bone marrow rescue [25] are examples of methods that may be used for individual patients if cell survival studies suggest that higher than normal drug doses may be beneficial.

Caution must be used when planning treatment by continuous drug exposure. If the agent is truly stable (over days to weeks) in culture, then the survival of cells will reflect a resistant cell population rather than a true dose-response relationship. Furthermore, it is unlikely that such 'continuous exposure to stable drugs' would parallel the in situ tumor cell exposure because agents that do not undergo chemical degredation would nevertheless be eliminated by other routes (e.g., kidney and liver).

Continuous treatment may result in a different rate of chemical degradation than short-term exposure (1–2 h) if complete medium with serum added is not used for the short-term studies. An example is the

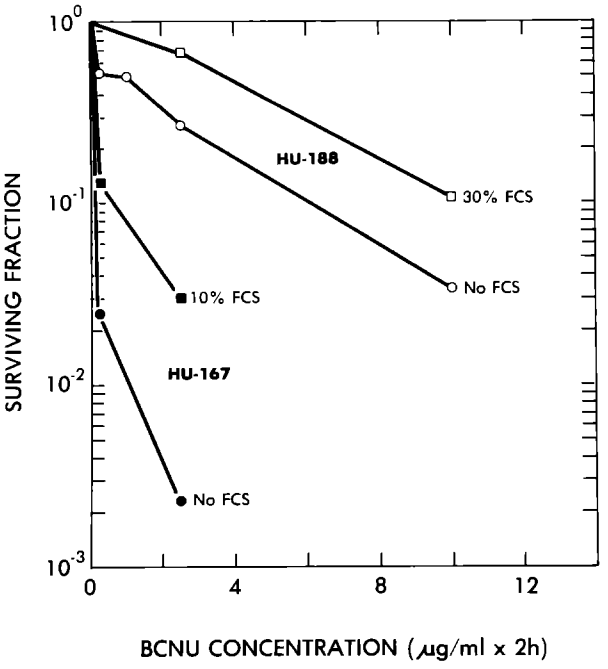

Fig. 7. Comparison of cell survival for glioblastomas nos. 167 and 188 after in vitro treatment with BCNU for 2 h with and without the presence of fetal calf serum ("FCS). Less cell killing occurs in the presence of FCS, probably as a result of a shorter $t_{1/2}$ for BCNU and consequently a smaller cell exposure dose (drug exposure integral)

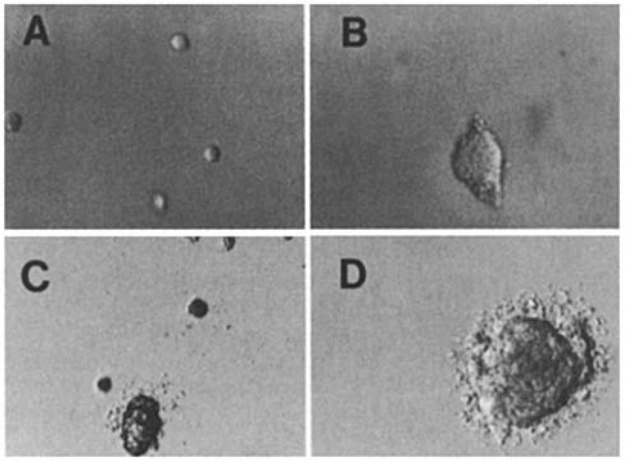

Fig. 8A–D. Possible confusion of nonclonogenic giant cells with small colonies or clusters of stem cells in agar cultures is demonstrated at 14 days of incubation for three different cell types. Single cells were originally seeded at day 0 (**A**); similar larger masses were present in cultures incubated for 14 days: (**B**) 9L rat heavily irradiated (4,000 rad), nonclonogenic cells; and (**C**) untreated glioblastoma no. 126 cells. The masses present in the 9L feeder plates (**B**) represent giant cells that might be confused with clusters of cells from glioblastoma no. 126 (**C**) at 14 days. The growth of cells in agar from glioblastoma no. 159 (**D**) was more suggestive of a cluster of individual cells

influence of serum on the $t_{1/2}$ of BCNU: In the absence of serum, the $t_{1/2}$ is 64 min, but in the presence of serum, the $t_{1/2}$ decreases to approximately 15 min, which reflects serum protein catalyzed degradation of BCNU [28]. Therefore, tumor cells in a plate containing complete medium to which BCNU had been added for continuous treatment may be exposed to less total drug (lower exposure integral [12, 30]) than if drug were administered in serum-free medium for a short period (2–4 h), which is our standard procedure. Treatment of two early passage human glioma cell lines with BCNU for 2 h with and without serum showed that there is less cell kill in the presence of serum (Fig. 7).

Analysis of Cell Clusters

Our assay would be potentially more beneficial in the clinical setting if it could be carried out in less time than is now needed. It has been suggested that if cell clusters rather than colonies were scored, the assay time could be shortened; use of this modification would require that the number of cell clusters developing early was related to the number of colonies that develop during the standard incubation period. There are two possible problems inherent in scoring clusters: First, perturbed cell division may be interpreted as cell kill, as mentioned above for the case of DAG [12, 13], and second, clonogenic cells that are 'killed' by the treatment may develop into giant cells that might be judged to be a colony in agar (Fig. 8). This mislabeling would be especially likely if machines are utilized to count colonies, because light-scattering techniques will not differentiate giant cells from a group of dividing cells of a similar size.

Criteria for in vitro Sensitivity

The criteria for in vitro sensitivity to chemotherapy are based on a comparison of in vitro and in situ response in a selected patient population [21]. A decrease in tumor size after drug treatment depends on clonogenic cell death, lysis and removal, and an inherent cell loss factor; different tumor types have different cell loss factors. Furthermore, the rate of cell lysis and removal depends on the tumor site; Kumar demonstrated that dead cells are removed from the brain more slowly than from intramuscular or subcutaneous sites [10]. Therefore, discrimination between cell sensitivity and resistance in vitro must be independently determined for each tumor type and location for the most valid interpretatioin of results.

System Errors

Errors will occur at various stages during the stem cell assay procedure. The coefficient of variation (CV) for colony counts of ten malignant gliomas was 21% for four untreated plates and 35% for four plates treated at a single BCNU dose. This error includes the variability caused by cell dilution, colony growth, and colony counting. The possible error for analysis of cell kill at a single drug dose will be a composite of these two CVs. Additional errors are imposed if a single cell count is made before dispensing cells into treatment vials, instead of counting cells immediately before aliquoting the contents of treatment vials into culture dishes. We have determined this variability by counting glioma cells before BCNU treatment and re-counting the vials before plating. A comparison of treated cell counts to counts of control cells from eight glioma specimens demonstrated a CV of 30% and no dose-dependent relationship. Elimination of these three primary sources of error has practical limitations; however, errors could be minimized by using duplicate controls for each experiment and by counting cells only once, immediately before plating. In any case, knowledge of the possible inherent errors is mandatory because cell sensitivity has been defined as 30%–70% cell kill [17, 22, 26] and single drug doses have been used in some studies [26].

Potentials for Human Stem Cell Analysis

An understanding of the basic requirements for and possible pitfalls of clonogenic cell analysis of human tumors should lead to significant improvements in the treatment of cancer. A reliable predictive assay for response to chemotherapeutic agents may permit individualized patient treatment. Different classes of agents can be screened against various types of tumors to identify tumor-selective drugs. Structural modifications of active agents might improve their activity and pharmacologic characteristics, which could produce increased tumor drug levels. Evaluation of different agents with various mechanisms of action should increase knowledge of tumor cell resistance and cross resistance. Cell sensitivity measurements will help explain the clinical failure, because failure is due to either inherent cell resistance, to cells residing in drug-insensitive phases of the cell cyle during treatment, or to inadequate drug delivery of chemotherapeutic agents. The mode and schedule of therapy administration can be altered to provide larger than usual doses of agents to which the tumor cells are potentially sensitive. Finally, clonogenic cell analysis will provide a method for the isolation and study of tumor stem cells; analysis of their kinetics, markers, and sensitivity to perturbing factors should increase our knowledge of tumor biology.

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