Compositionality of Artificial Heterogeneous Tumors in Vivo: Use of Mitomycin C as a Cytotoxic Probe

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ABSTRACT

A major part of the overall response of solid cancers to cytotoxic treatments will be due to the differential sensitivities of the neoplastic cell subpopulations present. To quantitatively investigate this, artificial heterogeneous human colon xenograft tumors were constructed using two clonally related cell lines (A and D) which were mixed to create compositions of approximately 9:1 or 1:9 A:D cells. Then, the pure A, D, or admixed tumors were challenged with mitomycin C which kills A cells more efficiently than D cells by a factor of about 2.3 as determined by in vivo survival curve inactivation slopes. This difference was also exhibited in vitro by the post-mitomycin C cell survival responses determined by excision assay and by the shapes of the regrowth curves of the pure A and D or admixed tumors. By approximately 30 days after treatment, artificial heterogeneous tumors had reached a new stable cellular composition which could be quantitatively predicted based on the individual survival of A and D cells from pure clonal tumors measured 24 h after treatment. Thus, in this model system, cytotoxic treatment of heterogeneous neoplasms produced predictable and stable, albeit altered, percentage admixtures of subpopulations. This result is consistent with the observed decreased clinical responsivity of primary tumors to sequential courses of therapy due to selection of preexisting resistant subpopulations.

INTRODUCTION

Both rodent and human solid tumors have been demonstrated to contain multiple subpopulations of clonally related cells which arise through the processes of mutation or aberrant differentiation, with environmental selection, to produce a condition of intraneoplastic diversity (1-6). For colorectal cancers, such diversity in stemline number has been documented by Hiddeman et al. (7). Clinically, it has been noted that successive cycles of chemotherapy of both solid and disseminated cancers are often accompanied by decreasing efficacy (8-10). Part of the explanation for such decreased clinical response must include production of a more resistant state by selection of drug-resistant subpopulations (1, 10, 11), a conjecture supported by in vitro studies which have demonstrated marked differential sensitivities between cancer cell subpopulations (1). To date, however, there has been little quantitative information concerning the relationship between such intrinsic intraneoplastic diversity and response to cytotoxic therapy of primary neoplasms, although work has been performed related to the stability of the metastatic phenotype (12, 13). This is a difficult problem because (a) intratumoral subpopulations will generally exhibit a distribution of sensitivities to any given agent (reviewed in Ref. 14), and (b) intraclostral interactions have been postulated to exist which might modulate cell survival responses in unpredictable ways in vitro (15-17).

To address these considerations, artificial heterogeneous tumors have been constructed using 2 clonal cell lines (A and D) originally isolated from the histopathologically heterogeneous DLD-1 human colon adenocarcinoma (18). These subpopulations differ significantly in their chromosomal content and in the morphology of colonies produced in tissue culture. We have shown that it is possible to admix varying proportions of these two lines to create solid xenograft neoplasms in nude mice (19, 20). The compositional stability, clonogenicity, and zonal characteristics of these AHTs have been described (20) in unper- turbated neoplasms after enzymatic disaggregation of solid tumors followed by clonogenic assay in vitro. With such base-line information, it is consequently possible to experimentally perturb AHTs by acute exposure to some agent which produces a marked degree of differential cytotoxicity between the A and D subpopulations. One may then assay the kinetics of cell death and repopulation and establish whether or not new equilibrium states are produced. Such information is important in the design of clinical strategies. In the A:D model system, we used the bifunctional alkylating agent mitomycin C as a probe to study responses of heterogeneous tumors to therapeutic intervention, as this agent produces significantly more cell killing in A cells than in D cells (18, 21).

MATERIALS AND METHODS

Tumor Lines. We have previously described in detail the DLD-1 adenocarcinoma from which the clone A and clone D subpopulations were obtained by soft agar cloning (18). Clone A cells produce poorly differentiated tumors, while clone D cells produce moderately differentiated tumors. In monolayer culture, colonies of either A or D cells can easily be identified due to their respective morphologies (18-20, 22). The A and D lines are maintained in our laboratory according to previously published procedures (19, 20) and are replenished from frozen stock every 3 to 4 mo.

Tumor Disaggregation Procedures. Full details on enzymatic disaggregation of either pure A or D or admixed tumors have been published (19, 20). The sampling design has been previously described (19, 20) and was based on the original work of Wallen et al. (23). Generally, 6 to 12 samples at 3- to 4-day intervals were taken from each tumor for cell yield and compositional and clonogenic studies. Samples were minced by scalpel and disaggregated with 0.05% trypsin-EDTA (Grand Island Biological Co., Grand Island, NY) (GIBCO) for 40 min at 37°C. Cells were centrifuged (4°C, 7 min, 1000 rpm), resuspended in RPMI-1640 medium containing 10% fetal bovine serum (GIBCO), counted by hemacytometer, and seeded into 60-mm plastic dishes (Becton-Dickinson Labware, Rutherford, NJ). Colonies developed at 37°C while in a humidified incubator (NAPOCO, Portland, OR) under a 95% air-5% CO2 environment for about 14 days. Colonies were then fixed and stained using 0.5% crystal violet in absolute methanol. Colonies containing more than 50 cells were counted by eye for estimation of the overall CFE from each tumor.

Each dish was visually inspected during the course of colony develop-

1The abbreviations used are: AHT, artificial heterogeneous tumor; CFE, colony-forming efficiency; ID50, concentration of drug required to cause a 50% inhibition of cell growth in vitro; CY, cell yield.

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ompent using phase-contrast microscopy, and colonies were characterized as being either A or D, based on morphology. This approach and its validation have been previously described (18–20, 22). Therefore not only could the overall CFE from each tumor be measured, but we could also determine the relative proportions of A and D cells for each admixture condition.

Production of Xenograft Tumors. CD-1 mice bearing the su/nu gene on an outbred Swiss background were obtained from the Charles River Breeding Laboratories, Wilmington, MA, and were maintained in the Animal Care Facilities of Brown University, Providence, RI. Mice were kept in a laminar flow hood (Thoren Industries, Pittsburgh, PA) under specific-pathogen-free conditions, with sterilized food, bedding, and water. Mice of both sexes of approximately 5 to 7 wk of age were used.

For injection, exponentially growing tumor cells were enzymatically removed (0.03% trypsin-EDTA; Gibco) from plastic flasks and suspended in Hank's basic salt solution (GIBCO). A total of 1 x 10⁷ cells was injected into the upper hip region in a volume of 0.25 ml. Mice were ear tagged for individual monitoring and were separated into groups on a random basis.

Solid tumors were obtained after injection of (a) either pure clone A or clone D cells alone, (b) a 94.2% A:5.8% D admixture, or (c) a 7.0% A:93.0% D admixture. As we have published previously, the composition of the injection admixtures was verified by (a) determination of the clonogenic efficiencies of aliquots of cells from the initial injection admixtures and (b) by integration under the volume curves of size distributions of A:D admixtures (19, 20). The morphological and cell volume differences between A and D cells which allow these procedures have been described (18–20). Colonies were identified by scanning of dishes under phase contrast at x10 magnification.

Measurement of Tumor Size. The solid tumors were measured by calipers in two orthogonal diameters, and the volumes were calculated using the formula for a prolate ellipsoid

\[ V = \frac{L \times W^2}{2} \]

where L and W are the major and minor diameters (in mm), respectively. We have used this technique in previous work (19, 20, 24, 25). The average volumes along with standard errors for each tumor group were plotted as a function of time to obtain growth curves. Volume measurements began at about Day 7 postinjection and extended over about the next 40 days. All measurements for all tumor groups were made by one person.

In Vivo Mitomycin C Injections. Mitomycin C (Mutamycin; Bristol-Myers Co., Syracuse, NY) was injected in sterile saline. Mice received a single i.p. dose of 5 µg/g when average tumor volumes were 500 mm³. There was no mortality produced. Mice in control groups received sterile saline injections.

In Vivo Mitomycin C Studies. Dexter et al. (18) and Calabresi et al. (21) have previously described the differential responses of A and D cells to mitomycin C and showed that A was significantly more sensitive than D. However, these studies were done using the ID₅₀ approach in which the relative contributions of cytostatic and cytotoxic drug activities cannot be distinguished. We therefore performed clonogenic survival studies on A and D cells. Exponentially growing cells in monolayer cultures were exposed to graded concentrations of mitomycin C for a period of 1 h at 37°C. The drug was dissolved in complete RPMI-1640 medium containing 10% fetal bovine serum (GIBCO). After exposure, the drug-containing medium was decanted, the cell monolayer was washed twice with ice-cold phosphate-buffered saline, and the cells were detached using 0.03% trypsin-EDTA (GIBCO) (5 min, 37°C). Cells were centrifuged (1000 rpm, 7 min, 4°C), resuspended in complete RPMI-1640 medium, counted using a Model ZM Coulter Counter (Coulter Electronics, Hialeah, FL), and seeded into 60-mm plastic dishes (B-D Labware, Trenton, NJ) at appropriate concentrations for assessment of colony formation. Colonies were allowed to develop for approximately 14 days, washed once with phosphate-buffered saline, fixed, and stained using 0.5% crystal violet in absolute methanol. Colonies containing more than 50 cells were counted, and linear least-squares regression analysis of the survival responses was performed (26).

Analysis of Tumor Growth Curves. Regrowth delay was defined for the mitomycin C treatments using the time in days needed for tumors to grow to 3 times their volume at the time of injection of mitomycin C, as we have done previously (24, 25).

RESULTS

The in vitro survival responses for the 1-h exposures to the graded concentrations of mitomycin C are shown in Fig. 1. Linear least-squares regression analysis of the data indicated that the mean inactivation slopes (concentrations required to reduce survival to 1/e of incident levels) were, respectively, 0.176 and 0.400 µg/ml for lines A and D (26). Using the ratio of slopes as a measure of relative sensitivity, A cells are therefore 2.3 times more sensitive to mitomycin C than D cells. Fig. 2 shows the volumetric growth of these neoplasms for either sham-injected controls or for mice given injections of the single dose of 5 µg/g, and it demonstrates the difference in growth which would be expected based on the differential in vitro cell killing data (Fig. 1). The time in days needed for tumors of the various treatment (treated – control) groups to grow to 3 times their volume at the time of treatment for the pure A and D neoplasms (Fig. 2, A and B) was approximately 11 and 8 days, respectively, indicating that the greater sensitivity of A cells is also exhibited in vivo. The regrowth delay time for the 94.2% A plus 5.8% D or 7.0% A plus 93.0% D tumors was approximately 10 and 8 days, respectively (Fig. 2, C and D). These values are about what would be predicted based on the independent sensitivity of each cell type (Fig. 2, A and B).

Cell yield data obtained from the pure or admixed tumors as a function of time after treatment were essentially the same as we have previously reported for these pure or admixed tumors (13, 14); therefore the data are not tabulated. For the pure clone D tumors, cell yields were in the range of 4 to 7 x 10⁵ cells/mg, while for clone A, values were in the range of 1 to 4 x 10⁶ cells/mg. For clone A tumors, there was a decline in CY over the 35-day assay period, similar to previously reported kinetics (20). The neoplasms comprised of 7% A plus 93% D cells yielded CY values not different than those seen in the pure line D tumors, and the 94.2% A plus 5.8% D AHTs showed CY values different from pure line A tumors. There was no
apparent effect of the mitomycin C injections on the cell yield.

In Fig. 3, A and B, we show the normalized survival responses for the pure or admixed tumors (each point in the figure represents the average survival of a single tumor obtained from 6 to 12 samples). Several features are apparent. First, the absolute level of cell killing is much more marked in A than in D neoplasms. The relative survival of cells from either pure A or D tumors assayed at 24 h posttreatment was about $5 \times 10^{-4}$ for A cells and about $3 \times 10^{-2}$ for D cells (colony-forming efficiencies from unperturbed A and D tumors were both about 10%). The relative survival of D:A cells is therefore about 60:1 which agrees reasonably well with $ID_{50}$ data in which D cells were about 40 times more resistant than A (18, 21).

The relative survivals for 24 h posttreatment (Fig. 3) generate a hypothesis about the final composition of these mitomycin C-treated AHTs. If the 24-h survival levels in vivo represent the relevant cell survival of A and D cells (including potentially lethal damage recovery from mitomycin C and excluding cellular repopulation) (27, 28), then by multiplying the relative percentage of A and D cells in each of the 2 admixtures by the relative surviving fraction, we can predict potential new compositional AHT states. From such calculations, values of about 18% A plus 82% D for neoplasms that were initially 94.2% A plus 5.8% D are obtained. Similarly, for the neoplasms initially 7% A plus 93% D, one obtains predicted values of about 0.1% A plus 99.9% D. The relative cellular compositions for the two different admixtures as a function of time posttreatment are shown in Fig. 4. By about 30 days posttreatment, the mitomycin C-treated tumors appear to reach the predicted new equilibrium states. An unexpected result was the time-dependent oscillation in the compositional data of Fig. 4. The biological basis for such behavior is probably because there is a large difference in the relative repopulation rates of A and D cells within these AHTs due to the large differential cell kill of mitomycin C (Fig. 3). The pattern of differential repopulation of A and D cells leading to the nonlinear changes seen from Day 0 to Day 15 post-mitomycin C treatment is schematically illustrated in Fig. 5 in which we have plotted the relative repopulation rates using the data from Fig. 3 from pure A and D tumors. It is possible to appreciate how the nonlinear compositional kinetics shown in Fig. 4 would result from the shape of the response of the A:D repopulation ratio.

**DISCUSSION**

To our knowledge, this work represents the first attempt to quantitatively describe dynamic responses of heterogeneous
scatterplots. These new compositions appear to be predictable based on clonogenic cell survival assayed 24 h post-mitomycin C treatment. Further, these new states appear stable for as long as assays were practical (to about 35 days posttreatment). The production of such “new” heterogeneous tumors by therapeutic intervention would seem to be an expected result based on empirical clinical experience. It would also appear logical to extrapolate these results to solid tumors containing more than 2 subpopulations, although these may represent only 5 to 10% of all colorectal carcinomas (7).

Goldie and Coldman (29) have stated that “clinical resistance to chemotherapy in responding tumors frequently occurs at a time when there has been significant regression of measurable disease.” It is apparent from the data presented herein that differential cell killing in heterogeneous neoplasms has, as a corollary, differential repopulation rates of surviving cells (illustrated by data in Figs. 3 and 5) and that the most sensitive subpopulations exhibit the largest repopulation rebound. Supporting this is the differential recruitment from quiescent cell kinetic compartments experimentally documented in heterogeneous mouse mammary carcinoma sublines by Wallen et al. (30, 31). The marked acceleration in proliferation occurs at a time when the growth curves of treated tumors have not yet reached a nadir. By the time treated tumors have begun showing regrowth volumetrically, the internal composition is well along in terms of establishment of the new equilibrium state. Therefore, retreatment of solid tumors with the same agent as used in initial treatments should likely be performed in the period prior to overt increase in tumor size. Conversely, retreatment with the same agent, once regrowth has begun, might be ineffective as the resistant state has already been established.

We have been developing biomathematical models to describe the growth of heterogeneous tumors (32, 33). In these models, we define neoplastic volumetric growth in terms of relative subpopulation growth rates, host carrying capacities (stomal influences), and competition rates among subpopulations. Implicit values for the robustness of competition may be derived...
from our studies of unperturbed and perturbed (e.g., mitomycin C) tumors. Changes in the local microenvironment can clearly affect such competition, and we have found that the models predict the transient maximum in the relative percentage arising from the differential repopulation rates we show in our data (Figs. 4 and 5). Therefore, the response of a (heterogeneous) neoplasm to cytotoxic intervention is not just a cell-killing phenomenon, but also represents the changes in (subpopulation) recruitment and repopulation rates, host effects (damage to stroma or vasculature), plus the dynamics of interspecific competition of subpopulations in a potentially less suitable environment.

In summary, cytotoxic treatment of heterogeneous neoplasms has been found to produce predictable and stable, albeit altered, percentage compositions of subpopulations. This finding is in accord with observed clinical responses and shows that selection of resistant subpopulations can dramatically change the basic character of solid tumors.

REFERENCES

Compositional Stability of Artificial Heterogeneous Tumors in Vivo: Use of Mitomycin C as a Cytotoxic Probe


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