Growth Properties of Artificial Heterogeneous Human Colon Tumors

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ABSTRACT

Two clonal cell lines (designated as clones A and D), originally isolated from the heterogeneous DLD-1 human colon adenocarcinoma, were used to produce xenograft tumors in nude mice. Neoplasms produced from either A or D cells alone were compared to those produced from a range of percentage admixtures of the two subpopulations. Then, Gompertzian growth parameters (initial growth rates, retardation rates) were determined, along with estimation of the final asymptotic volumes. It was found that the growth kinetics of the various artificial heterogeneous tumors could not be predicted from knowledge of the growth parameters of the pure clonal xenograft tumors. Additionally, both pure clonal and artificial heterogeneous tumors were enzymatically disaggregated as a function of time postinjection, and it was found that the admixed tumors became more zonal in composition as time progressed. Further, admixtures of extreme composition (i.e., 9% A plus 91% D or 88% A plus 12% D) remained stable with time, while those of intermediate initial composition (i.e., 50% A plus 50% D) did not. All of these data (growth kinetics, zonality, compositional stability) indicate that the growth properties of heterogeneous tumors are very complex.

INTRODUCTION

The concept of intrinsic cellular tumor heterogeneity is well established and well described (1). Consequently, investigations are being made into the implications of intraneoplastic diversity. In particular, the concept of an "intratumor ecology" exhibiting clonal interactions (2-8) is thought to be an important component of neoplastic growth. Also, it has been shown that the architecture of heterogeneous tumors displays "zonal" properties (e.g., the exhibition of melanotic and amelanotic regions within an individual tumor) which are thought to be important for the long-term maintenance of intratumor diversity (9). There are therefore two interlocking aspects of intratumor heterogeneity that must be studied, i.e., the potential interactions between neoplastic subpopulations, and the localization and distribution of these subpopulations.

We have recently published data on appropriate experimental conditions for the enzymatic disaggregation of xenograft solid tumors grown from pure or admixed clonal subpopulations from a heterogeneous human colon adenocarcinoma (10). In this work, we also showed that the relative percentages of the two neoplastic subpopulations used (designated as clones A and D) in AHTs produced from an initial injection of 50% A:50% D cells were not stable. Marked enrichment in one of the cell types (clone D) occurred with increasing time postinjection (increasing tumor age), resulting in neoplasms composed of approximately 100% A and 90% D cells. This appeared to indicate a preferred tumor subpopulation composition.

To further experimentally define the compositional stability of AHTs we have extended this initial excision assay work to include more extreme compositional states: these being AHTs produced from initial admixtures of either 9% A:91% D or 88% A:12% D cells. The time-dependent composition and clonogenicity of these neoplasms were studied after disaggregation and compared to the original data from the 50% A:50% D AHTs as well as to data from pure clonal tumors. We also used the cellular sampling data from these AHTs to determine if zonality could be observed. Further, in vivo, we investigated 7 different admixture compositions to define how this variability in the initial percentages of cell subpopulations might be reflected in their in vivo growth kinetics using the Gompertz equation (11). These in vivo data were then used in conjunction with the cellular composition data to describe some overall characteristics of this heterogeneous tumor model system.

MATERIALS AND METHODS

Tumor Lines. We have previously described in detail the DLD-1 tumor system from which the clone A and clone D subpopulations were obtained (12-16). Briefly, the original tumor specimen obtained at biopsy was histologically heterogeneous, and the cell line established from it was designated as DLD-1. The A and D subpopulations were obtained by soft agar cloning of the DLD-1 parent line. These lines are distinct in morphology, in chromosome number, and in their responses to a number of chemical and physical agents (12-14, 16, 17). In vivo, clone A cells produce poorly differentiated tumors, while the D cells produce moderately differentiated colon cancers. Our results to date indicate that the clone A and D subpopulations are responsible for the morphological heterogeneity observed in the parent neoplasm (13, 15, 18). The clone A and D tumor lines are maintained in tissue culture in our laboratory according to previously published procedures (13, 16, 17), and they are replenished from frozen stock every 3 to 4 mo.

Tumor Disaggregation Procedures. We have previously published in detail the procedures for disaggregation of AHTs (10). In the studies reported here, we disaggregated solid tumors at approximately 3-day intervals throughout the growth period. The neoplasms were excised, and 6 (tumor weight, <700 mg) or 12 samples (tumor weight, >700 mg) were taken based on previously published sampling procedures (10). Samples were minced by scalpel, enzymatically dissociated (0.5% trypsin-EDTA, 40 min, 37°C; Grand Island Biological Co., Grand Island, NY), and counted by hemacytometer; and single cells were seeded into 60-mm plastic dishes (Becton-Dickerson Labware, Rutherford, NJ). Colonies developed at 37°C while in a humidified incubator (NAPCO, Portland, OR) with a 95% air-5% CO2 environment for about 14 days. Colonies were then fixed and stained using 0.5% crystal violet in absolute methanol, and colonies containing more than 50 cells were counted by eye for estimation of the overall CFE from each sample.

Each colony was visually inspected using phase-contrast microscopy during the course of development and characterized as being of either clone A or clone D morphology, as each colony type has a unique appearance. Photomicrographs of these clone A and D colonies have appeared in several publications (12, 13). To ensure the validity of this procedure, we selected colonies of each morphological type on a random basis from a number of different dishes and from different tumors disaggregated on different days. Individual colonies were trypsinized after isolation using sterile microwells, and the cells were transferred to a 25-cm2 flask (Becton-Dickerson Labware, Rutherford, NJ) and allowed to proliferate until a sufficient number were available for karyotyping. The cultures were treated with colchicine to collect mitotic cells, which were treated with hypotonic salt solution, vitally stained,
and spread on glass slides. The chromosome content of the cells was determined using a ×100 dry objective. Because clone D cells contain 45 to 46 chromosomes while clone A cells contain 70 to 90 chromosomes (13), it was possible to absolutely determine the ancestry of each colony and to correlate this with the morphological assessment. In no case was a colony of mixed chromosome content noted, and in no case was there any discrepancy between the morphological and the karyotypic assessment of colony identity. We were therefore confident that not only could the overall CFE from each tumor be measured, but that we could also determine the relative proportions of clone A and D cells as a function of time postinjection for each admixture condition.

Sampling Procedures and Mathematical Techniques. The sampling procedures have been previously described (10) and were based on the design of Wallen et al. (19). Briefly, 6 to 12 (generally 12) samples were taken from each tumor for cell yield, compositional, and clonogenic studies. We endeavored to distribute these samples throughout the tumor volume so as to measure, as "randomly" as possible, the geometrical distribution of cell types in the tumor. It is important to note that we could determine the actual proportions of A and D cells in these samples.

Dixon and Massey (20) have described techniques for sampling dichotomous populations and fitting observed proportions to normally distributed curves. In the fitting procedure, the two members of the sample populations are assigned values of either 0 or 1 (i.e., either clone A or clone D), and an estimate of the proportion of either type in the total population is made. In this approach, it is possible to derive a normal distribution (with the parameters, μ and σ²) for the distribution of sample proportions. Estimates for the sample distribution parameters (μ, σ²) are based upon the population proportion, variance, and sample size. The specific question that can then be addressed is whether the clonal tumor subpopulations (A and D) which are initially injected in a homogeneous mixture remain homogeneously distributed throughout the tumor volume with increasing tumor age. If not, one would predict the appearance of "zonality" as shown by Fidler and Hart (9). If this hypothesis is correct, the distribution of standard normal deviates derived from the tumor samples should itself be standard normal. Goodness-of-fit criteria for fitting observed data to the normal distribution are given by Dixon and Massey (20) using the χ² statistic. Therefore, we can test the null hypothesis by fitting the derived standard normal variates to the normal distribution with a mean of zero and a variance of one. If the χ² statistic is greater than the critical value (95% confidence limits), we may accept the alternate hypothesis, i.e., that the colony-forming efficiencies of these cells as determined from exponentially growing cultures are essentially the same (about 60%), these visual scans yielded the above quoted values of the actual percentage of A:D cells injected. Control experiments involving plating of various percentages of A and D cells in vitro yielded clonogenic results always consistent with the independent CFEs of the two cell lines.

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

\[ V = \frac{4}{3} \pi a b^2 \]

where \( a \) and \( b \) are the major and minor diameters (in mm), respectively. We have used this technique in previous work (21). The average volumes along with standard errors for each tumor group were then plotted as a function of time to obtain the tumor growth curves. Volume measurements began at about Day 7 postinjection and extended over the next 30 to 35 days. All measurements for all tumor groups were made by a single individual. The experiments done on the 7 different admixture conditions constitute a more complete verification experiment of preliminary data on compositionally dependent tumor growth that has been previously reported (22). These previous data were performed on admixtures that were identical in composition to the one admixture condition done on the tumors used in the experiments reported in the previous study (22). The data for the experiments done on the 7 different admixture conditions are given in Table I. The differences among these kinetic parameters for the various admixed and xenografted tumors are significant. The average CC values for the various admixed or xenografted tumors are shown in Table I. We also calculated a value designated as the asymptotic final volume or CC of the various tumor conditions. The value is determined by letting \( N(t) \) approach infinity and is calculated as

\[ CC = N(t) = \frac{N(t)}{N(t)} \]

Intuitively, this equation states that a population of cells, represented by the number \( N(t) \) at time \( t \), will grow exponentially at the instantaneous rate of \( g(t) \). However, in the Gompertz formulation of growth, this initial growth rate \( g_0 \) is itself decreasing exponentially at a constant rate of \( A \) (the so-called retardation factor). A standard derivative-free nonlinear regression package (BMDPAR) (23) was used to fit the observed data to the Gompertz equation using a weighted least-squares estimate with the weighting factor being equal to the variance of the observations at each step. The program generates a set of estimates for each of the 3 parameters which minimizes the weighted residual sum of squares from the fitted data. Other obtained results include the asymptotic standard deviations of each parameter estimate. Standard statistical techniques were used to test for significant differences among these kinetic parameters for the various admixed and homogeneous tumor xenograft conditions. We also calculated a value designated as the asymptotic final volume or CC of the various tumor conditions. The value is determined by letting \( N(t) \) approach infinity and is calculated as

\[ CC = N(t)/A \]

RESULTS

Tumor Disaggregation Studies. In Fig. 1A, we show data for the CYs obtained from either pure clone A or clone D tumors as a function of time postinjection. There is an increase in CY for both which reaches a maximum of 0.8 to 1.0 × 10⁶ cells/mg at about 30 to 35 days. After this time CY decreases, but clone A neoplasms show a more rapid drop in CY between 30 and 40 days postinjection with a more or less constant value of CY thereafter. At 60 days, the CY values are approximately equal (about 2 × 10⁴ cells/mg). Using the data from Fig. 1A, it
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is then possible to predict what the cell yields should have been from the various AHTs, and these data are shown in Fig. 1, B to D. Generally, the CY data from the AHTs are consistent with predicted values (indicated by the dotted lines), although the CY value in the early sampling period is always higher than the predicted values. A particularly good correspondence between predicted and actual values is illustrated by the 88% A:12% D tumor data in Fig. 1D.

In Fig. 2A, the CFEs of cells from disaggregated pure clone A and D tumors are shown. The CFE of these neoplasms is not equivalent over the sampling period. Clone D tumors at all times show a higher CFE than do clone A tumors, with maximum values of about 20% and 15%, respectively, being reached at about 20 days postinjection. Also, the CFE of clone D tumors remains at about this level for the entire sampling period, while the CFE of clone A tumors begins to steadily decrease at about 30 to 35 days, reaching a value of about 3% by 60 days postinjection. The data shown in Fig. 1A can then be used (as with the CY data) to predict the CFE values that should have been attained from the various AHTs, depending on the initial composition. The data on the overall CFE (i.e., without discrimination as to colony ancestry) from the AHTs are shown in Fig. 2B to D. Generally, as with the CY data, the relationship between predicted and observed CFE is good.

In Fig. 3, we show the specific tumor composition (i.e., the actual clonogenic survival of A and D cells) of each type of admixture as a function of time postinjection. Again, a set of predicted values can be made, based on the CFE values from each pure tumor type (Fig. 2A), and on the initial starting percentages of A and D cells in the various AHTs. In Fig. 3A, data for the 9% A:91% D tumors are shown, and the predicted percentages of A and D colonies agree well with the observed values. The percentages remain fairly constant over the duration of the sampling period, although there is some enrichment of clone D cells at long times postinjection, consistent with the decrease in the CFE of clone A cells (Fig. 2A). This does not mean that the actual number of clone A cells is decreasing; only that the relative percentage of A:D cells is changing. The data from the 50% A:50% D tumors are shown in Fig. 3B. Here, deviation from the expected result is seen, as the relative percentage of clone A colonies decreases more rapidly than predicted and approaches a constant value of about 10% by about Day 40 postinjection as we have previously pointed out (10). The data from the obverse mixture (88% A:12% D) are shown in Fig. 3C, and again significant deviation from expected responses is seen. Instead of a rapid reassortment to an intratumor situation where clone D cells predominate (cf. Fig. 2A) there is the establishment of a constant, stable percentage of clone A and D cells which is not different from the composition of the initial injection admixture.

Tumor Zonality Studies. In testing the spatial zonality of the tumors, we established a statistical hypothesis about the distributions of the normalized variates. Our assumption was that these were distributed in accordance with a standard normal distribution, and we tested that hypothesis by using a standard goodness-of-fit criteria (20). The results of this analysis are plotted in Fig. 4. Fig. 4A is a theoretical expected distribution. The plot represents the number of expected proportions observed on each day as a function of standard deviation (σ = 1 by definition). We segmented the standard deviation range (−2.2, 2.2) into intervals of length 0.2. Fig. 4B plots the accumulated normalized variates observed in the “natural” tu-
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Fig. 2. Colony-forming efficiencies (percentage) for either pure clonal tumors (A) or artificial heterogeneous tumors (B to D) after enzymatic disaggregation, as a function of time after initial tumor cell injection. In B to D, a predicted overall CFE is indicated (dashed lines) based on the data from the pure clonal tumors (A). Bars, SE.

Fig. 3. Cellular composition of artificial heterogeneous tumors originally injected with 9% A:91% D, 50% A:50% D, or 88% A:12% D admixtures (A to C) as a function of time. The open and closed circles indicate the average composition of A and D cells, respectively, found in disaggregated tumors, while the dashed line indicates the predicted composition based on cell yield and overall clonogenic data (Figs. 1 and 2). (B is based on previously published data: published with permission from Invasion & Metastasis).

In Vivo Growth Kinetic Studies. In Table 1 we have summarized the best fit Gompertzian parameters from the volumetric growth curves for the various tumor conditions studied along with their respective asymptotic standard deviations. From these results, we can then infer that the initial size, initial growth rates, and retardation rates for the various tumor populations vary significantly with composition. Using the Gompertz data listed in Table 1, it is possible to derive the carrying capacity of these various tumor conditions. These values are plotted in Fig. 5 and are, respectively, 8,870, 10,200, 18,720, 12,020, 12,440, 5,360, and 6,720 mm³, respectively, for the pure A, pure D, 10% clone A:90% clone D, 25% clone A:75% clone D, 50% clone A:50% clone D, 75% clone A:25% clone D, and 90% clone A:10% clone D tumors. These data clearly show a statistically significant dependence of the final predicted
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Fig. 4. Plots of the distributions of percentages of A and D cells found in artificial heterogeneous tumors as a function of time postinjection. In A, a theoretical distribution which is applicable to all experimental admixtures is shown, illustrating that if the subpopulations of cells within artificial admixtures were homogeneously mixed, a Gaussian distribution in time would be expected from disaggregation studies (4). In B to D, the observed distributions for 9% A:91% D (B), 50% A:50% D (C), and 88% A:12% D disaggregated tumors are shown. Note that the distributions are not Gaussianly distributed, indicating the presence of zonal heterogeneity.

Table 1: Gompertzian parameters of growth for artificial heterogeneous tumors produced from varying proportions of two human colon adenocarcinoma cell populations

<table>
<thead>
<tr>
<th>Cellular composition of xenograft tumor</th>
<th>Gompertz parameter</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( N_0 ) (mm(^3))</td>
</tr>
<tr>
<td>Clone A cells only</td>
<td>56.9 ± 3.94*</td>
</tr>
<tr>
<td>(25)</td>
<td></td>
</tr>
<tr>
<td>90% A + 10% D (31)</td>
<td>26.5 ± 1.33</td>
</tr>
<tr>
<td>75% A + 25% D (32)</td>
<td>66.8 ± 8.66</td>
</tr>
<tr>
<td>50% A + 50% D (28)</td>
<td>181.9 ± 11.0</td>
</tr>
<tr>
<td>25% A + 75% D (29)</td>
<td>232.4 ± 18.6</td>
</tr>
<tr>
<td>10% A + 90% D (30)</td>
<td>243.2 ± 12.5</td>
</tr>
<tr>
<td>Clone D cells only</td>
<td>93.2 ± 9.63</td>
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<tr>
<td>(25)</td>
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* Mean ± SD (asymptotic) derived from a nonlinear parameter estimation package (BMDPAR) (23), representing the SD of each estimator given an infinite sample size.

DISCUSSION

The primary finding of this study is that there are differences in the kinetic parameters of growth among artificial heterogeneous tumors that (a) are dependent on the relative initial percentages of clone A and D cells used to produce the solid neoplasms, and (b) cannot be predicted on the basis of growth kinetic data from the tumors produced by either clone A or D cells growing independently (22). These results are well exemplified in Fig. 5, where the calculated asymptotic final volumes are shown as a function of the composition of the initial injection mixture, and the dependence of volume on the percentage of clone A cells is clear. It should be noted that the original histology of the parental DLD-1 adenocarcinoma was predominantly of the well-differentiated D clonal type with a morphological minority of the poorly differentiated A clonal type (13). It was estimated that “the clone A subpopulation, (comprised) not more than 10 percent of the parent DLD-1 line” (13) (i.e., a 10% A:90% D admixture). While the relationship of this observation to our in vivo experimental findings may be fortuitous, it is worthy of special notice that the 10% A:90% D artificial heterogeneous tumors seem to have a growth advantage in terms of final attained volume (Fig. 5), as compared to any other condition (22). Also, the relationship of the asymptotic final volume to intratumor compositional stability is of interest. The two extreme compositional admixtures (i.e., 90% A plus 10% D or 10% A plus 90% D) have significantly different calculated final volumes as compared to the volumes of the pure A and D tumors, but are compositionally stable (Fig. 3, A and C). In contrast, the tumors comprised of 50% A plus 50% D cells have final volumes which are not different than either the pure A or D tumors, but are compositionally unstable (Fig. 3B).

Fig. 5. Plot of the carrying capacity (asymptotic final volume) for each of the xenograft solid tumors as a function of the original percentage of clone A cells in the tumor.

Our data may therefore indicate that some type of “clonal interactions” exists. However, as Heppner (7) and Heppner and coworkers (24–26) have pointed out, if clonal interactions do exist, multiple mechanisms are likely involved. While some of these mechanisms may require cell-cell contact, others may not, although an appropriate environment (e.g., solid tumor, spheroid, artificial capillary structure) appears needed for their optimum expression. In this regard, in a study relevant to our work (27), based on the analysis of diploid and tetraploid subpopulations from an Ehrlich ascites tumor line, Janssen and Revesz showed that a balance was maintained between subpopulations which appeared to be related to the combined volume of the diploid and tetraploid cells. Makino (28) also showed that two different asciites tumor lines could apparently control each other’s growth rates. Miller et al. (29) have shown potentiation of growth of a mouse mammary line (C1-S1) isolated from a hyperplastic nodule by a tumor subpopulation (68H) isolated from a different heterogeneous mouse mammary adenocarcinoma. Miller et al. (3) have also demonstrated that some mouse mammary adenocarcinoma subpopulations can alter the take rate, latency period, or actual growth kinetics of other subpopulations in vivo. A similar effect has been shown by Tofilon et al. (30) who cocultured a fast-growing line of rat brain tumor cells (9L) with a slower-growing variant (9LR3) using spheroids and found that the slow-growing line would attain the growth rate of the 9L line. In general, growth rate effects in admixed situations have previously indicated that one typically notes that either “slow-growing” subpopulations will speed up, or “fast-growing” subpopulations will slow down (7, 26, 31), although the direction of such changes is not a priori predictable. Our quantitative results do not agree with these quantal responses.

While we do not know the mechanism(s) by which the composition-dependent changes in growth rates or carrying capacity (Fig. 5; Table 1) are produced, there are a number of nonexclusive possibilities. As stated by Heppner et al. (31), “the heterogeneity of the tumor cell populations is mirrored by the heterogeneity of the infiltrating populations.” In this regard, Rios (32) has shown in heterogeneous mouse mammary tumors qualitatively and quantitatively different T-lymphocyte populations, and Wang et al. (33) have hypothesized that a mixed population of antigenically related cells may induce a qualitatively different response than do homogeneous tumor cell populations. Heppner et al. (7, 26, 31) have shown that preimmunization of mice with admixtures of mammary tumor cell subpopulations may yield an overall immunity that is different from that based on the cross-reactive patterns of individual clones. Further, Heppner et al. (31) have also shown that the macrophage content of clonal tumors can vary dramatically (e.g., by 100%). Although these differences in macrophage content did not per se appear to correlate with tumor growth rate, it was suggested that the relative percentage of activated macrophages is the important parameter which may bear upon in vivo growth rates. Therefore host cell responses may be involved in the generation of our results.

Recent data by Wallen et al. (34, 35) on the cell kinetics of the line 66 and 67 subpopulations from the heterogeneous mouse mammary adenocarcinoma model system, using plateau-phase monolayer cultures, have shown that the depth into quiescent state was markedly different between the two subpopulations. Further, the rate of reentry into the proliferative state varied, as did the clonogenicity of tumor cells recruited from the quiescent state. Therefore, if the percentage of quiescent cells differed between A and D clonal tumors, or if the condition of in vivo coculturing (i.e., AHT) affected the transition between the quiescent and proliferative states, differences in solid tumor growth kinetics could be envisaged. Concomitantly, differences in other parameters, such as the potential doubling time or cell loss factor, may be involved, particularly as Watson (36) has shown that these factors are strongly dependent on tumor volume.

As tumor cells can promote formation of new blood vessels via endogenously produced angiogenesis factors (37), potential differences in this ability between clone A and D cells may impact on vascular structure and function. Related to this is the suggestion by Burton (38) and Summers (39) that differences in tumor growth rates can be due to vascular insufficiency resulting in such conditions as lowered extracellular pH, local hypoxia, and/or focal necrosis. Rofstad (40) demonstrated strong correlations between morphometrically determined vascular parameters in human melanoma xenograft tumors and parameters such as doubling time, growth fraction, and cell loss factors. Also, Goldacre and Sylven (41), Lewis et al. (42), and Porschen et al. (43) have demonstrated that the presence of a large volume of necrotic tissue within tumors can lead to volume growth curves which overestimate the true viable tissue volume by a large extent. This effect was particularly evident in large tumors and therefore is relevant to our calculations of asymptotic final volumes (Fig. 5) (22).

In summary, we have produced data indicating that the in vivo growth kinetics of AHTs in this human colon adenocarcinoma model system is quite different from what would be expected on the basis of the behavior of the individual A and D subpopulations. We have indicated a number of processes through which such unpredictable behavior may be produced, which will be the basis for further research.

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