Proliferative and Clonogenic Heterogeneity of Cells from EMT6/Ro Multicellular Spheroids Induced by the Glucose and Oxygen Supply

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ABSTRACT

The culture of EMT6/Ro multicellular tumor spheroids in a 20-fold range of glucose concentrations and a 4-fold range of oxygen tensions had profound effects on the cell subpopulations which developed in the spheroids. As the spheroids increased in diameter, the rate of accumulation of cells with a G1-phase DNA content was much greater for those spheroids cultured in the lower oxygen and glucose concentrations. Growth fractions of 600- to 800-μm-diameter spheroids, calculated from either DNA content or [3H]thymidine labeling data, showed a direct correlation with the glucose concentrations in the culture medium. The cells from spheroids cultured in low glucose concentrations also showed a more rapid loss in clonogenicity as a function of increasing spheroid diameter. Lowering the oxygen tension enhanced these effects of reduced glucose concentrations. Selective dissociation of 600- to 800-μm-diameter spheroids demonstrated that both the proliferation index and the clonogenic capacity of the spheroid cells decreased with increasing depth into the spheroid cell rim. The rates of decrease of both of these parameters as a function of depth into the spheroid were greater for spheroids cultured in the lower oxygen and glucose concentrations. These results indicate that both the glucose and oxygen supply are critical in the development of nonproliferating and nonclonogenic cell subpopulations in spheroids.

INTRODUCTION

Heterogeneity in the cell population of solid tumors remains one of the most important, and most perplexing, problems in tumor biology. Tumors contain a mixture of cells which differ in almost every measurable biological parameter, including cell size, buoyant density, morphology, ploidy, antigenicity, proliferative index, clonogenicity, and metastatic potential. These differences are thought to be reflected in the variability in response to therapeutic agents; indeed, the development of subpopulations of resistant cells in a tumor is the primary reason for the failure of chemotherapy (2) and perhaps radiotherapy (3). Several investigators have succeeded in separating subpopulations of resistant cells in a tumor is the primary reason for the failure of chemotherapy (2) and perhaps radiotherapy (3). Several investigators have succeeded in separating subpopulations of cells from tumors on the basis of cell density (4), cell size (5), or DNA content (6), and they have shown that different therapeutic responses do exist within the total cell population. Tumor cells apparently can shift between these different states, i.e., quiescent to proliferating (7, 8), when conditions in the tumor mass change. Morphological studies have shown that some of the heterogeneity observed in a tumor cell population is a result of the locations of the cells relative to the blood supply (9, 10). Although evidence exists that specific nutrients, such as oxygen (11) or glucose (12), are depleted in certain areas of tumors, the complexity of the in vivo tumor has precluded an understanding of the mechanism(s) behind tumor cell heterogeneity.

To eliminate some of the variables present in tumors, cultures of tumor cells have been used as models of heterogeneous cell populations. As cells enter growth plateau, they undergo alterations in cell cycle distribution, cell size, density, RNA content, and clonogenicity (13–15). Several studies have suggested that these changes occur as a result of nutrient deprivation (13, 16), analogous to the microenvironment of cells remote from blood vessels in a tumor. Depriving cells specifically of oxygen (17–19) or glucose (20, 21) results in the development of a population of cells similar to a plateau-phase culture. However, many hormones and protein growth factors have been isolated which also regulate the cell cycle traverse of malignant cells (22–24). Studies with isolated quiescent cell populations in vitro have shown them to be both more and less resistant to radiation and chemotherapeutic drugs than proliferating cells (15, 25, 26). Alterations in the culture environment can induce tumor cells to switch back and forth between different states (8, 27). In spite of the numerous correlations between in vivo studies and the tumor cell subpopulations observed in vivo, it remains unclear which nutritional factor(s) is actually involved in the development of cellular heterogeneity in tumors.

The multicellular tumor spheroid has been proposed as a model of tumor cell heterogeneity which is intermediate in complexity between the tumor and monolayer cultures. Studies based on morphology (28), cell separation (29, 30), and selective dissociation (31) have demonstrated that this system exhibits a cellular heterogeneity similar to that observed in tumors. Spheroid cell subpopulations are arranged in a spherically symmetric pattern (28, 31), suggesting that their development is related to the penetration of nutrients and growth factors into the cell mass. Concentration gradients of oxygen (32) and hydrogen ions (33) have been directly demonstrated, and similar gradients of glucose (34) and toxic waste products (35, 36) have been postulated. Although the general influence of the culture conditions on the cellular characteristics of spheroids has been recognized (37, 38), there are no reports on the influences of specific nutrients on the development of cellular heterogeneity in this system. In conjunction with the experiments on the regulation of growth and viability presented in the companion paper (36), this paper reports the effects of the external glucose and oxygen concentrations on the proliferative and clonogenic status of cells in EMT6/Ro spheroids.

MATERIALS AND METHODS

Spheroid Culture and Assays. Spheroids of the EMT6/Ro mouse mammary tumor cell line were cultured in suspension in eight combinations of media containing either 0.28 mM (20%) or 0.07 mM (5%) oxygen and 16.5, 5.5, 1.7, or 0.8 mM glucose as described in detail in the preceding study (36). The number of spheroids in the flask and the feeding schedule were maintained such that the concentrations of these two metabolites were within 90–95% of the desired value throughout the growth period. The methods used for sampling, dissociating and sizing spheroids, counting cells per spheroid, and preparing histological sections are described previously (36).

Flow Cytometric DNA Content Analysis. Cell suspensions derived from spheroids were fixed in 70% ethanol in distilled water prior to staining for flow-cytometric DNA content analysis. Mouse spleen cells fixed in ethanol were added to the tumor cells at approximately 1:5
spleen:tumor cell ratio to serve as a staining standard. The fixed cells were then centrifuged and resuspended in a solution of chymomycin A1 (1 mg/ml; Sigma Chemicals) in 50 mM MgCl2 buffer at pH 7.4 at a concentration of 1 × 106 cells per ml. The stained cells were passed 3 times through an 18-gauge needle and then analyzed on an EPICS IV flow cytometry system (Coulter Electronics). Immediately prior to analysis, fluorescein polystyrene microspheres were added to the cell suspension to serve as a laser power standard. The excitation wavelength was 457 nm at 500 mW power; the fluorescent signal was split using a 560 dichroic mirror such that the spleen and tumor cell fluorescence was detected through a 560-nm-long wavelength pass filter by the red photomultiplier tube, and the microsphere signal was detected through a 560-nm-short wavelength pass filter by the green photomultiplier tube.

The spleen cells had a fluorescent peak approximately half that of the tumor cells; ≈1 × 106 tumor cells were collected in each histogram. All data within a given experiment were corrected for any shifts in the peak channel of the spleen cells or the microspheres using a computer algorithm developed by Dr. James Leary. The tumor cell DNA content histograms had coefficients of variation of the G0 peak of 4–5%. Percentages of cells in each cell cycle stage were estimated using a polynomial fitting routine (39); the percentage of S-phase cells determined by this method compared well with determinations of the percentage of cells labeled with [3H]thymidine (see Table 1).

[3H]Thymidine Labeling Index. To determine the percentage of DNA-synthesizing cells, 50–200 spheroids were incubated in 50 ml of complete medium containing the appropriate oxygen and glucose concentrations and 20 μCi of [3H]thymidine per ml (20 Ci/mmol; Amersham-Searle) at 37°C for 30 min, then washed twice with 50 ml of unlabeled medium and, if necessary, incubated in unlabeled medium at 4°C for 1 h. The spheroids were then washed with medium without FBS and dissociated completely as described previously (36). The details of the autoradiography and counting the labeled cells have been given previously (31).

[3H]Thymidine in Spheroid Sections. A group of 50–60 spheroids from a given growth condition was exposed to [3H]thymidine in complete medium as described above. These spheroids were then fixed in Bouin’s fixative and processed for paraffin sectioning as described previously (36). The paraffin sections were then placed on acid-cleaned, gelatin-coated glass slides. The unstained slides were observed under a microscope, and those with the largest spheroid sections were deparaffinized in xylene and alcohol, dried, then coated with NTB-3 emulsion (Kodak), and processed for autoradiography as described previously (31). These emulsion-coated slides were developed when the single-cell labeling index slides showed a plateau in the number of labeled cells, at 7–12 days after dipping in emulsion. The thickness of the [3H]thymidine-labeled rim was estimated by measuring the distance from the surface of the spheroid to the location of the labeled cell located furthest into the spheroid. Measurements were done on 3 serial sections for each of 20–25 spheroids as described previously for the measurements of the thickness of the viable cell rim (36).

Estimation of Growth Fraction. The growth fractions of cells from these spheroids were estimated in two different ways. The first involved the use of both the flow-cytometric DNA content data (Figs. 1 and 2) and the [3H]thymidine labeling index data (Table 1). If we assumed that the growth fraction was ≥100% for exponentially growing monolayer cultures, then a population of completely proliferating cells has an S-phase compartment of 51%. As the spheroids grew larger, the percentage of S-phase cells decreased, while the percentage of G0-phase cells increased. We therefore assumed that the nonproliferating cells in the spheroid became arrested predominantly in the G1 and G2 phases of the cell cycle. Thus, the GF can be estimated by the reduction in the size of the S-phase fraction (40):

\[
GF = \frac{\text{% of S-phase spheroid cells}}{\text{% of S-phase exponential cells}}
\]

Similarly, an estimate of the growth fraction could be obtained from

\[
GF = \frac{\text{LI of spheroid cells}}{\text{LI of exponential cells}}
\]

\[
GF = \frac{[3H]\text{thymidine-labeled vol.}}{\text{total nonnecrotic vol.}}
\]

The second method we used to estimate the growth fraction used the measurements of the [3H]thymidine-labeled cell rim (Table 2). To make this calculation, we assumed that the number of cells per unit volume was the same throughout the spheroid necrotic area (31, 41). One can then equate the fraction of the total spheroid volume which contains labeled cells with the fraction of the total spheroid cell population which is actively proliferating.

\[
GF = \frac{[3H]\text{thymidine-labeled vol.}}{\text{total nonnecrotic vol.}}
\]

Since these two different methods of estimating the growth fraction use entirely different assumptions, both were used, and the data obtained are compared in “Results.”
Calculating the Proliferating Cell Zone. The growth fraction values obtained from the S-phase DNA content data as explained above can be used to estimate the thickness of the proliferating cell region in the spheroid as follows. Based on previous work (31) and observation of the \(^{3}H\)thymidine-labeled spheroid sections, we assumed that the proliferating cells are primarily confined to a discrete zone near the spheroid surface (see also Ref. 35). Assuming that the cells are distributed throughout the cellular zone in a fairly uniform manner, the percentage of the cellular volume in the spheroid which corresponds to the proliferating cell layer can be approximated by the growth fraction; e.g., if the growth fraction is 50%, then the proliferating cell zone constitutes \(\approx\)50% of the volume of the total viable cell zone. Thus, the thickness of the proliferating layer \(T\) can be calculated knowing the diameter of the spheroid \(D_s\), the diameter of the necrotic center \(D_n\), and the GF from the following equation (see Ref. 31).

\[
T = \frac{1}{2} [D_s - (D_s^2 + GF (D_s^2 - D_n^2))]^{1/2}
\]  

This calculation allows a comparison of spheroid kinetic measurements to histological observations of \(^{3}H\)thymidine labeling in spheroid sections.

Clonogenicity Assay. Cells to be assayed for colony-forming ability were centrifuged, then resuspended in fresh medium with 15% FBS, counted as described previously (36), and diluted to a concentration of approximately 200 cells per ml. Each of 10 60-mm dishes containing 4 ml of warmed medium was then inoculated with 1 ml of the cell suspension. These dishes were incubated for 6-7 days and stained with methylene blue, and the number of colonies with greater than 50 cells was counted. The means and standard deviations of the 10 dishes were determined to yield a percentage of colony-forming cells.

Selective Dissociation Technique. The technique used to dissociate spheroids into populations of cells from different locations in the cell rim was a modification of that described in detail previously (31). Spheroids to be dissociated were sorted by hand to yield populations of 50 spheroids with mean diameters of 600-800 \(\mu m\) and 3-5% diameter coefficients of variation. After sizing as described previously (36), the spheroids were divided into 2 groups of 25 and washed once with medium without FBS, and then 4 ml of 0.25% recrystallized trypsin (Worthington) in sodium citrate buffer, 22-24°C and pH 7.4, were added. These spheroids were gently agitated in specially designed dishes (31) for various lengths of time, and then medium with 15% FBS at 4°C was added to stop the trypsin action. The cells released from the spheroids were then removed from the spheroids by pipet, the spheroids were rinsed with medium without FBS, and the procedure was repeated on the remaining spheroid-associated cells. Cells released during the rinsing step were added to the previous population. Since spheroids of the same outside diameter had very different thicknesses of viable cell rims and total cell contents (36), the typsination times were determined by prior experimentation such that approximately 25 \(\pm\) 5% of the total spheroid cell content was released with each sequential trypsin exposure. The cells were stored on ice until the procedure was complete, and then the cells were counted and prepared for flow cytometric DNA content analysis and clonogenicity assay as described above. The original locations of the cell fractions in the spheroid were calculated from the cell counts and measurements of the spheroid diameter and the thicknesses of the viable cell rims as described previously (31).

RESULTS

The previous study (36) reported the growth rates of EMT6 spheroids as a function of culture in different oxygen and glucose concentrations. The distribution of cells in different cell cycle phases is shown in Figs. 1 and 2 as a function of the spheroid diameter. A similar trend can be seen in each case, i.e., an accumulation of cells with a G1-phase DNA content with growth, due to a depletion in the S-phase fraction as well as a somewhat smaller decrease in the percentage of G2 + M-phase cells. These data are not different among any of the culture conditions up to a spheroid diameter of 300-400 \(\mu m\), which represents the first 3-4 days of growth in the respective media. After attaining this size, the rate of accumulation of cells in the G1 phase as a function of spheroid diameter and the concurrent decrease in S-phase cells were greater for those spheroids cultured in the lower glucose and oxygen concentrations. Spheroids cultured in 0.07 mM oxygen and 0.8 mM glucose had a rate of accumulation of cells in the G1 phase which was \(\approx\)8 times greater than that for spheroids cultured in 0.28 mM oxygen and 16.5 mM glucose; the difference in the rate of depletion of S-phase cells was \(\approx\)6-fold. As reported in the companion paper (36), these spheroids reached a saturation in growth which was dependent on the culture conditions. The data in Figs. 1 and 2 indicate that, at the diameter of sphere at which growth saturation was attained, the cell population consisted of approximately 85% G1-phase, 10% S-phase, and 5% G2-phase cells in each culture condition.
As an independent measure of the proliferative status of these cells, spheroids were exposed to [3H]thymidine and dissociated, and labeling indices of the cells were determined. Table 1 shows these values for cells from different size spheroids cultured in the different glucose and oxygen concentrations. The same trend can be seen in these data as was discussed above: a depletion of [3H]thymidine-labeled cells as a function of increasing spheroid diameter. Although these data are not as extensive as those shown in Figs. 1 and 2, they do demonstrate that the percentage of [3H]thymidine-labeled cells in spheroids of a given diameter was dependent on the culture conditions. Cells from spheroids of 500 to 600-µm diameter grown in the highest glucose and oxygen conditions had a labeling index of ~40%, compared to ~12% for cells from spheroids cultured in the lowest nutrient concentrations. There were no significant differences in these total spheroid cell populations between the S-phase cell compartments determined by [3H]thymidine labeling or by DNA content analysis.

In order to determine the spatial distribution of the DNA synthesizing cells in these spheroids, autoradiographs of spheroid sections labeled with [3H]thymidine were prepared. The labeled cells were concentrated in the outer region of the cell rim in each spheroid observed, with a cell zone near the necrotic center containing no labeled cells. Table 2 shows the estimated thickness of the cell layer containing [3H]thymidine-labeled cells as a function of the glucose and oxygen concentrations. There is a considerable error in these data due to variability in the spheroid sections and the inherent difficulty in precisely measuring the extent of the labeled cells. Although many of the differences shown in Table 2 were not significant (P > 0.05), the thickness of the labeled cell rim did show a tendency to decrease when the spheroids were cultured in reduced oxygen and glucose concentrations. These reductions are significant (P < 0.03) when comparing spheroids cultured in either 16.5 mM or 5.5 mM glucose to those grown in 1.7 mM or 0.8 mM glucose. In order to compare this histological assay of the proliferative status of cells in spheroids to the data on cells isolated from spheroids, the percentages of S-phase cells given in Figs. 1 and 2 were used to calculate an equivalent thickness of the proliferating cell zone as explained in "Materials and Methods." These estimations, shown in the last column in Table 2, agree well with the measured values, generally falling within the error range of the measurements. Interestingly, the calculated proliferating cell rims were all smaller than those measured by autoradiography. This would perhaps be expected, since the labeled cell rims measured by autoradiography extended to the labeled cell located farthest into the spheroid; all of the cells within this layer were probably not proliferating.

Another method of determining the position of the proliferating cells in spheroids is to selectively dissociate cells from different positions in the cell rim and measure the cell cycle distribution of the resulting suspensions. At a diameter of ~600 µm, spheroids cultured in each of the different oxygen and glucose conditions were dissociated into four equal cell fractions. Fig. 3 shows the percentage of cells with an S-phase DNA content as a function of distance into the spheroid cell rim at which the cell populations were located prior to dissociation. Selective dissociation of the spheroids and estimation of the percentage of S-phase cells from flow-cytometric DNA content histograms are detailed in "Materials and Methods." Points are single determinations on cells from 600- to 800-µm-diameter spheroids cultured in 16.5 (○), 5.5 (●), 1.7 (○), or 0.8 (□) mM glucose and the indicated oxygen concentrations; lines are linear least-squares best fits to each data set.

![Fig. 3. Percentage of cells with S-phase DNA contents as a function of distance into the spheroid cell rim at which the cell populations were located prior to dissociation. Selective dissociation of the spheroids and estimation of the percentage of S-phase cells from flow-cytometric DNA content histograms are detailed in "Materials and Methods." Points are single determinations on cells from 600- to 800-µm-diameter spheroids cultured in 16.5 (○), 5.5 (●), 1.7 (○), or 0.8 (□) mM glucose and the indicated oxygen concentrations; lines are linear least-squares best fits to each data set.](image)

was 5.5 or 16.5 mM, but decreasing the oxygen tension also decreased the number of S-phase cells at a given location for spheroids cultured in 1.7 or 0.8 mM glucose. Although 80–95% of the total number of S-phase cells were contained in the outer cell fractions, the innermost fractions contained 5–20% S-phase DNA content cells. We have no direct measurements of the [3H]thymidine labeling indices of these different cell fractions, but observations of the autoradiographs of spheroid sections (see Table 2) indicate that these inner region cells with an S-phase DNA content did not label with [3H]thymidine, even though this DNA precursor penetrates quickly to the center of spheroids (34, 42). It is possible that some of these S-phase cells were not exposed to a sufficient amount of [3H]thymidine for a sufficient time to be labeled.

A uniform method to make use of the flow-cytometric and [3H]thymidine labeling data for comparison of the proliferative status of cells in spheroids cultured in these different oxygen and glucose concentrations is to calculate the growth fraction at a given size spheroid. Fig. 4 shows the growth fraction as a function of the glucose concentration in the culture medium for spheroids cultured in the two different oxygen tensions. These growth fraction values have been calculated from both the cell cycle distribution data (Figs. 1 and 2) and from the [3H]thymidine-labeled cell rim data (Table 2). Growth fractions calculated from [3H]thymidine labeling index data (Table 1) did not differ from those calculated from the data in Figs. 1 and 2 and are thus not shown. The data in Fig. 4 demonstrate that the growth fraction is correlated with the logarithm of the glucose concentration in the culture medium (r² > 0.94). This dependence was...
larger for 1000- to 1200-\(\mu\)m-diameter spheroids grown in 0.28 mM oxygen than it was for 500- to 600-\(\mu\)m-diameter spheroids in the same culture conditions. In each case, culture in the lower oxygen concentration at a given glucose concentration resulted in a lower growth fraction. Comparison of the growth fractions calculated by the two different techniques shows that the data in Table 2 gave a consistently higher estimate. This may be due to an overestimation of the volume of the proliferating cell zone by this method, as discussed above.

The cell suspensions prepared from these spheroids during growth were also assayed for their colony-forming ability. Fig. 5 shows the cellular clonogenic efficiency as a function of the spheroid diameter for spheroids cultured in the different glucose and oxygen conditions. In every culture medium, the percentage of clonogenic cells decreased with growth. The rate of this decrease as a function of the spheroid diameter was dependent on the culture conditions, varying from -0.8% per 100 \(\mu\)m of spheroid growth in 0.28 mM oxygen and 16.5 mM glucose to -11% per 100 \(\mu\)m of growth in 0.07 mM oxygen and 0.8 mM glucose. These rates were correlated with the glucose concentration in the culture medium for growth in both 0.28 and 0.07 mM oxygen \((r^2 > 0.92)\). These data demonstrate that there was no significant effect of the oxygen supply for spheroids cultured in 16.5 or 5.5 mM glucose, but lowering the oxygen supply for spheroids cultured in the lower glucose concentrations resulted in a reduction in the clonogenicity. The data in Fig. 5 also demonstrate that there was no effect of the oxygen concentration on the rate of decrease in clonogenicity with growth for

![Graph showing growth fraction of cell populations derived from spheroids as a function of glucose concentration.](image)

![Graph showing clonogenic efficiency of spheroid-derived cells as a function of spheroid diameter.](image)

![Graph showing clonogenic efficiency of spheroid-derived cells as a function of the distance into the spheroid cell rim.](image)
spheroids cultured in the two highest glucose levels. However, lowering the oxygen concentration from 0.28 mM to 0.07 mM changed this rate from -3.4% to -7.8% per 100 μm of spheroid growth in 1.7 mM glucose, and from -4.1% to -11% per 100 μm of growth in 0.8 mM glucose.

We have also measured the clonogenic potential of cells released from different locations in the spheroid by selective dissociation. Fig. 6 shows the clonogenic efficiencies of these cell fractions as a function of the location of the cells in the spheroid. As reported previously (31), there was no effect of the dissociation procedure itself on the colony-forming ability of these cells. The clonogenicity decreased with increasing depth into the spheroid structure in every culture condition. The rate of this decrease was inversely correlated with the glucose concentration in the culture medium for spheroids grown in both oxygen concentrations ($r^2 > 0.93$). Lowering the oxygen tension had no effect on this rate for spheroids cultured in 16.5 mM glucose, but reduction in oxygen supply did increase this rate for spheroids cultured in the other glucose concentrations. Except for cells from spheroids cultured in 0.28 mM oxygen and 16.5 mM glucose, the innermost cell fractions had clonogenicities of 18–24% in all culture conditions.

**DISCUSSION**

The data presented here on proliferative status of cells in spheroids cultured in a range of oxygen and glucose concentrations correlate with the growth rates of these spheroids as reported in the preceding paper (36). The data in Figs. 1 and 2 demonstrate that up to a diameter of 300–400 μm, or for the first 3–4 days of growth, there was no significant difference in the cell cycle distribution of cells from spheroids cultured in any of these glucose and oxygen levels. As discussed previously (36, 57), there undoubtedly were large differences in the oxygen and glucose concentrations in the spheroids of these various groups within the first few hours of transfer to the different media. The facts that the spheroids initially grew at the same rate and that the cell cycle distributions of the cell populations were very similar strongly imply that neither glucose nor oxygen had a direct effect on the proliferation of the cells. Previous monolayer studies with lowered oxygen (43, 44) and glucose (21) concentrations have indicated that much lower levels of these nutrients than were used in this study are necessary to significantly inhibit cell proliferation. Note, however, that over this initial period of spheroid growth, the percentage of S-phase cells decreased from 50–55% to 35–40% in every culture condition, with a consequent rise in the percentage of G1-phase cells. This implies that some growth factor(s), present at the same concentration in each of these different media, was regulating the proliferation of the cells, and that the penetration of this substance into the spheroids was already limited at a diameter of 300–400 μm.

After the first 4 days of growth the proliferative status of the cells in these spheroids began to show a dependence on the composition of the culture medium. The rate of accumulation of cells in the G1 phase of the cell cycle increased greatly in the spheroids cultured in 0.07 mM oxygen and 1.7 or 0.8 mM glucose. The data in Table 1 also indicate that, by a diameter of 400–500 μm, the percentage of $^3$Hthymidine-labeled cells in the spheroids was correlated with the glucose and oxygen concentrations. These changes in the proliferative status of the cell populations were reflected in the decreased growth rates of the spheroids (36). The onset of central necrosis in these spheroids, used as an indicator of necrotic development and progression, was also strongly correlated with the glucose and oxygen concentrations in the culture medium. The model of cellular growth regulation presented in the previous paper (36) postulates that, after central necrosis has developed, cytostatic factors produced or released by cellular lysis diffuse through the spheroid cell mass and reduce the thickness of the proliferating cell layer. This model predicts that cells in spheroids cultured in the lower glucose and oxygen concentrations would show alterations in the cell cycle distribution at a smaller spheroid diameter than spheroids cultured in the higher nutrient levels. This is indeed the observation from Figs. 1 and 2 and Table 1. The limited data presented on direct measurement of the thickness of the proliferating cell zones (Table 2) show that this growing cell rim was decreased significantly at a spheroid diameter of 400–500 μm for spheroids cultured in either 1.7 or 0.8 mM glucose. Even at 700- to 800-μm diameter, spheroids cultured in the higher glucose concentrations have a proliferating cell rim 70–80 μm thick, which is not significantly different from that found under normal growth conditions previously (31). The data presented here support the concept that the expansion of necrosis leads to a progressive diminution in the proliferating fraction of cells in the spheroid and thus results in growth saturation (see Ref. 36).

There are several limitations to this interpretation, some of which have been discussed previously (36). The data given in Tables 1 and 2 are not that extensive, and thus it is not possible to make precise correlations between the development of necrosis and the proliferative status of the cells. The data in Figs. 1 and 2 are most extensive, but the rigid comparison of this information to the progression of necrosis is also not straightforward. The percentage of cells in the whole spheroid cell population which is in a particular phase of the cell cycle is affected not only by changes in the thickness of the proliferating cell rim, but is also altered by the size of the viable cell zone. For instance, the percentage of G1-phase cells will increase as a spheroid enlarges even if the thickness of the proliferating cell layer remains constant, due to the expansion of the volume of the nonproliferating zone. Knowing the thickness of the proliferating and viable cell layers allows a simple geometric calculation of the relative percentages of proliferating and nonproliferating cells in the spheroid. The cell cycle distribution data for spheroids cultured in 16.5 mM glucose in both oxygen concentrations fit well with this model if it is assumed that the thickness of the proliferating cell zone remained constant in these spheroids throughout the growth period measured. In order to obtain a good fit to the remainder of the data in Figs. 1 and 2, one has to assume that at a certain spheroid diameter the thickness of the proliferating cell layer becomes progressively smaller. A model is currently under development which will use data such as those shown in Figs 1 and 2 to estimate the thickness of the proliferating cell layer at a given spheroid diameter. Clearly, more data will be needed on the cell cycle distribution of this and other cell lines before a cause-and-effect relationship can be firmly established between the development of necrosis and the reduction in the thickness of the proliferating cell layer.

In addition to supporting the growth regulation model presented previously (36), the cell proliferation data presented here have some important implications about the development of cellular heterogeneity in the spheroid system. It is clear that the culture conditions can radically alter the cell cycle distribution of the cells in a spheroid of a given diameter. This variation is illustrated in Figs. 3 and 4. Although we have not used any technique to specifically indentify quiescent cells, such as continuous $^3$Hthymidine labeling (45) or DNA and RNA content analysis (46), the percentage of nonproliferating cells
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can be estimated as one minus the growth fraction shown in Fig. 4. There are limitations to the methods used to obtain these growth fraction estimates, such as the assumptions that the cell cycle transit times do not change and the concept that only the outer region of cells in a spheroid is proliferating, but the values obtained are useful for comparisons between the different growth conditions since the assumptions used in each case are the same. It is apparent from these data that the quiescent cell fraction is influenced by the glucose and oxygen concentrations in the culture medium. As was discussed above, the percentage of proliferating cells in a given spheroid is controlled by the thickness of the proliferating cell layer, as well as by the total thickness of the viable cell zone. By lowering the glucose or oxygen concentration in the culture medium, the size of the proliferating layer is decreased, leading to an increase in the percentage of quiescent cells. However, the thickness of the viable cell zone is also reduced by expansion of the necrotic core, which results in a decrease in the total number of cells in the spheroid (36). The interaction between these two phenomena can be illustrated by the fact that 600- to 800-μm-diameter spheroids differ greatly in their growth fractions depending on the culture conditions (Fig. 4), but multiplying the total number of cells per spheroid by the growth fraction reveals that there are 2–3×10⁶ nonproliferating cells per spheroid in each case. One of the values of the spheroid model system is that there is this dependence of cell cycle distribution on both cell growth and cell loss, similar to the situation in tumors (40). An advantage of this system is the ability to accurately manipulate the culture environment. For EMT6/Ro cell spheroids, glucose and oxygen are nutrients which directly control cellular viability (36) as well as indirectly regulating cellular proliferation, presumably as mediated by the development of necrosis. This interaction makes the precise determination of the relative importance of these individual biological effects difficult. These studies still have relevance to the understanding of cellular proliferative heterogeneity in tumors, since oxygen and glucose are known to be limited in tumor tissue in vivo. There are hormones, growth factors, and trace elements which directly alter the proliferation of cells at concentrations which do not affect cellular viability (47–50), and the spheroid system should prove valuable in gaining a better understanding of the regulation of tumor cell growth and viability through studies with such factors. The data presented in Figs. 5 and 6 also demonstrate that the glucose and oxygen supplies to spheroids are involved in the regulation of the clonogenic potential of the cells in spheroids. The fact that the inner region cells are less able to form colonies than the outer region cells suggests that the decrease in plating efficiency with increasing spheroid diameter seen in Fig. 5 was due to an increase in the fraction of inner region cells which were inherently less clonogenic. Comparison between Figs. 3 and 6 demonstrates that the cells which were less efficient at forming colonies were also largely nonproliferating in the spheroid microenvironment. Similar results have been reported previously for unfed plateau phase monolayer cultures (13, 14, 16). One hypothesis to explain this effect is that the reduced levels of oxygen and glucose in the inner regions of spheroids had a direct effect on the clonogenicity of the cells located there, similar to the effects of these metabolites on cellular viability (36). It has been shown in several systems (51) that cell death is a multistep process. Cells exposed to low oxygen and glucose concentrations may lose their colony-forming ability as a step toward lysis. Suspensions of EMT6/Ro single cells deprived of both oxygen and glucose lose their clonogenic capacity within a few hours, but they remain intact for a much longer period before lysis. This suggests that the induction of quiescence and the loss of clonogenic potential are not directly related. This would appear to be the case for plateau-phase monolayer cultures, since replenishing the medium can sustain cell clonogenicity without inducing any further cell growth (13); monolayer cultures also become growth arrested prior to any decrease in clonogenicity (14, 16). This hypothesis appears likely in spheroids: careful comparison of the data in Fig. 3 and those in Fig. 5 shows that, in many culture conditions, the growth fraction was reduced to a greater extent, and at a smaller spheroid diameter, than was the clonogenicity. Such a biological effect could be explained if the ability to form a colony is related to the energy metabolism of the cell, as appears to be the case for cellular viability (36). We cannot rule out the possibility that once central necrosis has appeared, toxic products produced or liberated by cell lysis diffuse through the intact cell rim and further reduce the clonogenic potential of the viable cells. We have, in fact, demonstrated such an effect with extract from spheroids with large necrotic centers (52). Little work has been done to understand what factors are relevant to the maintenance of cellular clonogenicity in the absence of any therapeutic treatment. The investigation of factors which directly control cell proliferation without altering cellular clonogenicity would be valuable to an understanding of the regulation of these cellular functions.

The effects of the oxygen and glucose supply on cellular heterogeneity shown here have several implications for the use of spheroids as in vitro therapeutic models of tumor cell subpopulations (see also Ref. 36). Since both oxygen and glucose are rapidly consumed by most tumor cells, it is critical that the total number of cells per volume of medium in the culture is carefully controlled so that the concentrations of these nutrients do not change greatly during growth. This limitation has been documented previously for oxygen (53, 54), but it is clear from the present study that the consumption of nutrients which are not renewed except by medium exchange may be as important. The situation is even worse in static cultures of spheroids (55), in which case the concentration of oxygen at the spheroid surface is often much lower than that in the bulk medium. Since spheroids in such systems depend on diffusion for their nutrient supply, this problem may be exaggerated for molecules larger than oxygen (M, 32), such as glucose (M, 180), which have diffusion coefficients many times lower (34, 56). The data shown in Figs. 3, 4, and 6 illustrate that variation in the oxygen and glucose concentrations in the culture medium can have large effects on the internal composition of the spheroids, which will in turn affect the response of the spheroid to therapeutic treatment. The alterations in the cell cycle distribution (Figs. 1 and 2) and the clonogenicity (Fig. 5) of the cell population as a function of the spheroid diameter also stress the importance of having a uniformly sized spheroid population for experimentation. Differences of 50–100 μm with a spheroid population, or between different experimental groups, can have a significant effect on the cell subpopulations which comprise the spheroids. Although the results presented in this study clearly demonstrate the usefulness of the multicellular spheroid as a tool to study the development of tumor cell heterogeneity, they also indicate that precise control over the culture environment is critical to the results obtained with any particular spheroid system.

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