Role of Necrosis in Regulating the Growth Saturation of Multicellular Spheroids

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ABSTRACT

Growth curves for multicellular spheroids of 15 different tumor and normal cell lines were characterized by doubling times which decreased with increasing growth until a stable saturation was attained. In spite of the identical and constant conditions during growth, the size at saturation varied by factors of 67 in spheroid volume and 75 in cell content. These saturation sizes showed no correlation with the monolayer doubling times or clonogenic efficiencies, the initial spheroid growth rate or clonogenic capacity at saturation, the cell packing density, or the species of origin and type of cell line. There was a strong correlation between the maximal spheroid size and the size at which necrosis initially developed, suggesting control by necrosis. Crude extracts prepared from spheroids with extensive necrosis showed dose-dependent cytostatic and cytotoxic activities against monolayer cultures, while similar extracts from spheroids without necrosis had little effect. This activity was also detected in the culture medium to which the large spheroids had been exposed prior to preparation of extracts, suggesting that the responsible factor(s) can diffuse through the spheroid. The extract from spheroids of one cell line inhibited the growth and clonogenicity of four other cell lines, including human diploid fibroblasts. DNA content profiles measured during exposure to this extract showed that the cytostatic effect was not due to the arrest of cells in a specific cell cycle phase. The cell volumes were increased during culture in medium containing the extract from spheroids with extensive necrosis. These data support the hypothesis that growth saturation in spheroids is regulated by factors produced, released, or activated during the process of necrosis and suggest that these toxic factors have potential therapeutic use.

INTRODUCTION

Recently, considerable knowledge has been gained concerning the regulation of tumor cell growth and viability. Tumor cells can respond to a wide range of growth stimulatory substances, either generated externally or produced by the tumor cells themselves (1-5). Cell viability can be affected by a variety of environmental factors, usually those involved with energy metabolism (6-8). Substances have been isolated from both tumor and normal cell cultures which inhibit the proliferation and viability of tumor cells (9-14); similar inhibitors have been identified in several in vivo systems (15-17). Factors which stimulate growth can be inhibitory for other cellular processes (18). It has also been known for some time that inhibitory substances can be produced or released in the necrotic regions of tumors in vivo (19-22). There are several distinct substances known to be produced by immunological cells in vivo which specifically inhibit the viability of tumor cells (23-25). Taken together, these findings suggest that the growth and viability of cells in a tumor may be regulated as much by the microenvironmental conditions as by the intrinsic genetic or epigenetic properties of the individual cells. Due to the complex nature of the tumor microenvironment in vivo, the exact interactions among stimulatory and inhibitory substances in tumors are poorly understood, as is the relationship between the regulation of cell proliferation and viability.

The multicellular tumor spheroid culture system is a useful model to investigate these relationships in vitro (26). The onset of necrosis in spheroids, used as an assay of the loss of cell viability, has been shown to be regulated by the availability of several compounds involved in cellular energy metabolism (27-29). An advantage of this system is that the intraspheroid concentrations of several of these substances can now be assayed (30-31). Much less is known about the regulation of tumor cell proliferation in this system. Several reports have demonstrated a correlation between the external concentrations of low-molecular-weight nutrients and spheroid growth (32-34). In other reports, cell proliferation is considerably reduced at relatively high metabolite concentrations (28, 31, 35). Models for the growth of spheroids assume that the extent of the proliferating cell layer is controlled by the limited inward penetration of growth-stimulatory substances (36, 37), but the discovery that many tumor cells produce such substances themselves casts some doubt on the validity of this hypothesis.

One aspect of the growth of spheroids which may be exploited to study the relationship between proliferation and viability is the phenomenon of growth saturation, whereby the spheroid growth rate decreases with increasing diameter until a stable maximal size is attained (28, 38). A similar spheroid growth retardation occurs in vivo if vascularization is inhibited (39). Although growth saturation was originally attributed to surface area limitations for nutrient supply, consumption/diffusion models have shown that this is not the case. The spheroid growth model proposed by Landry et al. (37) suggests that growth saturation is due to the production of growth-inhibitory substances. Recent studies by Freyer and Sutherland (28, 35) demonstrate that spheroid growth saturation can be manipulated by altering the external growth conditions. A correlation was found between the onset of central necrosis and the spheroid saturation size. This led to the development of a model in which cellular proliferation and viability are regulated by independent mechanisms at small spheroid sizes; as the spheroid grows, these cellular processes become connected through the formation of toxic products during the process of necrosis. This manuscript validates the generality of the relationship between growth saturation and necrosis and demonstrates the cytostatic and cytotoxic properties of extracts from spheroids with extensive necrotic centers.

MATERIALS AND METHODS

Cell Lines. The EMT6/Ro mouse mammary carcinoma and V79 Chinese hamster fibroblast cell lines were originally obtained from Dr. Robert Sutherland of the University of Rochester Cancer Center and have been maintained in our laboratory for 5 yr. The human colon adenocarcinoma, lung carcinoma, and melanoma cell lines were obtained from the American Type Culture Collection; the human fibrosarcoma cell line was obtained from Dr. Robert Tobey of Los Alamos. The mouse adenocarcinoma, lung carcinoma, and fibrosarcoma cell lines were obtained from Dr. Nobuhiko Tokita at Los Alamos. The mouse colon carcinoma cell line was obtained from Dr. Harry Crissman at Los Alamos. The Chinese hamster lung fibroblast cells were obtained from Dr. Jacques Pouyssegur of the University of Nice, France, while the two Chinese hamster ovary cell lines were obtained from Dr. David Chen of Los Alamos. The 9L rat brain gliosarcoma cells were supplied...
by Dr. Peter Keng of the University of Rochester Cancer Center, while the rat fibrosarcoma cell line was established in our laboratory from a radiation-induced tumor isolated by Dr. Albert van der Kogel at Los Alamos. The human skin fibroblasts were obtained from Andrew Ray of Los Alamos.

Monolayer Growth Conditions. All cells were maintained in monolayer culture in a humidified incubator equilibrated with 5% CO₂ in air at 37°C; minimum essential medium, D-formulation (Grand Island Biologicals) containing 10% (V:V) fetal bovine serum (Hyclone Laboratories), 100 units/ml potassium penicillin, and 100 μg/ml streptomycin sulfate. Cultures were passaged twice weekly by treatment for 10 min with 0.25% trypsin (Sigma Chemical Co.) in Hank’s balanced salt solution containing 25 mM EDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (pH 7.4), washing by centrifugation, then plaque 5 × 10⁶ cells in 75-cm² culture flasks (Corning) containing 15 ml of complete medium. All cell lines were found to be free of mycoplasmal contamination using a simple DNA staining technique (40); the cells routinely carried at Los Alamos were also found to be contamination free using a culture technique (41). The clonogenic efficiencies of these different cell lines were assayed using standard techniques (28).

Spheroid Culturing. Spheroids were initiated and cultured essentially as described previously (28). The medium in the spheroid culture flask was changed on the second and fourth days and daily thereafter. The spheroids were assayed for mean size and cell content as described below, and spheroids were removed from the cultures during medium change such that the total number of cells in the spinner flask never exceeded 2 × 10⁶ per ml of medium. Previous studies with the EMT6/Ro cell line have demonstrated that these procedures ensure fairly constant medium conditions throughout the growth period, with the oxygen and glucose concentrations and the pH never decreasing by more than 5% of the values in fresh medium (28).

Spheroid Growth Curve Measurement. Spheroids were assayed for growth at medium replenishment times by measuring the population mean diameter and the number of cells per spheroid as described previously (28). The spheroid cell number at saturation was also combined with the spheroid volume and the extent of necrosis to estimate the mean cell packing density in terms of the number of cells per unit of viable spheroid volume.

Histological Measurement of Necrosis. Spheroids were fixed, mounted in paraffin, sectioned, and stained for histological observation as described in detail elsewhere (28). Measurement of the thickness of the viable cell layer was done using a calibrated reticle in a microscope; measurements of the central sections of each of 25–35 spheroids 800–1200 μm in diameter were made as described previously (28). This previous work demonstrated that for spheroids cultured in normal concentrations of oxygen and glucose there was no significant variation in the viable rim size over this range of spheroid diameters.

Necrotic Extract Preparation. In order to test for the presence of any toxic properties of necrosis in spheroids, extracts were prepared in the following manner. Three hundred EMT6/Ro spheroids were cultured to a size of ~1500 μm; at this size the thickness of the viable rim was ~250 μm, so that these spheroids were composed of ~36% necrotic material by volume. The spheroids were removed from the spinner flask and placed into a 15-ml centrifuge tube and allowed to settle, the medium was removed, the spheroids were washed quickly with 10 ml of PBS, then resuspended in 10 ml of double-distilled water. This spheroid suspension was homogenized using a tissue homogenizer (Sorval Instruments) for 5 min, then placed into a ~20°C freezer for storage. Extracts from spheroids without central necrosis were prepared in a similar manner using 1 × 10⁶ spheroids of ~450 μm diameter, which represents an equivalent volume of material as was used for the spheroids with necrosis. After defrosting, various dilutions of these extracts were added directly to complete culture medium and mixed for 1 h. After mixing, the pH of the medium/extract mixtures was adjusted to 7.4 using 1 N NaOH, then they were sterilized by passage through 0.22-μm nylon membrane filters (Millipore).

In order to be able to quantitate the amount of extract being added to the cultures, a second method of preparation was used. The spheroids were grown, isolated, washed, and frozen in PBS³ as described above. These were then placed into a freeze-drying apparatus, lyophilized, and stored at ~20°C until use. The freeze-dried spheroids were then ground to a fine powder and added to culture medium at a concentration of 1 mg/ml. After mixing for 1 h at room temperature, the medium with extract was adjusted and filtered as described above.

Monolayer Growth Curve Measurement. In order to initiate monolayer growth curves, cells obtained from exponentially growing monolayer cultures were washed by centrifugation and 10⁶ cells were inoculated into each of 30 60-μm dish dishes containing 5 ml of the medium of interest which had been preequilibrated to the proper pH and temperature. At various times after plating, three dishes were removed from each experimental group, the medium was removed, the dishes were washed with 5 ml of PBS without calcium or magnesium, and 1 ml of standard trypsin solution was added to each dish. These were incubated at 37°C for 10 min, then 2 ml of complete medium was added, the cell suspensions were mixed with a Pasteur pipet 10 times, and an aliquot of each dish was added to a counting vial containing PBS. Three counts of the cell suspension from each dish were then made using the Coulter counter and pulse-height analyzer. The pulse-height analyzer was also used to obtain an estimate of the mean cell volume using computer analysis described elsewhere (28). The log-normal distributions of the mean cell numbers per dish as a function of time of growth were then fit to a linear equation using a least squares method to obtain an estimate of the exponential growth rate. The remainder of the cell suspensions from the three dishes in each experimental group was then pooled and prepared for flow cytometric DNA content analysis as described below. In some experiments, the cells were allowed to attach in normal medium for 24 hours, then the medium was replaced by either control or extract-containing medium.

Flow Cytometric DNA Content Analysis. Cells were analyzed for total DNA content using a flow cytometric technique which has been detailed previously (28). The cell suspensions were analyzed for total DNA content on a Los Alamos flow cytometer (42) with the laser operating at 457 nm and collecting wavelengths longer than 512 nm. Histograms containing >5 x 10⁴ cells were collected and analyzed to determine the percentages of cells in each of the cell cycle phases using standard techniques (43).

RESULTS

Spheroid Growth Curves. In order to study the generality of the relationship between the saturation of growth and the onset of central necrosis, spheroids of 15 different cell lines were cultured under identical nutrient conditions until a stable maximal size was attained. Representative growth curves for spheroid volume and total cell content are shown in Fig. 1. In every case, spheroid growth was characterized by an initial exponential phase, a second phase in which the growth rate was continually slowing, and a final plateau in growth. These data were fit to the Gompertz equation by a nonlinear least squares best fit routine in order to arrive at objective estimates of the initial spheroid growth rate and the spheroid size at plateau. The estimated values of these parameters are shown in Table 1 for all of the cell lines. As can be seen from these data and the curves in Fig. 1, there was a considerable variation in the initial growth rates and the saturation sizes among these different cell lines. The initial doubling times varied by a factor of 4.4 when measured by volume growth and 3.7 in terms of spheroid cell content. The saturation sizes varied by factors of 67 and 75 for volume and cell number growth, respectively. There was no significant correlation between the initial growth rate and the saturation size, either in terms of spheroid volume (r² = 0.00095) or total cell number (r² = 0.013). The means and confidence limits for the maximal size attained by spheroids of a given species showed that there was no significant difference

³ The abbreviation used is: PBS, phosphate-buffered saline.
in growth saturation among the spheroids derived from these different mammals. Similarly, spheroids of different cell types in those instances in which more than one measurement was available (colon and lung carcinomas, adenocarcinomas, fibrosarcomas, and fibroblasts) also showed no dependence of saturation size on cell type.

Several other cellular parameters were measured for these different cell lines, as shown in Table 1. The monolayer doubling times were correlated with the initial spheroid growth rates ($r^2 > 0.85$), but there was no correlation between the monolayer growth rate and the size at saturation ($r^2 < 0.14$). Measurements of both the monolayer clonogenic efficiency and the spheroid cell clonogenic capacity at growth saturation also showed no correlation with the initial spheroid doubling times ($r^2 < 0.15$) or the growth saturation sizes ($r^2 < 0.19$). Note, however, that there was a 10–30% reduction in colony-forming ability comparing cells from large spheroids to those from monolayers. Calculations of the cell packing density in the spheroids at saturation also showed no correlation with the saturation size ($r^2 < 0.028$). Comparison of any of these cellular parameters with the logarithm of the maximal sizes also revealed no significant correlations ($r^2 < 0.32$).

There was a significant correlation between the thickness of the spheroid viable cell rim and the growth saturation size, both in terms of spheroid volume ($r^2 = 0.55$) and total cell content ($r^2 = 0.56$). Previous studies with the EMT6/Ro cell line grown under different culture conditions showed that the spheroid size at which necrosis initially developed could be reliably estimated over a wide range of values by doubling the measured thickness of the viable cell rim (28). Fig. 2 shows a semilogarithmic plot of the estimates of the spheroid size at the onset of necrosis and the spheroid size at saturation. These parameters were highly correlated, both in terms of spheroid volume ($r^2 = 0.85$) and total cell number ($r^2 = 0.72$). There was no correlation between the spheroid size at the onset of necrosis and the initial spheroid growth rate ($r^2 < 0.21$). Additionally, there was no correlation between the size at which necrosis initially developed and any of the cellular parameters detailed above ($r^2 < 0.21$). Also shown in Fig. 2 are values obtained for the spheroid diameter at the onset of necrosis and the saturation of growth in the EMT6/Ro cell line grown under different glucose concentrations (28). Pooling these data together with the measurements on the different cell lines in the same medium increases the correlation between necrosis formation and growth saturation both for spheroid volume ($r^2 = 0.92$) and total cell content ($r^2 = 0.89$).

Effects of Necrotic Extract. In order to directly test the toxic properties of necrosis in spheroids, extracts were prepared from large (~1500 μm diameter) and small (~400 μm diameter) EMT6/Ro spheroids as detailed in “Materials and Methods.” EMT6/Ro monolayer cells were then exposed to complete medium to which various dilutions of these extracts had been

### Table 1 Monolayer and spheroid growth, plating efficiency, saturation size, viable rim and cell packing parameters

<table>
<thead>
<tr>
<th>Species/cell line</th>
<th>Cell type</th>
<th>Doubling time (h)</th>
<th>Plating efficiency (%)</th>
<th>Initial doubling time (h)</th>
<th>Maximal spheroid volume (μm³)</th>
<th>Viable spheroid size (cells)</th>
<th>Viable rim (μm)</th>
<th>Plating efficiency (%)</th>
<th>Viable cell rim (mm)</th>
<th>Cell packing (cm³/cm³)</th>
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<tr>
<td>Mouse</td>
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<td>12</td>
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<td>1.7 x 10⁸</td>
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<td>63</td>
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<td>180</td>
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<td>CHL-O23</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>12</td>
<td>71</td>
<td>18</td>
<td>1.8 x 10⁸</td>
<td>22</td>
<td>4.7 x 10³</td>
<td>139</td>
<td>50</td>
<td>5.7 x 10⁸</td>
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<tr>
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<td>64</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>5.5 x 10³</td>
<td>18</td>
<td>3.2 x 10⁴</td>
<td>104</td>
<td>56</td>
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</tr>
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<td>3.1 x 10³</td>
<td>135</td>
<td>48</td>
<td>1.2 x 10⁸</td>
</tr>
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</table>

* ND, not done.
Fig. 2. Relationship between spheroid saturation size and the size of spheroid at the onset of central necrosis. The size of spheroid at the onset of necrosis was estimated from measurements of the thickness of the spheroid viable cell rim as explained in the text. Maximal size values are individual determinations from best fits to the Gompertz equation for data similar to that shown in Fig. 1. Lines, linear least squares best fits to all data points, ○, data for 15 different cell lines cultured in the same medium; ○, comparable data for EMT6/Ro spheroids cultured in medium containing four different glucose concentrations (28). Top, spheroid saturation size in terms of the maximal spheroid volume attained; bottom, this in terms of the maximal spheroid cell content.

Fig. 3. Representative monolayer growth curves for control EMT6/Ro cultures (○) and identical cultures exposed to a 1:1 dilution of spheroid necrosis extract (●) as explained in “Materials and Methods.” Points, mean of three dishes (SE within the size of the data points); lines, linear least squares best fits to all data points.

Fig. 4. Monolayer culture doubling times of EMT6/Ro cells as a function of the dilution either of the spheroid extract (A) or of the medium from spheroid cultures (B). ○, extract/medium from spheroids without necrosis; ●, data for extract/medium from spheroids with extensive necrosis, as detailed in “Materials and Methods.” Values are slopes of linear least squares best fit lines to growth curve data such as that shown in Fig. 3; bars, one SE of the best fit estimate (within the size of the data point where not shown); lines, hand-drawn fits to the data.

The effect of the spheroid extracts and the spheroid-exposed media on the clonogenic efficiencies of EMT6/Ro monolayer cells was also measured, as shown in Fig. 5. In this case, the cells were plated at low density (200 per dish) and exposed to the appropriate media throughout the 7-day period required to
grow colonies. Again, the extract or medium from spheroids without central necrosis had no significant effect on the ability of the cells to form colonies, even at the highest concentrations. The extract and medium from spheroids with extensive central necrosis showed a dose-dependent inhibition of colony-forming ability. At the highest concentration (a 1:1 dilution), the cell clonogenic efficiencies were reduced by factors of 2.9 and 1.8 for the extract and spheroid-exposed medium, respectively. Again, note that the clonogenic efficiency of the cells was slightly reduced even at the lowest concentration of the extract from spheroids with extensive necrosis.

The data in Figs. 3–5 were all obtained with extracts or medium from EMT6/Ro spheroids and tested against EMT6/Ro monolayer cells. In order to test the generality of these effects, monolayer cells of several different cell lines were exposed to medium which had been mixed with a lyophilized extract from EMT6/Ro spheroids with extensive necrosis, as explained in "Materials and Methods." For these experiments, the cells were inoculated at high (5 × 10^4 per dish) and low (200 per dish) density in complete medium and incubated for 24–26 h. Then the medium on the dishes was removed and replaced with control medium or medium mixed with 1 mg/ml of the lyophilized extract, and the dishes were handled for growth curve analysis and clonogenic efficiency determination. Fig. 6 shows the monolayer growth curves obtained for 9L rat gliosarcoma cells. All of the monolayer growth curves had this appearance, i.e., a reduced but still exponential growth rate in the extract-containing medium. The magnitude of the growth-inhibitory effect was cell-line dependent, as shown in Table 2.

For exposure to the identical extract-containing medium, the monolayer doubling times were increased by factors of 1.2 to 5.6. Comparison of the doubling times in these two conditions using a Student's t test shows that the monolayer growth rates are significantly different for each cell line (P < 0.005). In every case except the 9L cells in the extract-containing medium, the cells were cultured until they reached a plateau. There were no significant differences in the number of cells per dish at plateau (P > 0.5). Table 2 also shows the clonogenic efficiencies of these different cell lines in the two conditions. Again, in every case there were significantly fewer colonies formed in the extract-containing medium (P < 0.005). In the case of the 9L cells, there were no colonies seen out of the total of 2000 cells plated; for the HSE cells, there were no colonies seen after plating 5000 cells.

During the monolayer growth curve measurements, the cells removed from the dishes as a function of time were assayed to obtain cell volume and DNA content distributions as detailed in "Materials and Methods." The mean cell volumes were increased by 16–35% in the cells cultured in the extract-exposed medium for the EMT6/Ro, HT1080, MEL28, and HSF cells but not for the 9L cells (P > 0.05). There were no significant differences in the cell cycle distributions of cells cultured in these two conditions during the exponential phase of the growth curves, despite the significant differences in growth rates (Table 2).

**DISCUSSION**

One of the important results of this study was that a large number of basic cellular and spheroid parameters had no relationship to the spheroid saturation size. There was no correlation between the spheroid saturation size and the species or cell type from which the spheroids were cultured. The tumor cell

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**Table 2 Monolayer growth and clonogenicity in control and extract-treated medium**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control Doubling time (h)</th>
<th>Control Plating efficiency (%)</th>
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<tbody>
<tr>
<td>EMT6/Ro</td>
<td>9.25 ± 0.055</td>
<td>9.82 ± 0.15</td>
</tr>
<tr>
<td>HT1080</td>
<td>10.2 ± 0.072</td>
<td>12.2 ± 0.11</td>
</tr>
<tr>
<td>MEL28</td>
<td>9.82 ± 0.082</td>
<td>13.1 ± 0.11</td>
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<tr>
<td>9L</td>
<td>17.6 ± 0.15</td>
<td>19.2 ± 0.60</td>
</tr>
<tr>
<td>HSF</td>
<td>25.3 ± 0.67</td>
<td>36.7 ± 0.69</td>
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</table>

Comparison of the effect of an extract from EMT6/Ro spheroids with extensive necrosis on the growth and clonogenicity of monolayer cells of different cell types. Doubling times are given as best fit values ± SE of the fit for growth curves similar to those shown in Figs. 3 and 8 (n = 6–8). Control cultures were grown in normal complete medium; experimental cultures were exposed to medium containing an extract from spheroids as detailed in "Materials and Methods."
Therefore, it may be invalid to invoke the mechanism of limited conditions, as a spheroid should be able to grow indefinitely (37). The observation that the only parameter which correlated with the saturation size was the thickness of the viable cell rim. Despite the fact that all of the spheroids were cultured in identical and constant conditions, there was a large variation (85–257 μm) in this critical parameter. There is considerable evidence that the onset of necrosis is determined by the availability of sufficient metabolites for the maintenance of the energy requirements of the cells in the inner spheroid region (27–29). Thus, the range in viable rim sizes observed in the present study may be due to cell line differences in any of several parameters, including metabolite consumption, energy production and utilization, and the sensitivity to low metabolite levels. In addition, several metabolites and catabolites may interact in determining the viability of cells in spheroids (26).

The data in Fig. 2 demonstrate that the onset of necrosis is closely coupled with the later development of growth saturation. Calculations from the data in Table 1 show that, in spite of the variation in rim sizes, spheroid growth saturation was attained at a size at which central necrosis constituted 50–70% of the total spheroid volume.

The observation that the only parameter which correlated with the spheroid saturation size was the diameter at which necrosis developed argues strongly against the hypothesis that growth saturation is due to the limited surface area available for supplying nutrients to the cell volume (38, 39). The surface-to-volume ratios at growth saturation measured in the present study varied by a factor of 4.2. It is difficult to invoke a nutrient-supply mechanism to explain spheroid growth saturation in some cases at 970 μm and in others at ~4000 μm in the same medium. Mathematical models (36) have shown that as a spheroid grows, the nutrient supply by diffusion from the medium becomes similar to the situation in an infinite sheet of a thickness equal to that of the viable cell rim. Under these conditions, a spheroid should be able to grow indefinitely (37). Therefore, it may be invalid to invoke the mechanism of limited inward penetration and supply to explain spheroid growth saturation.

Growth saturation in spheroids is more likely related to the expansion of central necrosis through the formation, release, or activation of cytostatic factors. One of the most striking aspects of Fig. 2 is the similarity in the data for different cell lines cultured in the same medium and for a single cell line cultured in different media. This clearly suggests that the relationship between the development of necrosis and growth saturation is a fundamental one. The nature of this relationship was first detailed in a model developed by Landry et al. (37) in which cellular proliferation in the outer rim of the spheroid is reduced by the action of cytostatic factors diffusing out of the necrotic core. As later expanded by Freyer and Sutherland (28), the model proposes that spheroid cell proliferation and viability are regulated independently at small spheroid sizes, but that these processes become indirectly related when products generated during the process of necrosis inhibit cell proliferation. This may be an important consequence of the fact that cell loss in spheroids (and primarily in tumors) occurs through the process of necrosis, as opposed to the more orderly mechanism of apoptosis (44). The data in this manuscript, especially the finding that central necrosis is necessary in order to demonstrate a cytostatic effect in spheroid extracts (Fig. 4), strongly support this model. The discovery of a cytostatic effect of the medium exposed to spheroids with extensive central necrosis (Fig. 4) also supports the critical requirement that the putative cytostatic factors diffuse through (and out of) the spheroid. The connection between the regulation of proliferation and viability is an important contribution of the spheroid system to an understanding of tumor cell biology and should have relevance to the analogous situation in tumors in vivo.

The data presented allow some limited characterization of the mechanism of action of the toxic extracts from spheroids. The information in Fig. 6 and Table 2 demonstrate that the extract from spheroids of one cell line is active against other cell lines. This may indicate that the responsible factor(s) has a general mechanism of action. Such a cross-reactivity of tumor cell inhibitory factors has been found in other systems (13, 14); interestingly, the spheroid necrosis extract also inhibited the growth of human diploid fibroblasts. Preliminary data with extracts from necrosis-containing spheroids of the HT1080 human fibrosarcoma cell line demonstrate the presence of both cytostatic and cytotoxic activities, suggesting that the toxic effects of spheroid necrosis are also a general phenomenon. An interesting finding of the present studies is the fact that the spheroid necrosis extract does not induce the arrest of cells in a particular cell cycle phase. Two reports of growth inhibitors produced by normal cells show specific inhibition of the G1/S transition (10, 15), but at least one tumor cell growth inhibitor found in normal human serum shows no cell cycle phase specificity (16). The fact that the spheroid extract growth-inhibitory activity slows traverse through all phases of the cell cycle may have some relationship to the presence of nonproliferating S- and G2-phase cells in some spheroid systems (45, 46).

There are two other findings in the present study which may have some implications for the mechanism of action of the growth inhibitory activity. The first is that the volumes of the cells exposed to the extract were significantly increased. This effect has not been reported previously for tumor cell growth inhibitory factors and suggests some mechanism involving the cell membrane or perhaps the general cellular metabolism. The second interesting effect is the cytotoxic activity of the extract, as measured by a clonogenicity assay. Cytotoxic activities of tumor cell growth inhibitors have been reported in other systems (14, 17). This could occur by the same mechanism which causes the growth inhibition, or it may be an inhibition of cell attachment as has been reported for extracts from necrosis in tumors (21).

There are several possibilities to explain the origin of toxic factors which are specifically present in spheroids with central necrosis. The spheroid cell mass generates metabolic waste products which can be found in the spheroid extracellular space (26, 30), and such catabolites can inhibit growth (47). It is unlikely that common catabolites are responsible for the observed growth and viability inhibition, however. There was little effect of the extract from spheroids without central necrosis, yet these spheroids carry out metabolism and presumably contain catabolites. Also, there was a significant effect of the extract from spheroids with necrosis even at very high dilutions in which the amount of any added catabolites would be quite low.
We also have preliminary data showing that the cytostatic activity in necrotic extracts is found in a high-molecular-weight fraction. A second possible source of the toxic activity is the release of materials from the cells during the process of necrosis, for example, lysosomal enzymes (19, 22). Again, the finding of activity only in extracts from spheroids with necrosis suggests that such a mechanism would have to be specific to cell death by necrosis. A third intriguing possibility is that substances released or generated during necrosis could themselves generate a toxic compound through enzymatic or chemical action. Such a modification of growth-inhibitory activity by proteolytic action has been shown in several systems (15, 19, 48, 49). The microenvironment in the necrotic region of spheroids is poorly understood; it is plausible that toxic factors are released or activated in this region and then alter the environment around the viable cells.

Regardless of the mechanism, the presence of growth-inhibitory factors in spheroid necrotic regions has some interesting implications regarding the concept that growth saturation in spheroids is analogous to the situation of a nonvascularized tumor or metastatic nodule in vivo (39). The current results suggest that the size to which a nodule can grow without vascularization can vary greatly, there is no doubt a greater variation in the complex in vivo situation than is reported here for spheroids in vitro. Secondly, it is possible that the original impetus for the vascularization of a tumor nodule is the formation and expansion of necrosis. Studies with the spheroid as an immune response stimulus in vivo suggest that several types of host cells are attracted by the necrotic region (50). Factors released from the necrotic region of a small tumor nodule may actually assist in the future expansion of the tumor by inhibiting the further growth of the tumor cells in a reversible manner while concurrently serving as a signal for the expansion of the vascular system through angiogenesis. The spheroid system provides a suitable model for investigating the initial events in tumor nodule vascularization and the role of necrosis in this process.

Another critical area that the present results have important implications for is the use of spheroids as a therapeutic test system. The spheroid growth model proposed by Freyer and Sutherland (28), and supported by this manuscript, assumes that growth regulation in spheroids is a competition between stimulatory and inhibitory factors; the relative importance of these factors changes as the spheroid grows. Thus, it may be improper to interpret results obtained with therapeutic agents at one spheroid size as applying in general, especially if the agent used is dependent on cellular proliferation. This is an important caution, as the majority of published reports using therapeutic agents in spheroids have not investigated a range of spheroid diameters. Another consideration is that many therapeutic modalities can alter the spheroid microenvironment and cell subpopulations, possibly altering the balance between growth inhibition and stimulation. Again, there is considerable work which could be done with the spheroid system to better understand the changes in cell growth regulation which occur after therapeutic treatment. This would be especially true for treatments which themselves induce the formation of necrosis. Finally, it should be productive to investigate the possibility of enhancing the cytostatic and/or cytotoxic properties of necrosis as a new therapeutic approach. Seen in this context, the nutrient-deprived conditions (such as hypoxia) in spheroids and tumors, which are generally seen as an advantage in surviving treatment, could be made harmful to the tumor by manipulating the toxic properties of the necrosis that develops in such regions. This approach would have the advantage of being specific to tumor microregions, as these are generally the only locations of necrosis in vivo.

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Role of Necrosis in Regulating the Growth Saturation of Multicellular Spheroids

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