Proliferation and Viability in Cellular Spheroids of Human Origin

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ABSTRACT

The capacity of cells to form growing tumor-like colonies in culture was tested by a new and unlabourious suspension technique. Six of nine tested cell lines formed spheroids, five of which started to grow. The growing spheroids reached a maximal size determined by balanced cell gain in a proliferative, superficial layer and cell death, particularly at larger depth. Sections of spheroids of different origin showed large variations in the thickness of the viable cell layers and in the shape of the proliferative gradients. The data, taken together with earlier published information, indicate that a difference between rodent and human cells generally exists, the former showing thinner viable and proliferative layers.

INTRODUCTION

Cultured tumor-like colonies of mammalian cells have gained interest for several reasons. Spinner flask cultures of hamster cell spheroids, resembling nodular carcinomas and containing radiation-resistant hypoxic cells, have been used in radiobiological studies [cf. Sutherland and Durand (16)]. The role of the surface area limitation in self-regulation of growth has been studied by Folkman et al. (8), using spherical colonies of hamster lung or tumor cells cultured in agar gel; they showed that a maximal spheroid size is reached in such cultures. McAllister et al. (11) cultured cells from virus-induced hamster tumors in agar gel and found that the 3-dimensional colonies were morphologically similar to the original tumors. A comparison of the fine structure between central and peripheral regions in agarose-cultured human glioma spheroids was done by Carlsson and Brunk (3), and radial variations in the cellular amount of mitochondria and vacuoles were found. These studies showed that the central regions of the colonies had a lowered proliferation and viability probably due to lack of oxygen or other metabolites (or accumulation of catabolic products). Thus, the 3-dimensional colonies seem to mimic the growth pattern in the small nodules, present in many types of tumors, where proliferative cells near the capillaries shield regions of proliferation-inhibited or degenerating cells (18, 20).

In this work we have studied the formation, growth, and morphology of spheroids from some human and hamster cells that usually grow as monolayer or single-cell suspension cultures. The spheroids were sectioned, and local variations in proliferation and viability were studied. Two techniques have been used previously in spheroid culture. One uses spinner flasks with liquid medium in which the cells first aggregate to form the spheroids. The spheroids thereafter increase in volume mainly due to proliferation. This method has been shown to work well for the V-79 strain of hamster lung cells (17). The other method, which allows repeated growth measurements on individual spheroids, uses cultivation in agar of agarose gel [e.g., Folkman et al. (8)]. However, some drawbacks with the gel culture method have been reported. Folkman et al. (8) had to subcultivate spheroids in new gel every 2 or 3 days to prevent a drop in pH. In another study, in which the top layer of the agarose gel was flushed with humid air containing 5% CO₂, permitting normal pH values to be maintained for up to 4 weeks, it was found that a continuous increase in osmolality occurred, concomitant with inhibition of proliferation after about 10 days (2). Thus, repeated medium changes seem to be necessary to provide adequate physiological conditions in cultures growing for more than 2 weeks.

By using agarose-coated (to prevent cell attachment) minidishes that permit easy medium change, we could establish a less laborious method to culture cell spheroids that also allows single spheroids to be followed during growth.

MATERIALS AND METHODS

Cell Lines. The characteristics of 2 human malignant glioma cell lines (118 MG and 251 MG) have been described previously in detail by Westermark et al. (22). The strain of HeLa cells studied has been described by Crowell et al. (4) and the human glia cells (622 CG), taken from adult brain biopsies, were studied by Pontén et al. (12). For comparison, Chinese hamster cells, a diploid clonal derivative (CHEL) of male embryonic lung cells (10), were also used. All these cells were normally growing as monolayers in Eagle’s MEM* (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% bovine calf serum and antibiotics [penicillin (100 IU/ml) streptomycin (50 μg/ml), and amphotericin B (1.25 μg/ml), all from Kemila, Stockholm, Sweden].

The glia and the CHEL cells, which had a limited life span, were studied during the first 10 to 15 passages. Both lines showed signs of being normal diploid cell lines since they had a high serum requirement and a low cytoplasmic overlapping in dense monolayer cultures.

Some suspension-cultured human cells were also tested for their ability to aggregate and grow as spheroids. Cells from a solid lymphoma (U-715), melanoma cells (U-226) (15), Burkitt lymphoma cells (P3) (9), and leukemia cells...
Preparation of Minidishes. Clear plastic trays (Linbro FB 48-TC with 48- x 0.5-ml wells; Flow Laboratories) were, with sterile instruments (scissors and tweezers), cut into pieces ("minidishes") containing 4 wells each and transferred into 5-cm plastic dishes (Nunclon) (Fig. 1). One % agarose (Indubiose A37; L'Industrie Biologique Française) in distilled water was boiled, and about 0.15 ml was added in each well (Chart 1). The agarose was allowed to solidify at room temperature for 1 hr. and then the gel was washed twice with 0.3 ml Eagle's MEM. Minidishes containing about 0.3 ml Eagle's MEM were finally stored in the refrigerator, for several weeks if necessary.

Cell Culture in Minidishes. Cells cultured in monolayer were suspended with 0.25% trypsin (Difco Chemical Co., Detroit, Mich.). For some cell types pretreatment with 0.02% EDTA (Difco) in phosphate:physiological saline (3 g sodium phosphate and 8 g salts in 1 liter distilled (K,0) was necessary to ensure that a single-cell suspension was obtained. The concentration of the suspended cells was determined in a Coulter electronic cell counter. The wells in each minidish were washed with fresh medium (Eagle's MEM containing 10% calf serum), and 0.3 ml medium containing the suspended cells was added to each well. The initial number of cells in each well was, if not otherwise stated, about 10^3 cells. The minidishes were incubated at 37° in humid air containing 5% CO_2. When cells aggregated they formed nearly spherical colonies within a few days (the exact time being dependent on cell type and the number of seeded cells). The medium was changed 3 times a week with the use of a bent Pasteur pipet with a plastic mouth tube that allowed convenient handling of colonies and medium.

Growth Curves. Individual spheroids were repeatedly measured by means of an ocular scale manipulated by a micrometer screw, in an inverted Olympus microscope. The volume of each colony was calculated by the relation \( V = \frac{4}{3} \pi \cdot (ab)^{3/2} \) where \( a \) and \( b \) were the observed minimum and maximum radii measured at right angles.

pH and Osmolality. pH in the culture medium was measured with a digital pH-meter (Radiometer) equipped with a Radiometer GC 2303 C electrode calibrated at 37°. During measurement the electrode was mounted inside the incubator with the tip immersed in the medium.

Osmolality was measured by the freezing point method on 0.15 ml cell culture medium in a Knauer type M electronic osmometer.

Administration of [3H]dThd. The cells were labeled with [3H]dThd with a specific activity of 5 Ci/mmol (The Radiochemical Centre, Amersham, England). Prewarmed medium containing 1 𝜇Ci/ml was added to the spheroids, and the dishes were incubated for 1 hr. Immediately after incubation, the cells were washed and fixed as described under "Histological Technique."

Histological Technique. Prior to fixation the spheroids were washed twice in phosphate physiological saline and were thereafter fixed with methanol:acetic acid (3:1) for 2 hr. The spheroids were finally stored in 70% alcohol.

The spheroids were embedded in a small piece of 0.5% agarose gel to facilitate further manipulations. After dehydration in 80, 90, and 100% alcohol for at least 1 hr in each bath, infiltration with glycol:methacrylate (Solution A) with catalytic powder (Sorvall JB-4 embedding kit) was done overnight. Finally, the agarose piece, containing the spheroid, was molded in a standard plastic frame containing Solution A, catalytic powder, and Solution B (hardening solution) (14). Sections of 2 𝜇m were cut with a glass knife. Only sections from the central area of the colonies (sections with maximal diameter) were studied. The sections were mounted with Euparal. Some sections were in addition stained with eosin (2 min).

Zones of Viability and Necrosis. The zone of massive central necrosis was easily recognized in sections (cf. Fig. 3). The zone outside the massive necrosis was, although sometimes a few pyknotic nuclei could also be seen there, called the zone of viability. With an ocular scale the zones...
were measured in at least 3 sections from the center of each spheroid.

**Autoradiography.** The sections were processed for autoradiography by dipping into emulsion Ilford K5 and were left in the dark for 30 days before development in Kodak D19-B and fixation.

**Evaluation of Labeling Index.** The numbers of labeled and unlabeled cells were counted in a rectangle, covering $40 \times 80$ $\mu$m of the colonies, mounted in the eyepiece of a Leitz Ortholux microscope. The evaluation proceeded stepwise, at 40-$\mu$m intervals, along 2 diameters at right angles (2). Several sections from at least 2 spheroids were analyzed for each cell type.

**RESULTS**

**Spheroid Formation.** Many of the cell lines tested could form solid spheroids (see Table 1). However, 3 of the hemopoietic cell types (P3, CEM, and 266) did not form spheroids. Instead they sedimented to a 2-dimensional layer on the bottom of the wells. For the cells cultured in monolayer, the capacity to form spheroids was not affected by trypsinization prior to seeding. Trypsinization for 5 to 30 min was tried without any visible changes. EDTA treatment prior to trypsinization did not affect the spheroid formation. Typical spheroids are shown in Fig. 2.

The initial size of the spheroids depended on the number of cells that were seeded in each well. The cells aggregated and arranged themselves in such a way that, in most cases, spherical colonies were obtained within 2 or 3 days. The spherical shape of the colonies was easily tested by rotating the spheroids through mild agitation of the medium. In a few cases, especially when less than 100 cells were plated, more than 1 spheroid was formed in each well. In these cases all the spheroids were rejected.

**Initiation of Growth.** Growth was initiated in 5 types of spheroids tested (Table 1). For the human cells it was generally found that about 10 cells at least were needed in each aggregate for onset of growth. When seeded with only 1 cell/well, fewer than 1 of 50 human cells started growth. The spheroids of human glia cells (622 CG) did not grow at all. The hamster cells (CHEL) showed a growth fraction of 30 to 50% when seeded with only 1 cell/well.

**Growth Curves.** The growth of spheroids from some of the cells tested is shown in Charts 2 and 3. The human glioma 118 MG cells were plated at different concentrations (1 to $100 \times 10^3$ cells/well); plateaus are reached in all curves regardless of the initial size of the spheroids. The final size of the spheroids could not be increased by increasing the frequency of the medium changes (changes every day had no effect) or by transfer into larger medium baths. Neither did increasing amounts of serum affect the growth pattern (up to 30% bovine calf serum was tested).

**pH and Osmolality.** The pH of newly added medium (with 10% calf serum) was about 7.9. After equilibration for about 5 min with humid air containing 5% CO$_2$, the pH dropped to 7.6. After about 1 hr it had decreased to 7.4 and then remained stable. This pattern of pH variation is similar to that previously reported by Eagle (6) for monolayer cultures.

The osmolality varied between 290 and 300 mosmol in the exponential growth phase and between 290 and 310 mosmol in the plateau phase.
mOsmol in the plateau phase; these values are well within the recommended range for cell culture media (21).

**Volume-doubling Times.** The measured volume values during the first 6 to 8 days (i.e., the phase of exponential growth) for the glioma 118 MG spheroids were adapted to an exponential function by the method of least squares. The doubling times thus obtained were plotted against the diameter at 3 days after plating (Chart 4). It is seen that the doubling rate is slower for large spheroids. For comparison the values obtained for the same type of cells growing as small spheroids in agarose culture (2) are included; the new method described gives comparable growth rates.

**Cellular Arrangement.** Cells that were nonpolar when grown as monolayers (HeLa and CHEL) also displayed a nonpolar shape when growing as spheroids. The glioma 118 MG cells, which are extremely polar and fibroblast-like in monolayer culture, also were elongated and polarized inside the spheroids (Fig. 3). The lymphoma 715 cells were to a large extent rounded inside the spheroids.

**Zones of Viability and Necrosis.** The glioma 118 MG spheroids could reach rather large sizes (to about 700 µm) before any visible signs of central necrosis occurred. The spheroids from the glioma line 251 MG and the HeLa cells showed a somewhat less good tolerance in this aspect because central necrosis was introduced at diameters of about 500 µm. The central necrotic zones in glia and HeLa spheroids (also those that had been in the plateau phase for up to 3 weeks) contained randomly distributed pyknotic nuclei. The Chinese hamster cell spheroids, which showed central necrosis at diameters of about 250 µm, contained necrotic regions containing a homogeneous amorphous mass with a few pyknotic nuclei (Fig. 3).

In Chart 5 the thickness of the zone of viability has been plotted against the diameter of spheroids tested in this work and for spheroids cultured in agarose or spinner flask culture as reported in the literature. The viable zone for all rodent cells was in the region of 25 to 200 µm, while all human cell spheroids had viable layers of 200 to 350 µm. If only tumor cells are considered, the thickness of the viable layer varied between 75 and 350 µm, while for the few nontumorous cells thus far investigated (which all were of rodent type) the thickness was 25 to 125 µm.

In the plateau phase the thickness of the viable layer generally decreased with time. For example, in the case of glioma 118 MG cells, it decreased from 300 µm at 20 days to about 200 µm at 40 days.

**Labeling Index.** The fraction of labeled cells (after 1 hr incubation with [3H]dThd) at different distances from the surface of the spheroids is shown in Chart 6. The data for both the glioma cell lines and the HeLa cells were adapted to exponential curves by the technique of least squares (the fit was in all 3 cases less good when straight lines were adapted). In the case of CHEL cells too few points were obtained to allow adaptation. The radial distances at which the labeling indices changed by a factor of 2 (the half-values) were about 80 and 40 µm for the glioma 118 MG and 251 MG cells, respectively; 55 µm for the HeLa cells; and 20 µm for the CHEL cells.

Small fractions of proliferative cells always existed rather
close to the necrotic areas, although the position of these areas in relation to the outer surface varied strongly between the different types of spheroids.

The labeling indices in the plateau phase were, for all cell types studied, only slightly lowered, indicating that volume growth stopped due to balance between cell gain and cell loss.

**DISCUSSION**

Although the investigated types of spheroids looked similar and also had somewhat similar growth rates, they revealed clear microscopic differences. Some types had very thick viable layers (especially the human glioma 118 MG cells), while others had thinner layers (especially the Chinese hamster cells). It seems as if a general difference between the studied rodent and human cells exists (Chart 5). Comparison of neoplastic and nonneoplastic cells was difficult from these data because all nonneoplastic cells were of rodent origin. Testa and Lord (19) reported that human nonneoplastic hemopoietic stem cells could grow in agar, but the histological characteristics of the colonies were not investigated. In the rodent group no difference between tumorous and nontumorous cells could be seen.

Several factors can be responsible for the variations in the thickness of the viable layers. It is generally assumed that the cells in central regions suffer from a low oxygen tension. Thus, differences in the central oxygen tension due to differences in the oxygen consumption of the peripheral cells may possibly explain the variations. In fact rodent tissues usually have a higher oxygen consumption than do human tissues (1). Another possible explanation is differences in the capacity to utilize anaerobic glycolysis for energy production. Such differences have recently been reported to exist between rabbit epithelium and human fibroblast cells (13). Differences in the capacity to stand the stress of low pH and high osmolality in the central regions might also be important. From the literature it is not possible to obtain information on the proportions at which these factors contribute to the observed variations. Comparison of oxygen consumption and ability to stand hypoxia, low pH, and high osmolality for the studied cell types in conventional cell culture could hopefully give some answers. We are presently trying to measure the local PO2 in the spheroids using platinum needle electrodes with internal reference (the internal reference makes corrections for disturbances due to membrane potentials possible).

It has been shown previously that an exponential decrease of proliferative activity like that seen for the human tumor cells in Chart 6, a to c, gives rise to a nearly exponential growth (2). This is in agreement with the growth curves seen in Charts 2 and 3b. The hamster spheroids, which had a very thin layer of proliferative cells, had a very short period of nearly exponential growth (Chart 3a).

The growing spheroids studied had a final maximal size to which they could grow. This is in parallel with the findings by Folkman et al. (8), who reported that V79 hamster cells and 2 lines of nonspecified tumor cells could reach maximal diameters of about 2 to 4 mm. The maximal diameters obtained for our spheroids were about 1.0 to 1.5 mm regardless of the amount of nutrients added. Folkman et al. (8) did, however, continue to grow their cultures for several months after the cells had left the initial phase of fast growth. A slow residual growth that might have occurred could explain the difference.

The culture technique described here allows easy testing of the capacity of cells to form and grow as spheroids. The rounded shape of the bottom layer of the agarose gel (Chart 1) favored the chance that only 1 spheroid was formed in each well by forcing the cells to come into contact during sedimentation. Large amounts of equally sized spheroids could thus easily be obtained.

Cell spheroids will probably be valuable model systems for studies of reoxygenation in tumor tissue and other effects of changes in the physical and chemical environment that are of clinical interest [cf. Sutherland and Durand (16)]. The results in Charts 5 and 6 imply that the fraction of hypoxic and poorly nourished cells in a spheroid might vary largely depending on cell line. Thus, the choice of cell type in such studies seems to be of importance.

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**REFERENCES**

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Fig. 3. Sections (2 μm thick) of plastic-embedded spheroids fixed in the phase of rapid growth. a, human glioma 118 MG cells; b, human glioma 251 MG cells; c, HeLa cells; d, hamster embryonic lung cells; e, solid lymphoma 715 cells. H & E.
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