BIOPHYSICAL AND MORPHOLOGICAL CORRELATES OF KINETIC CHANGE AND DEATH IN A STARVED HUMAN MELANOMA CELL LINE

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SUMMARY

An investigation was made of the sequential biophysical and morphological changes that occur as cultured human melanoma cells (MM96) outgrow their supply of nutrient. Simultaneous buoyant-density and velocity-sedimentation fractionation experiments were used to characterize cells from 3 kinetically differing types of culture. Cells from exponential cultures were large, moderately dense and rapidly sedimenting; cells from post-exponential cultures were of intermediate size, less dense and much more slowly sedimenting; and dye-excluding cells from reproductively non-viable late post-exponential cultures were small, of widely variable though generally high density and sedimented moderately rapidly. Although reductive viability was high in cells from both exponential and post-exponential cultures, depletion of clonogenic cells was seen at the extremes of the distribution profiles of cells fractionated by either method. This was particularly evident at the low-density extreme of the buoyant-density profiles where cells retained viability despite their loss of proliferative potential.

As cells became post-exponential, nuclear size diminished in parallel with cell size, the number of microvilli declined, mitochondria condensed, cytoplasm vacuolated, the frequency of osmiophilic vacuolar inclusions rose, chromatin clumped and nucleoli became prominent. Progression to a reproductively non-viable late post-exponential state resulted in a continued parallel fall in nuclear size, increased cytoplasmic blebbing, further mitochondrial condensation, an increased proportion of cytoplasmic vacuoles containing osmiophilic material, the major part of which was melanin, and further clumping and margination of chromatin. Cells progressed rapidly from this newly described pre-apoptotic state to death by apoptosis, a process characterized by the budding and division of cells into a number of ultrastructurally well-preserved membrane-bound fragments.

INTRODUCTION

A major factor limiting the rate at which a tumour will grow is often the vasculature. Actively proliferating cells are confined to the well-nourished regions in the vicinity of blood vessels. Many of the progeny of these cells may migrate or be displaced into less well-nourished regions where proliferation is slow or absent (Tannock, 1968; Hirst & Denekamp, 1979). Further movement brings the cells into an environment where reproductive viability is lost. Beyond lies a region of necrosis.

A simple and effective model of this tumour vasculature-related proliferation gradient is provided by the multicellular spheroid culture system (Sutherland, McCredie & Inch, 1971). With it, all zones may be represented simultaneously.
An alternative in vitro model is one in which, as a result of progressive crowding and nutrient depletion, tumour cells growing as monolayers in unreplenished medium sequentially mimic the populations that occur within successive zones of tumour. By this means we recently described the kinetic changes that occur as cultured human melanoma cells (MM96) outgrow their supply of nutrient (Sheridan & Simmons, 1981). In that study cells from 3 stages of unfed culture were examined in detail. Cells studied were from exponential cultures, post-exponential cultures and reproductively non-viable late post-exponential cultures; exponentially growing cells corresponding to the proliferating cells in the well-nourished areas, post-exponentially growing cells corresponding to the reversibly non-proliferative or slowly cycling cells in areas of marginal nutrition, and late post-exponential cells corresponding to the irreversibly non-proliferative cells of the least well-nourished areas of viable tumour.

Using this culture system, it was shown that as a result of crowding and nutrient depletion the cells proliferated progressively more slowly. The mechanism of this slowing growth rate was a progressive non-uniform increase in phase durations of the cell cycle. Manifestations of this kinetic change were: a moderate increase in the proportion of cells with a $G_1$-like DNA content, a fall in the proportion of cells labelling with $[\text{methyl-}^{3}H]$thymidine and a fall in mitotic index; and in cells separated by velocity sedimentation, a change in the pre- and post-fractionation continuous thymidine-labelling patterns. When cells from post-exponential cultures were replated under favourable conditions, many gradually resumed active proliferation. Continuation of adverse culture conditions resulted in irreversible loss of proliferative potential (late post-exponential cultures) followed shortly by cell death as indicated by permeability to trypan blue dye. DNA estimates showed that although the proportion of cells with a $G_1$-like content was highest in cells from late post-exponential cultures, a sizeable fraction of these dying cells was located elsewhere in the cell cycle. Although a lack of mitoses in the more advanced cultures precluded chromosomal analysis, the observation that the $G_1$-like DNA content did not differ significantly between cells of differing kinetic state suggested that chromosome complement was unaffected by nutritional state.

The present study was designed to investigate the relationship between the bio-physical and the morphological characteristics of cultured neoplastic cells as they outgrow their supply of nutrient. It was also designed to examine the nature and extent of change in the cells at the time reproductive viability is lost and to determine the mode of cell death in nutritionally compromised cells. The study was done to provide a basis for comparison between cultured tumour cells of differing kinetic state and their equivalents in solid tumours.

**MATERIALS AND METHODS**

**Tumour cell line**

The MM96 human malignant melanoma cell line was used in this study because of its relative karyotypic homogeneity mean of $43 \pm 2.3$ (s.d.) (Parsons & Morrison, 1978), range $32-59$ (Pope et al. 1979) and stability, its ease of culture, its ability to melanize (Whitehead &
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Little, 1973) and its ability, under certain circumstances, to show loss of proliferative potential coupled with obvious signs of morphological differentiation (Sheridan & Simmons, unpublished data). The retention of this latter ability was important as it would enable the relationship between unfavourable culture conditions and irreversible differentiation to be investigated. The line, which was established in 1970 from a melanized lymph node metastasis in a 66-year-old Caucasian woman, has been partially characterized with respect to its morphological and cultural characteristics (Whitehead & Little, 1973), its virus-like inclusions (Parsons, Goss & Pope, 1974), its ultraviolet resistance (Chalmers et al. 1976), its DNA repair mechanisms (Goss & Parsons, 1976; Lavin, Willett, Chalmers & Kidson, 1977), and its interactive behaviour with normal fibroblasts (Stephenson & Stephenson, 1978). At the time of this study the cell line had been passaged twice weekly in culture for a cumulative period of about 5 years with no changes detected in its character.

Cell culture and cell counts

The culture medium used in this study was iso-osmotic with human serum (290 mmol/kg H2O) and had a pH of 7.3. It consisted of RPMI 1640 (Gibco), 10.4 g; HEPES, 6.2 g; NaOH, 125 mg; NaHCO3, 2.0 g; tyrosine, 60 mg; thymidine, 2.5 mg; penicillin G, 100,000 units; and streptomycin 60,000 units, in distilled water to 1045 ml. The tyrosine supplement was included to permit melanization. Prior to use the medium was completed by the addition to 10% of foetal calf serum (FCS). Incubation was at 37 °C in a fully humidified atmosphere of 5% O2, 5% CO2 and 90% N2. Preparation of single-cell suspensions and methods used to obtain cell counts were as previously described (Sheridan & Simmons, 1981). The criterion for viability was exclusion of trypan blue by cells with refractile cytoplasm.

Protocol of unfed-cell-culture experiment

The experimental protocol has been fully described elsewhere (Sheridan & Simmons, 1981). Only those aspects essential to the results of this paper are given here. Exponentially growing cells were seeded into 4 sets of replicate 50-mm Petri dishes to yield cultures containing 3.13 x 10^6, 6.25 x 10^6, 1.25 x 10^7 and 2.50 x 10^7 viable cells in 5 ml medium. Incubation was for intervals of 24, 48, 72 or 96 h. At daily intervals cultures were photographed, harvested and counted. In addition to clonogenic cell assays and thymidine-labelling studies, cells were subjected to cell-size analysis, and light and electron microscopy.

Protocol of cell-separation-aided unfed-cell-culture experiments

Exponentially growing cells were seeded in complete medium into 150 cm^2 flasks at the same concentration and to the same depth as used in the unfractionated unfed-cell-culture experiment outlined above. Incubation was for 48, 72, 96 or 120 h, the last 24 h of which was in the presence of 0.04 μCi [2-14C]thymidine. Following photography the cells were harvested and total and viable counts made. After setting aside a sample of the unfractionated cells for later comparison, cells were prepared for both isopycnic centrifugation and unit gravity velocity sedimentation (Sheridan & Simmons, 1981).

The results of 3 simultaneous buoyant-density and velocity-sedimentation cell-separation studies are reported in detail. The first and second concerned cultures that were initiated simultaneously at a viable cell concentration of 1.25 x 10^6 cells/ml, harvested at 48 and 96 h, respectively, and then fractionated. The third was initiated separately at a viable cell concentration of 5 x 10^6 cells/ml, harvested at 120 h and then fractionated. Once fractionated the cells, together with samples of the unfractionated cells, were counted, adjusted in concentration to 250,000 viable cells/ml and then aliquots subjected to a number of investigations including secondary 24- and 48-h continuous [methyl-3H]thymidine-labelling, clonogenic cell assay, cell-size analysis, and light and electron microscopy. Methods used in obtaining the results presented in this paper are described below.

Isopycnic centrifugation

The procedure of Shortman (1968) as modified by Sheridan & Finlay-Jones (1977) was used. Separation was in 10%–20% (w/w) linear albumin density-gradients at pH 5.2, centrifugation being at 4000 g for 60 min at 4 °C. Fraction collection and density determinations...
were, with minor modification (Sheridan & Simmons, 1981), as previously described (Sheridan & Finlay-Jones, 1977).

**Unit gravity velocity sedimentation**

Separation was in 0.3%–2% (w/w) albumin gradients at pH 5.2, contained within a Sta-Put (SP-180) sedimentation chamber. Cell loading, generation of the stabilizing albumin gradient fraction collection and calculation of sedimentation rate were as previously described (Sheridan & Simmons, 1981).

**Albumin salt solution**

A stock albumin salt solution (40%, w/w, pH 5.2, 290 mmol/kg H2O real osmolality) was prepared and stored as previously described. Prior to use, it was diluted to the appropriate concentration with unbuffered balanced salt solution of human serum osmolality (Sheridan & Finlay-Jones, 1977; Sheridan & Simmons, 1981). In order to enable comparison between results, the same batch of albumin salt stock solution was used in all experiments, no matter which method of cell separation was used.

**Clonogenic cell assay**

Cells were mixed thoroughly with soft agar medium (Sheridan & Simmons, 1981) to give a concentration of 250 viable cells/ml. Aliquots (1 ml) of this suspension were dispensed into quadruplicate 35-mm plastic Petri dishes, allowed to gel and then incubated in a fully humidified atmosphere of 5% O2, 5% CO2 and 90% N2 at 37 °C. Aggregates of more than 50 cells at 10 days were scored as colonies.

**Post-fractionation [methyl-3H]thymidine labelling and measurement of radioactive thymidine incorporation**

Cells were cultured at 12,500 viable cells per 250 µl of [3H]thymidine-supplemented medium (2.0 µCi/ml) in quadruplicate wells of 2 microtitre trays. Cultures were harvested at 24 and 40 h and the cells deposited onto glass-fibre mats. Once dry the glass-fibre-adherent samples were placed in vials, scintillant was added and both high- (14C) and low-energy (3H) emissions counted. Results were expressed as c.p.m. per 50,000 viable cells (at time primary culture was harvested) per hour exposure to isotope within the period of interest.

**Cell and nuclear-size analysis**

Immediately prior to electronic sizing 50,000 viable cells were suspended in 10 ml of Ca- and Mg-free phosphate-buffered saline of human serum osmolality (HPBS). Electronic cell-sizing was done with a Celsoscope particle counter (Particle Data, Illinois) fitted with a 120 µm orifice. Cell-size information was processed by computer.

Cell and nuclear-size determinations were also made at ×1000 on stained cytocentrifuge preparations (30,000 cells/slide, 20 g, 10 min), using a calibrated ocular micrometer as reference. One hundred sequential cells and their nuclei were measured in each sample.

**Morphological studies**

For light microscopy, samples were cytocentrifuged and then stained with May–Grünewald (BDH) and Giemsa (BDH) stains for general morphological examination or by the Masson–Fontana method (Pearse, 1972) for the demonstration of melanin. Preparations were examined at magnifications of ×400 and ×1000.

For electron microscopy, cells were fixed by immersion in 3% glutaraldehyde for 1–2 h at 4 °C, post-fixed in osmium tetroxide for 1–2 h at room temperature and then embedded with centrifugation in 2% agar. Small blocks of cells in agar were dehydrated in ethanol and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H300 electron microscope.
Table 1. Effect of cell crowding and progressive nutrient depletion on cell and nuclear size

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Size assessed by electronic sensing zone method</th>
<th>Size visually assessed on stained cytocentrifuged preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median cell diameter (μm)</td>
<td>Mean cell diameter ± S.D. (μm)</td>
</tr>
<tr>
<td>Exponential culture</td>
<td>17.75</td>
<td>28.03 ± 4.47</td>
</tr>
<tr>
<td>Post-exponential culture (early)</td>
<td>14.42</td>
<td>23.87 ± 3.58</td>
</tr>
<tr>
<td>Post-exponential culture</td>
<td>14.10</td>
<td>22.27 ± 4.90</td>
</tr>
<tr>
<td>Late post-exponential culture</td>
<td>12.86</td>
<td>20.53 ± 5.22</td>
</tr>
</tbody>
</table>

|                             | Mean nucleus diameter ± S.D. (μm) | Mean cell diameter ± S.D. (μm) |
| Exponential culture         | 16.64 ± 2.91                  | 0.60 ± 0.08                   |
| Post-exponential culture (early) | 13.55 ± 2.58                  | 0.57 ± 0.07                   |
| Post-exponential culture    | 12.86 ± 2.06                  | 0.59 ± 0.09                   |
| Late post-exponential culture | 12.60 ± 2.76                  | 0.62 ± 0.09                   |
RESULTS

Studies on unfractionated cells

In the unfed-cell-culture experiment, cells from exponential cultures were shown to be large, those from post-exponential cultures intermediate and those from reproductively non-viable late post-exponential cultures small. Microscopic examination of cells from this experiment showed that nuclear size remained proportional to cell size (Table 1).

Light-microscopic examination of these cells, prior to their detachment from the plastic surfaces on which they had grown, revealed no evidence of alignment. In sparse exponential cultures polygonal cells with 2-5 dendrites predominated (Fig. 1 A). As crowding increased, the cells became smaller and their processes more difficult to visualize. Occasional cells with up to 4 dendrites were seen (Fig. 1 B). In addition to large numbers of detached rounded-up cells, many of which were permeable to dye and often heavily melanized, unfed late post-exponential cultures contained a sparse coverage of generally lightly melanized, shrunken attached cells each with 2 or 3 thin dendritic processes (Fig. 1 C).

Electron microscopy of the harvested, previously attached cells revealed clear differences between unfed cells of differing kinetic state. The nuclei of cells from exponential cultures, in which 89-94% of the cells were viable (and of these 61-79% clonogenic) generally had finely dispersed chromatin and prominent nucleoli (Fig. 2 A, B). Their cytoplasm contained well-developed Golgi complexes, small amounts of rough endoplasmic reticulum and moderate numbers of sausage-shaped mitochondria. A small amount of cytoplasmic vacuolation was also seen, where, in some cases, appeared to be melanin deposition. Microvilli were numerous, with a low proportion of cells showing cytoplasmic blebbing. Cells from post-exponential cultures, in which 86-93% were viable (and of these 44-63% clonogenic), differed in frequently having clumped chromatin with less prominent nucleoli (Fig. 2 C, D). Golgi complexes and endoplasmic reticulum were still clearly defined but the mitochondria were condensed. Cytoplasmic vacuolation was increased and frequently extensive. While there was an increased deposition of osmiophilic material, the majority of the vacuoles contained little visible material, microvilli were less numerous and cytoplasmic blebbing was common. Cells from late post-exponential cultures were of variable morphology. Cultures initiated with 1-25 x 10^6 viable cells, when harvested at 96 h, contained 74% viable cells, of which 3% were clonogenic. These cells showed clumping and margination of chromatin, mitochondria that were

Fig. 1. 'In situ' morphological appearance of unfed MM96 cells. A. An exponential culture (initially 1-25 x 10^6 viable cells per 50-mm Petri dish) at 48 h. Cells were polygonal with 2-5 dendrites. x 80. B. A post-exponential culture (initially 2-5 x 10^6 viable cells per 50-mm Petri dish) at 72 h. Cells were smaller, more closely packed and processes more difficult to see. x 80. C. A late post-exponential culture (initially 1-25 x 10^6 viable cells per 50-mm Petri dish) at 96 h. Attached cells were generally lightly melanized and shrunken with 2-3 thin dendritic processes. Large numbers of detached rounded up cells, many of which were heavily melanized and permeable to dye, were also present. x 80.
Fig. 2. Electron micrographs of enzymatically detached unfed MM96 cells. A, B. Cells from an exponential culture (initially $0.325 \times 10^6$ viable cells per 50-mm Petri dish) at 48 h. $\times 2000$ and $\times 5000$, respectively. C, D. Cells from a post-exponential culture (initially $2.5 \times 10^6$ viable cells per 50-mm Petri dish) at 72 h. $\times 2000$ and $\times 5000$, respectively.
Fig. 2. Cont. E, F. Cells from a predominantly pre-apoptotic late post-exponential culture (initially $1.25 \times 10^8$ viable cells per 50-mm Petri dish) at 96 h. $\times$ 2000 and $\times$ 5000, respectively. G, H. Cells from a predominantly apoptotic late post-exponential culture (initially $2.5 \times 10^6$ viable cells per 50-mm Petri dish) at 96 h. $\times$ 2000 and $\times$ 5000, respectively.
grossly shrunken and dense, and a high proportion of cytoplasmic vacuoles containing heavy deposits of osmiophilic material, the major part of which was melanin. Microvilli were no longer seen and cytoplasmic blebbing remained common (Fig. 2E, F). Cultures initiated with $2.5 \times 10^6$ viable cells, when harvested at 96 h, contained 60% viable cells, of which 1% were clonogenic. These cells showed extreme morphological changes. Many showed a further increase in clumping and margination of chromatin, nuclear and cytoplasmic condensation, and formation of large protuberances that separated to form membrane-bound ultrastructurally well-preserved fragments, a process that has been termed apoptosis (Kerr, Wyllie & Currie, 1972). Due to the heavy deposits of melanin and cytoplasmic condensation, Golgi complexes and endoplasmic reticulum were rarely seen in these cells (Fig. 2G, H). Examination of the dye-permeable detached rounded up cells and debris from late post-exponential cultures showed these to be apoptotic cells and apoptotic fragments (apoptotic bodies) that had undergone secondary disintegration with membrane disruption. Light-microscopic observation on stained cytocentrifuge preparations yielded results that were consistent with the electron microscopic findings.

**Studies on fractionated cells**

In those studies reported in detail, viable cell numbers in cultures begun with $1.25 \times 10^6$ cells/ml had increased 3.39-fold by 48 h. At this time the cells were growing exponentially. Continuation to 96 h resulted in a further increase in viable cell numbers of only 1.65-fold at which time the cells were post-exponential. Cells from neither type of culture were visibly melanized when harvested. Viable cell numbers in cultures initiated with $5 \times 10^6$ cells/ml had peaked and were falling rapidly at 120 h. Counts on the remaining adherent cells in these late post-exponential cultures showed that viable cell numbers had fallen to 0.98 of the number originally seeded. At this time the majority of the cells were melanized. The microscopic appearances immediately prior to harvest of the exponential, post-exponential and late post-exponential cultures were indistinguishable from those shown in Fig. 1. With all 3 types of culture over 94% of the harvested adherent cells were classified as viable according to our criteria.

The size-distribution profiles of the unfractionated cells from the exponential, post-exponential and late post-exponential cultures are shown in Fig. 3. It can be seen that, although the cells became smaller as culture conditions deteriorated, little change occurred in the heterogeneity of the volume of the cells. The difference in size between the cells of Fig. 3 and those of comparable kinetic state represented in Table 1 is thought to be due to the rate at which nutrition-related kinetic change occurred. Cells from cultures initiated at low concentration progressed more slowly to a late post-exponential state and in doing so showed greater changes in size than cells from cultures initiated at high cell concentrations (fig. 2, Sheridan & Simmons, 1981).

Fig. 4 shows that cells became less dense as they passed from an exponential to a post-exponential state. Transition to a late post-exponential state was accompanied by a return to an even higher density than that of cells from exponential cultures.
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Viable cell recoveries from these experiments were calculated to be 98, 100 and 97%, respectively, of the numbers loaded. Five additional buoyant-density experiments, 2 involving unmelanized exponential cells and 3 involving unmelanized post-exponential cells, gave results that were in agreement with the graphically presented findings.

Relative pre-fractionation [³⁴Cl]thymidine incorporation corresponded well with the distribution of clonogenic cells except at the low-density extreme, where a disproportionately low number of cells subsequently proliferated (Fig. 5). Mean clonogenicities of the viable fractionated cells were 51, 45 and 1%, respectively, for cells from the 3 kinetically differing types of culture. In none of the 8 buoyant-
Fig. 4. Total viable cell and clonogenic cell buoyant-density distribution profiles of the unfed MM96 cells that are described in detail in the text. The clonogenic cell profiles are drawn in proportion to their contributions to the total viable cell profiles. Modal diameters of cells from several evenly spaced fractions are indicated above the profiles. Vertical solid lines indicate the medians of the total viable cell numbers; vertical broken lines the medians of the total clonogenic cell numbers.

A. Exponential culture. B. Post-exponential culture. C. Late post-exponential culture.
Total viable cells (●—●); total clonogenic cells (○—○).

density experiments were clonogenic cells significantly enriched in any particular fraction. Although clonogenic cells were depleted at both extremes, particularly at the low-density extreme, in each case the median density of the clonogenic cells corresponded closely to the median density of the viable cells. Cells from the low-density extreme of fractionated exponential and post-exponential cultures were of interest because the majority of these cells that had synthesized DNA in the previous 24 h had, on fractionation, lost the capacity for continued proliferation; yet, as
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Fig. 5. Clonogenicity, 24-h pre-fractionation [2-14C]thymidine incorporation and o to 24 and 24 to 40-h post-fractionation [methyl-3H]thymidine incorporation buoyant-density distribution profiles of the unfed MM96 cells that are described in detail in the text. Clonogenicity is expressed as % cells forming colonies/fraction. Incorporation is expressed as c.p.m./50,000 viable cells at time of fractionation per h exposure to isotope per fraction. A. Exponential culture. B. Post-exponential culture. [14C]thymidine pre-fractionation label (•—•); [3H]thymidine post-fractionation label, 0–24 h (A—A), 24–40 h (A—A). 14C incorporation rate on left and 3H incorporation rate on right of scale.

shown by the absence of a fall in the levels of pre-fractionation incorporated [14C]-thymidine between 24 and 40 h after fractionation, they had retained viability. These findings are in agreement with observations on murine solid tumours, where it was found that the least dense cells, although viable, were predominantly non-clonogenic and non-tumourigenic (Sheridan & Finlay-Jones, 1979). The failure of the vast majority of the cells from late post-exponential cultures to form colonies, or to survive in secondary culture, showed that they, by contrast, were a dying population.

Examination of the cell-size distribution profiles of 5 evenly spaced fractions from a total of 9 buoyant-density experiments revealed an overall tendency, irrespective of kinetic state, for low-density cells to be larger than high-density cells. However, size ranges of cells from individual fractions were often as broad as those of the unfractionated cells and occasional fractions were found, particularly towards the density extremes, that were not in agreement with the general trend. The modal diameters of cells in selected fractions from the buoyant-density experiments, described in detail in this section, are shown in Fig. 4. Microscopic examination of stained cytocentrifuge preparations failed to reveal significant differences in the mean
Fig. 6. Total viable cell and clonogenic cell velocity-sedimentation distribution profiles of the unfed MM96 cells that are described in detail in the text. The clonogenic cell profiles are drawn in proportion to their contributions to the total viable cell profiles. Modal diameters of cells from several evenly spaced fractions are indicated above the profiles. Vertical solid lines indicate the medians of the total viable cell numbers; vertical broken lines the medians of the total clonogenic cell numbers. A. Exponential culture. B. Post-exponential culture. C. Late post-exponential culture. Total viable cells (●—●); total clonogenic cells (○—○).

nucleus diameter:cell diameter ratios of cells from different fractions, a result in accord with an observed lack of sorting according to cell-cycle location (Sheridan & Simmons, 1981).

Electron microscopy of exponentially growing cells revealed a general tendency for both microvilli and nucleoli to become more numerous as cell density increased. Also with post-exponential cells nucleoli became more numerous as density increased. With both post-exponential and late post-exponential cells, increasing density was associated with an increase in electron density of both cytoplasmic and nuclear
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matrix, and an increase in vacuolation and accumulation of osmiophilic material. Apoptotic cells were confined to the high-density fractions from the late post-exponential cultures. In no case were density-related differences noted in the number of mitochondria or other cytoplasmic components.

The velocity-distribution profiles of viable cells from the same cultures as used in the previously described buoyant-density experiments are shown in Fig. 6. The changes in sedimentation rate that occurred as cells passed from an exponential to a post-exponential and finally a late post-exponential state were complex. Initially, sedimentation rate decreased as a result of the combined fall in cell size and density. Terminally, sedimentation rate again increased due to an increase in density that was insufficiently offset by the continued fall in cell size. Recoveries of viable cells from these experiments were calculated to be 96, 87 and 92%, respectively, of the numbers loaded. Repeat experiments, whilst supporting the sedimentation rates found for the unmelanized cells, indicated that melanized cells sedimented about 1 mm/h faster than otherwise comparable unmelanized cells.

Mean clonogenicities of the viable fractionated cells were calculated to be 39, 43 and 1%, respectively, for cells from the 3 kinetically differing culture types. In none of a total of 8 separations were clonogenic cells significantly enriched, although moderate depletion was seen at both extremes of sedimentation rate. The median velocities of the clonogenic cells were therefore close to the median velocities of the viable cells (Fig. 6).

Estimates of cell size showed that, within each experiment, the faster the sedimentation rate the larger the cells. For a given sedimentation rate, however, cells from late post-exponential cultures were significantly smaller than those from any less-advanced culture, a finding related to the higher density of these cells. Microscopic examination of stained cytocentrifuge preparations showed that in each case the rapidly sedimenting cells had larger nuclei and slightly lower nucleus diameter:cell diameter ratios than the slowly sedimenting cells, presumably due to the relationship between sedimentation rate and cell-cycle location.

Electron microscopy of exponentially growing cells showed, with increasing sedimentation rate, a tendency for blebbing to decrease and vacuolation to increase. With post-exponential cells, increasing sedimentation rate was associated with increasing vacuolation and accumulation of osmiophilic material. Late post-exponential cultures showed an increase in osmiophilic material and increased cellular budding with increasing sedimentation rate. Sedimentation rate-related changes in such characteristics as microvilli (when present) or number of mitochondria and other cellular organelles were not found in any instance.

DISCUSSION

As exponentially growing MM96 cells became crowded, cell size decreased. This fall in cell size continued as cells entered a post-exponential and then a late post-exponential state. Similar observations were made by Meisler (1973) with respect to daily-fed 3T3 cells and their simian virus 40-transformed derivative. It was,
however, also found with the MM96 cell line that nuclear diameter diminished in a way that closely paralleled that of cell diameter. Although slowly cycling or stationary cells in post-exponential MM96 cultures were distributed throughout all phases of the cell cycle, their smaller nuclear diameter and their characteristic of gradually resuming active proliferation when reseeded under favourable conditions (Sheridan & Simmons, 1981), were in keeping with the finding of Yen & Pardoe (1979) that correlated nuclear size of $G_0$ $3T_3$ cells with cell-cycle re-entry rate.

Light- and electron-microscopic studies showed that changes in cell and nuclear size were accompanied by marked morphological changes. With the exception of melanization, these changes were not characteristic of differentiation. Thus this common mode of loss from the growth fraction (Cooper, Bedford & Kenny, 1975) was not seen despite the capacity of this line to express profound morphological signs of differentiation in response to high doses of [H]thymidine (Sheridan & Simmons, unpublished data).

Although many of the progressive changes described in unfed MM96 cells were similar to those observed under unfavourable conditions in other systems, the relationship of these changes to the kinetic state of the cells has never before been examined in detail.

Cytoplasmic blebbing has been described under normal culture conditions (Sauk, White & Witkop, 1975), although it also increased as a manifestation of membrane damage following chemical injury (Cooper et al. 1975; Trump & Arstila, 1975) and mechanical trauma (Sheridan & Finlay-Jones, 1979). Vacuolation and production of osmiophilic dense bodies have been observed in ‘stationary phase’ L cell cultures where they are postulated to result from autophagic activity (Gordon, 1977). Condensed mitochondria have also been observed in such cultures (Gordon, 1977) as well as in early hypoxic cell cultures (Penttila & Trump, 1975). Clumping and margination of chromatin have been found as a result of chemical injury, as a result of ischaemia (Trump & Arstila, 1975) and, together with loss of microvilli, as a result of hypoxia in vitro (Penttila & Trump, 1975). In these examples, however, cells progressed to an eventual swollen state that terminated in rupture of plasma and organelle membranes with dissolution of organized structure, a process that has long been termed coagulative necrosis.

Cellular changes identical to the most extreme changes observed in the late post-exponential cultures have been described in untreated human solid tumours, where they are considered to represent a common mode of cell death (Kerr & Searle, 1972). Although this mode of cell death, apoptosis, is maximal in regions bordering on areas of coagulative necrosis, it occurs classically as a scattered phenomenon (Kerr et al. 1972). The cause of scattered apoptosis in solid tumours is unknown. It seems likely, however, that the zonal apoptosis observed at the periphery of areas of coagulative necrosis is related to slowly progressing ischaemia. By contrast, rapidly induced cell death, as caused by infarction, generally results in coagulative necrosis.

In unfed MM96 cultures, apoptosis was by far the most common mode of cell death. Unlike the situation in solid tumours, where apoptotic bodies are rapidly
engulfed by neighbouring cells (Kerr et al. 1972), cells dying by this means in tissue culture became detached and shortly afterwards underwent secondary disintegration. It would seem likely that the less extreme changes observed in the majority of the reproductively non-viable, yet refractile and trypan blue dye-excluding, cells from the late post-exponential cultures may be representative of a previously undescribed pre-apoptotic state.

The progressive changes observed in the sedimentation rate of unfed MM96 cells, although complex, are explicable on the basis of the known relationship of this parameter to cell size and density (Miller & Phillips, 1969).

Density-distribution profiles of MM96 cells, although narrower, resembled those of exponential and post-exponential Chinese hamster ovary (CHO) and EMT6 cells in being unimodal, a finding that contrasted with the often multimodal density-distribution profiles of cells taken directly from murine solid tumours (Grdina, Milas, Mason & Withers, 1974b; Ng & Inch, 1978; Sheridan & Finlay-Jones, 1977, 1979). In addition, with the exception of cells from late post-exponential cultures, the buoyant-density distributions of MM96 cells were very much narrower than similarly fractionated cells obtained directly from a variety of murine solid tumours (Sheridan & Finlay-Jones, 1977, 1979). Similar observations have been made by others (Grdina, Meistrich & Withers, 1974a; Grdina et al. 1974b; Ng & Inch, 1978).

It is probable that the homogeneity of the relative density of monolayer culture-derived cells results from both their uniform nutrition, with consequent kinetic uniformity, and their single-cell lineage; whereas the heterogeneity of the density of tumour-derived cells results from both their previously variable nutrition, with consequent kinetic variability, and their mixed neoplastic and normal-cell lineage.

Why the density of MM96 cells was affected in a biphasic manner as nutritional conditions deteriorated and crowding increased is unclear. Presumably the fall in density that occurred as cells entered a post-exponential state was due to a disproportionate loss of high-density cellular components and/or a relative increase in low-density components. With unfed L cell cultures, entry into a post-exponential state was shown to be characterized by a fall in cell size that was accompanied by large falls in cellular DNA, RNA and protein content (Glinos, Werrlein & Papadopoulous, 1965). In other studies both total cellular lipid and lipid to protein ratios were found to increase as L cell cultures became post-exponential (Gordon, 1977). Changes such as these could produce a fall in cell density. Except for the fact that the DNA contents of unfed MM96 cells were little reduced on entering the post-exponential state (Sheridan & Simmons, 1981), electron microscopy revealed changes that were similar to those demonstrated by Gordon (1977) in post-exponential L cells. It is of interest that monolayer cultures of unfed CHO (Grdina et al. 1974a) and daily-fed EMT6 cells (Ng & Inch, 1978) increased in density as they entered a post-exponential state. Presumably the balance of biochemical events that determine cell density in the post-exponential state varies from cell type to cell type. Such a balance may also be affected by the nutrition of the culture (Glinos et al. 1965). In the present study a considerable proportion of the osmiophilic material observed in the late post-exponential cultures was melanin. The higher sedimentation rates of cells from
melanized cultures than cells of similar size and kinetic state from unmelanized cultures suggests that melanin or related products may exert an effect on cell density. The increased osmiophilia of the nuclear and cytoplasmic matrices of many of these late post-exponential cells also suggested an increase in density.

A clear analogy exists between the sequential kinetic changes that occur in unfed MM96 cell cultures (Sheridan & Simmons, 1981) and the kinetic state of tumour cells at varying distances from blood vessels (Tannock, 1968; Hirst & Denekamp, 1979). The morphological relationship between cells of known kinetic state growing in unfed cultures with those growing in vitro as solid tumours is, however, less clear. Detailed morphological examination, coupled with cell separation and kinetic studies on tumours arising from xenografted MM96 cells, is expected to clarify this relationship.

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REFERENCES


Kinetic change and death in starved cells


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