Growth of HeLa Cells in Diffusion Chamber Cultures in Vivo

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SUMMARY

In order to investigate some characteristics of Millipore diffusion chamber cultures (DCC) and their potential for the quantitation of tumor cell growth kinetics, HeLa S-3-cells were grown in diffusion chambers implanted i.p. in mice. HeLa cells grew pseudologarithmically in DCC with a population-doubling time of approximately 2.8 days when 9.2 to 12.0 x 10^6 cells were placed in the chambers initially. The population-doubling time varied as a function of the initial inocula size but was always longer than the in vitro population-doubling time of 1.2 days. Comparable harvests of cells from experiment to experiment varied quantitatively by no more than a factor of 1.8. This variation somewhat limits the use of DCC for the quantitation of subtle effects. The fate and morphology of HeLa cells in DCC were determined from scanning electron microscopy. DCC might be exploited in clinical studies of the growth of patients' tumors and the effects of various therapies in a host-mediated system.

INTRODUCTION

Conceptually, the use of diffusion chambers to grow human tumor cells in vivo may present several advantages over other in vivo and in vitro model systems. Potentially advantageous features of DCC include: (a) tumor cells may be quantitatively grown in an in vivo environment analogous to the avascular areas of some solid tumors; (b) the effects of chemotherapeutic drugs and their host-mediated metabolites on the entire population of tumor cells in vivo may be assayed; and (c) host response to acellular products of growing tumor cells, such as antigens, might be measured. With these potential advantages of using DCC, it is perhaps surprising that few researchers have investigated tumor cells grown in DCC. Evgenjeva (4–6) found that 1 experimental and 4 human tumors grew in diffusion chambers and were morphologically similar to the original tumor. Lauerman et al. (7) studied the proliferation of 4 types of malignant cells in diffusion chambers and observed a slower experimental growth rate of all cell types in diffusion chambers as compared to corresponding in vitro cultures. They suggested that the mode of tumor cell proliferation in DCC may be more representative of tumor cell growth in vivo than the proliferation of tumor cells in vitro. In order to investigate further the DCC of tumor cells, the experiments described below were designed to determine: (a) the reproducibility of cellular recovery as a function of time after implantation, (b) the influence of host irradiation on the growth curve, (c) the growth rate as a function of initial inoculum size, and (d) the morphological fate of HeLa cells in DCC.

MATERIALS AND METHODS

In general, HeLa cells were taken from a log-phase suspension culture and grown in diffusion chambers that were implanted into the peritoneum of mice. The suspension culture of HeLa cells was continuously stirred in a medium of 92.1% minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.; Catalog No. 238), 6.5% fetal calf serum (Grand Island Biological; Catalog No. 614), 0.5% penicillin-streptomycin (Grand Island Biological, Catalog No. 514), and 0.9% L-glutamine (Grand Island Biological; Catalog No. 503-L). Diffusion chambers consisted of Millipore filters (Millipore Corp., Bedford, Mass., Catalog No. GSWP01300) of mean pore size ≤0.22 μm cemented to both sides of a Lucite ring (10 mm diameter x 2 mm) with an access hole drilled in the side. Each diffusion chamber (approximate volume, 130 μl) was filled through the access hole with 100 μl of HeLa cells suspended in culture medium; the diffusion chambers were sealed with a nylon plug cemented into the access hole, and 2 of the chambers were implanted into the peritoneum of each 8- to 12-week-old, male, Swiss albino (Hale-Stoner, BNL strain) host for 30 min, 1 to 7 days, or 14 days. Fourteen-day DCC were removed from their hosts after 7 days, wiped clean, and reimplemented into secondary hosts. In the quantitative growth studies, 6 to 10 diffusion chambers were recovered at the appropriate times, wiped clean, and shaken for 60 to 90 min in a Pronase solution (0.15% Pronase, Calbiochem, San Diego, Calif., Catalog No. 53702; 5.0% Ficoll, Pharmacia, Uppsala, Sweden, in a 0.9% NaCl solution buffered to pH 7.2 by 20 mM KH₂PO₄ and 13 mM NaOH) to facilitate the harvest of cells. Cells were harvested with repeated washings of the chamber with approximately 1 ml of isotonic (Coulter Diagnostics, Inc., Hialeah, Fla., Catalog No. B3157-11). The number of cells recovered per chamber was calculated from the mass of the chamber washings and from Coulter counts of the washings. The morphology of the cells in culture was studied with light and SEM.
Study 1 consisted of 7 experimental runs and was designed to quantitatively compare the reproducibility of cellular recovery from experiment to experiment. Four experimental runs utilized unirradiated hosts. Cells were harvested from 6 to 10 cultures with an initial inoculum of 9.2 to 12.0 \times 10^5 cells/chamber, 30 min, 1 to 7 or 14 days after implantation. Parallel determinations were made by comparing 3 of the runs with identical cultures implanted into mice irradiated with 750-rad X-rays (250 kVp, 30 ma, 1.0 mm Al, 0.5 mm Cu, 120 rads/min) 18 hr prior to implantation. In each case, cellular recovery was calculated as the percentage of the 30-min recovery, and the population-doubling time during Days 0 to 7 was calculated from a linear regression of the weighted means of the percentage of cells recovered from the various runs plotted as a function of time (Charts 1 and 2). The population assayed included all cells, viable and necrotic, counted by the Coulter counter. A total of 463 DCC were assayed for these 2 determinations.

Study 2 was designed to determine the growth rate, expressed as population-doubling time, of HeLa cells in DCC hosted by unirradiated mice as a function of the initial inoculum size of cells per chamber. Population-doubling times were calculated on the basis of the mean percentage of cells recovered on Days 0 to 7 after implantation. Each study was designed to determine the growth rate, expressed as a function of the initial inoculum size of cells per chamber. Population-doubling times were calculated on the basis of the mean percentage of cells recovered from experiment to experiment. Four experimental runs utilized unirradiated hosts. Cells were harvested from 6 to 10 cultures with an initial inoculum of 9.2 to 12.0 \times 10^5 cells/chamber, 30 min, 1 to 7 or 14 days after implantation. Parallel determinations were made by comparing 3 of the runs with identical cultures implanted into mice irradiated with 750-rad X-rays (250 kVp, 30 ma, 1.0 mm Al, 0.5 mm Cu, 120 rads/min) 18 hr prior to implantation. In each case, cellular recovery was calculated as the percentage of the 30-min recovery, and the population-doubling time during Days 0 to 7 was calculated from a linear regression of the weighted means of the percentage of cells recovered from the various runs plotted as a function of time (Charts 1 and 2). The population assayed included all cells, viable and necrotic, counted by the Coulter counter. A total of 463 DCC were assayed for these 2 determinations.

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Study 3 was designed to determine the morphological fate of HeLa cells after 30 min or 5 to 6 days in DCC, and in other DCC to ascertain the effects of Pronase on the cells. Thirty min after implantation, 4 groups of 3 or 4 DCC with an initial inoculum size of 10^5 or 7.4 \times 10^5 cells/chamber were taken at random from other diffusion chamber studies. The 1st 2 groups consisted of cultures with an initial inoculum of 10^5 or 7.4 \times 10^5 cells/chamber and were taken after 30 min of culture. The 3rd group had an initial inoculum of 7.4 \times 10^5 cells/chamber and was recovered 6 days after implantation. Recovered chambers were wiped clean and gently opened with a pointed scalpel blade. The interiors of the chambers were gently rinsed with Isoton and fixed in 3% buffered glutaraldehyde. The 4th group of cultures had an initial inoculum of 7.4 \times 10^5 cells per chamber and was recovered 6 days after implantation. This last group was wiped clean, shaken in the Pronase solution for about 70 min, washed with Isoton for a quantitative recovery of the cells, and fixed in 3% buffered glutaraldehyde.

Some of the 6-day cultures without Pronase treatment were imbedded in paraffin, sectioned for light microscopy, and stained with hematoxylin and eosin. Portions of the chambers from all 4 groups were processed for SEM. Cells from DCC were washed in cacodylate buffer and then washed in distilled water containing 1 to 2% chloroform. The specimens were freeze dried for SEM by blotting away excess water and plunging the specimen into liquid nitrogen. The frozen specimens were placed in a chilled test tube which was then inserted into a brass block at liquid N\textsubscript{2} temperature. The specimens and assembly were placed under vacuum in a glass vessel with a thermistor attached. After 8 hr, the water sublimed away and the specimens and assembly reached room temperature. The specimens were then mounted on SEM specimen plugs and coated lightly with carbon and silver. HeLa cells from the initial suspension culture were used for the control SEM study, and were fixed with an excess of 3% buffered glutaraldehyde at 2\textdegree C for 2 weeks. Fixed cells from suspension culture were dehydrated with cold acetone and processed through the Sorvall critical-point drying apparatus (Ivan Sorvall, Inc., Newtown, Conn.) according to the technique described by Porter et al.
RESULTS

In Study 1, the mean cellular recovery 30 min after implantation of DCC was 65.8 ± 5.5% (S.E.) of the initial inoculum, with a range of 54.5 to 94.8%. The number of cells recovered from DCC in both unirradiated and irradiated hosts increased in an apparent exponential manner from 0 to 7 days after implantation (Charts 1 and 2). After 14 days of culture, there was either no increase in cellular recovery, compared with the 7-day culture (Chart 1), or there was a lower recovery rate than would have been expected from a continued exponential growth (Chart 2). A linear regression of the weighted means of the 4 and 3 runs, respectively, weighted according to the number of chambers per run, yielded a population doubling time of 2.8 days with a correlation coefficient of 0.85 in unirradiated hosts and 2.7 days with a correlation coefficient of 0.94 in irradiated hosts. The mean doubling time of the stock in vitro suspension culture was approximately 1.2 days. Plots of cellular recovery versus days in DCC for single runs gave a smooth curve, provided that the same individual harvested the cells on every day of the assay. The variation of the cellular recovery, measured by mean ± S.E., on any particular day’s assay was less than 12% of the mean value (Charts 1 and 2). However, on specific days in parallel runs, means varied by as much as a factor of 1.8. The growth rate measured for individual runs was relatively constant, with a doubling time of 2.8 ± 0.1 days (range, 2.6 to 3.2 days) for cultures in unirradiated hosts and doubling time of 2.6 ± 0.1 days (range, 2.5 to 2.7 days) for cultures in irradiated hosts. Growth rates calculated from the weighted means of all DCC in unirradiated hosts or irradiated hosts yielded doubling times of 2.8 and 2.7 days, respectively. The apparent discrepancy of doubling times of DCC in irradiated hosts is due to compensating variations between the individual runs. In separate experiments performed with diffusion chambers made with Nuclepore or Millipore filters washed with 100° glass-distilled water to remove detergents (2), no significant decrease in the population-doubling time was observed (unpublished data).

From Study 2, it was found that the more cells put in the chambers initially, the longer the population takes to double. Chart 3 illustrates a linear relationship between cellular inoculum size and population-doubling time. The linear regression (correlation coefficient, 0.82) of the doubling time versus initial inoculum size for 8 experiments indicated that the doubling time would increase from 1.5 to 3.0 days in DCC with initial inocula in the range of 1.5 to 12 × 10⁶ cells per chamber. Extrapolation of the regression line to 0 cells per chamber intercepts the doubling-time ordinate at 1.3 days (Chart 3).

In Study 3, HeLa cells with several types of surface topography were found in both suspension culture and DCC. The suspension cultures were comprised mainly of cells densely covered with microvilli. Cells with microvilli and/or blebs or flasks were also found in suspension culture. After 30 min in DCC, most cells were spherical with microvilli forming attachments to the Millipore substrate. Some cells had a “fried-egg” configuration with and without blebs. The maximum length of microvilli was approximately 15 μm. After 5 or 6 days of DCC, most of the cells were found next to the filter surface, and a proteinaceous clot had formed over the cellular layer. Necrosis was extensive as judged from the light microscopy. The cells in the 5 to 6 day cultures were somewhat flattened, in multilayers, and interconnected with numerous processes; cells were covered with microvilli. An extensive network of processes interconnected the cells in 6-day DCC. Treatment of the cells with Pronase and Isoton washings resulted in the disappearance of microvilli and intercellular processes.

DISCUSSION

The growth curves of HeLa S-3-cells in DCC show an apparent logarithmic increase in the number of cells recovered during the 1st 6 to 7 days, followed by a stationary phase. Furthermore, the growth curves of cultures in irradiated and unirradiated hosts do not differ markedly (Charts 1 and 2). This observation is in contrast to the stimulated growth of hemic cells in DCC hosted by irradiated mice or goats (1, 8). Apparently the stimulatory effect of host irradiation on DCC of hemic cells is limited to specific cell types or is more pronounced in hemic cell cultures. The percentage of cells recovered at a particular time varied by no more than a factor of 1.8 from experiment to experiment where the initial inoculum ranged from 9.2 to 12.0 × 10⁵ cells/chamber. Variations of recovery did not correlate with differences in initial inocula size. The scatter of the means of separate runs around the linear regression was less in irradiated hosts (correlation coefficient, 0.94) than in unirradiated hosts (correlation coefficient, 0.85). It is concluded from these growth curves that the variation from experiment to experiment limits the usefulness of DCC to detect subtle quantitative effects on growth rate; however, the variations are at least as precise, if not more, than estimates of tumor-doubling volumes in vivo. DCC are sufficiently reproducible to use as a model system for the relative growth of cells in a host-mediated environment. Further refinements in handling the DCC during the initial stages are expected to improve the reproducibility of DCC from experiment to experiment.

Since there was no apparent advantage of host irradiation for DCC of HeLa cells, the change of doubling time with initial inoculum size was performed in unirradiated hosts. From Study 2, we consider an initial inoculum of 5 × 10⁵ cells/chamber optimal for recovering a sample large enough for reliable counting statistics while maintaining a relatively short doubling time of approximately 2.0 days. A shorter mean population-doubling time of 1.2 days was observed in the stock suspension culture which was maintained in log phase during the DCC experiments. The longer population-doubling times of DCC as compared in the in vitro cultures probably reflect an increasing cell generation time due to the increasing concentration of cells in the DCC (Chart 3). It is noted that tumor cells that normally would be...
lost by migration or phagocytosis in in vivo cultures are contained and recovered in DCC. This recovery of cells which otherwise would have migrated and/or necrotic cells from in vivo tumor cultures provides a system in which the "cell-loss" factor can be measured.

SEM studies provided additional information on the fate of HeLa cells in DCC. Apparently, some cells ruptured soon after they were introduced into culture and were among the more than 30% of cells lost within 30 min after implantation. Many cells appeared to round from a flattened morphology until they assumed a spherical configuration with microvilli attached to the substratum. The Pronase treatment of the interconnected, multilayers of cells apparently aided the cellular recovery by digestion of the clot and intercellular processes.

Most of the HeLa cells in suspension culture or DCC had surfaces densely covered with microvilli, as Porter et al. (10) found. Cells covered with microvilli and blebs or flakes were occasionally seen in both suspension cultures and DCC. Microvilli on cells from suspension cultures quickly attached to the Millipore filter substrate and were likely sources of the processes that interconnect the cells in DCC. Blebs have been associated with cells in the G1 phase of the cell cycle (3). The flakes may represent flattened precursors or remnants of blebs. The metabolic and/or structural functions of these surface features have yet to be elucidated.

In summary, it has been shown that HeLa cells can be grown in DCC with a useful but unrefined reproducibility between experiments. The observed differences in population-doubling time and surface morphology depended on the in vitro or in vivo culture conditions, and serve to illustrate that model systems of tumor growth must be chosen according to the characteristics to be investigated. Host irradiation does not markedly affect the doubling time of HeLa cells in DCC, but may lead to better reproducibility between individual runs. It appears the DCC might prove especially useful in investigations such as the assay of the kinetics and response of individual patient's tumors to therapy in a host-mediated system.

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