Novel Oxygenation System Supports Multilayer Growth of HeLa Cells

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ABSTRACT

We have developed a novel substratum in which gelatin is bonded to a reservoir of perfluorodecalin using a perfluoroalkylating technique. This forms a stable substratum supporting good adhesion for cells. HeLa cells cultured on this substratum continued to grow exponentially after the surface was covered with a monolayer forming a tissue-like structure of more than 19 layers of cells. Histological sectioning and staining of the block of tissue formed revealed the presence of mitotic figures deep within the structure. Every cell was surrounded by other cells, similar to growth of cells in vivo. This technique opens up a new approach to studying problems involved in cell-cell interaction and development of histotyptic structures in vitro.

INTRODUCTION

Growth of anchorage-dependent cells in culture has usually been restricted to a monolayer with relatively low cell densities per square centimeter. This limitation is thought to be due to the low solubility of oxygen and slow rate of diffusion in growth medium. The use of gas permeable membranes and hollow fibers perfused with oxygenated medium have been used in an effort to improve oxygenation at the substratum. These methods have resulted in significant improvements in function and morphology of the cells, and under certain conditions, cells can be grown two or three layers deep (5–8, 10). However, the amount of oxygen available to the cells in these systems...
might still be limited since a large fraction of the cell surface is occluded by adhesion to the solid areas of the membrane and the mesh of the hollow fibers.

Giaever and Keese (4) investigated the use of microspheres prepared by emulsifying perfluorocarbons and stabilizing the surfaces with polylysine as microcarriers for tissue culture. These were found to support some growth of several types of cells. However, this was found to be due to a film of protein that was desorbed from the serum used in the nutrient medium. The films were weak and easily broken by the traction exerted by the cells during growth. However, these studies of Giaever and Keese raised the possibility of using perfluorocarbons in a substratum so that oxygen could be delivered more directly to the cells at the interface.

We have developed a system that overcomes the limitation of standard cell cultures and allows HeLa cells to grow in three-dimensional (3-D) structures at least 19 cell layers deep. This system includes a substratum consisting of gelatin bonded by means of a perfluoroalkylation technique (2) to a reservoir of perfluorodecalin (PFD). It is well known that PFD has nearly 16 times the solubility for oxygen than growth media (9,14). Perfluorocarbons have previously been used as oxygen delivery systems to increase oxygenation of cells grown in suspension (5–7).

**MATERIALS AND METHODS**

**Experimental**

Two configurations of this system have been investigated (Figure 1). The first, using 15-mL tissue culture polystyrene test tubes, is described as follows and shown in Figure 1, a–c. In Figure 1a, a volume (0.88 mL) of PFD (Product No. P990-0; Aldrich Chemical, Milwaukee, WI, USA) is placed in the bottom of the test tube. This volume fills the round bottom of the tube, giving a top surface area of 1.33 cm². This is covered by a sterile preparation of gelatin, which has been perfluoroalkylated using perfluorooctylpropyl isocyanate in a moisture-free sterile atmosphere. Gelatin (Bloom 300; Sigma Chemical, St. Louis, MO, USA) was partially fractionated and sterilized by precipitation overnight at 4°C in 50% vol/vol isopropyl alcohol (EM Sciences, Gibbstown, NJ, USA). The isocyanate group reacts with surface amino groups on the gelatin in a 20% vol/vol acetonitrile solution as discussed by Boedecker and Lenhoff (1). The perfluoroalkylation substitutes a chain of 8 perfluorocarbons for one of the hydrogens on the free amino groups. The hydrophobic tails sink into the PFD so that the gelatin spreads across and bonds firmly to the perfluorocarbon surface. Tensiometric studies
showed that complete adsorption and bonding takes several hours (12). The system is allowed to stand at least 12 h in a sterile hood at room temperature. This time also allows the PFD to become saturated with oxygen from the air. At the end of 12 or more hours, any unadsorbed perfluoroalkylated gelatin (PF-gelatin) is gently aspirated, and then growth medium (11 mL), with or without cells, saturated with air is added. Other test-tube configurations are shown in Figure 1, b and c.

In one set of experiments designed to investigate different substrata, 11 mL of a suspension of HeLa cells were added to test tubes prepared with (i) PF-gelatin/PFD substratum, (ii) PFD with gelatin that had not been perfluoroalkylated and (iii) PFD alone. Eleven milliliters of a suspension at $2.17 \times 10^4$ cells/mL were added to each of a series of tubes. Adhesion of the cells was complete within an hour giving an initial cell density of $1.8 \times 10^5$/cm$^2$. Finally, a “control” was set up using a 2-cm$^2$ well polystyrene tissue culture plate (Limbro, available from Flow Laboratories, McLean, VA, USA). These were inoculated to give the same cell density/cm$^2$ as in the tubes, using 2 mL of medium per well. At certain intervals, a test tube or well was chosen, the cell layers trypsinized, the number of cells determined by hemocytometer and the results are given as cells per cm$^2$.

A second configuration of the system (Figure 1d) using glass U-tubes (Fisher Scientific, Pittsburgh, PA, USA) was tested in an effort to allow some re-oxygenation of the PFD. The bottom of the tube was filled with 9.0 mL of PFD. This raised the level of the fluid into the arms of the tube, thus facilitating any pipetting needed for preparing the PF-gel surface and replenishing the medium during growth. Only 5 mL, instead of 11 mL, of medium could be added on each side due to the length of the tube. The cells were grown in one arm of the U-tube, and the other arm was filled with 5 mL of the growth medium. This was done using a double-barreled pipet shaped like a tuning fork. This made it possible to add or withdraw medium from both arms of the tube simultaneously so as to not disturb the position of, and possibly, damage the cell layer.

**Cell Culture**

HeLa cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with glutamine (350 mg/L), pyruvate (110 mg/L), 10% bovine serum (HyClone Laboratories, Logan, UT, USA), 30 mM HEPES, penicillin (100 U/mL), streptomycin (100 µg/mL) and 33 mM NaHCO$_3$ in a humidified 5% CO$_2$ air incubator at 38°C ± 0.5°C. Suspensions of HeLa were prepared by trypsinization from a stock culture 2 days after the culture had reached confluency. The tubes were replenished with medium every two days during the first 8 days and more frequently after that to keep the pH between 7.3 and 7.4.

**Chemicals**

DMEM and other chemicals used for culture were obtained from Sigma Chemical. Perfluorodecalin was purchased from Aldrich Chemical. The perfluoroalkylating agent was kindly provided by Du Pont (Wilmington, DE, USA) but can be synthesized using the procedures described by Urata et al. (13).

**Histology**

The cell layer from a U-tube culture was fixed with glutaraldehyde and dehydrated through ethanol and finally 100% acetone. The cell layer was cut loose from the sides of the tube. Using an automatic syringe, PFD was then
pumped slowly into the opposite arm to raise the layer to the top of the tube, where it was floated off into a small beaker and embedded in glycolmethacrylate (PolySciences, Warrington, PA, USA). The tissue block was sectioned and stained in the Histology Laboratory of the University of Utah Hospital.

RESULTS

The results of the experiments on different substrata are shown in Figure 2. It is seen that after a lag of about 2 days, the cells on the PF-gelatin/PFD grew exponentially for eight days and continued to grow slowly, attaining a density of \(8.8 \times 10^6/\text{cm}^2\) after 17 days, which was as long as the cultures were carried. It is also seen that this depended on using PF gelatin. Cells did not attach or survive on “naked” PFD. Microscopic observation revealed that the cells scattered off the PFD, accumulating on the side of the tube. This also occurred on surfaces to which untreated gelatin had been added. The limited growth that was observed was restricted to a rim of cells around the wall, probably on gelatin stuck to the polystyrene. The rate of growth of cells in the PF-gelatin/PFD test tube cultures was about the same as in the polystyrene wells. A closer comparison of growth rates cannot be made because of the differences in the two systems. The striking finding is that in the “control” polystyrene well cultures, the exponential phase stopped after 6 days when cell density was \(2.5 \times 10^6/\text{cm}^2\). This was maintained for 4–5 days, after which, in spite of daily replenishments, the number of cells declined.

Cells growing in polystyrene wells obtain oxygen by diffusion from air through the medium. The 21% concentration of oxygen in air has been found optimal for HeLa cells (2,11). Increasing the concentration of oxygen in the gas phase to 30% has been found to be without effect or slightly inhibitory (11,12). This would seem to indicate that the higher cell densities in the PF-gel/PFD system were the result of improved oxygenation at the cell substratum interface because of diffusion from the PFD and not from within the medium. An alternate explanation was that bonding gelatin to PFD produced a growth-promoting configuration that, in some way, permitted cells to grow to very high densities.

These two possibilities could be distinguished by determining the effect of decreasing the volume of the PFD used as a reservoir. If the high cell densities were dependent on the amount of oxygen available from PFD, decreasing the

Figure 2. Comparison of growth obtained on PFD, (i) with PFD only, (ii) with PFD with untreated gelatin and (iii) on PF-gelatin/PFD. Growth obtained on polystyrene surfaces is included for comparison. The error bars are based on an average of 10 repeat runs and are drawn using one standard deviation above and below the average.
volume used should result in a significant decrease in the densities reached. If, however, the configuration of gelatin was the critical factor, the final cell density should be unaffected by a decrease in volume of PFD. A series of tubes were set up with smaller volumes of PFD. Since 0.3 mL of PFD did not cover the bottom of the tube, small glass beads (diameter 50–100 µm) were added to provide the same top surface area as in tubes with 0.88 mL. PFD has a very low surface tension, so that the 0.3 mL permeated between the beads, leaving a thin layer on top, to which the PF-gelatin was bonded. A slightly smaller volume (0.25 mL) was also tested using specially designed flat-bottom glass test tubes, which were made to have the same cross-sectional area.

It is shown (Figure 3) that in tubes with 0.88 mL of PFD, the cells continue to grow for 10 days, reaching a final cell density of $8.8 \times 10^6$ cells/cm$^2$, as in the previous experiment. In contrast, in the tubes with only 0.30 and 0.25 mL of PFD, both round- and flat-bottom, growth stopped at 10 days at a density of only $2.2 \times 10^6$ cells/cm$^2$. This finding suggests that the high cell densities that can be reached using PF-gel/PFD surface are not because of the state of the collagen. They are most reasonably ascribed to better oxygenation at the substratum because of diffusion from PFD. The findings also suggest that cells stop growing in the polystyrene wells as a result of oxygen depletion at the polystyrene interface.

In an effort to improve the re-oxygenation of the PFD, cultures were set up in U-tubes. The tubes required 9 mL of PFD to fill the bottom. A growth curve of HeLa cells grown in the U-tube (Figure 4) shows that the rate of growth was about the same as in the test tubes. However, the exponential phase lasted one day longer. The final cell density in the U-tubes was also higher, e.g., $1.1 \times 10^7$/cm$^2$ compared to $8.8 \times 10^6$/cm$^2$.

The condition of the cells after 15 days of growth in the U-tubes was evaluated histologically. A photograph of a 3-µm vertical section stained with hematoxylin and eosin is shown in Figure 5. It is seen that cells had grown to form a tissue-like structure of more than 19 layers. The structure has three significant features. The top layer is parallel to the bottom layer, which was attached to the PF-gel/PFD substratum. This shows that the architecture is the result of cells growing in layers on top of each other and is not just a conglomeration of aggregates or spheroids. The section is also seen to be free of any necrotic areas as seen in spheroids (3). The most important feature is the presence of mitotic figures deep within the section. This indicates that the level of oxygen needed for growth was maintained across the multilayers.

**DISCUSSION**

A novel system for oxygenation of anchorage-dependent mammalian cells has been developed. The cells are grown on a reservoir of perfluorodecalin, which has been covered with PF gelatin. This provides a stable and very adhesive surface, while at the same time...
time, making oxygen available to the cells at the cell substratum interface. Many studies have indicated that poor oxygenation at the substratum contributes to metabolic and morphological changes that occur in culture (8,10). The studies presented here indicate that depletion of oxygen at the interface may also account for the general finding that many cells stop growing at relatively low cell densities when the substratum has been covered by a monolayer, e.g., density-dependent inhibition. In this new PF-gel/PFD system, even in this very preliminary stage of its development, HeLa cells grew to form a 3-D structure of more than 19 layers. This technique opens up new approaches to investigating cell-cell interactions and developing histotypic structures in vitro.

Density-dependent inhibition has been a major impediment to advancement of tissue culture. The growth of cells in monolayers deprives the cells of the benefits to be derived from uniform contact with other cells. This limits their physiological repertoire and potential. The PF-gel/PFD substratum was developed on the supposition that poor oxygenation at the substratum was a major factor in limiting growth to a monolayer. The finding that obtaining high cell densities depended on providing a critical volume of the oxygen carrying perfluorocarbon at the substratum supports this supposition. However, further studies with primary cells are needed and are being undertaken.

REFERENCES


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