Multilayers of Cells Growing on a Permeable Support

An In Vitro Tumour Model

Andrew I. Minchinton, Karen R. Wendt, Kathy A. Clow and Karen H. Fryer

From the Department of Medical Biophysics, B.C. Cancer Research Center, Vancouver, BC, Canada

Correspondence to: Andrew I. Minchinton, Department of Medical Biophysics, B.C. Cancer Research Centre, 601 West 10th Avenue, Vancouver, B.C. V5Z 1L3, Canada

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A system for growing three-dimensional cell culture has been developed which exhibits many features of solid tumours. This system comprises cells growing as a thick mat on a semipermeable membrane suspended in stirred media. SiHa cells grown as these multilayered cell cultures (MCCs) have produced cultures up to ca. 20 cell diameters in thickness. The MCCs, like solid tumours growing in vivo, develop diffusion-dependent necrosis and hypoxia and the cell packing acts as a barrier to the diffusion of drugs. These cultures can, therefore, be used to study aspects of cancer biology and drug transport that are difficult to study using other techniques.

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Oxygen, nutrients and drugs are delivered to solid tumours by blood vessels. These agents diffuse from the blood vessels and are consumed or depleted as they diffuse through successive cell layers. The resultant heterogeneity in oxygen, nutrients and drug concentration has important implications for anticancer therapies. The depletion of oxygen in cells distal from blood vessels results in the generation of a sub-population of cells which are resistant to radiation and therefore to radiotherapy. These same cells are likely to be exposed to low concentrations of chemotherapeutic agents and, therefore, may limit the effectiveness of cancer chemotherapy.

The need to model the heterogeneity of solid tumours in vitro has led to the development of three-dimensional histoculture (1, 2), the Sandwich culture (3) and the multicellular spheroid (4). Multicellular spheroids have become the predominant in vitro model for the study of three-dimensional characteristics of tumours such as gradients of oxygen tension (4, 5) and nutrients (6, 7) and the development of hypoxia and necrosis. Radiation (4, 5) and drug resistance (8–12) caused by diffusion gradients of oxygen and drugs respectively have also been successfully studied in multicellular spheroids.

In the present article, we describe an alternative means of modelling solid tumours in vitro. This model, which we call multilayered cell culture (MCC), involves the culturing of cells on a semi-permeable membrane in such a way that they form a thick mat of cells. Several cell types have been cultured in this way and this article describes several properties that the multilayered cell cultures (MCCs) have in common with solid tumours growing in vivo.

MATERIAL AND METHODS

Chemicals and materials

SiHa (human cervix squamous cell carcinoma) cells were obtained from American Type Culture Collection. SCCVII (murine squamous cell carcinoma) cells were obtained from Stanford University Division of Radiation Biology. Collagen type I (C-9791), bisBenzimide trihydrochoride (Hoechst 33342, B-2261) and trypsin/EDTA (T-5775) were purchased from Sigma. Minimum essential media (MEM) with Earle's salts (41500–018) and fetal bovine serum (FBS) were purchased from Gibco/BRL. Millicell™ CM 0.4 μm 12 mm tissue culture inserts were obtained from Millipore. Tirapazamine was obtained from Sterling Winthrop Inc., Collegeville, P.A., USA was dissolved in phosphate-buffered saline at a concentration of 1 mg/ml just prior to use. In the experiment involving tirapazamine, multilayered cell cultures were treated with 2 μmol/l tirapazamine for 90 min and 10 μmol/l Hoechst 33342 for 60 min prior to dissociation on ice. The cells were resuspended and sorted into 10 fractions on the basis of fluorescence (see flow cytometry method below). The cells were then plated and incubated for 12 days and the number of colonies which developed were counted.

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Cell culture

The luminal surface of the tissue culture insert membrane is coated with 50 μl of 5 g/l collagen dissolved in 60% ethanol 40% 1 mol/l HCl and allowed to dry overnight. Approximately 1×10^5 cells in MEM containing 10% FBS is inoculated onto the coated surface of the membrane in a volume of 400 μl and incubated for 2–4 h to allow the cells to attach. Silicone o-rings placed around the inserts are used to support the insert between two plates held in a frame above a magnetic stir bar in a rectangular chamber containing 90 ml media (see Fig. 1). The inserts with attached cells are then completely immersed in the stirred media and incubated for up to 10 days in a humidified environment gassed with 5% O_2, 5% CO_2 and 90% N_2 at 37°C. Cells were dissociated with 0.5% trypsin/EDTA for 5–10 min at 37°C or 30 min on ice. Cell survival in the radiation experiment was assessed by plating appropriate numbers of cells in media with FBS and incubating in humidified 5% O_2, 5% CO_2 and 90% N_2 at 37°C for 12 days. Survival was assessed by counting colonies containing at least 50 cells.

Histology

The photomicrographs shown in Fig. 2 were obtained by photographing 10 μm cryostat sections. The photofluoromicrograph in panel b shows the distribution of the fluorescent stain Hoechst 33342 obtained via UV excitation at 376 nm and imaging the emission via a 418 nm long band pass filter. The same section is shown after haematoxylin and eosin staining in panel a.

Radiation

Radiation treatment was given with a Philips RT250 x-ray unit operating at 250 kVp, filtered with 0.5 mm Cu, at a dose rate of approximately 1.7 Gy/min. Cells irradiated in suspension were gassed for 50 min prior to irradiation with either humidified gasses, 95% N_2 5% CO_2 for anoxic conditions or 95% air 5% CO_2 for oxic conditions.

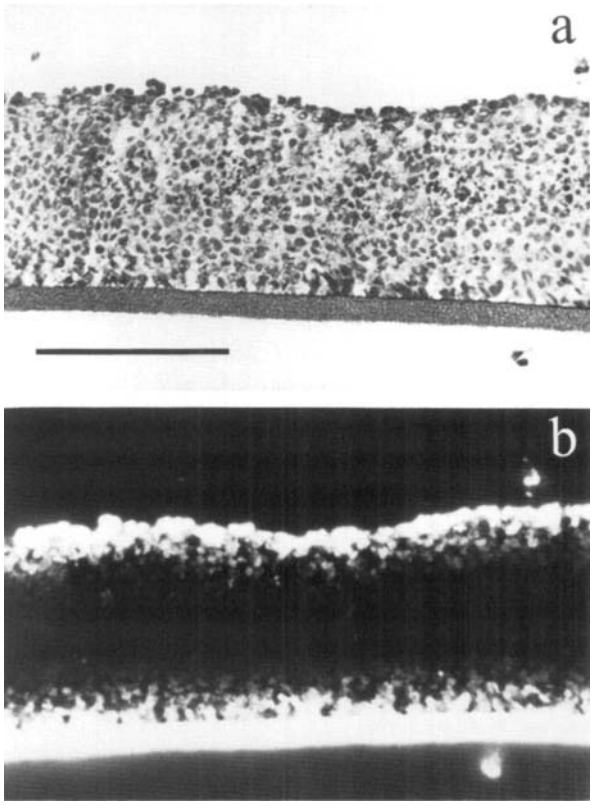

Fig. 2. Histological sections of a multilayered cell culture comprising SiHa cells grown for 8 days on a Millipore Millicell CM membrane. a) photomicrograph of a section stained with haematoxylin and eosin. b) fluorescence photomicrograph of the same section showing the distribution of Hoechst 33342 within the culture after the membrane had been incubated in media containing Hoechst 33342 (2 μmol/l) for 30 min. Scale bar = 250 μm.

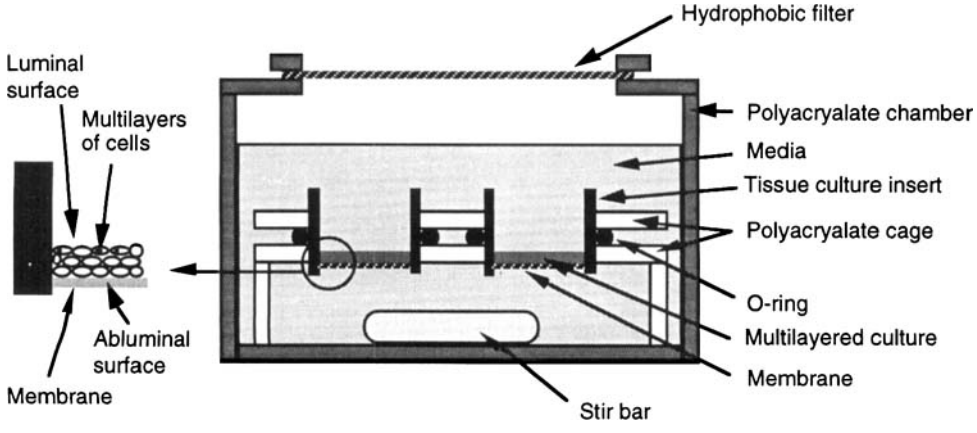

Fig. 1. Diagrammatic representation of the apparatus used to culture MCCs. Tissue culture inserts are held suspended in stirred media by a polyacrylate cage. The assembly is placed in a humidified incubator gassed with 5% O_2, 5% CO_2, 95% N_2.

Flow cytometry

The multilayered cell cultures and monolayers of cells were incubated with Hoechst 33342 (2 μmol/l) in media with FBS for 30 min. The cells were analyzed and sorted on the basis of 'concentration', dividing Hoechst 33342 derived fluorescence by the peripheral light scatter as described elsewhere (13).

RESULTS

The device used for growing the multilayered cell cultures is shown in Fig. 1. Approximately 10^5 cells are seeded onto the luminal surface of the collagen-treated membrane of the tissue culture insert. After the cells have attached, the insert is transferred to the growing chamber. A silicone o-ring holds the insert in place between two plates suspended in stirred media.

Photomicrographs of a typical 8-day-old SiHa multilayered cell culture is shown in Fig. 2. The culture shows growth of about 20 cell layers and exhibits frank necrosis developing equidistant from the surfaces of the culture. Panel 'a' shows a photomicrograph of a section stained with haematoxylin and eosin and panel 'b' is a fluorescence photomicrograph of a culture after it had been incubated for 30 min with media containing 2 μmol/l Hoechst 33342. A diffusion gradient has been established where cells proximal to both the luminal and abluminal surfaces can be seen to be intensely fluorescent whereas cells in the centre of the culture are faintly fluorescent. This shows that the multilayered cell culture acts as a barrier to the penetration of Hoechst 33342 in a manner similar to that of solid tumours and multicellular spheroids (14). We also analyzed cells dissociated from MCCs using flow cytometry as described previously (13). The fluorescence profile of the MCC cells was compared with that of similar cells dissociated from monolayer culture and stained in an identical manner. Fig. 3 shows that the distribution of fluorescence in the cell suspension derived from multilayered cell cultures (a) is significantly more heterogeneous than the fluorescence of the cell suspension derived from the monolayer cell culture (b) of the same cells. This indicates that cells dissociated from the membrane retain their Hoechst 33342 derived fluorescence and are amenable to flow cytometric sorting. In order to determine the presence of radiobiologically hypoxic cells within the multilayered cell cultures we performed a radiation cell survival experiment. In this experiment multilayered cell cultures were irradiated in situ and their radiosensitivity compared to cells irradiated in suspension under fully oxic or anoxic conditions. Fig. 4 shows the survival curves for the three cell populations. The cells derived from the multilayered cell culture were found to have a cell survival curve characteristic of a mixed population of oxic and hypoxic cells similar to cells derived from murine tumours.

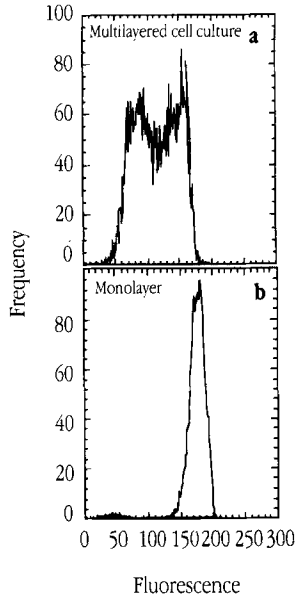

Fig. 3. Cellular fluorescence of SCCVII cells dissociated from multilayered (a) or monolayer cell culture (b) after incubation with 2 μmol/l Hoechst 33342 for 30 min.

The final experiment aimed to assess the sensitivity of cells at different depths into the multilayered cell culture to the bioreductive cytotoxic agent tirapazamine. Cells were exposed to tirapazamine for 90 min and Hoechst 33342 for 60 min. The multilayered cell culture was dissociated to a single cell suspension on ice to prevent the cytotoxic action of tirapazamine continuing after the culture was removed from the media. Fig. 5 shows the effect of exposure to tirapazamine on cell viability as a function of depth into the culture and shows that the dimly fluorescent cells situated deep within the culture were more effectively

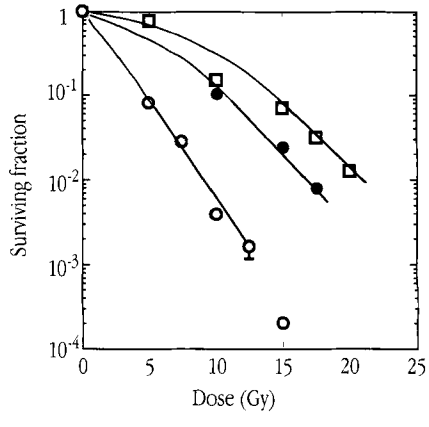

Fig. 4. Cell survival plotted as a function of radiation dose for SCCVII cells derived from a multilayered cell culture (●), from cells irradiated in suspension and gassed with air/CO_2 mixture for oxic conditions (○) or Nitrogen/CO_2 mixture for anoxic conditions (□).

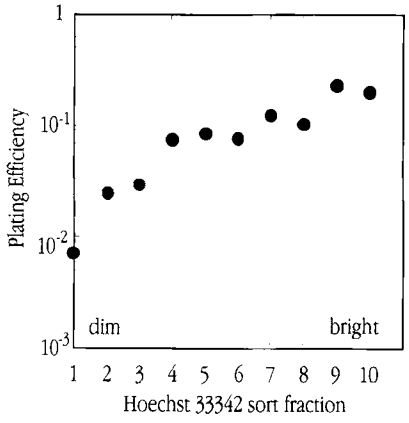

Fig. 5. Cytotoxicity of tirapazamine as a function of 10% 'sort fractions' based on Hoechst-derived fluorescence. Sort fraction 1 (dim cells) represents the 10% least fluorescent fraction of SCCVII cells derived from a multilayered cell culture exposed to 10 μmol/l tirapazamine for 90 min and 2 μmol/l Hoechst 33342 for 60 min.

killed by the treatment with tirapazamine than the brightly fluorescent cells situated close to the surface of the culture.

DISCUSSION

We have described an in vitro model of solid tumours that may complement other models in the ability to represent some of the features of solid tumours. The multilayered cell culture acts as a barrier to the diffusion of small molecules as evidenced by the gradient of fluorescence seen when the culture is incubated with Hoechst 33342. Histological examination of the MCCs reveal that necrosis develops distal from the media, similar to cells in tumours located distal from nutritive blood vessels. Radiobiological experiments showing that cells from MCCs exhibit survival curves characteristic of a mixed population of oxic and hypoxic cells similar to that seen in experimental mouse tumours. Studies with tirapazamine, a drug which exhibits selective toxicity to cells at low oxygen tension, has shown more toxicity to cells in the central layers of the MCC than in the layers close to the media, suggesting that there is a gradient in oxygen from the outer to the inner cells. In conclusion, the results of these experiments suggest that multilayered cell cultures may represent a useful in vitro laboratory model of solid tumours.

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