GROWTH OF ANIMAL CELLS ON FLEXIBLE INTERFACE OF LIQUID FLUOROCARBON

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Abstract

The aim of this work was to study BHK-21 fibroblasts cultured on the interfacial area of two immiscible liquids: perfluorodecalin (hydrophobic; PFD) and cell culture medium (aqueous; DMEM) what allows creating 3-D multicellular structures. We showed that the robust growth of 3-D aggregated animal cells could be achieved on flexible PFD/DMEM interfacial area. We also indicated that 3-D aggregates of BHK-21 cells could be successfully subcultured on solid hydrophobic surface and cells could migrate from those multicellular structures, spread on solid surface and further grow in typical monolayered form. Results of our experiments showed that the PFD/medium system is simple and ready-to-use method without need of any inserts traditionally used for 3-D cultures of animal cells. Formed multicellular 3-D aggregates could be directly used in the same culture system for inoculation of biomaterial elements or scaffolds.

Keywords: *perfluorochemical (perfluorocarbon), liquid/liquid culture system, flexible interfacial area, animal cell aggregates*

[Engineering of Biomaterials 114 (2012) 12-14]

Introduction

The traditional systems of in vitro culture of anchorage-dependent animal cells use solid surfaces, i.e. polystyrene dishes or flasks, as well as hydrophobic microcarriers and macroporous carriers. However, their application is strongly limited by the fact that cells grown under such conditions usually form surface-attached monolayers [1,2]. Intensive development of tissue and implant engineering involving biocompatible scaffolds testing requires new methods of in vitro culture of multicellular aggregates [3]. Insufficient cell migration into the scaffolds and limited oxygen transfer inside cell aggregates/tissue containing implants are remaining problems. Such elaborate and susceptible to hydrodynamic shear stress culture systems cannot be directly aerated by traditional ways (e.g. bubble aeration or air-lift aeration) commonly applied to cultures of suspended cells. Supplying sufficient amount of oxygen to the living multicellular or aggregated structures is essential for survival and growth of cells, otherwise necrosis occurs. In the case when in vitro cultured cells form multilayer colonies, sheets or aggregates, the oxygen supply to every cell might be a limiting factor [4,5].

Liquid perfluorochemicals (perfluorocarbons, PFCs) are synthetic, fully or partially fluorine-substituted derivatives of hydrocarbons. Such compounds are characterized by a high solubility of respiratory gases (oxygen, carbon dioxide) and other non-polar gases. Liquid PFCs have raised much interest in biomedical and technical applications [6-8]. One of the biotechnological approaches of PFCs involves culture of 3-D anchorage-dependent animal cells aggregates on a flexible liquid/liquid interfacial area created between PFC and culture medium layers [9-11]. Due to PFCs hydrophobicity and immiscibility with water animal and human cells adhere gently to the flexible PFC/medium interface, spread and create multicellular aggregates or sheets. In contrast to the traditional methods of proteolytic treatment where cell to cell junctions and also ECM are disrupted the multicellular formations can be easily collected from liquid/liquid culture system in intact 3-D forms by centrifugation or by pipetting, along with extracellular matrix elements, and their structure can be preserved during subcultivation. Thus, multicellular aggregates, produced in such a 2-phase PFC-containing system will be an ideal starting point for inoculation of a biodegradable scaffolds or implants.

The previously published data of animal cell cultures on PFC/medium surface [12,13] are brief and only indicate that cells could be cultured on flexible interface of liquid/liquid system supported with protein based layer formed between two immiscible liquids. The aim of this work was to study mammalian cells cultured on the interfacial area directly between PFC and culture medium what allows creating 3-D multicellular structures. The fibroblast cell line (BHK-21) has been chosen to study the effects of flexible interface. It has been also indicated that formed multicellular 3-D aggregates of analyzed cells could be directly used in the same liquid/liquid culture system for inoculation of biomaterial elements or scaffolds.

Materials and Methods

Liquid perfluorinated phase

Perfluorodecalin (PFD; $C_{10}F_{18}$; 1,1,2,2,3,3,4,4a,5,5,6,6,7,7,8,8,8a-octadecafluorodecalin; ABCR GmbH & Co. KG, Karlsruhe, Germany) was used as a liquid hydrophobic phase. PFD was sterilized by autoclaving, cooled to 37°C and additionally it was filtered using membrane filters (0.2 μm; Sartorius, Germany) to remove any microbial or solid contaminations. Then PFD was saturated by compressed air and pure oxygen in aseptic conditions [8] to obtain the oxygen concentration as 10.08 μMO_2 mL^{-1} PFD. Such oxygen level in PFD has been determined as favourable for animal cell cultures in our previous investigations [14].

PFD created separate phase on bottom of culture dishes after pipetting. PFD was collected after experiments, recovered (first by filtration to remove solid remains and then by washing with ethanol and deionized water), and then reused after sterilization and gas saturation procedure.

Animal cells, culture medium and inoculum preparation

The anchorage-dependent, i.e. adherent, hamster BHK-21 fibroblasts were evaluated in this work. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (0.05 u mL^{-1} penicillin, 0.05 u mL^{-1} streptomycin) at 37°C in a humidified, 5% CO_2 incubator. The culture medium was also supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as buffering agent. The culture medium, FCS, and all chemicals were obtained from Invitrogen Co. (USA) and were of animal cell culture quality.

Inoculum was prepared from standard nearly, i.e. 75-80% confluent cultures. The cells were washed with PBS (Invitrogen Co.; USA), and then they were enzymatically detached by using 0.25% trypsin (Invitrogen Co.; USA) and incubated for 2-3 minutes at 37°C. Next the cells suspended in culture medium were counted in Malassez hemocytometer (Brand, Germany) to obtain initial cell density as $0.48 \cdot 10^5$ cells mL^{-1}. Finally, the cells were plated into the culture dishes.

Experiment procedure

The BHK-21 cells were cultured on PFD/DMEM interface in closed and sealed 24-well plates (24-WPs) and vertically oriented 25 cm^2 culture flasks (Becton-Dickinson, USA). 2 mL of PFD and 2 mL of DMEM have been used in 24-WPs and 6 mL of PFD and 6 mL of culture medium were poured into vertically-oriented culture flask to create flexible PFD/medium interfacial area. The cell cultures on solid surface of 24-WPs were used as reference system. In the case of reference cultures, the cells were passaged every 3-4 days. In the case of the liquid/liquid culture system cells were passaged every 3 days by simply pipette out (without trypsinization needed) used DMEM and PFD and addition of fresh liquid elements of culture system to preserve multicellular aggregates formed during experiment. At least 5 independent cultures have been performed for each variant of experiment.

The multicellular aggregates of BHK-21 cells were also passaged from PFD/DMEM interface directly onto solid surface of 24-WPs. First, almost all amount of DMEM medium has been removed out from BHK-21 cultures conducted in liquid/liquid system for 7 days. Then all phase of PFD has been gently pipetted out from culture system. The aggregates of BHK-21 cells fell down on solid surface of culture dish. Next, 2 mL of fresh DMEM were added to cells very gently to preserve the 3-D structure of BHK-21 aggregates. Such subcultured multicellular aggregates of BHK-21 cells were incubated at 37°C for next 2 days.

Cultures of cells and aggregates were monitored and digitally photographed with Eclipse TS-100/F (Nikon, Japan) inverted light microscope supported with digital camera (Nikon, Japan) and Nikon CoolView software.

Results and Discussions

Cells of BHK-21 line which were referentially cultured on solid polystyrene surface of 24-well culture dishes had typical morphology of monolayered cells which strongly adhere to solid surface. Next, these cells were cultured on the flexible interfacial area of PFD and DMEM in the liquid/liquid culture system with the presence of air saturated and oxygen enriched liquid PFD on the bottom of each well of 24-well culture plate. FIG. 1 presents the representative image of the BHK-21 cells cultured on flexible PDF/DMEM interface.

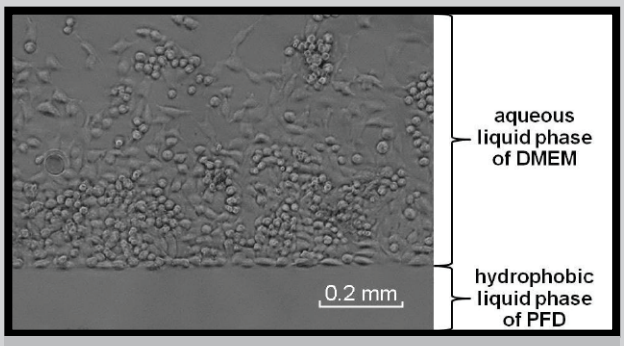

FIG. 1. Growth of the BHK-21 cell aggregates on flexible interfacial area formed between PFD (hydrophobic) and DMEM (aqueous) layers. The scale bar indicates 0.2 mm.

In the case of all experimental cultures on the liquid/liquid interfacial area the cells were located on the interface of two immiscible liquid phases, i.e. PFD and DMEM, at the hydrophilic side (i.e. DMEM) of the interface. Growth of none cells was noted at hydrophobic side (i.e. PFD) of interfacial area. The BHK-21 cells also grew on the culture dish walls, in the immediate vicinity of interfacial area of two immiscible liquids. The number of cells on walls diminished along the distance from the interface. The cells located on PFD/DMEM interface and on the solid walls of culture dish have been counted separately. Almost all of them were localized on the interfacial area of the PFD/PDMS system. Not more than 1% of analyzed cells grown on the solid walls, so they were neglected in further investigations. In comparison to the BHK-21 cells cultured on solid surface in the control cultures the cells which grew directly on flexible PFD/DMEM interface had isometric 3-D morphology, i.e. were closely packed in 3-D multicellular aggregates. In our studies suspension of single isometric cells was also observed after gently shaking of the liquid/liquid culture system. Just cells which attached to side-surface of culture plate were adhered and elongated, i.e. similar to cells in control cultures.

Next, we compared control BHK-21 cells cultured on solid surface and those ones cultured in flexible PFD/DMEM system (FIG. 2). Cells cultured on the flexible interface of two immiscible liquid phases did not adhere to the PFD surface but remained spherical for as long as 7 days of culture. Multicellular aggregates were formed after 48 hours of culture. Finally, over 1 mm diameter 3-D aggregates of the BHK-21 cells were visible in studied liquid/liquid system. Obtained aggregates were localized directly on the flexible interface of two contacting liquid phases of PFD and DMEM and floated if culture medium was pipetted or plate was shaken. It proves that adherent BHK-21 cells in the liquid hydrophobic/aqueous culture system grow without the attachment to the hydrophobic interface. Thus, in the case of BHK-21 cells PFD/medium system could be classified as 3-D cell culture. As a result, harvesting cells in order to analyze their viability and growth was greatly facilitated. Disintegration of aggregates to smaller pieces or single cells could be easily obtained by simply shaking or pipetting of culture before sample harvest. So, the enzyme-mediated detachment of aggregated BHK-21 cells was unnecessary in the case of culture them in the presented PFD/medium system.

The morphology of BHK-21 cells observed in this study is in accordance with results obtained for mouse-derived L-929 fibroblasts cultures on layer of liquid perfluorinated amines previously presented by Shiba et al. [12]. Shiba et al. did not use PFCs saturated with any gas and the growth of L-929 was rather minor and strongly limited to small aggregates consisting of few cells. Rappaport [13] reported more traditional monolayered morphology of human liver carcinoma HepG2 cells cultured on layer of perfluorinated aldehyde. But in the case of Rappaport's study the cells were cultured not directly on layer of liquid PFC but on thin layer of collagen covering the flexible interfacial area of PFC/medium system.

To document that morphology changes of aggregated cells in PFD/DMEM system are fully reversible we checked the ability of cells forming aggregates on layer of PFD to be grown on solid surface. To address this issue the multicellular aggregates of BHK-21 cells were passaged from PFD/DMEM interface directly onto solid surface of 24-WPs. The aggregates of BHK-21 cells fell down on solid surface of culture dish after gently pipetted out of PFD from culture system. Microscopic analysis performed after 24 hours revealed that BHK-21 cells located in the middle of aggregates retained their 3-D spherical morphology. But some of the cells located near the edge of aggregates became flatten, elongated and started to adhered to solid surface (FIG. 3).

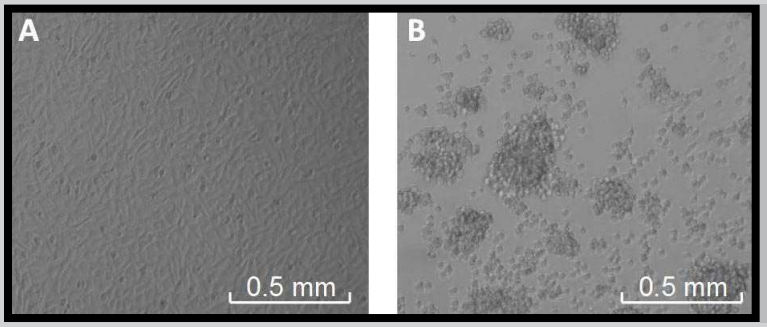

FIG. 2. Morphology of the BHK-21 cells (4th day of cultures): typical monolayer of cells in control culture on solid surface (A) and 3-D multicellular aggregates in control on flexible interfacial area of the PFD and DMEM layers (B). The scale bars indicate 0.5 mm.

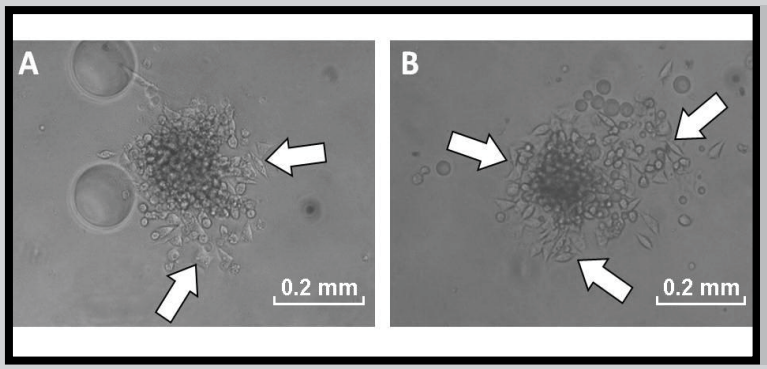

FIG. 3. Morphology of the BHK-21 cells after subcultivation of 3-D aggregates from the PFD/DMEM interface onto the solid surface: 12 (A) and 24 (B) hours after transfer. The typically elongated fibroblasts migrated from the multicellular aggregates which adhered to the bottom of 24-well plate have been marked with arrows. Closely packed 3-D aggregated BHK-21 cells remained inside the multicellular aggregates. The scale bars indicate 0.2 mm.

Such effect has not been discussed previously by any published data concerning the results of animal cells cultured on layer of liquid PFC [12,13].

Conclusions

Based on our observations we conclude that the proposed flexible interfacial area of PFD/DMEM culture system may have practical application in cultures of multicellular aggregates or single or in any animal cell cultures where the 3-D structure of cells/aggregates should be retained. The PFD/medium system is simple and ready-to-use method without need of any inserts traditionally used for 3-D cultures of animal cells and also without any needed coating or supporting of the liquid/liquid interfacial area. We have also revealed the fully reversible morphology changes of 3-D aggregated BHK-21 fibroblasts cultured on flexible interface of PFD and ability of such aggregates to undergo the changes in morphology and their further growth on solid surface.

To sum up, the robust growth of 3-D aggregated animal cells could be achieved on flexible PFD/DMEM interfacial area. We have concluded that hypothetical benefit of the PFC/medium system is a possibility to use them directly for testing and improving of cell growth on any solid biomaterial examined for use as scaffolds. For example, the two-phase liquid/liquid system culture system could be used as simple device for culture of multicellular aggregates of chondrocytes or osteoblasts. Formed multicellular 3-D aggregates could be directly used in the same culture system for inoculation of biomaterial elements or scaffolds. Then after migration of cells from aggregate onto solid surface of scaffold such implant could be in vitro or in vivo analyzed.

Acknowledgments

This work was partially supported by MNT-ERA.NET Project "ArtiCart" financed by The National Centre for Research and Development (NCBiR; Poland).

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