研 究 速 報

A PDMS microfabricated bioreactor for perfused 3D primary rat hepatocyte cultures

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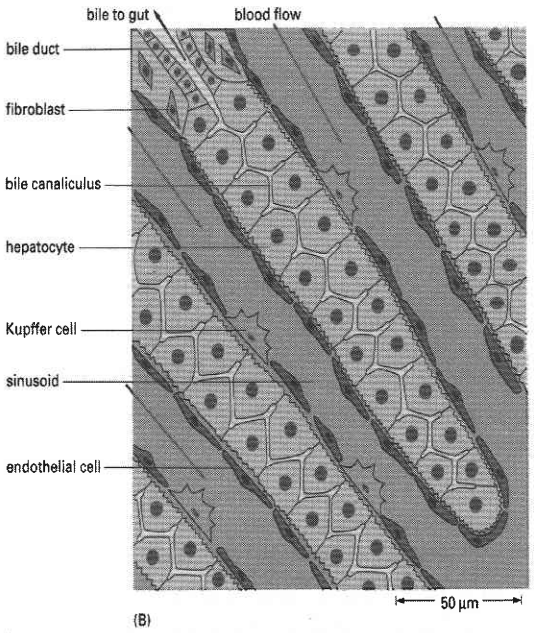
1. Introduction

The parenchymal cells or hepatocytes are highly differentiated epithelial cells involved in many liver functions. Their major roles range from blood detoxification to blood and bile components secretion as well as from protein, steroid or fat metabolism to vitamin, iron or sugar storage. This multi-functionality implies a great number of parameters, which are difficult to reproduce *in vitro* and hepatocytes rapidly lose liver specific function when maintained under standard *in vitro* culture conditions[1]. So many strategies to restore and maintain normal hepatic structure and function *in vitro* culture have been investigated[2]. Among them different culture methods have been used as protein coated culture dishes[3], collagen gel sandwich[4], co-culture with other liver-derived or non liver cell types[5,6], culture on porous membranes[7], culture in spheroids[8], or bioreactors[9]. Still, not all the important functions of liver can yet be replicated at desired levels *in vitro*, prompting continuous development of new approaches to create physiological mimics of liver. From several years our laboratory masters a microtechnology based on a plastic material named polydimethylsiloxane (PDMS). One of our research fields is the building of biomicroelectromechanicalsystems (bioMEMS). We have shown recently the possible use of PDMS in building microbioreactors and its efficiency in the maintenance of hepatocarcinoma cell cultures in terms of favorable environment[10]. The aim of this study is to extend the use of PDMS to normal hepatocyte cultures and to build a microbioreactor, which mimics nearer the *in vivo* liver architecture, suitable for the hepatocyte functionality. In this paper we present this PDMS microbioreactor and its application.

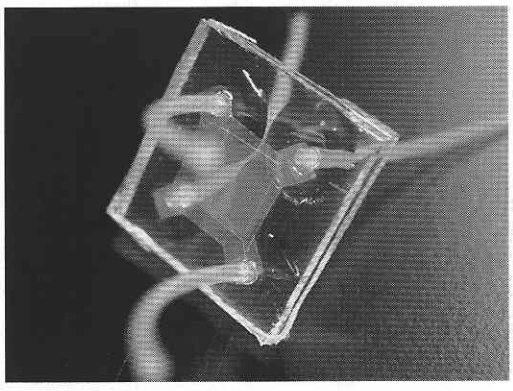
2. Materials and methods

Microdevice design

In vivo liver parenchyma comprises a hierarchical structure where hepatocytes interact with each other and with non parenchymal cells (NPC) like stellate cells (myofibroblast-like cells), Kupffer cells (resident liver macrophages)and endothelial cells[11]. Hepatocytes are connected to each other laterally to form



A



B

Fig. 1 A) Liver pattern taken from Molecular biology of the cell. Alberts B. *et al.* Garland Publishing, Inc. B)Microbioreactor with clear polyester membrane sealed inside. Cell culture chamber 1 cm × 1 cm × 100μm.

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a plate-like structures lined with NPC on the basal surfaces creating sinusoids for blood flow, whereas on their apical surface they are connected to bile canalicular. So after focused on one cellule, we decided to build a microreactor constituted by two bonded microstructured PDMS layers forming a large cell culture chamber separated in two parts by a membrane (Fig. 1). We then connected each side of the chamber with silicone tubes outlet and inlet to have the opportunity to perfuse hepatocytes on their apical side (upper chamber) and on their basal side (lower chamber).

Microdevice building

Typically a SU-8 mold master is firstly built. After the cleaning step, a SU-8 50 photoresist is spin-coated on a glass substrate at 2000 rpm during 30 seconds to reach a 50 μm thickness. This SU-8 layer is pre-baked at 95°C during 30 minutes then patterned by 50 second UV exposition through a photomask and then post-baked at 95°C during 30 minutes. Development in methoxy 1 propyl 2 acetate during 10 minutes followed by isopropanol 2 rinse during 3 minutes is then proceeded.

The dry mold master is then treated by CHF_3 plasma with RIE apparatus (Samco, RIE-10NR) to get an easy release of the PDMS layer later.

PDMS prepolymer is mixed with curing reagent with a 10:1 mass ratio and pored in the mold master. A degassing process is done in a vacuum chamber and then by heating at 70°C during 1 hour 40 minutes in an oven the PDMS is cured. PDMS structure is then obtained by pealing it off from the master.

Holes are drilled into the PDMS layers and silicone tubes as inlet and outlet are fixed. To obtain the bioreactor a permanent bonding between two microstructured PDMS layers is done by applying an O_2 plasma treatment on each bonding surfaces with RIE apparatus whereas a clear polyester membrane (taken from an insert of a transwell plate/Corning incorporated/code 3450/0.4 μm pores size) is sealed between the two layers.

Perfusion circuit

The bioreactor is fixed in the loop of a perfusion circuit composed of a culture medium tank, a peristaltic pump, a bubble trap and a magnetic agitator (Fig. 2).

The perfusion circuit is sterilized by autoclaving whereas the microdevice is sterilized by ethanol 30 minutes. Then the microdevice is rinsed with sterile water and coated with a 0.3 mg/ml collagen type 1 (In Vitrogen) solution before use. Just before cell inoculation the microdevice is rinsed with phosphate buffered saline (PBS) to remove free extra collagen and loaded with culture medium. When perfuse a 5–15 μl/minute flow speed was used. In these experiments the perfusion circuit was only plugged on the inlet and outlet tubes of the upper side of the cell culture chamber whereas the inlet and the outlet of the lower side were simply connected each other.

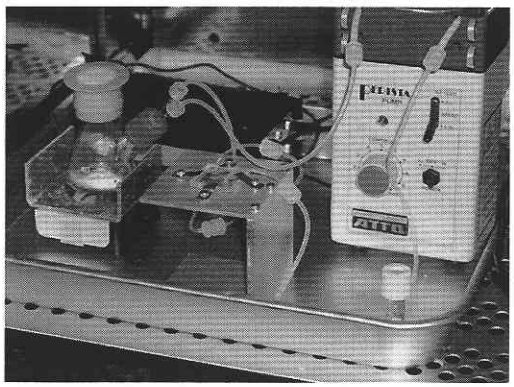

Fig. 2 microdevice and perfusion circuit.

Cells and culture medium

A male Wibstar rat aged 6–8 weeks is anaesthetized and the liver is treated by the collagenase perfusion method[12]. Hepatocytes are extracted and purified by Percoll centrifugation[13]. Viability by trypan blue exclusion was 91,6% and the total cell number was 3.5 10^7.

Cell culture was performed in bottom well (Sumilon MS80120), in insert (Corning Incorporated 3460) and in microbioreactor with a cell density inoculations of 4.10^5 cells/well, 10^5 cells/insert and 5.10^5 cells/bioreactor, respectively. The experiments were performed during 18 days in an incubator at 36°C, 5% CO_2, with humidity and the culture medium was changed each three days.

Leibovitz L15 medium (Gibco, code 11415-064) has been chosen as base because its formulation is known, it is a good serum free medium for long term culture and it is highly amino acid enriched which avoid autophagic lysosomal protein degradation problems[14]. We supplemented it with 20 mM HEPES, 100 units-penicillin/ml, 100 μg streptomycin/ml, 1 μg amphotericin/ml, 20 mM sodium hydrogenocarbonate ($NaHCO_3$/Sigma-Aldrich 28-1850-5), 10 ng/ml mouse epidermal growth factor (EGF/Takara, Tokyo, Japan), 0.1 μM insulin (Takara, Tokyo, Japan), 0.1 μM dexamethasone (Wako, Osaka, Japan), 0.8 μM cupper sulfate ($CuSO_4$, $5H_2O$/Wako 033-04415), 2 nM selenious acid (H_2SeO_3/Wako 196-00331), 2.6 μM zinc sulfate ($ZnSO_4$, $7H_2O$/Wako 269-01052), 0.3 μM manganese sulfate ($MnSO_4$, $5H_2O$/Wako 130-13182), and pH was adjusted around 7.4 with 750 μl sodium hydroxide 1N (NaOH/Wako 197-02125).

Albumin measurement

Rat albumin concentration in the culture medium was sampled at each medium exchange and measured by ELISA as previously described[15].

3. Results

After the microdevice was filled uniformly with the cell sus-

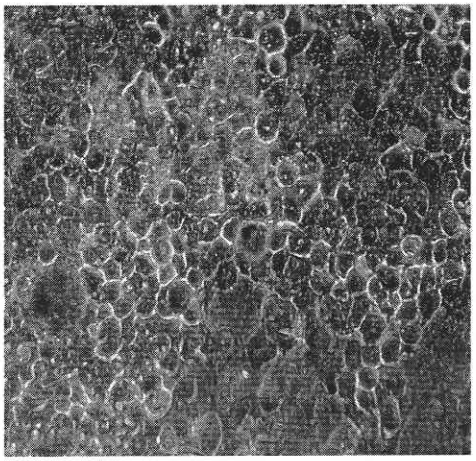

Fig. 3 Primary rat hepatocytes in perfusion at day 11 of culture.

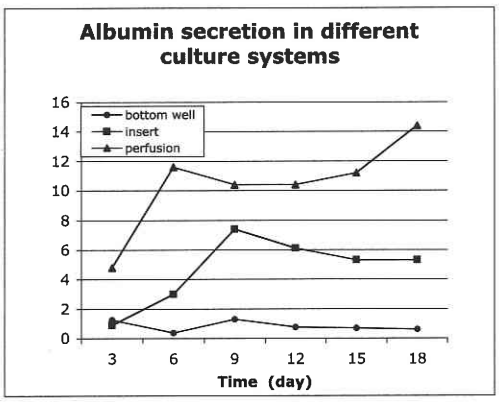

Fig. 4 Albumin secretion with different culture methods.

pension we kept it during one night into an incubator without flow perfusion to let the cells attach on their substrate. We observed a good cell attachment and little by little the cells began to combine and form a tissue-like structure. After some days we observed a beautiful aggregation plate of cuboidal and polygonal cells (Fig. 3) with apparition of a bile canalicular network characterized by the presence of a translucent band around the periphery of nearly all cells[2]. We could again observed this aggregation plate after one month of culture maintenance.

Albumin secretion have been measured and standardized with the number of cells inoculated (Fig. 4). Cell culture in insert strongly increases albumin secretion versus standard bottom well culture, whereas again much higher albumin amount is produced in perfusion culture.

4. Discussion

In the goal to maintain hepatocyte functionality *in vitro* many efforts are made to mimic *in vivo* environment. In this study for example, it appears clearly through albumin measurement (Fig. 4) that hepatocyte cultures with inserts are better than standard bottom well cultures certainly because the hepatocytes are more bathed with the culture medium and so their supply in nutrients, in oxygen and their exchanges are increased. However perfusion culture in microfluidic device is more advantageous compared to standard or insert cultures because of the permanent flow of fresh medium and the microenvironment offered to the cells, which mimics more precisely *in vivo* situation. In this study we built a PDMS microbioreactor containing a clear polyester membrane to mimic nearer the *in vivo* liver architecture. In addition this membrane allows us to well visualize the cells under a microscope whereas two perfusion circuits can be plugged on the microdevice to perfuse the apical and the basal side of hepatocytes. Amongs the problems encountered in hepatocyte culture, the uniform distribution of the cells and their attachment on their substrate are major questions. In addition, in many cases it has been observed that after few days of culture, whereas a nice layer of cells is formed, a part of hepatocytes detach and die and the cell layer become like a rag[16]. The use of our microbioreactor looks to overcome this type of problem since we observed a beautiful cell aggregation plate during long time (Fig. 3). The presence of bile canalicular network is also a sign that the hepatocytes are well reorganized and look to appreciate their environment, which is confirmed by the albumin secretion curve. As already reported by others this type of perfused microenvironment leads to enhance cell activities[17].

5. Conclusion

We have extended the use of PDMS to primary adult rat hepatocyte culture and built a microbioreactor containing a clear polyester membrane as a scaffold for the cells and different inlets and outlets allowing the perfusion of the apical and the basal sides of hepatocytes. With this microbioreactor a very good cell aggregation and maintenance have been observed as well as an increase of the albumin production compared to static cultures. Further investigations are now underway to study more precisely the functionality of normal hepatocytes in this microenvironment.

Acknowledgments

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