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Human hepatocytes and endothelial cells in organotypic membrane systems

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ABSTRACT

The realization of organotypic liver model that exhibits stable phenotype is a major challenge in the field of liver tissue engineering. In this study we developed liver organotypic co-culture systems by using synthetic and biodegradable membranes with primary human hepatocytes and human umbilical vein endothelial cells (HUVEC). Synthetic membranes prepared by a polymeric blend constituted of modified polyetheretherketone (PEEK-WC) and polyurethane (PU) and biodegradable chitosan membranes were developed by phase inversion technique and used in homotypic and organotypic culture systems. The morphological and functional characteristics of cells in the organotypic co-culture membrane systems were evaluated in comparison with homotypic cultures and traditional systems. Hepatocytes in the organotypic co-culture systems exhibit compact polyhedral cells with round nuclei and well demarcated cell–cell borders like *in vivo*, as a result of heterotypic interaction with HUVECs. In addition HUVECs formed tube-like structures directly through the interactions with the membranes and hepatocytes and indirectly through the secretion of ECM proteins which secretion improved in the organotypic co-culture membrane systems. The heterotypic cell–cell contacts have beneficial effect on the hepatocyte albumin production, urea synthesis and drug biotransformation. The developed organotypic co-culture membrane systems elicit liver specific functions *in vitro* and could be applied for the realization of engineered liver tissues to be used in tissue engineering, drug metabolism studies and bioartificial liver devices.

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1. Introduction

In living organism tissue development is orchestrated by numerous regulatory factors, dynamically interacting at multiple levels, in space and in time. Recent developments in the field of tissue engineering are aimed at designing a new generation of systems with an *in vivo* like but fully controllable cell environment. So, the primary aim in the tissue engineering design process is the fabrication of an optimal analog of the *in vivo* scenario. This is achievable applying biofabrication approaches in which the configuration and composition of cells and bioactive matrix components can recapitulate the three-dimensional microenvironments that promote cell–cell and cell–matrix interactions. In the *in vivo* physiologic environment, liver tissues assume complex well-organized three-dimensional architectures where parenchymal and non-parenchymal cells are in tight contact and supported by an extracellular matrix highly organized and composed by proteins, glycoproteins, proteoglycans and glycosaminoglycans.

An *in vitro* strategy to realize a liver model closer to the *in vivo* situation is represented by the co-culture of primary hepatocytes with endothelial cells. Hepatocytes and endothelial cells together account for more than 80% of the liver mass. In conventional primary culture hepatocytes lost their polarized shape and interactions with non-parenchymal cells [1]. It was largely demonstrated that hepatocytes in co-culture with endothelial cells remain well differentiated and functionally relatively stable improving their liver specific functions [1–3] and retaining and increasing their ability to transcribe liver specific genes [3–6]. Supported by endothelial cells hepatocytes not only enhanced their primary metabolism, drug clearance and gene expression but maintained a differentiated shape and established a functional apical and basal polarization [7,8]. Some co-culture systems were used to investigate the effect of pharmacological stimuli for a sustained period. Berthou et al. utilized a co-culture model to investigate the influence of retinoic acid on the regulation of apolipoproteins Apo A-I and A-II gene expression [2] while Ohno et al. to study the induction CYP gene expression by Phenobarbital treatment [6]. Different techniques have been adopted for the realization of co-culture systems: two-dimensional layered configuration [1–7], spheroid layered configuration [9], three-dimensional matrix of fibrin gel

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[10], micropatterned processes by capillary force lithography [11] or by laser-guided direct writing [12]. Some dynamic co-cultures using multi-compartmental connected cells [13] or a mixture of hepatocytes and non-parenchymal cells in a capillary membrane bioreactors [14] were realized to investigate the crosstalk between the two different cell types through soluble ligands. Among the layered systems some co-cultures were realized seeding endothelial cells on a monolayer of hepatocytes directly [1] or by means of a temperature-responsive patterned surfaces and a sheet of modified polyvinylidenfluoride (PVDF) membrane [3–7]. Berthou et al. inversely realized the layered co-culture system by seeding hepatocytes on confluent epithelial cells to obtain contacts between both cell types immediately following hepatocyte seeding [2]. All the investigated co-culture systems have used different kind of epithelial cells and different species of hepatocytes (e.g., primary rat or mouse) or cell lines (HepG2, Fao cells) or fetal liver progenitor cells [10]. Few papers reported on primary human hepatocytes in co-culture with endothelial cells [8,14] and no data are available about human co-cultures in biodegradable membrane systems. In liver tissue engineering an additional challenge is represented by developing a hepatic tissue by using biodegradable materials that allow the maintenance of a stable phenotype. Polymeric membranes would be able to modulate the adhesion, proliferation and differentiation of cells by controlling the mass transfer of molecules and by providing the physical and chemical instructive signals to the cells [15–19]. Our recent developments allowed us to identify the membranes that are able to support the differentiated functions of primary human hepatocytes [16–19]. As previously reported a synthetic membrane by a polymeric blend constituted of modified polyetheretherketone (PEEK-WC) and polyurethane (PU) with properties of thermal and mechanical resistance, elasticity, permeability, selectivity and well-defined geometry, supported the long-term maintenance of primary human hepatocytes [17]. Recently a biodegradable chitosan membrane was able to promote the expansion and functional differentiation of embryonic liver cells [19]. In this study we developed liver organotypic co-culture systems by using synthetic and biodegradable membranes with primary human hepatocytes and human umbilical vein endothelial cells (HUVEC). The realization of these systems was aimed to obtain an *in vitro* physiological model for drug testing and for studying of metabolic disease as well as for therapeutic applications.

2. Materials and methods

2.1. Membrane preparation

Chitosan membranes were prepared as previously described [19] by dissolving 4% (w/v) chitosan (Sigma, Milan, Italy) in acetic acid solution 2% (v/v), adding polyethylene glycol (PEG) with a molecular weight of 6000 Da (Merck-Schuchardt, Hohenbrunn, Germany) at a ratio of 4:1 and stirring for 2 h. The solution was cast on a glass plate using a commercial applicator (Adjustable Bird Applicator 0–250 μm, Elcometer) and dried at room temperature and then immersed in a solution of 1% NaOH.

Synthetic membranes were prepared from a polymeric blend of modified PEEK-WC or poly(oxa-1,4-phenylene-oxo-1,4-phenylene-oxa-1,4-phenylene-3,3-(iso-benzofurane-1,3-dihydro-1-oxo)-diyl-1,4-phenylene) and Polyurethane PU by the inverse phase technique using the direct immersion–precipitation method as previously described [17].

2.2. Membrane characterization

The morphological properties of the membrane were characterized by SEM's observation. The wettability properties of all membranes were characterized by using water contact angle (WCA) measurements obtained by the sessile drop method and water sorption at ambient temperature using a CAM 200 contact angle meter (KSV Instruments, Helsinki, Finland). Results are the means of 10 measurements of different regions of the sample surface. All measurements were repeated six times. Dissolution tests were performed on the chitosan membranes. Membrane samples (1 × 1 cm) were weighed and then placed in 1 ml of phosphate-buffered saline (PBS) at 37 °C. After drying at 37 °C for 48 h samples were weighed and the solubility percentage was calculated as $\%S = (Wi - Wd)/Wi \times 100$, where Wi and Ws are the sample weights before and after incubation in PBS, respectively and Wd is the dried sample weight after the dissolution test. Each test consisted of four replicate measurements.

Mechanical properties of the membranes were assessed via tensile test device Zwich/Roell Z2.5 (Germany). A pre-load of 0.1 N was applied before starting tensile tests at constant elongation rate of 5 mm/min. Real-time longitudinal deformation measurements were acquired by PC and analyzed by testXpert® testing software. Tensile tests were carried out at 20 °C. Young's Modulus E evaluated from the slope of the linear portion of the stress-strain curve, tensile strength (Rm) and the elongation at break were determined. For each membrane at least 10 strips (1 × 5 cm) were used.

2.3. Cell cultures

2.3.1. Primary human hepatocytes

For cell culture experiments primary human hepatocytes (Lonza Sales Ltd, Basel, Switzerland) isolated from non-transplantable tissue of young single donors were used. The purity of isolated hepatocytes is 95% and non-parenchymal cells are present in a very low percentage (5%). Cryopreserved human hepatocytes were quickly thawed in a 37 °C water bath with gentle shaking. Then, the cell suspension was transferred slowly into a tube containing 30 ml of cold hepatocyte culture medium (HCM™, Lonza Sales Ltd, Basel, Switzerland), and centrifuged at 50 *g* at 4 °C for 5 min. The HCM™ is constituted of hepatocyte basal medium (HBM™, Lonza Sales Ltd, Basel, Switzerland) together with all the components provided in HCM™ bulletkit® (Lonza Sales Ltd, Basel, Switzerland): human epidermal growth factors (hEGF), insulin, ascorbic acid, transferrin, hydrocortisone 21-hemosuccinate, gentamicin sulfate 50 μg/ml, amphotericin B 50 ng/ml. The cell pellet was suspended in HCM™ and tested for the cell viability by Trypan blue exclusion. Human hepatocyte homotypic cultures were obtained seeding the cells at a density of 1.8×10^5 cell/cm^2 on PEEK-WC-PU and chitosan membranes previously conditioned with hepatocyte culture medium HCM™ supplemented with bovine serum albumin-fat acid free 2% (BSA-FAF). Polystyrene culture dishes (PSCDs) coated with type I collagen (5 μg/cm^2) from rat tail (Roche Diagnostics, Mannheim, Germany) were used as reference substrates for homotypic cultures. Cells were incubated at 37 °C in a 5% CO_2; 20% O_2 atmosphere (v/v) with 95% relative humidity in hepatocyte culture medium HCM™ containing BSA-FAF 2% for the first 24 h, thereafter under serum-free condition and in presence of Diazepam 10 μM for the whole culture time.

2.3.2. Human endothelial cells

Human Umbilical Vein Endothelial Cells (Cascade Biologics, Mansfield, UK) cryopreserved at the end of the primary culture stage were quickly thawed in a 37 °C water bath with gentle shaking and tested for the cell viability by Trypan blue exclusion. HUVECs were cultured in Medium 200 (Cascade Biologics, Mansfield, UK) together with all the components provided in the Low Serum Growth Supplement kit, LSGS kit (Cascade Biologics, Mansfield, UK): hydrocortisone1 μg/ml, human epidermal growth factor (hEGF) 10 ng/ml, basic fibroblast growth factor (bFGF) 3 ng/ml, heparin 10 μg/ml, gentamicin 10 μg/ml, amphotericin B 0.25 μg/ml and supplemented with fetal bovine serum 2% (FBS). HUVEC homotypic cultures were obtained seeding cells with 4–5 population-doubling levels at a density of 1.25×10^4 cell/cm^2 on PEEK-WC-PU and chitosan membranes previously conditioned with culture medium. PSCD was used as the reference substrate. Cells were incubated at 37 °C in a 5% CO_2; 20% O_2 atmosphere (v/v) with 95% relative humidity in Medium 200 supplemented with LSGS kit and FBS 2% for the whole culture time.

2.3.3. Organotypic co-culture systems

The organotypic layered co-culture systems of human hepatocytes on HUVECs was obtained seeding HUVECs with 4–5 population-doubling levels on membranes previously conditioned and on PSCD as reference substratum at a cell density of 1.25×10^4 cell/cm^2; after 24 h human hepatocytes were overlayed at a cell density of 1.25×10^5 cell/cm^2. Cells were incubated at 37 °C in a 5% CO_2; 20% O_2 atmosphere (v/v) with 95% relative humidity in 1:1 HCM™ and Medium 200 culture medium containing 2% BSA-FAF and 2% FBS respectively for the first 24 h; thereafter under serum-free conditions and in presence of Diazepam 10 μM for the whole culture time.

2.4. Cell morphology

Cell morphology on all investigated substrates was observed by Laser Confocal Scanning Microscopy (LCSM) after 3 and 13 days of culture by cytoskeleton and ECM protein immunostaining. In particular cells were visualized for actin staining and HUVECs were identified for the glycoprotein CD31 staining. ECM proteins were investigated by laminin and fibronectin staining. Morphology of cells cultured on PSCD was further observed by light-inverted microscopy for the whole culture time.

2.4.1. Cell staining for LCSM

Samples were washed with PBS, fixed for 15 min in 3% paraformaldehyde in PBS at room temperature (RT), permeabilized for 5 min with 0.5% Triton-X100 and saturated for 15 min with 2% Normal Donkey Serum (NDS).

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