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Long-term maintenance of human hepatocytes in oxygen-permeable membrane bioreactor

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Abstract

An oxygen-permeable membrane bioreactor utilizing human hepatocytes has been tested in this study. In the bioreactor, human hepatocytes were cultured between flat-sheet gas-permeable polymeric membranes, which ensure the diffusion of O_2 and CO_2 providing a support for cell anchorage and growth and permit the online observation of the cells with an inverse microscope. This bioreactor allows a direct oxygenation of cells adhered on membranes and of the medium overlaying cells simulating in vivo sinusoidal organization. Human hepatocytes were cultured in the presence of some therapeutic molecules to assess the temporal liver-specific functions of the cells. Interleukin 6 (IL-6), which is a multifactorial proinflammatory cytokine involved in a variety of host defences and pathological processes, and diclofenac, an arylacetic non-steroidal anti-inflammatory drug, were used as therapeutic molecules.

The aim of this study was to evaluate the in vitro performance of the small oxygen-permeable membrane bioreactor in the long-term maintenance and differentiation of human hepatocytes under in-vivo-like conditions.

The fluid dynamics of the bioreactor were characterized before using it for human cell culture. The functional response to a step challenge in the medium of IL-6 (120 pg/ml), diclofenac (80 μM) and IL-6 and diclofenac together was investigated. The ability of hepatocytes to perform liver-specific functions in terms of urea and albumin synthesis, as well as secretion of total proteins, was maintained for 32 days. Also, the diclofenac biotransformation functions were sustained as the formation of the metabolites 4′-OH-diclofenac and 5-OH-diclofenac lactam demonstrated.

This study attested the feasibility of the membrane bioreactor as an in vitro simple model system that allows human hepatocytes to be maintained in a differentiated state similar to that in vivo.

Keywords: Membrane; Human hepatocytes; Bioreactor; Functions; Viability

1. Introduction

In recent years, numerous hepatocyte bioreactors using different materials and configurations have been developed [1–7] and most attempts were focused on adapting bioreactor technologies to the culture of primary hepatocytes in the development of an extracorporeal hybrid liver support device. Previously, a full-scale flat membrane bioreactor utilizing porcine hepatocytes was developed as a bioartificial liver reconstructing the plate architecture of the liver and supporting the expression of tissue-specific functions [8,9]. An oxygen-permeable membrane bioreactor, based on the same principle, but with different configuration and utilizing human hepatocytes, is assessed in this study. Human hepatocytes were cultured in the bioreactor between flat-sheet gas-permeable polymeric membranes, which directly provide support for the anchorage and the growth of the cultured layers of cells and ensure the diffusion of O_2, CO_2 and H_2O vapour.

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The membrane oxygen-permeable bioreactor allows a direct oxygenation of cells adhered on membranes and of the medium overlaying cells simulating the in vivo sinusoidal organization. The physical membrane property of transparency also permits the online observation of the cells with an inverse light microscope. Human hepatocytes were cultured under an in-vivo-like microenvironment in basal conditions and in the presence of some therapeutic molecules to assess the maintenance of liver-specific functions of human hepatocytes. Interleukin 6 (IL-6) and diclofenac were used as therapeutic molecules.

IL-6 is a multifactorial proinflammatory cytokine involved in a variety of host defences and pathological processes [10]; specifically, in the liver, IL-6 promotes hepatic survival by stimulating liver regeneration after injury or partial hepatectomy [11] and providing protection in a variety of liver-injury models [12]. IL-6 concentrations in the serum are elevated after partial hepatectomy in rats and human [13–15] and in patients with end-stage cirrhosis or fulminant hepatic failure [16–18]. IL-6 may directly induce cell-cycle progression and proliferation in hepatocytes [19] increasing activation of STAT3 and its target genes c-myc [17] and cyclin D1 [20] both essential in cell-cycle progression. STAT3 also blocks apoptotic injury by induction of anti-caspase regulators and by reduction of oxidative injury via up-regulation of Ref-1, an antioxidant protein [12,21]. The activation of ERK2 by IL-6 results in gene expression to promote growth, differentiation or mitosis [22]. Furthermore, in the liver, IL-6 is a major inducer of hepatic acute phase (AP) response and an activator of the AP proteins following infection.

Diclofenac is an arylacetic non-steroidal anti-inflammatory drug approved for clinical use in treating several rheumatic diseases and as an analgesic [23], which undergoes an extensive hepatic biotransformation involving aromatic hydroxylation and conjugations. Previous studies on porcine hepatocytes showed that diclofenac could induce cytotoxicity at concentrations higher than 100 μM and affect metabolic functions also at low concentrations [24,25].

The aim of this study was to evaluate in vitro performance of the oxygen-permeable membrane bioreactor in the long-term maintenance and differentiation of human hepatocytes under in-vivo-like conditions. We studied the IL-6 and diclofenac effects on liver-specific functions as well as on xenobiotic metabolism over a prolonged period of culture time.

2. Materials and methods

2.1. Human hepatocytes culture

Primary human hepatocytes (Cambrex Bio Science) isolated from non-transplantable tissue of young single donors were used for cell culture experiments. 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 25 ml of cold hepatocyte culture medium (HCM™, Cambrex Bio Science), and centrifuged at 50*g* at 4 °C for 3 min. The HCM™ is constituted of hepatocyte basal medium (HBM™) together with all the components provided in HCM™ bulletkit® (Cambrex Bio Science, Milan, Italy): epidermal growth factors, insulin, ascorbic acid, transferrin, hydrocortisone 21-hemosuccinate, bovine serum albumin-fat acid free 2% (BSA-FAF) and gentamicin sulphate 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 hepatocytes were seeded in the bioreactor chamber on a gas-permeable surface, previously conditioned with HCM™ containing BSA, to give a concentration of 5.5×10^4 cells/cm^2 and incubated for the first 24 h at 37 °C in a 5% CO_2: 20% O_2 atmosphere (v/v) with 95% relative humidity in HCM™ containing 2% BSA. Thereafter, the culture was continued in a 5% CO_2: 10% O_2 atmosphere (v/v) under serum-free conditions for the whole culture time. Hepatocytes were cultured also in batch system on collagen gel, which was used as the reference substratum. Type I lyophilized collagen from rat tail (Roche Diagnostics, Mannheim, Germany) was dissolved with 2% sterile acetic acid to the final concentration of 2 mg/ml; pH was adjusted to 7.4 with 10× concentrate Dulbecco's modified Eagle medium diluted 1:10 with the collagen solution. Solution of collagen gel was added to obtain a coating density of 5 μg cm^{-2}. Cells and controls were incubated at 37 °C in a 5% CO_2: 20% O_2 atmosphere (v/v) with 95% relative humidity for the duration of the experiments.

Experiments were performed in basal condition and in the presence of human IL-6 120 pg/ml, diclofenac 80 μM and IL-6 and diclofenac in combination in the culture medium.

The morphology and the concentration of the cells cultured inside the membrane bioreactor were assessed by inverted light microscopy every day of the culture.

The liver-specific functions of the human hepatocytes cultured in the bioreactor were investigated in terms of albumin production, urea synthesis and secretion of total proteins for the whole culture time. Proteins secreted by cells in the medium were identified by gel electrophoresis and quantified. The ability of cells to perform drug biotransformation functions was evaluated by investigating the diclofenac elimination in presence of 80 μM diclofenac and the formation of its metabolites.

2.2. IL-6

Human IL-6 was produced by peripheral blood mononuclear cells (PBMC) isolated from healthy subjects after mitogenic stimulation. PBMC were isolated by Ficoll-Hypaque (Flow, Irvine, UK) gradient density centrifugation (400*g* for 30 min) [26], then the cells were washed twice with PBS (Sigma, Milan, Italy) and resuspended in RPMI medium (Sigma, Milan, Italy) supplemented with 1% heat-inactivated FCS (Sigma) and antibiotics (penicillin, streptomycin). After viability assessment, the cells were incubated in culture tubes (Falcon) at a concentration of 1×10^6 cells/ml and stimulated with 200 ng/ml bacterial (Escherichia Coli) lipopolysaccharide (LPS) (Sigma). After incubation for 24 h at 37 °C in a 5% CO_2 atmosphere, samples were collected and centrifuged at 1100 rpm for 5 min. Dosages of IL-6 with ELISA test (kit Quantikine, R&D Systems, Minneapolis, MN) were performed from supernatants [26].

2.3. Membrane bioreactor

The bioreactor consists of a membrane housing supported by an acrylic ring fitted with inlet and outlet ports. The chamber for the hepatocytes culture was delimited by an upper and lower area of a 25 μm thick gas-permeable (CO_2, O_2 and H_2O vapour) fluorocarbon foil membrane bioFolie (IVSS GmbH, Germany), with a surface area of 21.24 cm^2 and a volume of 10 cm^3. The bioreactor was connected to the perfusion system consisting of a glass medium reservoir, tubing, a microperistaltic pump and a glass medium waste (Fig. 1). Medium and cells were directly oxygenated in an incubator by the diffusion of humidified air across the