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Maintained function of primary human hepatocytes by cellular interactions in coculture: implications for liver support systems

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Abstract The application of primary hepatocytes in hybrid artificial liver systems has been hampered by the gradual loss of differentiated morphology and function in vitro. Therefore, we have established a coculture model of autologous human hepatocytes and biliary epithelial cells (BEC) in collagen gel in the presence of hepatotrophic growth factors. Furthermore, we examined the effect of hepatocyte cell perfusion in a woven multicompartment capillary membrane system. Normal hepatocytes isolated from human liver produced albumin for more than 2 weeks in serum-free media, and were further stimulated by conditioned medium. When cocultured with BEC, albumin secretion was greatly enhanced, suggesting that cellular interactions promote tissue-specific differentiation. When perfused in bioreactors, albumin levels were maintained at steady state for longer than 2 weeks. These data indicate that differentiation of primary hum hepatocytes can be maintained by coculture interactions and three-dimensional hybrid organ devices, providing appropriate growth factors and matrix for tissue regeneration.

Key words Human hepatocytes · Biliary epithelial cells · Coculture · Growth factors · Bioartificial liver

Introduction

Hepatocytes perform many complex functions in the human organism, including synthesis of proteins (e.g. albumin and clotting factors), metabolism and elimination into blood or bile. However, application of primary hepatocytes is hampered by a gradual loss of differentiated morphology and function of these cells in vitro. In several culture systems, efforts have been made to maintain specific functions such as albumin synthesis by the application of extracellular matrix sandwich layers, addition of growth factors or coculture with other liver epithelial cells, but these factors are generally not provided by hybrid organ systems. Accordingly, normal human hepatocytes have been precluded from use in bioartificial liver systems in spite of their potential benefits: (1) synthesis of homologous proteins, (2) defined metabolic properties and (3) no risk of transmitting xenogenic infections.

In order to provide an environment that resembles more closely organotypical conditions, we have combined these three factors for the culturing of primary human liver cells. Using albumin synthesis as a parameter of differentiated cellular function, we have examined human hepatocytes in dependence of coculture with human biliary epithelial cells (BEC), addition of growth factors and culture in collagen gels or three-dimensional capillary systems. All of these experiments were performed in serum-free medium to evaluate the effect of defined growth factors [hepatocyte growth factor (HGF)] in relation to a conditioned medium (CM) prepared from previous cocultures of cocultured liver epithelial cells. Using this culture model, we wanted to determine if substances released by autologous non-parenchymal liver cells can promote hepatocyte function and cell organisation.

It was the aim of this study to investigate if the described factors enhance the hepatocyte function, and if

primary human hepatocytes can be maintained in a hybrid organ system which allows subsequent application of these factors. For this purpose, human hepatocytes were perfused in a woven multicompartment capillary system developed by Dr. J. Gerlach, as described before [4]. The three-dimensional framework enables decentralised cell perfusion with metabolic gradients, decentralised oxygenation and carbon dioxide removal, and capillary compartments for additional cell populations. Here, we describe our extended in vitro coculture results and the initial findings of primary human hepatocytes perfused in the bioreactor system.

Materials and methods

Normal liver tissue (150–250 g) was obtained from segments surgically removed from donor organs ($n = 15$) before reduced-graft transplant into pediatric recipients, as approved by our ethics committee and with the consent of the appropriate persons. Hepatocytes were isolated by enzyme perfusion as described previously [5]. Cells were plated at 4×10^5 cells/ml into 35-mm culture plates coated with collagen type I gel in serum-free Williams medium E supplemented with insulin (100 nmol/l), hydrocortisone (5 μmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml). Williams was used alone or with 10 ng/ml human recombinant HGF or epidermal growth factor. For preparation of CM, supernatants of cocultures grown in Williams medium were collected after 24 h, filtered and mixed with 50% fresh Williams medium. Intrahepatic human BEC were isolated from 30–50-g segments of liver as described before [6] and spread onto subconfluent hepatocytes at a concentration of $1–2 \times 10^4$ BEC/ml culture medium, resulting in a final proportion of 1–5% BEC/hepatocytes as found in vivo. Cocultures were cultured between two layers of collagen type I gel in a sandwich configuration, as described before [7].

The bioreactor developed by Dr. J. Gerlach consists of a woven multicompartment capillary membrane system enabling plasma inflow, oxygenation and carbon dioxide removal, plasma outflow, and another compartment for the addition of non-parenchymal liver cells [4]. For perfusion in serum-free Williams medium E $1–1.5 \times 10^8$ primary human hepatocytes were introduced between and on the capillaries, and, in this capillary array, decentralised metabolite and gas exchange with low gradients is possible. Cell morphology was assessed in vitro by light microscopy. Albumin levels of 24-h periods were determined immuno turbodimetrically. Results are expressed in means of triplicates ± SD of 13 independent coculture experiments and three independent bioreactor runs.

Results

Human hepatocytes plated in a sandwich collagen DG exhibited typical polygonal morphology and formed cord-like structures within the first 2 weeks in culture with bile canaliculi between adjacent cells (Fig. 1). Hepatocytes could successfully be subpassaged on collagen gel following collagenase digestion of gels, and monolayers could be maintained for more than 4 weeks in serum-free and growth factor-free Williams medium. Cocultures of hepatocytes and BEC typically demonstrated

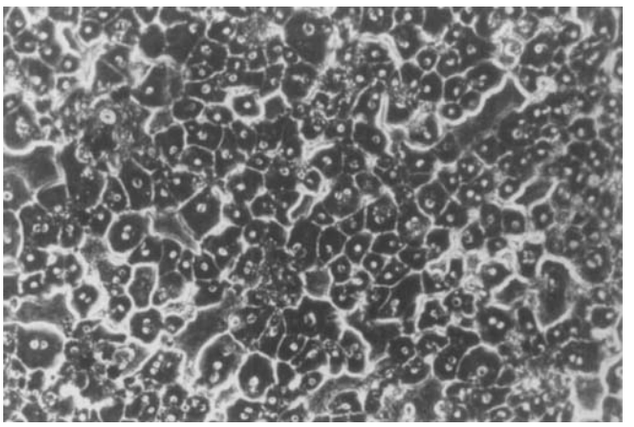

Fig. 1 Light microscopic picture of human hepatocytes plated in a sandwich collagen DG exhibiting typical cord-like structures after 2 weeks in culture in serum-free Williams medium. Original magnification × 300

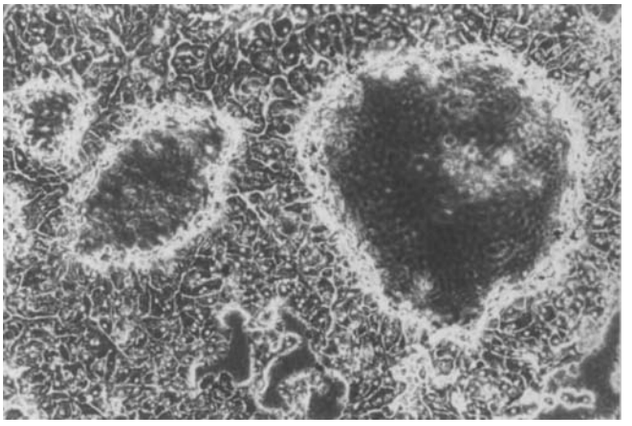

Fig. 2 Light microscopic picture of cocultured human hepatocytes and biliary epithelial cells (BEC) in serum-free Williams medium after 5 days. Cystic aggregates of BEC forming a hollow lumen are surrounded by a subconfluent layer of adjacent hepatocytes within the collagen sandwich gel. Original magnification × 150

layers of subconfluent hepatocytes surrounding islands of BEC aggregates. Within the collagen sandwich gel, BEC reorganised into three-dimensional cystic aggregates with a hollow lumen (Fig. 2). From these spheroidal structures, BEC grew out to establish ductular formations with identifiable lumena, progressing to a three-dimensional network of anastomosing ducts which could be maintained for more than 7 weeks in culture [8].

During the 1st week in culture in collagen sandwich gel albumin secretion of hepatocytes remained stable in serum-free Williams medium, but declined during the 2nd week. The peak levels could be extended and slightly increased, when HGF was added to the culture medi-

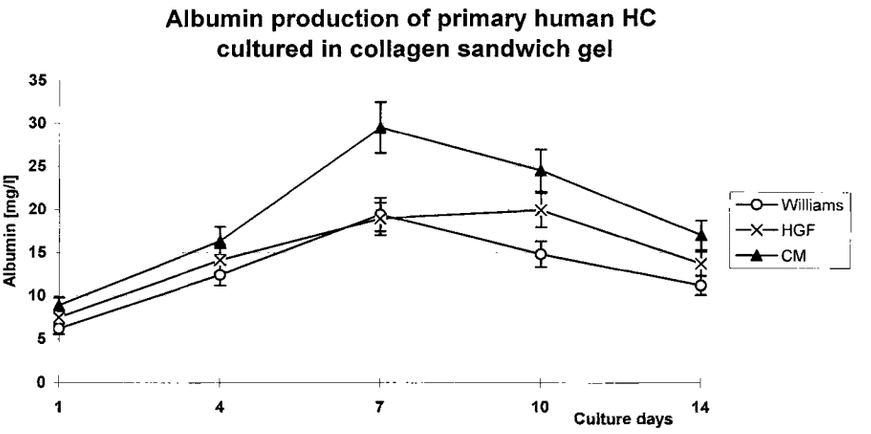

Fig. 3 Albumin production of primary human hepatocytes *(HC)* cultured in a collagen sandwich gel. Levels are most significantly enhanced by conditioned medium *(CM)* from previous cocultures as compared to Williams with or without hepatocyte growth factor *(HGF)*. Results are means of triplicates of six independent experiments ± SD

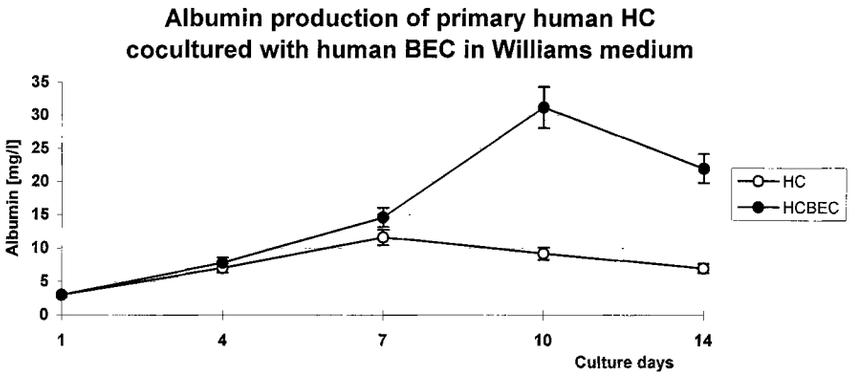

Fig. 4 Albumin production of primary human hepatocytes cultured alone or in coculture with autologous human *BEC* in serum-free Williams medium. Albumin levels are strongly enhanced and maintained at these higher levels in coculture configuration. Results are means of triplicates of seven independent experiments ± SD

um. However, albumin secretion was greatly enhanced when hepatocytes were cultured in CM from previous cocultures and remained elevated after 2 weeks, indicating that CM contains factors capable of improving the differentiated hepatocyte function (Fig. 3).

When comparing the production of albumin levels of hepatocytes alone in collagen gel with cocultures of autologous hepatocytes and BEC, it could be demonstrated that albumin levels were not only enhanced, but also maintained at high levels in coculture conditions (cocultures of hepatocytes with BEC measuring 22 mg/l as opposed to hepatocytes alone with only 7 mg/l after 14 days in serum-free Williams medium; Fig. 4). Therefore, the positive effect of cocultures may be partly related to specific cellular interactions, as suggested by some authors [9]. It should be noted, that all of these investigations were obtained by the use of serum-free medium under chemically defined conditions, thus demonstrating only the effect of liver epithelial cells within a three-dimensional collagen matrix.

Finally, we examined if normal human hepatocytes can be cultured and maintained in a three-dimensional perfused capillary system without extracellular matrix supply. Using the woven, multicompartment capillary system of Dr. J. Gerlach, we were able to show that the albumin levels remained at steady state over the whole 2 weeks examined in serum-free Williams medium without added growth factors (Fig. 5). In all three independent experiments, hepatocytes remained viable and demonstrated a well-differentiated ultrastructure over this period.

Therefore, the differentiated function of parenchymal human liver cells is improved by the addition of growth factors, coculture with liver epithelial cells and three-dimensional matrix composition. When adequate oxygenation is provided in a three-dimensional setting, as provided by the bioreactor system, hepatocytes can also maintain their differentiated function for at least 2 weeks. The metabolic properties of these cells are currently under investigation.

Discussion

Recent studies have demonstrated the critical role of cell environment as well as growth factors on the maintenance of cell functions [10]. The complexity of liver functions implies the difficulty of establishing and main-

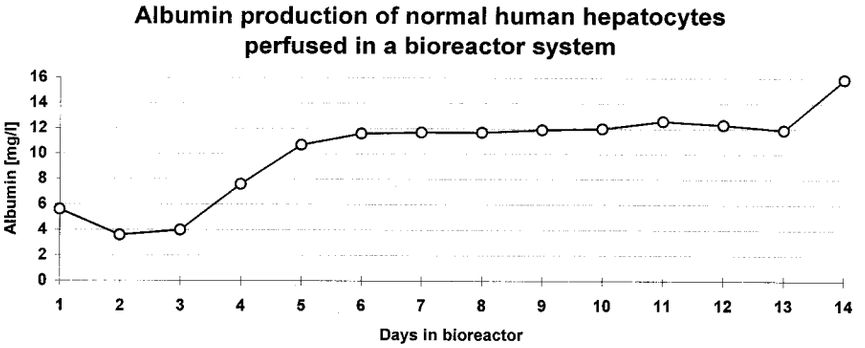

Fig. 5 Albumin levels of 1.5×10^8 hepatocytes in a woven, multicompartment capillary system perfused over 2 weeks with serum-free Williams medium. Albumin levels of normal human hepatocytes remain at steady state, indicating that nutrition and oxygenation support the differentiated cellular function in this hybrid organ system. Data represent means of duplicates of a representative experiment of three independent reactor runs

taining differentiated hepatocytes in vitro. However, it is desirable to use human hepatocytes for studies of liver repair, regeneration and even bioartificial liver support. In previous studies it has been demonstrated that collagen sandwich techniques [1], application of HGF [2] and coculture of hepatocytes with a liver epithelial cell type [3] improve some aspects of hepatocyte function, e.g. albumin synthesis. However, direct cell-cell and cell-matrix interactions occur in vivo simultaneously and may be modified by mediators locally released by adjacent non-parenchymal cell types.

In our experiments we have been able to demonstrate the effect of these factors alone and in combination, resulting in an increased albumin secretion of human hepatocytes. This enhancement, resulting from a combination of applied collagen DG, growth factors and coculture with BEC, confirms the significance of an appropriate cell microenvironment. It must be emphasised that cell-derived factors contained in CM have even exceeded the effect of HGF, suggesting that a combination of different growth factors and mediators is involved in liver growth and repair.

The addition of autologous BEC to human hepatocytes may have similar effects on the metabolic function and growth of primary hepatocytes, which is subject to further investigations. Although isolation yield of autologous BEC is restricted in initial availability, we have not used other cell types for coculture studies because there is evidence that cellular interactions depend on particular cellular membrane receptors [9–11].

In the second part of our studies, we have been able to show that human hepatocytes can be maintained in a hybrid organ system for more than 2 weeks in serum-free medium, continuously producing albumin. Apart from that, we have also detected other human proteins and clotting factors in culture supernatants of human hepatocytes, emphasising the potential use in liver support therapy. Production rates and persistence of other synthetic and metabolic functions, including cytochrome P450-mediated metabolism of lignocaine (MEGX test), are currently investigated. The application of growth factors and different non-parenchymal liver cells in this bioreactor system offers new strategies for the design of liver support therapies. The success of joining the various liver-typical components will determine the feasibility of a bioartificial liver system using normal human liver cells.

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