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Isolated Hepatocytes Versus Hepatocyte Spheroids: In Vitro Culture of Rat Hepatocytes

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The use of hepatocytes that express liver-specific functions to develop an artificial liver is promising. Unfortunately, the loss of specialized liver functions (dedifferentiation) is still a major problem. Different techniques, such as collagen entrapment, spherical multicellular aggregates (spheroids), and coculture of hepatocytes with extracellular matrix, have been used to improve the performance of hepatocytes in culture. The aim of this study was to compare two different models of hepatocyte isolation in culture: isolated hepatocytes (G1) and hepatocyte spheroids (60% hepatocytes, 40% nonparenchymal cells, and extracellular matrix) (G2). To test functional activity of hepatocytes, both synthetic and metabolic, production of albumin and benzodiazepine transformation into metabolites was tested. G2 showed a high albumin secretion, while a decrease after 15 days of culture in G1 was noted. Diazepam metabolites were higher in G2 than in G1 in all samples, but had statistical significance at days 14 and 21 ($p < 0.01$). The glycogen content, after 30 days of culture, was very low in G1 ($14.2 \pm 4.4\%$), while in G2 it was $72.1 \pm 2.6\%$ ($p < 0.01$). Our study confirms the effectiveness of a culture technique with extracellular matrix and nonparenchymal cells. Maintenance of a prolonged functional activity has been related to restoration of cell polarity and close cell-to-cell contact. We showed that isolated hepatocytes maintain their functional activity for a period significantly reduced, when compared to the hepatocyte spheroids. We confirmed the role of extracellular matrix as a crucial component to promote hepatocyte homeostasis, and the close link between cellular architecture and tissue-specific functions.

Key words: Hepatocyte spheroids; Isolation; Culture; Matrix; Benzodiazepine; Albumin

INTRODUCTION

In past studies, nonbiological liver support devices for critical liver failure, such as plasmapheresis, haemoperfusion, and ultrafiltration techniques, did not significantly improve survival (10). Therefore, the use of hepatocytes that express liver-specific functions to develop an artificial liver is crucial. Unfortunately, the loss of specialized liver functions, together with changes in cell morphology, in cultured hepatocytes (dedifferentiation) is still a major problem (3,13). Isolated hepatocytes in a stationary suspension culture lose their differentiated functions within hours. Hepatocytes in monolayer culture lose their differentiated functions within 2–3 days (6). Therefore, different techniques, such as collagen entrapment, microcarrier-induced aggregates, spherical multicellular aggregates (spheroids), and coculture of hepatocytes with extracellular matrix, have been used to improve the performance of hepatocytes in culture (1, 2,5,17).

Recently, several studies have investigated different techniques to form spheroids, using proteoglycans, synthetic polymers, and various culture substrata (5,6). The aim of this study was to compare two different models of hepatocytes isolation in culture: isolated hepatocytes and hepatocyte spheroids.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 300–350 g, were used as donors. Animal studies were performed in compliance with National and International Guidelines for the Human care of experimental animals. The two models were: 1) isolated hepatocytes (IH): 95% pure

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(group G1); 2) hepatocyte spheroids (SH): 60% hepatocytes, 40% nonparenchymal cells, and extracellular matrix (group G2).

For IH a standard collagenase technique was used. The technique for SH has been previously described (2). Briefly, the liver was perfused by collagenase type IV (Gibco) at concentration of 0.05 g/ml, with 5 mmol of calcium chloride added at 37°C, and at the flow of 200 ml/min. The perfused segments were shred and passed trough a Ricordi digestion chamber. The liver cells (60% hepatic cells and 40% nonparenchimal cells) were filtered through 3 silk mesh at different porosities (from 500 to 200 μm). Then the liver cells, without shaking or spinner flasks, were suspended in a DMEM cold solution with fetal calf serum (FCS, Sigma) added at a concentration of 10% and then washed with calcium-free Hank's balanced salt solution (HBSS). Cell viability as assessed by the trypan blue dye exclusion test was 90–94%.

Samples of IH (G1) and SH (G2) containing 3×10^6 liver cells were plated on petri dishes (60 mm diameter) in 3 ml hormonally defined Williams E medium (Biochrom) and cultured for 30 days. Culture medium was changed every 2 days. Cells were cultured at 37°C with 5% CO_2 and water vapor saturated.

Enzyme-linked immunoadsorbent assay (ELISA) for rat albumin was performed every 2 days, to test the specific functions of the hepatocytes.

The culture medium was supplemented with 20 μg/ml of diazepam (DZP) on days 6, 13, and 20, and metabolite generation was investigated to test the maintenance of monoxygenase activity and metabolism in vitro. DZP and metabolite concentrations were assessed by HPLC analysis (Merck) on days 7, 14, and 21, monitoring the UV absorption of the benzodiazepines at 236 nm. Glycogen content was detected after 30 days of culture.

All values are expressed as mean ± SD. The different groups were compared using one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

ELISA assay (Fig. 1) showed a high albumin secretion in G2, while in G1 a decrease, more evident after 15 days of culture, was noted. In G2, the amount of secretion increased continuously in the first 24 days. A little decreased was noted from day 24 to day 30. In addition, the expression of albumin mRNA (Northern blot) also increased with time of culture, differently in the two groups (data not shown).

The metabolism of DZP is shown in Figure 2. Typical DZP metabolites (*N*-desmethyl-diazepam, oxazepam, and temazepam) were generated. Metabolite concentrations were higher in G2 that in G1 in all samples, but had statistical significance at days 14 and 21 ($p < 0.01$).

The glycogen content, after 30 days of culture, was very low in G1 (14.2 ± 4.4%), while it was 72.1 ± 2.6% in G2 ($p < 0.01$).

Light microscopy showed isolated hepatocytes dispersed in the medium in G1, with 60% of dead cells. In G2, the spheroids appeared in colonies surrounded by an extracellular matrix. The main diameter of spheroids was 84.4 ± 33.1 μm. The dead cells were less than 10%. An abundant rough endoplasmic reticulum was also found by transmission electron microscope, suggesting an active synthesis and secretion of serum proteins (data not shown).

DISCUSSION

Use of hepatocytes for the treatment of hepatic failure has many advantages (19). There are different possible sources of hepatocytes (cell lines, xenogenic hepatocytes, and stem of fetal cells), with limited availability of hepatocytes from discarded livers (16). Unfortunately, hepatocytes rapidly lose their differentiated functions when cultured as monolayers in vitro (3). In addition, the quality of the hepatocytes depends on the nature of the tissue and on the technique used for isolation, and porcine hepatocytes were found to be the most appropriate (11,18). However, isolated hepatocytes, under specific conditions, self-assemble into tissue-like structures called spheroids (8). Hepatocyte spheroids are ultrastructurally similar to liver tissue in vivo and maintain their differentiated function (albumin synthesis, cytochrome 450 activity, urea genesis) compared with monolayer cultures (15,20).

Our study confirms the effectiveness of a culture technique with extracellular matrix and nonparenchymal cells. Maintenance of a prolonged functional activity has been attributed to restoration of cell polarity and close cell-to-cell contact (1,2,12).

To test functional activity of hepatocytes, both synthetic and metabolic, we used two proven techniques: the production of albumin and the DZP transformation into metabolites. Because detoxification is one of the major functions of the liver, maintenance of full biochemical capacity is crucial for an artificial liver support. For this reason, DZP biotransformation, which reflects the cytochrome P450 activity of hepatocytes, is particularly useful and is considered to be crucial for recovery from hepatic failure (4).

Maintenance of albumin secretion is another essential biosynthetic function. It has been reported that albumin synthesis increases during the first 4 days before declining in both cultured and encapsulated hepatocytes (7). In our study we showed that protein synthesis was main-

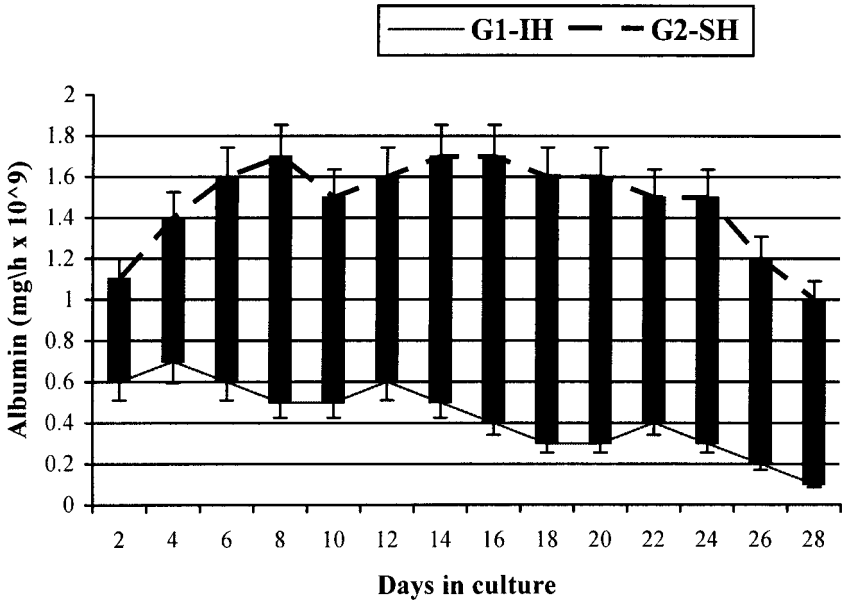

Figure 1. Albumin production in G1 IH (isolated hepatocytes) and G2 SH (hepatocytes spheroids).

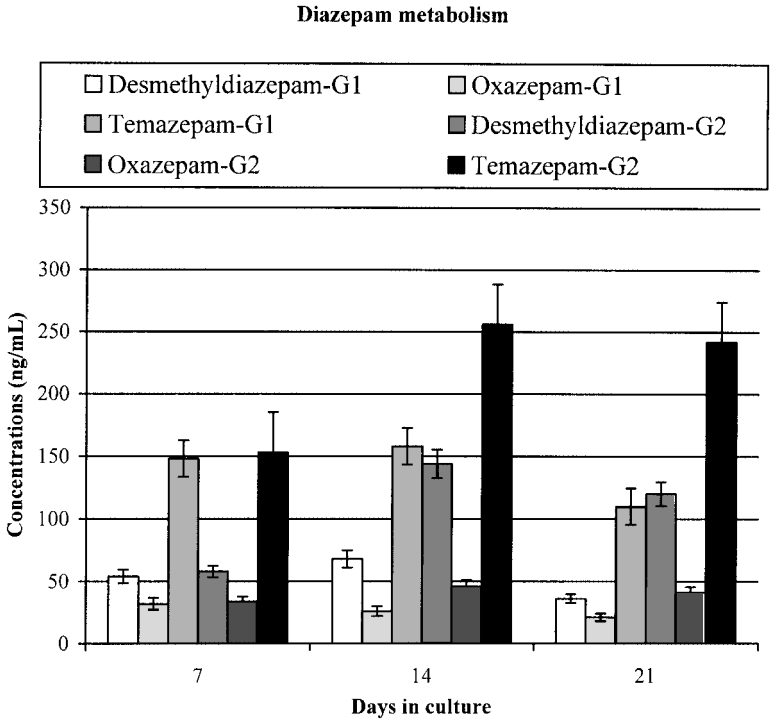

Figure 2. Diazepam metabolism in G1 and G2.

tained, and even increased, in G2. In G1 a progressive decreasing of function (both synthetic and metabolic) and detectable mortality rate were observed. In this group the technique of isolation and purification excludes the extracellular component, and the creation of the mentioned physiological structure. In contrast, in G2 extracellular component is maintained and interactions, through this network, are possible. A culture technique that allows the in vitro preservation of aggregate cell morphology, cell-to-cell contact, and interactions (e.g., formation of tight junctions) is crucial for growth and functionality of hepatocytes, and may be essential for the design of a bioartificial liver.

Many factors (hormones, growth factors, etc.) have been used to maintain cell viability and functions of hepatocytes. Bovine serum, in particular, is usually added to culture medium to promote growth and attachment of hepatocytes (9). However, an easy culture technique, without complex additives, may facilitate bioartificial supports where serum-free medium is used. Our data suggest the major role of nonparenchymal liver epithelial cells and extracellular matrix (including different growth factors and cytokines) in maintaining differentiated functions of hepatocytes.

Concerning the dimension of spheroids, it is interesting to note that with our technique we found main diameters <100 μm, considered to be the limit to avoid necrosis of hepatocytes in the central portion. In fact, oxygen is thought to permeate only up to 100 μm depth in the liver tissue (14). Nevertheless, the relation between size of the spheroids and our technique can be here only supposed.

In conclusion, we showed that isolated hepatocytes maintain their functional activity for a period significantly reduced, when compared to the hepatocytes spheroids, obtained with our technique. We confirmed the role of extracellular matrix as a crucial component to promote hepatocyte homeostasis, and the close link between cellular architecture and tissue-specific functions. Using the technique of coculture of hepatocytes with nonparenchymal cells and biomatrix is very attractive, but careful investigations are needed to confirm the exciting conclusion of our preliminary results.

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