TISSUE ENGINEERING
Volume 10, Number 1/2, 2004

Influence of Flow Conditions and Matrix Coatings on Growth and Differentiation of Three-Dimensionally Cultured Rat Hepatocytes

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ABSTRACT

Maintenance of liver-specific function of hepatocytes in culture is still difficult. Improved culture conditions may enhance the cell growth and function of cultured cells. We investigated the effect of three-dimensional culture under flow conditions, and the influence of surface modifications in hepatocyte cultures. Hepatocytes were harvested from Lewis rats. Cells were cultured on three-dimensional polymeric poly-lactic-co-glycolic acid (PLGA) matrices in static culture, or in a pulsatile flow-bioreactor system. Different surface modifications of matrices were investigated: coating with collagen I, collagen IV, laminin, or fibronectin; or uncoated matrix. Hepatocyte numbers, DNA content, and albumin secretion rate were assessed over the observation period. Culture under flow condition significantly enhanced cell numbers. An additional improvement of this effect was observed, when matrix coating was used. Cellular function also showed a significant increase (4- to 5-fold) under flow conditions when compared with static culture. Our data showed that culture under flow conditions improves cell number, and strongly enhances cellular function. Matrix modification by coating with extracellular matrix showed overall an additive stimulatory effect. Our conclusion is that combining three-dimensional culture under flow conditions and using matrix modification significantly improves culture conditions and is therefore attractive for the development of successful culture systems for hepatocytes.

INTRODUCTION

ALTERNATIVES TO LIVER TRANSPLANTATION as a treatment for liver diseases are currently under investigation.[1] These approaches are based on the use of single liver cells instead of the whole organ for the replacement of liver function. Mainly, two strategies were designed: (1) intracorporeal application (transplantation) of isolated hepatocytes for long-term treatment,[2] or (2) use of extracorporeal devices, such as bioartificial liver (BAL) systems containing hepatocytes. This tool is aimed at "bridging" until organ transplantation is possible or regeneration occurs.[3] However, a period of cell/tissue culture is crucial for both strategies: for cell transplantation, a cell culture period may permit an increase in cell numbers and function of hepatocytes; furthermore, a culture period may allow the genetic correction of, for example, diseased autologous hepatocytes[4] or the cryopreserva-

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tion[5] of cells. For BAL the achievement of enhanced specific function is attractive. Therefore, the optimization of hepatocyte-specific function and cell growth is the main objective of cell culture improvements.

To develop potent culture systems for hepatocytes, hepatotrophic stimulation of the cells *in vitro* is necessary.[6] Several stimulatory mechanisms were evaluated in hepatocyte cultures; (1) coating of culture dishes with isolated extracellular matrix (ECM) molecules,[7,8] (2) the addition of growth hormones and cytokines to the culture medium,[9,10] or (3) coculture with other cell types.[11–15] In particular the culture configuration was shown to have a major impact on cellular differentiation: cultures in "sandwich" configuration could achieve a significant elongation of the culture period, as well as an increase in specific function and cell growth.[8] A variety of novel culture systems for hepatocytes, including hydrogel microspheres, hollow fibers, and macroporous polymer scaffolds, were developed and shown to promote specific functions, such as albumin secretion or detoxification capacity.[16,17] Furthermore, initial data suggest a strong positive influence caused by flow in a bioreactor system for hepatocyte culture: hepatocytes cultured under flow conditions show new tissue formation and high albumin production.[18] This approach seems to be attractive, because it may permit the creation of a functional bioartificial liver tissue for transplantation.

The aim of this study was to evaluate the effect of flow conditions on hepatocytes cultured using polymeric matrices. In addition, we investigated the impact of coating the polymeric matrix with ECM molecules and analyzed various ECM components.

MATERIALS AND METHODS

Hepatocyte isolation

Hepatocytes were isolated from Lewis rats by two-step collagenase perfusion described by Seglen.[19] Donor animals received 250 units of heparin (Liquemin; Hoffmann-La-Roche, Nutley, NJ) before cell isolation. After cannulation of the portal vein the liver was perfused with a calcium-free buffer solution (1000 mL of distilled water, 8.3 g of NaCl, 0.5 g of KCl, 2.38 g of HEPES [Sigma, St. Louis, MO], pH 7.4; flow, 30 mL/min) at 37.0°C for 7 min. The liver was then perfused with collagenase solution (1000 mL of distilled water, 8.3 g of NaCl, 0.5 g of KCl, 2.38 g of HEPES, 0.7 g of $CaCl_2 \cdot 2H_2O$, 7.5 mg of trypsin inhibitor [ICN, Eschwege, Germany], and 500 mg of collagenase [Collagenase H; Roche, Mannheim, Germany], pH 7.35, flow, 30 mL/min) at 37°C for 8–11 min.

The perfused liver was resected, and the cells were released by gentle shaking and collected in 20 mL of William's medium E without L-glutamine (GIBCO-BRL, Eggenstein, Germany). The cell suspension was filtered through a 200-μm pore size nylon mesh and washed two times with William's medium E (centrifugation at 50 *g*; 4°C for 3 min). Cells were purified by Percoll (density, 1.13 g/cm^3; Sigma) gradient centrifugation (400 × *g*; 4°C for 12 min) and washed two times in William's medium E.

Poly-lactic-co-glycolic acid matrix fabrication and coating with ECM

Polymer matrices were fabricated from poly-lactic-co-glycolic acid (PLGA). In brief, an 85:15 copolymer (Resomer RG 858; Boehringer Ingelheim, Ingelheim, Germany) was ground after freezing polymer pellets in liquid nitrogen. After grinding, polymer was sieved, and particles sized between 108 and 250 μm were used for matrix fabrication. Sodium chloride was sieved in a large sifter, and particles of a size between 250 and 425 μm were used for matrix fabrication. A mixture of 760 mg of salt and 40 mg of polymer per matrix was filled in a die. After this, a pressure of 1000 lb/in^2 was applied to the mixture for 1 min. For gas foaming, sponges were foamed for 24 h in CO_2 (850 lb/in^2) gas atmosphere on a Teflon dish. Polymer matrices with entrapped salt particles were then leached in water for 48 h to remove the salt particles; afterward the matrices were dried on absorbent paper overnight. Highly porous polymer sponges resulted, with a defined porosity of 95 ± 2%, and a pore size of 250 ± 120 μm. For coating with ECM, sponges were placed in a solution of type I collagen from calf skin (3 μg/mL; Sigma), laminin from EHS mouse sarcoma (3 μg/mL; Sigma), type IV collagen from EHS mouse sarcoma (3 μg/mL; Sigma), or fibronectin from human plasma (3 μg/mL; Sigma) in pH 9.4 carbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate). All matrices were lyophilized to remove residual solvents and were sterilized by γ irradiation before use.

Culture medium, hepatocyte seeding, and culture conditions

Culture medium. For culture a hormone-stimulated serum-free culture medium was used, consisting of 500 mL of William's medium E without L-glutamine (GIBCO-BRL) and supplemented with L-glutamine (50 mg/L), penicillin–streptomycin (100 IU/L), 20 mM HEPES, 20 mM sodium pyruvate (GIBCO-BRL), 5 nM dexamethasone (Sigma), epidermal growth factor (10 ng/mL; GIBCO-BRL), and insulin (20 mU/mL; GIBCO-BRL).

Seeding of hepatocytes on polymers. Hepatocytes (5 × 10^6) were initially seeded on a cylindrical disk (Q = 12.5 mm, d = 3 mm) of polymeric PLGA matrix. Matrices

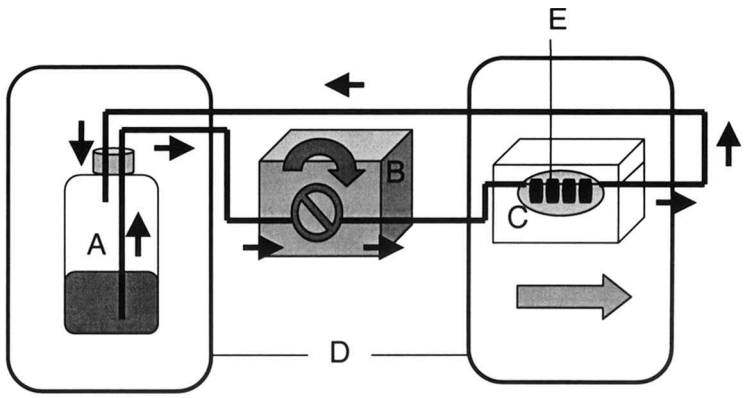

FIG. 1. Scheme of the flow bioreactor system. A 250-mL medium reservoir (A) containing culture medium was placed in the cell culture incubator (D; 37°C, 95% humidity, 5% CO_2 atmosphere). Silicone tubes allowing gas exchange connect the medium reservoir with a pulsatile pump (B), which provides a flow rate of 15 mL/min. Arrows indicate the direction of flow. Cell-seeded three-dimensional poly-lactic-co-glycolic acid (PLGA) matrices (E) were placed in the culture chamber (C; Minucells) in the culture incubator (D). The flow circuit used was continuous.

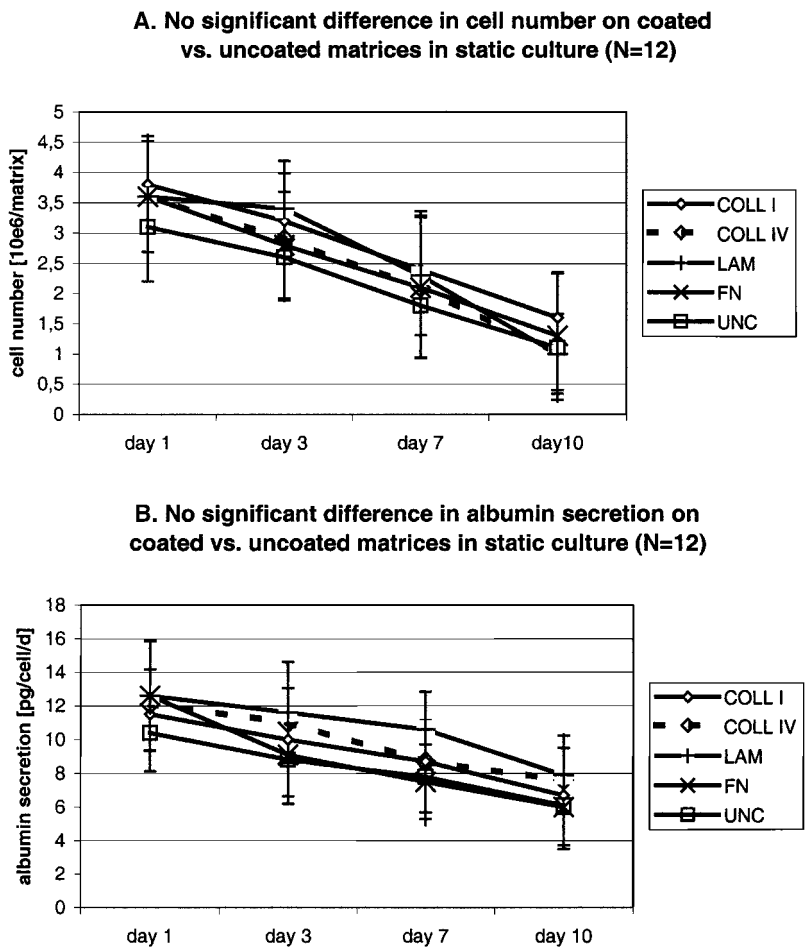

FIG. 2. Cell number (**A**) ([mean value ± SD] × 10^6 per albumin secretion rate (**B**) (mean value ± SD, in picograms per cell per day) of hepatocytes cultured on poly-lactic-co-glycolic acid (PLGA) matrices with different coatings under static conditions. No significant differences in cell number or albumin secretion rate were observed between cultures on coated versus uncoated matrices. COLL I, collagen I; COLL II, collagen II; LAM, laminin; FN, fibronectin; UNC, uncoated.

were either used uncoated, or after coating with collagen type I, collagen type IV, laminin, or fibronectin as described above.

Culture conditions. For static culture, seeded matrices were kept in six-well plates (Nunclon multidishes; Nalge Nunc International, Rochester, NY) with 2 mL of culture medium, and were kept in a cell culture incubator at 37°C, 95% humidity, and 5% CO_2 gas atmosphere. Culture media were changed every other day.

Culture in flow bioreactor system. For culture under flow conditions, seeded polymers were kept in flow culture chambers (Minucells, Regensburg, Germany), connected by gas-permeable silicone tubes with the medium reservoir (Fig. 1). Culture chambers were placed in a cell culture incubator at 37°C, 95% humidity, and 5% CO_2 gas atmosphere, with a pulsatile flow rate of 7.5 mL of culture medium per minute for seeding (first 12 h), and then 15 mL/min from a 250-mL reservoir (Ismatec pump; Ismatec, Glattbruch-Zurich, Switzerland). Culture media were changed every other day.

DNA quantification, cell number, and viability

Matrices were harvested and washed in Dulbecco's modified Eagle's medium without serum (DMEM), and were frozen in liquid nitrogen. Samples were then dried in a lyophilizer for 8 h. Samples were incubated with proteinase K for 10 h at 60°C in a water bath. After the preparation DNA was isolated, using a DNAQuant kit (Qiagen, Berlin, Germany) according to the manufacturer's instructions. DNA content was then measured with a photometer (Hoefer DyNA Quant 200; Amersham Biosciences, Uppsala, Sweden), at an extinction wavelength of 365 nm and an emission of 460 nm. DNA con-

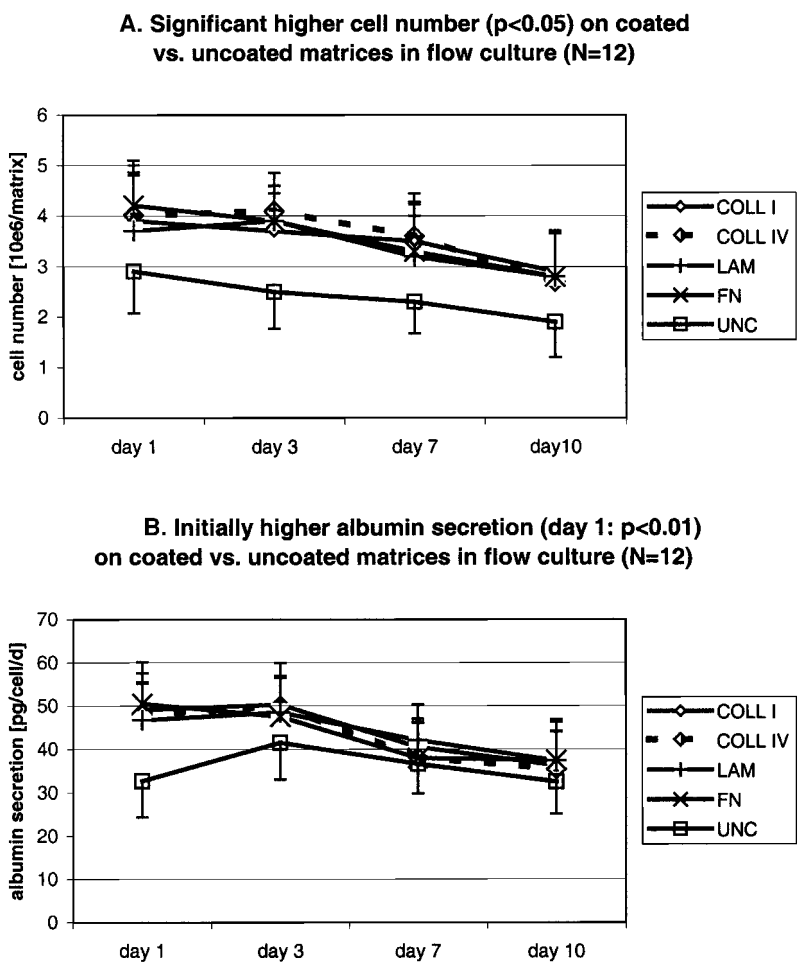

FIG. 3. Cell number (**A**) ([mean value ± SD] × 10^6 per matrix) and albumin secretion rate (**B**) (mean value ± SD, in picograms per cell per day) of hepatocytes cultured on poly-lactic-co-glycolic acid (PLGA) matrices with different coatings under flow conditions. Higher cell numbers ($p < 0.05$) and initially enhanced albumin secretion rate ($p < 0.01$) were observed on coated versus uncoated matrices under flow conditions. See Fig. 2 for abbreviations.

tent was calibrated with calf thymus DNA (Amersham Biosciences). A standard curve was calculated from DNA values of known hepatocyte numbers, and hepatocyte number was calculated from measured DNA values. For each group and time point 12 samples were evaluated. Cells were counted under standardized conditions, using a light microscope (Olympus, Hamburg, Germany). General metabolic activity of cultured cells was tested by their ability to reduce 3-(4,5-dimethyldiazol-zyl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma).

Albumin ELISA

Albumin secreted in the culture medium was measured in a standard enzyme-linked immunosorbent assay (ELISA).[20] Medium samples from static cultures were harvested from culture supernatant. To assess albumin secretion of cultures under flow conditions, seeded polymers were removed from the bioreactor system and incubated for 6 h in six-well culture dishes with 3 mL of culture medium. Medium samples were then harvested from the supernatant. Anti-rat albumin antibodies and albumin standards were purchased from Organon Teknikon-Cappel (Durham, NC). Briefly, 96-well plates (Nunclon multidishes) were coated overnight at 4°C with the sheep anti-rat albumin antibody. Afterward, 1% gelatin (Sigma) was added for 1 h at 37°C to block unspecific binding. After washing three times with 0.05% Tween 20, sample was added. After 1 h of incubation at 37°C the plates were washed, and peroxidase-conjugated sheep anti-rat albumin antibody (diluted 1:5000) was added for 1 h. The plates were then washed and color reaction with the substrate 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was allowed for 45 min at room temperature. Samples were measured in a photometer (MR 5000; DynaTech, Overath, Germany) at 450 nm. A standard

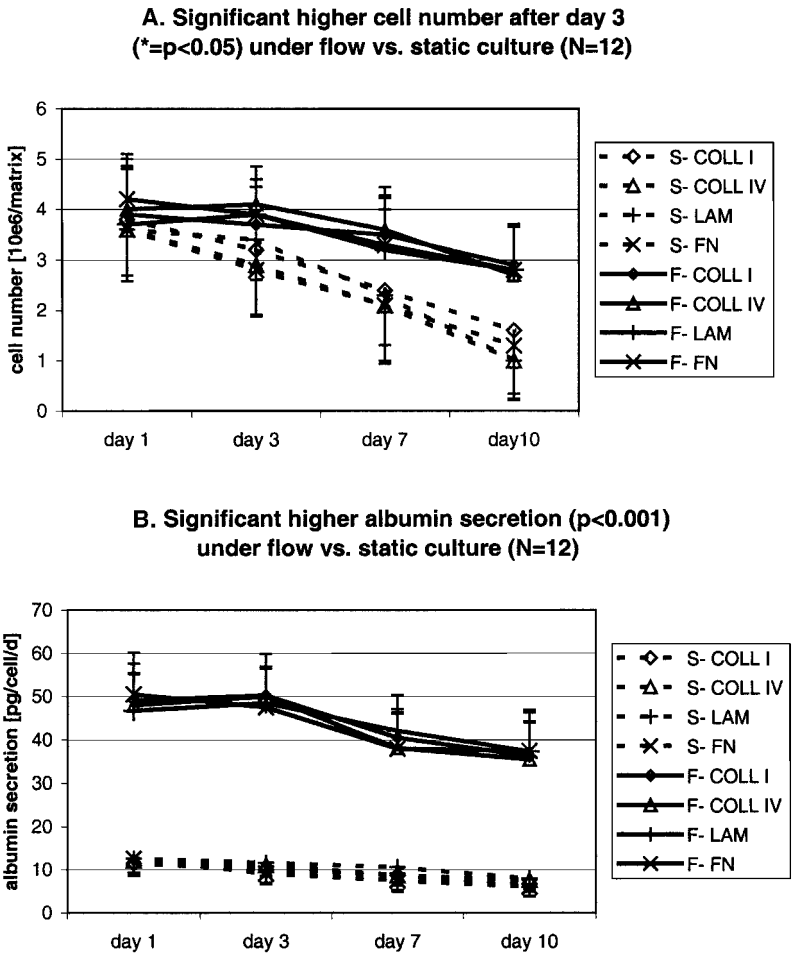

FIG. 4. Cell number (**A**) ([mean value ± SD] × 10^6 per matrix) and albumin secretion rate (**B**) (mean value ± SD, in picograms per cell per day) of hepatocytes cultured on poly-lactic-co-glycolic acid (PLGA) matrices with different coatings and under flow versus static conditions. Significant higher cell numbers were observed at the end of the culture period in cultures under flow versus static conditions ($p < 0.05$). Albumin secretion was strongly increased (up to 4- to 5-fold) under flow conditions, when compared with static conditions ($p < 0.001$). S, static; F, flow; see Fig. 2 for other abbreviations.

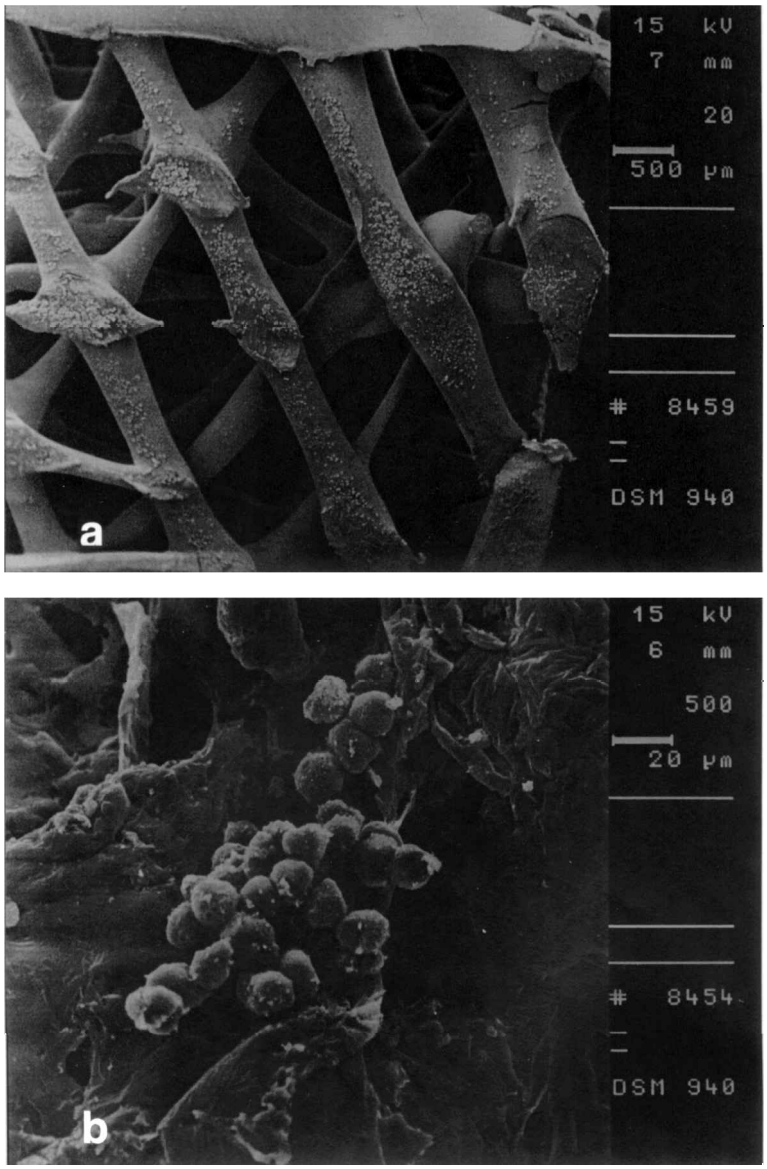

FIG. 5. Scanning electron microscopy (SEM) of hepatocytes seeded on three-dimensional PLGA matrices. (**A**) Cells directly after seeding on the PLGA matrix. Note the isolated cells attaching to the polymer fibers. (**B**) SEM of hepatocytes cultured for 3 days on PLGA matrices in flow culture. Cells tend to form small groups, and secreted their own extracellular matrix. (**C**) SEM of hepatocytes cultured for 7 days on PLGA matrices under flow conditions. Cells show spreading of podocytes into the surrounding tissue.

curve was established by measuring known amounts of purified rat albumin. Each probe was measured in duplicate, and the mean value was calculated. For each group and time point 12 samples were evaluated.

Scanning electron microscopy

Specimens were fixed in glutaraldehyde for 24 h and then microdissected in 80% ethanol. After dehydration, the specimens were dried by the critical point method, gold sputtered, and then examined and photographed with a DMS 940 scanning electron microscope (Carl Zeiss, Oberkochen, Germany) (Fig. 5).

Statistical analysis

Mean values and standard deviations for cell numbers (10^6 cells per matrix) and albumin secretion rate (picograms of albumin per cell per day) were calculated. Statistical analysis was performed on an MS-Windows system (Windows 98) using Excell 2000 software (Microsoft, Redmond, WA). Groups were analyzed for statistical significance by Student t test. p

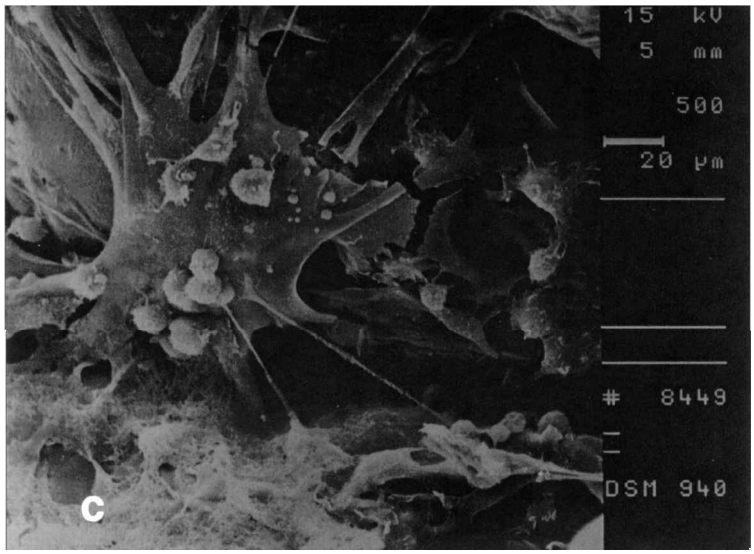

FIG. 5. (*continued*)

Values were two tailed and $p < 0.05$ was considered significant.

RESULTS

Results of hepatocyte isolation

Viability ranged from 80 to 95% as assessed by the trypan blue exclusion method. Cell number ranged from 3.0×10^8 to 4.0×10^8 viable hepatocytes per liver.

Influence of matrix coatings on cell number in static and flow cultures

Static culture. In all groups cell number decreased over time in culture. In cultures on coated PLGA matrices cell number decreased from $(3.8 \pm 0.8) \times 10^6$ cells per matrix (collagen I), $(3.6 \pm 1.02) \times 10^6$ cells per matrix (collagen IV), $(3.6 \pm 0.91) \times 10^6$ cells per matrix (laminin), and $(3.6 \pm 0.91) \times 10^6$ cells per matrix (fibronectin) on day 1 to $(1.6 \pm 0.73) \times 10^6$ cells per matrix (collagen I), $(1.0 \pm 0.79) \times 10^6$ cells per matrix (collagen IV), $(1.0 \pm 0.66) \times 10^6$ cells per matrix (laminin), and $(1.3 \pm 1.06) \times 10^6$ cells per matrix (fibronectin) on day 10 (Fig. 2A). There were no significant differences in cell numbers between all groups in static culture on coated polymers. Cell numbers of cultures on uncoated PLGA matrices also decreased from $(3.1 \pm 0.9) \times 10^6$ cells per matrix on day 1 to $(1.1 \pm 0.7) \times 10^6$ cells per matrix on day 10. No significant difference was observed between cultures on uncoated matrices and matrices coated with ECM (all coatings). MTT assays showed general metabolic activity of all attached cells at all times observed (data not shown).

Culture in a flow bioreactor. In all cultures cell number decreased over time. In flow cultures on coated PLGA matrices cell number decreased from $(3.9 \pm 0.96) \times 10^6$ cells per matrix (collagen I), $(4.0 \pm 1.0) \times 10^6$ cells per matrix (collagen IV), $(3.7 \pm 1.1) \times 10^6$ cells per matrix (laminin), and $(3.9 \pm 0.9) \times 10^6$ cells per matrix (fibronectin) on day 1 to $(2.9 \pm 0.81) \times 10^6$ cells per matrix (collagen I), $(2.7 \pm 0.95) \times 10^6$ cells per matrix (collagen IV), $(2.8 \pm 0.9) \times 10^6$ cells per matrix (laminin), and $(2.8 \pm 0.89) \times 10^6$ cells per matrix (fibronectin) on day 10 (Fig. 3A). There were no significant differences in cell numbers between all groups under flow conditions on coated polymers. Cell numbers of cultures on uncoated PLGA matrices also decreased from $(2.5 \pm 0.83) \times 10^6$ cells per matrix on day 1 to $(1.9 \pm 0.7) \times 10^6$ cells per matrix on day 10. Higher cell numbers on coated polymers compared with uncoated polymers were statistically significant, with p values of $p < 0.05$ (all coatings) on day 1, $p < 0.001$ (all coatings) on day 3, $p < 0.01$ (all coatings) on day 7, and $p < 0.05$ (all coatings) on day 10. MTT assays showed general metabolic activity of all attached cells at all times observed (data not shown).

Static culture versus flow culture. Significantly higher cell numbers were found in cultures under flow conditions (Fig. 4A) compared with static culture on collagen IV-coated matrices from day 3 on ($p < 0.01$), on fibronectin-coated matrices from day 3 on ($p < 0.05$), on collagen I- or laminin-coated matrices from day 7 on ($p < 0.05$), and on uncoated matrices at day 10 ($p < 0.01$).

Influence of matrix coatings and flow on hepatocyte albumin secretion rate

Static culture. In all groups the albumin secretion rate decreased over time in culture. In cultures on coated PLGA matrices albumin secretion decreased from 11.5 ± 2.67 pg/cell per day (collagen I), 12.1 ± 3.52 pg/cell per

day (collagen IV), 12.6 ± 3.24 pg/cell per day (laminin), and 12.6 ± 3.28 pg/cell per day (fibronectin) on day 1 to 6.7 ± 2.8 pg/cell per day (collagen I), 7.6 ± 2.7 pg/cell per day (collagen IV), 7.9 ± 2.32 pg/cell per day (laminin), and 6.0 ± 2.28 pg/cell per day (fibronectin) on day 10 (Fig. 2B). The albumin secretion rate of cells cultured on uncoated PLGA matrices also decreased from 10.4 ± 2.27 pg/cell per day on day 1 to 6.1 ± 2.61 pg/cell per day on day 10. No significant difference was observed between cultures on uncoated matrices and matrices coated with ECM (all coatings).

Culture in a flow bioreactor. In cultures on coated PLGA matrices albumin secretion decreased from 49.1 ± 8.47 pg/cell per day (collagen I), 48.0 ± 7.49 pg/cell per day (collagen IV), 46.7 ± 8.49 pg/cell per day (laminin), and 50.5 ± 9.63 pg/cell per day (fibronectin) on day 1 to 36.2 ± 7.81 pg/cell per day (collagen I), 35.4 ± 8.83 pg/cell per day (collagen IV), 37.3 ± 9.6 pg/cell per day (laminin), and 37.4 ± 8.89 pg/cell per day (fibronectin) on day 10 (Fig. 3B). The albumin secretion rate of cells cultured on uncoated PLGA matrices also deceased from 32.7 ± 8.37 pg/cell per day on day 1 to 32.5 ± 7.37 pg/cell per day on day 10. The differences in albumin secretion between all cultures on coated polymers and cultures on uncoated polymers were significant on day 1 ($p < 0.001$), but no statistical significance was found after day 3. No significant differences were observed when cultures on coated polymers with different ECM coatings were evaluated.

Static culture versus flow culture. A strongly enhanced albumin secretion was found in all cultures under flow conditions when compared with static cultures; the statistical significance was high ($p < 0.001$) at all time points and in all groups (Fig. 4B).

DISCUSSION

The results of this study indicate that flow conditions provide a useful stimulus for hepatocytes in culture. In this study we combined culture on three-dimensional polymeric matrices with the application of medium flow, using a bioreactor system. Hepatocytes on three-dimensional matrices under flow conditions showed better maintenance of cell number, when compared with hepatocytes cultured under static conditions. Also, specific function was significantly higher (4- to 5-fold) under flow conditions, as assessed by measuring the albumin secretion rate. This is consistent with the findings of other investigators, who showed a positive effect of physical flow on the detoxification capacity of cultured hepatocytes.[21] Stimulation of the albumin secretion rate was found in hepatocyte cultures, using scaffolds of PLGA under flow conditions, when a galactose modification-versus-glucose modification of the scaffold was performed.[22] Initial results with hepatocytes cultured on poly-L-lactic acid (PLLA) scaffolds without coating in a flow bioreactor system showed that hepatocytes constitute a "neotissue" by formation of spheroids, which depends on the medium and flow conditions used.[18] In this study, we compared the culture of hepatocytes on polymeric matrices under flow conditions and static conditions, and showed that flow alone is an important factor in improving culture conditions for hepatocytes. In previous studies, culture configuration was shown to have a strong influence on hepatocyte viability and function. Using the "sandwich" configuration, or culturing on three-dimensional polymeric scaffolds, could significantly enhance cell attachment and specific function.[8,17] However, liver-specific function decreased over time under all conditions. In this study we achieved stable cell function and a minimal decrease in cell number over a culture period of 10 days, when cells were cultured under flow conditions. The differences observed between cultures under flow and static conditions may be due to enhanced mass transport of nutrients, oxygen supply, and cellular waste products in the flow culture. In contrast, under static conditions the mass transport by diffusion is limited by a high cell density, and by the thickness of the polymeric scaffolds. Thus, culturing hepatocytes under flow conditions seems to be favorable over culture under static conditions, in order to overcome these problems of three-dimensional cell culture.

The role of specific ECM coatings of tissue culture plastic in regulating hepatocyte attachment and function has been clearly demonstrated in several other studies.[7,8,23,24] It was shown that matrix type and matrix concentration (configuration) mainly influenced growth and function of cultured hepatocytes: by the use of different concentrations of ECM it was possible to switch proliferation of liver cells to specific function and vice versa.[7] These effects may rely on the activation of mechanochemical signal pathways by induction of a distinct configuration of the cytoskeleton[23] as, for example, shown for the formation of tubular structures of cultured endothelial cells.[25] Previous studies have documented enhanced cell attachment and a loss of liver-specific function resulting from culture on collagen I-coated dishes, or on dishes coated with laminin or collagen IV.[7] However, culture in a collagen sandwich or in collagen gel strongly enhanced hepatocellular function.[8,17] The modification of three-dimensional scaffolds by ECM also showed a strong influence on cultured liver cells: treatment of PLGA sponges with collagen or gelatin coatings resulted in enhanced DNA content, and urea synthesis was decreased.[26] In our study, we found improved cell attachment and cell number on PLGA polymers coated with collagen I, collagen IV, laminin, or fibronectin,

when compared with uncoated polymers. Under flow conditions this effect was significant and comparable for all ECM types used, and was additive to the improvement of cell number caused by flow alone. Cellular function was also initially higher on ECM-coated polymers than on uncoated polymers, but little functional difference was observed toward the end of the culture period. Thus, we conclude that the ECM coating promotes cell attachment and maintenance of cell number, but has minimal effect on specific cellular function. However, it is worth noting that ECM coatings allowed stable albumin production and did not lead to a loss of cellular function.

In summary, we investigated the effect of flow provided by a bioreactor system on cultured hepatocytes, using three-dimensional polymeric PLGA scaffolds. Furthermore, we evaluated the impact of ECM coatings with different matrices on cell number and function in this culture model. Our results show that flow alone has a highly stimulatory effect on cell number and function of hepatocytes seeded on three-dimensional polymeric matrices. Coating with ECM molecules increased cell numbers in an additive manner, and allowed stable cell function over the whole observation period under flow conditions. Under static conditions, little effect was observed by ECM modification of the matrices. Combination of flow provided by a bioreactor system with the use of three-dimensional polymeric matrices coated with ECM molecules may help to overcome problems of conventional culture systems for hepatocytes.

ACKNOWLEDGMENTS

The authors thank Mrs. B. Appl and Mrs. B. Roth for technical assistance. This work contains parts of the doctoral thesis of J.H. and T.M. It was supported by grants from the Deutsche Forschungsgemeinschaft (DFG KA 971-3.1) (Bonn, Germany) and the Werner-Otto-Stiftung (Hamburg, Germany).

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