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Chitosan/gelatin composite microcarrier for hepatocyte culture

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Abstract

Solid and porous chitosan/gelatin (CG) composite microcarriers were prepared by a water-in-oil emulsion process with additional freezing and lyophilization. Adult rat hepatocytes (10^6 cells ml^{-1}) attached on CG microcarriers maintained at least 15 d of viability and differentiated functions. Over 15 d, unimmobilized hepatocytes released 1.34-fold less lactate dehydrogenase (LDH), and retained 1.63-, 1.51- and 1.28-fold higher albumin secretion, urea synthesis and 7-ethoxycoumarin deethylation activities, respectively, than those on collagen-coated microcarriers. The CG matrix is therefore a promising microcarrier for hepatocyte culture.

Introduction

Microcarriers are frequently used for large-scale animal cell culture (Kwon & Peng 2002, Grohn *et al.* 1997). The viability and function of anchorage-dependent mammalian cells are strongly related to the surface to which they are attached and depend on the interactions between the cells and the matrix (Reid 1990). Chitosan is a linear polysaccharide consisting of $\beta(1 \rightarrow 4)$ linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups, which are similar to glycosaminoglycans, the components of extracellular matrix. Gelatin, a hydrolysis product of collagen, contains carboxyl groups on its chain backbones and can bind to chitosan by hydrogen bonding (Taravel & Domard 1993). Chitosan bonded with gelatin results in a composite polymer (CG) with improved mechanical properties and cytocompatibility (Cheng *et al.* 2003) that can act as a matrix for cell attachment. The CG composite film can support many types of cells and maintain their functions (Mao *et al.* 2003, Cheng *et al.* 2003). In this study, we have fabricated microcarriers made of chitosan and gelatin to grow anchorage-dependent mammalian cells. Viabilities and liver specific functions of primary rat hepatocytes on the microcarriers have been evaluated for the possible utility of the microcarriers in an artificial liver device.

Materials and methods

Materials

Chitosan (Mr $\sim$400 000) and gelatin (pH 5, 180 Bloom) were from Fluka. Collagenase type IV and Dulbecco's modified Eagle medium (DMEM) were from Gibco BRL. LDH kit, Rat albumin ELISA kit and urea UV detection kit were from Boehringer Mannheim, Bethyl Laboratories and Centronic GmbH, respectively. All other materials used were from Sigma unless otherwise specified.

Preparation of CG microcarriers

CG microcarriers were prepared by a protocol based on the methods of Shu & Zhu (2001) and Wissemann & Jacobson (1985). Briefly, 2% (w/v) chitosan in acetic acid was mixed with an equal amount of gelatin in aqueous solution. The homogeneous mixture was added to mineral oil containing 2% (v/v) Span 80, resulting in a water-in-oil (w/o) emulsion

under mechanical stirring. Glutaraldehyde was incorporated into the mixture to give 1% (v/v). The formed microbeads were washed with 1% (v/v) Tween 80 until the oil was completely removed. Two stainless steel meshes with grid sizes of 100 μm and 280 μm were employed to collect the microbeads with diameters of 100–280 μm, which served as solid microcarriers. All glass ware was silanized with dimethyl dichlorosilane/chloroform (1:50, v/v). For preparation of the porous microcarriers, the microbeads with diameters of 450–600 μm were collected. The microbeads were frozen at -30 °C for 2 d and then lyophilized for 2 d. Both the microcarriers were washed with double distilled water and then were treated with 0.1 M glycine to block non-reacted aldehyde groups. The microcarriers were further stabilized with 1.2% (w/v) NaOH to remove residual acetic acid and thoroughly washed with double distilled water. Finally, the microcarriers were kept in phosphate buffer after being autoclaved (115 °C, 20 min). Before cell seeding, the microcarriers were dipped into DMEM overnight.

Determination of microcarrier density

One ml solid microcarrier suspension (containing approx. 5×10^4 solid microcarriers) was transferred into an Eppendorf tube. The volume of the microcarriers was calculated according to the supernatant volume measured by a 1 ml stripette (D = 0.01 ml) after sedimentation of the microcarriers. A balance (D = 0.01 mg) was used to determine the weight of microcarriers remaining in the tube.

Isolation and culture of hepatocytes

Hepatocytes were isolated from female Sprague–Dawley rats (200–250 g) as described by Aiken *et al.* (1990) and purified according to Dunn *et al.* (1991). Viability of the freshly isolated hepatocytes was 92–98% as determined by Trypan Blue exclusion assay. Non-parenchymal cell contamination was less than 1% as judged by its size and morphology.

Hepatocytes were cultured in serum-free medium, consisting of DMEM supplemented with (l^{-1}): 0.3 g L-glutamine, 25 μg $CuSO_4 \cdot 5H_2O$, 0.5 μg Na_2SeO_3, 14 ng $ZnSO_4 \cdot 7H_2O$, 39 μg dexamethasone, 200 U insulin, 20 μg epidermal growth factor (Pepro Tech), 4 μg glucagon, 6.25 mg transferrin (Calbiochem), 50 μg linoleic acid, 2 g bovine albumin, 3.6 g HEPES, 50 mg gentamycin and 100 mg chloramphenicol. Solid CG microcarriers were seeded at 10^4 beads ml^{-1} into Petri dishes containing 10^6 hepatocytes ml^{-1}. For porous CG microcarriers, the ratio of cell to bead was 500:1. Cytodex 3, a collagen coated microcarrier, was used as control. Its cell seeding was the same as that for the solid CG microcarrier. The cultures were gently swirled every 30 min for 3 h. After incubation for 6 h, unattached cells were washed away from the cultures by fresh medium. Monolayer culture of hepatocytes was carried out on 1:1 mixed CG film with 7×10^4 cells cm^{-2}. Medium was changed the following day and then every other day.

Scanning electron microscope (SEM)

After hepatocytes were cultured on porous CG microcarriers for 24 h, the microcarrier cultures were pretreated with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h, and then were postfixed in 1% (w/v) OsO_4 for 1 h followed by dehydration in an ethanol series (50% to 100%, v/v). Finally, the samples were dried in a critical point dryer and coated with gold. The coated samples were observed under scan electron microscope.

Viable and functional assays

Collected supernatant samples were analyzed for lactate dehydrogenase (LDH), albumin and urea content with corresponding kits according to the manufacturer's guidance. For 7-ethoxycoumarin-*O*-deethylation assay (Langsch & Bader 2001), hepatocyte cultures were incubated with 260 μM 7-ethoxycoumarin for 5 h. Formation of 7-hydroxycoumain (7-HC) was quantified by fluorometry (excitation 360 nm, emission 460 nm). The spectrofluorometer was calibrated using 7-HC standards. The DNA content of cultured hepatocytes was determined with a fluorometric DNA assay described by Rago *et al.* (1990), using Hoechst dye 33258.

Results and discussion

Characteristics of microcarriers

The procedure described above resulted in yellowish microcarriers with optically transparent and smooth surfaces (Figure 1a). The freezing and lyophilization process generated an open pore microstructure with a high degree of interconnectivity on the surface of the microcarriers. The pore sizes ranged from 50 to

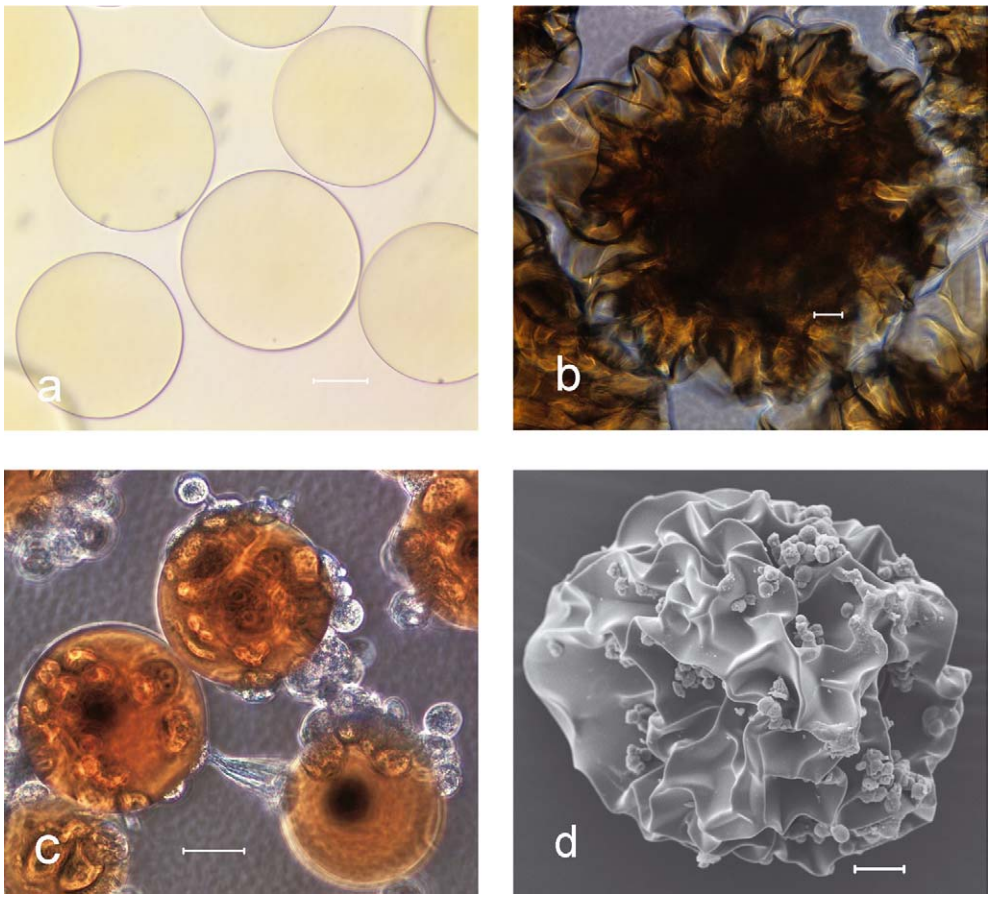

Fig. 1. Light microscope of (a) bare solid, (b) porous and (c) hepatocyte-seeded solid chitosan/gelatin microcarriers as well as scanning electron microscope of (d) hepatocyte-seeded porous chitosan/gelatin microcarriers. Hepatocytes were cultured in serum-free medium for 1 d. Bar = 50 μm.

200 μm (Figures 1b, d). The density of the microcarriers was approx. 1.02 g ml^{-1}, which was suitable for cell suspension culture and could be changed with different concentration of initial CG mixture. The microcarriers were stable in medium for at least two months and showed no indication of disintegration during the culture.

Hepatocyte culture on microcarriers

When incubated in serum-free medium, the hepatocytes attached to the solid microcarriers within 3–4 h and spreaded on them after 5 h. An increase in the ratio of chitosan to gelatin lowered the attachment and the spreading. A small percentage of close solid microcarriers formed aggregates joined together by hepatocytes (Figure 1c). The aggregates increased with time. For the porous microcarriers, most hepatocytes formed clusters in the cavities. Under SEM, the hepatocytes settled well in the cavities of the porous microcarriers with well-developed microvillus protrusions (Figure 2). After an initial peak on d 1, LDH activities in the supernatant decreased gradually and remained low from d 5 onwards. In comparison, LDH activity was highest for the Cytodex 3 cultures and lowest for the monolayer cultures (Figure 3a). The higher value of LDH in the microcarrier cultures was likely due to the mixing at the beginning of culture.

Functions of hepatocytes on microcarriers

The albumin secretion rate increased to stable levels after 7 d in the CG microcarrier cultures and was approx. 1.6-fold higher than that in the Cytodex 3 cultures, whereas it peaked on d 5 and decreased thereafter in the monolayer cultures. The urea levels in the supernatant from the solid, the porous CG microcarrier, the Cytodex 3 and the monolayer cultures

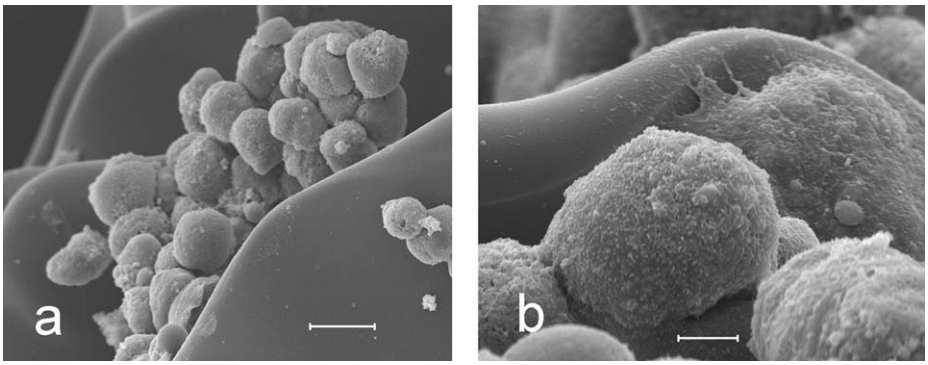

Fig. 2. Scanning electron microscope of hepatocytes in the pores of the chitosan/gelatin microcarriers. (a) Hepatocyte aggregates in the pores (bar = 15 μm). (b) There are plenty of microvillous protrusions on the surface of hepatocytes (bar = 5 μm). Hepatocytes were cultivated in serum-free medium for 1 d.

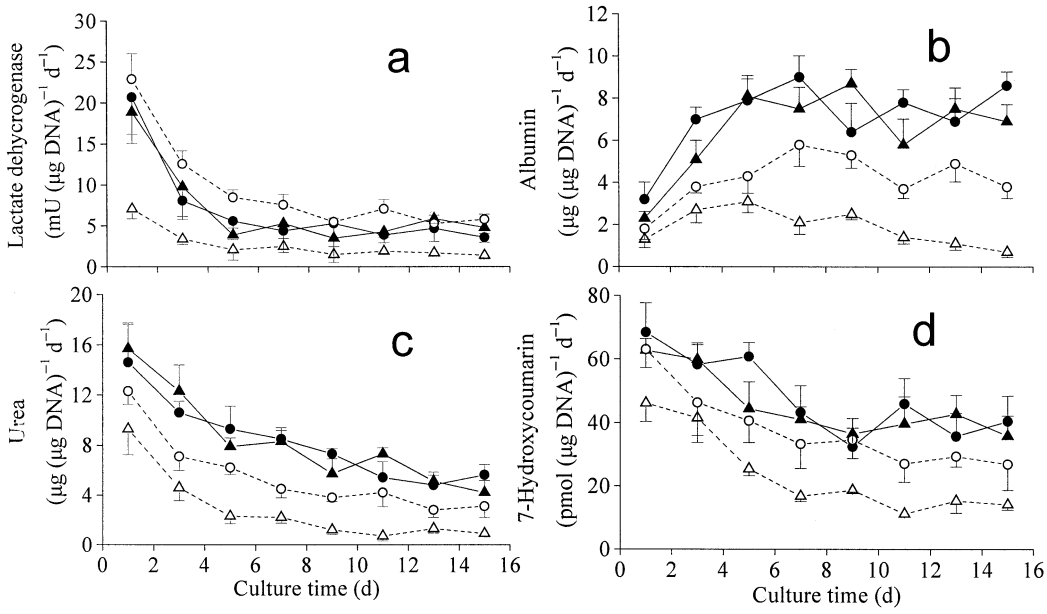

Fig. 3. Time course of (a) lactate dehydrogenase, (b) albumin, (c) urea and (d) 7-hydroxycoumarin levels in the supernatant obtained from cultured hepatocyte. Hepatocytes were cultured on (●) solid, (▲) porous, (○) Cytodex 3 microcarriers and on (△) CG films with serum-free medium. The error bars represent standard errors from triplicate culture.

decreased from 14.6 ± 3, 15.7 ± 2.1, 12.3 ± 1.1 and 9.3 ± 2.1 to 5.6 ± 0.8, 4.2 ± 0.9, 3.1 ± 0.9 and 0.9 ± 0.3 μg μg DNA^{-1} d^{-1}, respectively, throughout the culture period.

7-HC formation decreased in all cultures over the first week followed by the stabilization that was at the highest level in the CG microcarrier cultures and at the lowest level in the monolayer cultures. No difference was observed between the two types of CG microcarrier cultures though porous microcarriers could be seeded with more cells, which can save cultivation volume (Figures 3b–d). The prolonged cell functions observed in microcarrier-cultured hepatocytes probably are related to the formation of the multicellular aggregates. The cytoarchitecture and extensive cell-cell communication in aggregates, which resemble those in the liver, was hypothesized to contribute to extended liver-specific activities (Peshwa *et al.* 1996, Landry *et al.* 1985). Chitosan component of the CG microcarriers, owing to its analogy to natural hepatic matrix (Kawase *et al.* 1997), may play a important role in maintaining the differentiated liver functions superior to the Cytodex 3.

In conclusion, the CG microcarrier may have the potential application to scale up primary hepatocyte culture.

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