Effectiveness of Fructose-Modified Chitosan as a Scaffold for Hepatocyte Attachment

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Free amino groups of chitosan, which has previously been shown to be a good scaffold for hepatocyte attachment, were covalently modified with fructose. The modification significantly increased the number of cells that could be attached on the surface of chitosan gel. Rat hepatocytes cultivated on fructose-chitosan behaved similarly to those on unmodified chitosan, i.e., they retained the spherical shape they have in vivo, and released much less lactate dehydrogenase than cells attached on a collagen-coated surface. The modification with fructose did not alter the important characteristics of chitosan for hepatocyte culture: liver-specific functions such as urea synthesis and drug metabolism were stably maintained for 5 d in the hepatocytes cultured on fructose-chitosan. In sharp contrast, hepatocytes attached on a collagen-coated surface underwent a severe morphological change, from spherical to flat, and lost almost all their lidocaine-removal activity within 5 d. A very thin fructose-chitosan layer was also applied onto the collagen-coated surfaces of polystyrene plates and a dextran microcarrier by crosslinking free amino groups in the chitosan and collagen with glutaraldehyde to fix the thin layer. Hepatocytes on the fructose-chitosan-coated surface retained their spherical shape, masking the cell-flattening effect of the collagen layer. Perfusion culture was then carried out using a hollow-fiber cartridge in which hepatocytes attached on fructose-chitosan-coated microcarriers were suspended in the extracapillary space: the liver-specific functions were stably maintained during 4 d of the culture. A fructose-chitosan-coated surface thus appears to be a very promising scaffold for hepatocyte attachment which can be used in cellular biological studies of liver functions, especially in relation to cytochrome P450, as well as in bioartificial liver support systems.

Key words: chitosan; bioartificial liver; cytochrome P450; hepatocyte

Isolated mammalian cells, which have been used in clinical trials to treat diseases such as fulminant hepatic failure and diabetes, constitute what have come to be known as bioartificial organs.1–3) To develop effective bioartificial organs, a suitable microenvironment needs to be provided for the anchored cells. The viability and functions of the anchored-dependent mammalian cells depend mainly on the nature of the scaffold on which the cells are cultivated in vitro. In early studies, we entrapped hepatocytes within a gel matrix of Ca-alginate to maintain liver functions in vitro.4–6) However, although microencapsulated hepatocytes metabolize substances such as ammonia, phenol, indole, and short-chain fatty acids, synthesize and secrete plasma proteins, and synthesize glucose from various precursors, difficulty has been experienced in terms of the permeability of high-molecular-weight substances, such as secreted plasma protein, through the gel matrix. Furthermore, when a hollow fiber-type bioreactor, in which hepatocytes are packed in the extracapillary space, is used, the substances secreted from the cells have to permeate a patient’s plasma through the double barrier of both the gel matrix and the fiber pores. Creation of an attachment culture on the scaffolds is thus seen as being more expedient than microencapsulation in terms of permeability.

We previously showed that chitosan, a mucopoly-saccharide derived from chitin by deacetylation, is a suitable biomaterial to use as a scaffold for the attachment of hepatocytes.7) Chitosan is considered to be a very promising biopolymer for various biomedical and pharmaceutical uses because of its nontoxic and biocompatible nature. An additional reason for selecting chitosan as a scaffold for hepatocyte attachment is that its structure is similar to that of glycosaminoglycans, which are components of the liver extracellular matrix. Rat hepatocytes on glutaraldehyde-crosslinked chitosan (GA-chitosan) retained the spherical form that they exhibit in vivo, and released only a very small amount of lactate dehydrogenase (LDH). By contrast, hepatocytes on a collagen-coated surface became flattened, and released much more LDH than the cells on GA-chitosan. Since the area needed for the attachment of one hepatocyte on GA-chitosan is much less than that required to attach a flat cell on collagen, it is possible to attach cells to GA-chitosan at a high density. In the study reported here, free amino groups of chitosan were chemically modified by various sugars with the aim of increasing the number of cells that can be attached to the chitosan surface.

MATERIALS AND METHODS

Chitosan Gel Chitosan of 100% deacetylated grade was purchased from Funakoshi Co. (Tokyo, Japan). GA-chitosan was prepared as described previously.7) Chitosan was modified chemically using various sugars before crosslinking. Five hundred milligrams of chitosan, which contained 3.53 mmol of a free amino group, was dissolved in 20 ml of a solution consisting of 1% acetic acid and 50% methanol. The resulting solution was mixed with 5.33 mmol of an aldehyde group to form a Schiff base between the aldehyde in the sugar and the amino group in the chitosan. Reduction of the Schiff base was performed

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in the presence of sodium borohydride overnight at room temperature under dark conditions. After adding 2 ml of 5% glutaraldehyde to the reaction mixture, 3 ml of the chitosan solution was poured into each well of 6-well polystyrene culture plates (Sumilon; Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The resulting gels were washed twice with a solution consisting of 50 mM phosphate buffer (pH 7.0) and 50% methanol.

**Chitosan Coating** The surface of each 6-well polystyrene culture plate was coated with 0.03% collagen (Cellmatrix Type I-A, from porcine skin; Nitta Gelatin, Yao, Japan). One hundred milligrams of chitosan was dissolved in 20 ml of a solution consisting of 1% acetic acid and 25% methanol. This chitosan solution was mixed with 1 mmol fructose and 2 ml of 5% glutaraldehyde and maintained overnight at room temperature. Reduction was performed for 3 h by the addition of sodium borohydride, and the mixture was poured into the collagen-coated plates. A collagen-coated dextran microcarrier, Cytofex 3 (Pharmacia Biotech, Uppsala, Sweden), was also coated with fructose-modified chitosan by the same method described above. 10 ml of fructose-modified chitosan solution was mixed with 1 ml of 5% glutaraldehyde and kept for 15 min, after which 0.5 g Cytofex 3 was coated with stirring.

**Cell Isolation** Liver cells were isolated from male Sprague Dawley (SD) rats weighing 250—300 g by perfusion of the liver with 0.05% collagenase (182 units/mg, from Clostridium histolyticum; Wako Pure Chemicals, Osaka, Japan) by the method of Seglen. The total liver cell suspension was centrifuged at 50 × g for 30 s. The pellet was used as the hepatocyte fraction after further purification by centrifugations at 50 × g for 30 s each. The isolated hepatocytes were suspended at 5 × 10^5 cells/ml in a medium consisting of Williams E medium (WE; ICN Biochemicals, Costa Mesa, CA, U.S.A.), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungione (BM). Hepatocytes with more than 90% viability, assessed by trypan blue exclusion, were used for the experiments.

**Culture Conditions** Isolated hepatocytes were seeded at a density of 1.0 × 10^5 cells/cm² onto chitosan gel and the collagen-coated surface in the 6-well polystyrene culture plates. After 6-h cultivation in 3 ml of BM supplemented with 10% fetal bovine serum (FBS; ICN Biochemicals), 1 nm insulin (Sigma Chemicals, St. Louis, MO, U.S.A.), and 1 nm dexamethasone (Nacalai Tesque, Kyoto, Japan), the cells were cultivated for 18 h in BM. Subsequent cultivation was carried out in BM supplemented with 5 ng/ml epidermal growth factor (EGF, Toyoobo, Osaka, Japan) and 1 nm insulin, and the medium was exchanged every 24 h.

**Perfusion Culture** Cytofex 3 coated with fructose–chitosan was mixed with 1.6 × 10^6 cells at a final concentration of 3 mg/ml in BM, then incubated for 24 h without stirring at 37°C. The cell-attached microcarriers were injected into the extracapillary space of a hollow-fiber cartridge (Cellmax™; Cellco, Inc., Germantown, MD, U.S.A.). The medium was perfused inside the hollow fiber at a flow rate of 25 ml/min, and changed every 24 h with fresh medium.

![Fig. 1. Typical GC Profile of Lidocaine](image)

**Gas Chromatographic (GC) Analysis of Lidocaine** Five hundred microliters of a medium containing lidocaine and its metabolites was mixed with 1.0 ml of 50 mM borate buffer (pH 10.0). Lidocaine and its metabolites were extracted by 4 ml tert-butyl methyl ether containing 5 μg pentacosane as an internal standard. After the organic phase was evaporated, lidocaine and its metabolites were dissolved in 75 μl tert-butyl methyl ether and subjected to GC analysis: 0.4 μl organic solution was injected (split ratio, 1:50) into a Shimadzu GC-14A gas chromatograph equipped with an SGE-HT5 column (nonpolar, 20 m), with nitrogen as the carrier gas. The column temperature was programmed to increase by 15°C/min from 150°C to 300°C. The injection temperature was 285°C. A typical gas chromatogram is shown in Fig. 1.

**Assay** LDH activity was measured by the method of Bergmeyer et al. The amounts of urea and protein were determined by the methods of Ormsby and Lowry et al., respectively. The number of free amino groups in the chitosan was determined by colloidal titration.

**RESULTS**

Chitosan was modified with various sugars before cross-linking with glutaraldehyde to increase its affinity for hepatocyte attachment. Chemically modified chitosan gel was prepared and hepatocytes were seeded onto the gels, as described in Materials and Methods. Under phase-contrast microscopic observation, no change was discerned in the number of cells attached on the gels modified with safranin and glucosamine as compared with those attached on non-modified GA-chitosan; a slight increase was observed in the number of cells on the chitosan modified with glucose and galactose; however, modifications with fructose, lactose, and maltose resulted in
Fig. 2. Scanning Electron Micrographs of Hepatocytes Cultured on the Surfaces of GA- and Fructose Chitosan Gels

Hepatocytes were cultured for 3d on GA-(A) and fructose-(B) chitosan gels. Bars in the micrographs represent 100 μm.

Fig. 3. LDH Activity Released from Hepatocytes Cultured on Fructose Chitosan and Collagen-Coated Surfaces

At the times of medium exchange, aliquots of used media from cultures on fructose chitosan (●) and collagen-coated (■) surfaces were subjected to assay for the activity released during 24-h cultivation. Values are the means ± S.D. of three experiments.

significantly improved cell attachment. Taking the attached cell number into account, fructose was selected as the modification ligand for the subsequent experiments. The cell-densities on fructose-modified and unmodified chitosans were $5.3 \times 10^4$ and $2.2 \times 10^4$ cells/cm$^2$, respectively.

Figure 2 shows scanning electron micrographs of hepatocytes cultivated for 3d on either fructose-modified or unmodified chitosan. The hepatocytes did not spread flat, but retained their spherical form on both surfaces. The activity of the LDH released from hepatocytes cultured on fructose-chitosan gels and collagen coated polystyrene plates was measured with the aim of evaluating the extent of cell damage during the culture period. The activity in the culture supernatant was measured every 24h at the time of medium exchange. As shown in Fig. 3, hepatocytes cultured on fructose-chitosan gels released much less LDH than those on collagen-coated plates.

Urea synthesis and lidocaine metabolism, which represent the liver functions of detoxication and cytochrome P450 activity, were measured in hepatocytes cultured on fructose-chitosan and collagen. Cells were incubated for 2h in BM containing 1mM NH$_4$Cl and the amount of urea in the culture medium was determined (Fig. 4). Cells on fructose-chitosan had much higher activity of urea synthesis than those on collagen during the 5-d culture period. Lidocaine was added to BM at a final concentration of 10μg/ml, incubated for 4h, and the residual amount was then measured by GC as described.

Fig. 4. Urea Synthesis in Hepatocytes Cultured on Fructose-Chitosan and Collagen-Coated Surfaces

At the times indicated, hepatocytes attached on fructose chitosan (●) and collagen-coated (■) surfaces were incubated in 1mM NH$_4$Cl-containing BM for 2h, and urea synthesized in the medium was determined. The medium was then changed to BM supplemented with 5ng/ml EGF and 1μm insulin. Values are the means ± S.D. of three experiments.

Fig. 5. Lidocaine Metabolism in Hepatocytes Cultured on Fructose-Chitosan and Collagen-Coated Surfaces

At the times indicated, hepatocytes attached on fructose chitosan (●) and collagen-coated (■) surfaces were incubated in BM containing 10μg/ml lidocaine for 4h, and the residual amount in the medium was determined. The medium was then changed to BM supplemented with 5ng/ml EGF and 1μm insulin. Values are the means ± S.D. of three experiments.
in Materials and Methods. As shown in Fig. 5, cells on collagen had lost almost all of their lidocaine metabolism activity from day 5 and thereafter. In contrast, the activity was stably maintained during the entire culture period in cells on fructose-chitosan gels.

Since it is very difficult to apply a chitosan gel to 3 mm in thickness to construct a bioreactor, the effect on hepatocyte attachment of coating collagen-coated plates with a thin layer of chitosan was examined. The concentration of chitosan was lowered to one-fifth that used for gel formation and the resulting layer was fixed chemically onto the lower surface. Free amino groups of collagen were chosen to chemically fix the chitosan layer. The collagen-coated plates were further coated with fructose-chitosan by crosslinking the free amino groups in collagen and chitosan as described in Materials and Methods. Hepatocytes were seeded onto the very thin layer of fructose-chitosan and cultivated for 2 d (Fig. 6). Although hepatocytes cultivated on a collagen-coated surface without a chitosan layer spread flat, their spherical shape was retained when they were cultivated on the fructose-chitosan coated surface i.e., the cell-flattening effect of the collagen was completely masked by the very thin fructose-chitosan coating.

In additional experiments, the collagen-coated dextran microcarrier Cytodel 3 was further coated with fructose-chitosan for perfusion culture. Hepatocytes attached to the fructose-chitosan-coated microcarrier were injected into the extracapillary space of a hollow-fiber cartridge comprised of modified cellulose capillaries having a molecular weight cut off of 30 kDa. The cell density was approximately $1 \times 10^6$ cells/ml. The medium passed through the lumen of the fibers and returned to the 50-ml reservoir bottle. Perfusion culture was carried out with the BM containing 1 mm NH$_4$Cl and 10 pg/ml lidocaine for 4 d in a CO$_2$ incubator. The reservoir was replaced every 24 h by a new one containing fresh medium and substrates. The samples were taken from the reservoir containing media perfused for 24 h, and synthesized urea (○) and metabolized lidocaine (●) were determined at the time of medium-exchange. Values are the mean ± S.D. of three experiments.

**Fig. 7. Urea Synthesis and Lidocaine Metabolism in Perfusion Culture of Hepatocytes Attached on Fructose-Chitosan-Coated Microcarriers**

BM containing 1 mm NH$_4$Cl and 10 pg/ml lidocaine was perfused inside the hollow fiber through a 50-ml reservoir bottle for 4 d. The reservoir was replaced every 24 h by a new one containing fresh medium and substrates. The sample was taken from the reservoir containing media perfused for 24 h, and synthesized urea (○) and metabolized lidocaine (●) were determined at the time of medium-exchange. Values are the mean ± S.D. of three experiments.

DISCUSSION

Chitosan has already been shown to be a promising biopolymer for the culture of animal cells. An additional advantage of using chitosan as a biomaterial is that its sugar moiety has a free amino group that can be modified using various substances having an amino or aldehyde group. Since the effectiveness of a lactose-carrying polystyrene surface for hepatocyte cultivation has already been reported, in the present study various sugars were used for chitosan modification, in the expectation that the sugar might act as an anchor through the transporter on the cell membrane. The results showed that the number of attached hepatocytes was significantly increased by fructose modification, without any adverse effects on the cell shape or LDH release by the cultured hepatocytes. As determined by colloidal titration, only 9.4% (S.D. = 0.86) of the amino group was ascertained to be modified by fructose in our experiment. It should thus be possible to further increase the number of attached cells by improving the extent of fructose modification. The fructose modification seems to change only the attachment nature of chitosan because the specific activity of liver function was almost the same in cells on both fructose-modified and unmodified surfaces (data not shown). The binding of fructose and the transporter on the cell surface appears to be the main cause of the increase in the attached cell number. However, there was a difference in the effect of sugars used for the chitosan-modification at
the primary screening. The modification efficiencies of some other sugars are reported to be rather higher than fructose. Further investigation is therefore needed to analyze the additional interaction which may exist between fructose and the cell surface besides the binding with the transporter.

Cultivation on a chitosan-coated surface appears to be more promising than that on chitosan gel because various microcarriers can be coated with a chitosan layer for use in several types of bioreactors. In the present study, a chitosan layer was fixed chemically by crosslinking with free amino groups in collagen coated on a dextran microcarrier. However, surfaces that can be coated with chitosan are not limited to those possessing free amino groups. A fluidized-bed bioreactor is easy to increase in scale in order to supply oxygen to hepatocytes, as compared with the hollow fiber-type bioreactor. We tried coating the macroporous polyethylene microcarrier Cytoline I (Pharmacia Biotech, Uppsala, Sweden), which has been developed for use in a fluidized-bed bioreactor. Photoaffinity labelling, previously used for enzyme function improvement, was applied to crosslink chitosan and Cytoline I. The crosslinking was done by the method described by Bayley. A photoregenerated reagent, 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid, was used to label the sites with carbon–hydrogen bonds by irradiation with near-ultraviolet light. The free amino groups in the chitosan were then crosslinked with the activated sites in Cytoline I. Hepatocytes attached to fructose–chitosan-coated Cytoline I retained significantly higher lidocaine metabolism activity than those on the uncoated microcarrier (data not shown). A chitosan layer could thus be coated onto various surfaces possessing carbon–hydrogen bonds.

Hepatocytes cultured as a monolayer on a collagen-coated surface flatted and lose their cytochrome P450 activity very rapidly. The lidocaine-removal activity was shown to be lost completely within 5d in the monolayer culture in this study, while hepatocytes cultured on fructose–chitosan maintained both their normal cell morphology and function. In our earlier work, we showed that hepatocytes entrapped within Ca-alginate maintained both their spherical shape and many of their liver-specific functions. It has also been reported that hepatocytes cultured between collagen layers in a sandwich configuration did not spread, and they stably maintained albumin secretion for 2 months. Recently, evidence has been reported of a close relationship in hepatocytes between cell morphology and the expression of genes responsible for liver-specific functions. Therefore, the fructose–chitosan-coated surface is a very promising scaffold for hepatocyte attachment that can be used in cellular biological studies of liver functions, especially with respect to cytochrome P450, in addition to its applications in bioartificial liver support systems.

REFERENCES