Gene transfection of multicellular spheroid of hepatocytes on an artificial substrate*

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Abstract

The handling of hepatocytes, a major cell population in the liver, is an important technique in both liver tissue engineering and hepatology. However, these cells are so fragile that it has been impossible to harvest hepatocytes with high viability from tissue culture dishes after a period of culture *in vitro*. In this study, we employed an artificial substrate for transfection of multilayer hepatocytes and harvested these cells with high viability after transfection. Hepatocytes cultured on an amphiphilic artificial substrate form multilayer aggregates (spheroids) in the presence of growth factors during gene transfection with cation liposomes. Compared to cells cultured on a collagen-coated plate, these spheroids are easily harvested with high viability by pipetting in EDTA solution. In addition, these spheroids rapidly spread on collagen after transfer from the artificial substrate, demonstrating that hepatocytes in the center of the spheroids were viable. Epidermal growth factor (EGF) increased the transfection efficiency into hepatocytes while hepatocyte growth factor (HGF) alone did not increase the efficiency. However, HGF synergistically increased the effect of EGF on transfection. Interestingly, this transfection required the process of spheroid formation because the gene was not transfected once the spheroid formation completed or under conditions where hepatocytes did not form spheroids. This method using spheroidal hepatocytes for *in vitro* transfection is promising for the development of *ex vivo* gene therapy.

Introduction

Gene therapy is one of the most promising therapeutic methods for incurable diseases such as congenital genetic diseases. The liver has been considered to be a promising target organ for gene transfection because of its size and ability to proliferate *in vivo* after hepatectomy. Several trials to transfect the liver directly *in vivo* have been reported (Alino et al., 1994; Kaneda et al., 1989; Takehara et al., 1995). However, it has been a difficult problem to achieve highly efficient transfection and maintain the constitutive expression of the transfected genes *in vivo*. *Ex vivo* gene therapy is thought to be a proper method to solve these problems. However, only few trials using liver cells for *ex vivo* therapy have been carried out since hepatocytes are very difficult to

handle *in vitro* because of their fragility. In addition, primary hepatocytes do not proliferate actively *in vitro* even in the presence of growth factors, which results in the defect of transduction by retroviruses. However, in our previous study, we have reported that primary cultured hepatocytes can be highly efficient target cells for transfection by cation-liposomes *in vitro* (Watanabe et al., 1994).

It is undescribed, common knowledge among hepatologists that it is almost impossible to detach primary cultured hepatocytes from a tissue culture substrate such as collagen with high viability because these cells are extremely susceptible to mechanical stresses or are sensitive to proteases such as trypsin. These characteristic features of hepatocytes are a serious hindrance to tissue engineering, the study of these cells *in vitro*, and *ex vivo* gene therapy using these cells because even the passage of hepatocytes in culture has been

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impossible so far. Therefore, development of a method to detach hepatocytes from the culture substrate after transfection would both enable practical *ex vivo* gene therapy with hepatocytes and contribute to the application of hepatocytes such as in tissue engineering and cell biology of these cells.

Hepatocytes are the major population of liver cells. Primary hepatocytes attach to and spread on collagen and fibronectin, which are commonly used in the culture substrate as scaffolds of cells. Cell adhesion to a scaffold is necessary for cells to metabolize, survive or proliferate (Boudreau et al., 1995; Fang et al., 1996; Frisch and Francis, 1994; Meredith et al., 1993; Re et al., 1994; Zhu et al., 1996). Extracellular matrix (ECM) such as collagen or fibronectin are potential substrates for cells because these ECMs transduce essential biochemical signals into cells via integrin receptors (Hynes, 1992). However, several artificial materials have been tried to substitute for these natural substrates. Previously, we have developed some amphiphilic materials as artificial culture substrates for hepatocytes (Kobayashi et al., 1994a; Kobayashi et al., 1992; Kobayashi et al., 1994b; Tobe et al., 1992). On these artificial substrates, hepatocytes bind to a D-galactose polymer, poly-N-p-vinylbenzyl-D-lactonamide (PVLA) at the initial stage via their asialoglycoprotein receptors, which are hepatic lectins recognizing galactose residues for endocytosis (Spiess, 1990). Hepatocytes cultured on this substrate form multilayer aggregates (spheroids) in the presence of growth factors such as epidermal growth factor (EGF) (Tobe et al., 1992) and stably maintain the differentiated features for a long period after formation of the multilayer spheroids without any significant decrease of viability (Kobayashi et al., 1994a; Kobayashi et al., 1994b).

In this report, we applied this artificial substrate to *ex vivo* transfection of hepatocytes. Hepatocytes were efficiently transfected on PVLA in the presence of growth factors and the transfected hepatocytes were detachable with high viability from this substrate. This method is considered to be useful for both *ex vivo* gene therapy and cell biological research of hepatocytes.

Materials and methods

Reagents

Poly-N-p-vinylbenzyl-D-lactonamide (PVLA) was synthesized in our laboratory as described (Kobayashi

et al., 1983). Recombinant human HGF (HGF) was a gift from Snow Brand Milk Products Co., Ltd. (Tokyo, Japan). Recombinant human epidermal growth factor (EGF) was kindly gifted by Hitachi Chemical Co. Ltd. (Tokyo, Japan). Lipofectin (cation-liposome reagent) was purchased from GIBCO BRL (Bethesda, USA), Tfx-50 from Promega (Madison, WI). 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and p-nitrophenyl- β -D-galactopyranoside (pNGP) were purchased from Wako Corp. (Osaka, Japan) and GIBCO BRL, respectively. β -galactosidase expression vector, pCH110, was purchased from Pharmacia Co. (Uppsala, Sweden). Collagen type I for coating culture dishes was a gift from Kawasumi Laboratories, Inc. (Tokyo, Japan). Female ICR (6–12 wk) mice employed in the experiments of this study were purchased from Charles River Japan, Inc. (Kanagawa, Japan). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

Cell culture

Parenchymal hepatocytes were isolated from an adult mouse by the modified *in situ* perfusion method (Page and Garvey, 1979; Tanaka et al., 1978). Briefly, the liver was perfused *in situ* through the thoracic inferior vena cava with 0.0125% collagenase solution. After the liver was excised, cells were dispersed in cold Hank's solution. Parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 x g for 90 sec. The dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia). The live parenchymal hepatocytes were suspended in Williams' E medium containing antibiotics and were plated at a density of 2×10^4 cells/well in hydrophobic flat-bottomed 96-well plates (Iwaki Glass Co., Ltd., Chiba, Japan) pre-coated with PVLA or BSA. The purity of the hepatocytes was confirmed by microscopic observation counting and flow cytometry analysis.

Transfection and β -galactosidase assay

Cells were transfected with pCH110 plasmids by cation-liposomes (Lipofectin or Tfx-50), basically following the manufacturer's instructions with minor modification. Briefly, DNA and the reagent were first mixed and incubated for 30 min at room temperature. The turbid solution was then diluted with the culture medium. The sample was added to the freshly isolat-

ed cells and incubated at 37 °C for 48 hr on PVLA or collagen. Transfection efficiency was measured by β -galactosidase assay. At 48 hr after transfection, the supernatant was removed, 100 μ l/well of the substrate solution (3 mg/mL of pNGP or 1 mg/mL of X-gal in 50 mM phosphate buffer (pH 7.2), 1.5 mM MgCl₂ 0.1% Triton X-100) was added, and the plate was incubated at room temperature for 4 hr. The reaction was terminated by the addition of 50 μ l/well of 0.5 M Na₂CO₃, then the absorbance at 415 nm was measured with a micro plate reader, MTP-120 (Corona Electric Co., Ibaragi, Japan). pNGP was used as a general substrate for the evaluation of the protein expression of the transfected gene and X-gal was used to stain the β -galactosidase-expressing cells, because the degraded product was insoluble. When X-gal was used as a substrate, the stained cells were observed by a microscope.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

The viability of cells was evaluated by MTT assay (Mosmann, 1983). Briefly, MTT was added to cells plated in a 96-well flat-bottomed plate at a final concentration of 500 μ g/mL, and the cells were incubated for 4 hr at 37 °C. One hundred μ l of acidic isopropyl alcohol was then added to each well, and the solution was vigorously mixed to solubilize the reacted dye. The absorbance of each well at 550 nm was measured using a micro plate reader, MTP-120 (Corona Electronic Co., Ltd., Ibaragi, Japan).

Results

Recovery of viable primary hepatocytes by forming spheroids

Hepatocytes adhere to PVLA via the interaction between asialoglycoprotein receptors and galactose residues of this substrate at the initial stage. As shown in Figure 1, hepatocytes adhering to PVLA became round shape and these cells formed spheroids 48 hr after seeding in the presence of EGF and HGF. These spheroids attached to PVLA with only a part of the whole bodies. In contrast, hepatocytes plated on collagen attached to and spread on this substrate with the whole basolateral membranes. According to this morphology, we expected that these spheroids would be detached easily from PVLA with high viability. In fact,

gentle pipetting with EDTA solution detached these spheroids from PVLA and these detached cells were completely viable (Figure 2A). On the contrary, hepatocytes detached from collagen by trypsinization were seriously damaged according to both morphological observation (Figure 2A) and MTT assay (Figure 2B). However, it had not been known whether cells in the center of the aggregates are viable. Figure 3 shows that hepatocytes transferred to collagen started to migrate out from the spheroids 1 day after the transfer, and these cells spread out and adhered to collagen 3 days after the transfer. These data demonstrate that primary hepatocytes could be recollected without loss of cell viability from the primary culture on PVLA.

Transfection of spheroidal hepatocytes

We have previously reported that hepatocytes were transfected efficiently on collagen with cation-liposomes (Watanabe et al., 1994). However, hepatocytes on PVLA express many different features from those on collagen. Therefore, we examined the conditions of gene transfection into hepatocytes on PVLA. Figure 4 compares the effect of growth factors on transfection of hepatocytes. On collagen, EGF, HGF or their combination dose-dependently increased transfection efficiency to almost the same degree (Figure 4A). In contrast, EGF only slightly increased the transfection efficiency to hepatocytes cultured on PVLA, and HGF did not have any effect on the transfection efficiency (Figure 4B). However, the combination of EGF and HGF significantly enhanced the efficiency of gene transfection in a dose dependent manner (Figure 4B). These data demonstrate the different characteristics of hepatocytes in transfection on collagen and on PVLA, and that EGF and HGF have different mechanisms to enhance gene transfection. Furthermore, we examined the effect of sodium butyrate, a transfection enhancer (Gorman and Howard, 1983), on transfection of hepatocytes. As shown in Figure 4C, sodium butyrate increased the transfection efficiency in a dose-dependent fashion within the concentration of 16 mM. Concentration of more than 30 mM impaired the efficiency, probably because it damaged hepatocytes since the reagent is known as a DNA damaging molecule (Kruh, 1982). Therefore, the following experiments employed sodium butyrate at the concentration of 16 mM. Under optimal conditions derived from the experiments above, hepatocytes cultured on PVLA were transfected with lacZ genes and stained with X-gal. As shown in Figure 4D, almost all hepa-

Figure 1. Spheroid formation of hepatocytes on PVLA. Isolated murine hepatocytes were cultured on collagen-coated (100 $\mu\text{g/mL}$) or PVLA-coated (100 $\mu\text{g/mL}$) plates for 2 days in the presence of EGF (100 ng/mL) and HGF (50 ng/mL). Magnification: $\times 100$.

cytes in the spheroids cultured on PVLA were transfected.

Transfection on PVLA required growth factors such as EGF and HGF which are essential for spheroid formation. Accordingly, we examined the correlation between transfection efficiency and multilayer spheroid formation. Interestingly, transfection of hepatocytes on PVLA required the process of spheroid formation. As shown in Figure 5, the transfection was successful when it was performed during 0 to 48 hr, which is the period of spheroid formation. However, if the transfection was performed after the completion of spheroid formation (more than 48 hr after plating), the efficiency was extremely low (Figure 5). Compared to the results of transfection on PVLA, on collagen the timing of transfection did not influence its efficiency. Data in the next experiment are consistent with these results. Figure 6 shows the transfection efficiency into hepatocytes cultured on various types of substrate. Transfection into the hepatocytes cultured on collagen,

BSA-coated dish and PVLA were successful while it did not succeed for the hepatocytes cultured on a poly (HEMA)-coated or Sigmacoat-coated dish, on which hepatocytes do not form spheroids (data not shown). In addition, colchicine, an inhibitor of tubulin polymerization, completely inhibited both spheroid formation and transfection of hepatocytes on PVLA (Figure 7). Interestingly, colchicine also inhibited the spreading of hepatocytes on collagen and induced spheroid formation of hepatocytes on the substrate (Figure 7B).

Finally, we examined the possibility of harvesting viable hepatocytes from PVLA after transfection. Transfected hepatocytes harvested from a collagen-coated dish with trypsinization were seriously damaged and almost all the cells were dead, as expected from the results of Figure 2 (data not shown). On the contrary, transfected hepatocytes transferred from a PVLA-coated dish strongly expressed β -galactosidase protein by X-gal staining (Figure 8A). The expression of β -galactosidase protein in transfected hepatocytes

Figure 2a-b. Recovery of spheroidal hepatocytes from PVLA. Freshly isolated primary hepatocytes were cultured on either collagen-coated or PVLA-coated culture plate for 2 days. These hepatocytes were then detached by trypsinization or pipetting, respectively, followed by being replated on collagen-coated plates for 3 hr. (A) Morphological observation of recovered hepatocytes. Magnification: $\times 100$ (B) Cell viability was quantitatively measured by MTT assay as described. (Open bar) cells not detached, (Filled bar) detached cells.

Figure 2b.

Figure 3. Cell motility and spreading of hepatocytes from spheroids on collagen. Spheroidal hepatocytes after cultured on PVLA were transferred to a collagen-coated plate, then incubated for 3 days. Hepatocytes forming spheroids migrated, spread out from the spheroids, and formed a monolayer sheet. Magnification: $\times 100$.

Figure 4a-d. Gene transfection of spheroidal hepatocytes cultured on PVLA. Hepatocytes were cultured and transfected in the presence of EGF and/or HGF at various concentrations. The transfection efficiency was quantified by β -galactosidase assay as described. (A) Hepatocytes were transfected on a collagen-coated plated in the presence of sodium butyrate (15 mM). (B) Hepatocytes were transfected on PVLA in the presence of sodium butyrate (15 mM). (Open bar) EGF, (Filled bar) HGF, (Hatched bar) EGF + HGF. (C) Hepatocytes were cultured and transfected on PVLA in the presence of EGF (100 ng/mL) and HGF (50 ng/mL) with sodium butyrate at various concentrations. After 48 hr, β -galactosidase assay was carried out. (D) Morphological observation of transfected multilayer hepatocytes on PVLA. Hepatocytes cultured on PVLA were transfected in the presence of EGF (100 ng/mL), HGF (50 ng/mL) and sodium butyrate (15 mM). These cells were stained with X-gal 48 hr after the transfection as described in Materials and methods.

Figure 4c.

Figure 4d.

was quantitatively measured by β -galactosidase assay (Figure 8B). The degree of the protein expression in the hepatocytes on PVLA was almost the same as that of the cells cultured on collagen. The continuous expression of the transfected gene was observed at least until

4 days after transfer of the hepatocytes to a collagen-coated plate along with cell spreading (data not shown).

Figure 5. Transfection of hepatocytes is performed during spheroid formation. Transfection to hepatocytes was carried out during the process of spheroid formation in the presence of EGF (100 ng/mL), HGF (50 ng/mL) and sodium butyrate (15 mM) (0–48 hr after seeding cells) (*Open bars*) or after the completion of the formation (after 48 hr of plating cells) (*Hatched bars*). Compared to the transfection of hepatocytes on collagen, the transfection on PVLA was unsuccessful after the completion of multilayer spheroid formation.

Figure 6. Efficient transfection to hepatocytes requires the process of spheroid formation. Freshly isolated hepatocytes were cultured on various types of substrate and transfection was performed in the presence of EGF (100 ng/mL), HGF (50 ng/mL) and sodium butyrate (15 mM). Of these substrate, hepatocytes form multicellular spheroids on BSA-coated (1%) and PVLA-coated plate. On either poly (HEMA)-coated (100 ug/mL) or Sigmacoat-coated plate, hepatocytes did not form spheroids.

Figure 7a-b. Disturbance of microtubules inhibits both spheroid formation and transfection of hepatocytes. Transfection was performed in the presence of EGF (100 ng/mL), HGF (50 ng/mL), sodium butyrate (15 mM) and colchicine at various concentrations. (A) Transfection efficiency was measured by β -galactosidase assay. Primary cultured hepatocytes were transfected on a collagen-coated plate (*Open bars*) or on PVLA-coated plate (*Hatched bars*). (B) Morphological observations of hepatocytes cultured with colchicine (1 μM) for 2 days.

Figure 7b.

Discussion

Primary cultured hepatocytes have been extensively employed in numerous *in vitro* studies since the method of the preparation was established (Page and Garvey, 1979; Tanaka et al., 1978). However, the problem that

primary hepatocytes, because of their fragility, can not be detached with high viability from a tissue culture substrate such as collagen or fibronectin has hindered the progress of *in vitro* research and has limited the application of hepatocytes. For some reason, this technical problem which is undescribed common knowl-

Figure 8a-b. Transfer of gene-transfected multicellular hepatocyte spheroids from a PVLA-coated plate to a collagen-coated plate. Hepatocytes were transfected on PVLA, then these cells were transferred to a collagen-coated plate. The gene expression was measured 9 hr after the transfer to compare the efficiency to the untransferred control. (A) Microscopic observation. Transferred hepatocytes were stained with X-gal. Magnification: $\times 100$. (B) β -galactosidase assay after the transfer. (*Open bars*) non-transfected control hepatocytes. (*Hatched bars*) transfected hepatocytes.

Figure 8b.

edge among researchers studying hepatocytes has not been addressed. In this study, we applied a synthetic artificial substrate, PVLA, for the culture of primary hepatocytes and demonstrated that primary hepatocytes after a period of culture or even after gene transfection could be harvested from this substrate with almost 100% viability.

In this study, we employed cation-liposomes for transfection of hepatocytes. In the previous paper, we reported that this method is the most effective for primary cultured hepatocytes compared to other conventional methods such as calcium phosphate and DEAE-dextran (Watanabe et al., 1994). Recently, some types of viruses have been used as carriers of genes for transfection. However, the method using cation-liposomes has some advantages over virus vectors. First, the composition of liposomes can be easily modified. Therefore, the most effective composition of liposomes for different cell types can be adjusted without difficulty. Second, it has been reported that virus vectors trigger the host immune system and the cells transfected with virus vectors are eliminated by the activated killer T-cells (Yang and Wilson, 1995; Yang et al., 1995). Therefore, long-term expression of the transfected gene can not be expected with virus vectors. On the other hand, liposome transfection does not significantly activate any immune system in the host. Third, the handling of viruses in the laboratory always contains the risk of environmental contamination. Therefore, with these advantages of liposome-transfection method, we expect more development of its use for gene therapy in the future.

There are several interesting characteristics found in the transfection of spheroidal hepatocytes. The transfection was successful only in the process of spheroid formation. In addition, the efficient transfection required a combination of both EGF and HGF. Either EGF or HGF alone induced spheroid formation of hepatocytes on PVLA (data not shown) although neither cytokine alone performed efficient transfection into hepatocytes. Therefore, spheroid formation is essential but in itself is not a sufficient condition for effective gene transfection. EGF and HGF were originally found as an epithelial cell growth factor and hepatocyte growth factor, respectively. Both cytokines are now known as multifunctional cytokines (Carpenter and Wahl, 1990; Zarnegar and Michalopoulos, 1995). To hepatocytes, these cytokines are cell motility factors and strong mitogens, and they induce signals to regulate the cytoskeleton (Stolz and Michalopoulos, 1994). Our previous study shows that cellular microfilaments

are involved in liposome transfection of hepatocytes (Watanabe et al., 1994). Zabner *et al.* has also reported that gene transfection by cationic liposomes is mediated by endocytosis (Zabner et al., 1995), which is known to be regulated by microtubules in hepatocytes (Goltz et al., 1992). In fact, the data that colchicine inhibited the transfection of spheroids suggest the involvement of microtubules in transfection of hepatocytes on PVLA. Microtubules are also considered to be involved in cell spreading of hepatocytes on collagen because the spreading was inhibited by colchicine; in addition, the reagent induced spheroid formation of hepatocytes on collagen (Figure 7B). These data suggest the possibility that signals by EGF and HGF regulate both cell motility and endocytosis of liposomes in hepatocytes via a cellular cytoskeleton organization. Therefore, the combination of EGF and HGF might facilitate the occurrence of endocytosis of transfected genes or increase the endocytosis efficiency by reorganizing microfilaments. Alternatively, these cytokines might increase the expression of cation-liposome receptors, which are thought heparin/heparan sulfate (Mislick and Baldeschwieler, 1996). However, further experiments are required to investigate these hypotheses.

Transfection efficiency might be dependent on the mitogenic activities of EGF and HGF. However, this possibility is unlikely because of the following reasons: (1) we have previously shown that cytochalasin B, an inhibitor of cytoskeleton, inhibited cation-liposome mediated transfection (Watanabe et al., 1994). (2) even in the presence of growth factors, when hepatocytes could not form spheroids like on poly-HEMA (which suggests that cell motility was inhibited), transfection was not successful (Figure 6). (3) most importantly, when hepatocytes form spheroids on PVLA in the presence of EGF or HGF, the proliferation was almost completely suppressed (Kobayashi et al., 1994a), indicating that either EGF or HGF functions as a motogen but not as a mitogen to hepatocytes on PVLA. Therefore, motogenic activities of EGF and HGF are thought to be more important than the mitogenic activities for the gene transfection of hepatocyte spheroids.

Hepatocytes form multicellular spheroids under various conditions. A number of researchers reported different substrates or methods to form multicellular spheroids of hepatocytes (Kobayashi et al., 1994a; Koide et al., 1990; Landry et al., 1985). According to our result that transfection of hepatocytes was also successful into the spheroids formed on BSA-coated plates (Figure 6), it is likely that spheroid formation of hepatocytes is a more important condition for effi-

cient transfection of hepatocytes than the character of the culture substrate. Transfection into multicellular spheroids of hepatocytes is thought to be useful for tissue engineering and the study of hepatocyte biology because spheroidal aggregates of hepatocytes show differentiated characters reflecting *in vivo* situations in terms of protein synthesis and proliferation (Landry et al., 1985; Yuasa et al., 1993).

Ex vivo gene therapy using hepatocytes is a promising method, because implanted hepatocytes have the ability to proliferate enough times to replace the whole liver (Rhim et al., 1994; Rhim et al., 1995). In addition, replaced hepatocytes continue to function indefinitely (Ponder et al., 1991). Therefore, the development of techniques to transfect primary hepatocytes *in vitro* and recover these cells will enable practical *ex vivo* gene therapy. The detailed information of our present study to transfect primary cultured hepatocytes by cation liposomes without any significant loss of cell viability will be helpful for the development of techniques for *ex vivo* gene therapy.

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