PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Transduction of primary rat hepatocytes with bicistronic retroviral vector

Qing Xie[1], Dan Liao[1], Xia Qiu Zhou[1], Shu Bing Qian[2] and Shi Shu Cheng[2]

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INTRODUCTION

Hepatocellular transplantation (HCT) could provide a therapeutic alternative to orthotopic liver transplantation (OLT) in the treatment of hepatic metabolic defects and experimental hepatic failure[1-4]. Under appropriate conditions, the engrafted liver cells can continue to express liver-specific functions for an indefinite period of time. The major limitation of many animal studies in HCT is that, since the donor hepatocytes are often indistinguishable from those of the host, it has often been difficult to demonstrate a clear correlation between engraftment and the therapeutic effect. In order to verify engraftment dependent on the therapeutic response, a recombinant retroviral vector carrying marker genes is used to label the donor hepatocytes[5,6]. The vector is capable of transducing hepatocytes, integrating gene stably into the genome and directing expression. Efficient retroviral-mediated gene transfer has introduced the possibility of targeting genetic markers to hepatic cells and somatic gene therapy for liver diseases[7-11]. Stable integration and expression of retroviral genes is dependent upon active division of the infected cell[9-13]. Although hepatocytes maintain growth potential *in vivo* and are capable of substantial regeneration following partial hepatectomy, their ability to grow in culture is quite limited.

[1]Department of Infectious Disease, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
[2]Human Genetic Therapy Research Center, Shanghai Second Medical University, Shanghai 200025, China
Qing Xie, graduated from Shanghai Second Medical University in 1988, now associate professor of infectious diseases, engaged in the researches of therapy and mechanisms of viral hepatitis, having 20 papers published.
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Correspondence to: Qing Xie, Department of Infectious disease, Ruijin Hospital, Shanghai Second Medical University, 197 Ruijin 2nd Road, Shanghai 200025, China
Tel. 0086-21-64311242, Fax. 0086-21-64451757
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In the present study, we explored the optimal culture system for hepatocyte proliferation and the potential for retroviral- mediated gene transfer into primary hepatocytes. We successfully demostrated the efficient and stable transduction of primary culture of adult rat hepatocyte by replication of defective retrovirus carrying β-gal gene and NeoR gene.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 140g - 200g were provided by Experimental Animal Center of Shanghai Second Medical University.

Sources

Hepatocyte-specific collagenase and culture medium were purchased from GIBCO- BRL (Gaithersburg, MD). Insulin, dexamethasone, transferrin, polybrene and epidemal growth factor (EGF) were Sigma Chemicals products (St. Louis, MO). 4CL-5Bt-3indolyl-β-galactoside (X-Gal) was purchased from Hua Mei Biotech Co. ^{3}H-TdR was purchased from Neucleic Energy Institute. Tissue/cell DNA extract kit was purchased from Shanghai Hua Shun Biotechnical Limited Co. Culture plastic dishes were Nunclon Co product.

Isolation and culture of hepatocytes

Rat hepatocytes were prepared by the modified procedure of Seglen with a two-step collagenase perfusion combined with 49.5% Ficoll centrifugation[14-15]. The dissociated cells were suspended in hormonally defined medium: M199 containing 10% fetal calf serum (FCS), 10^{-8}M insulin, 10^{-6}M dexamethasone and 5mg/L transferrin. They were seeded at a density of 3×10^4 cells/cm^2 on a 35mm tissue culture plastic dishes, and grown at 37℃ in a 5% CO^2 environment. The medium was changed 4h after seeding, and replaced by different culture mediums: group A with M199 containing 5% FCS, 10^{-8}M insulin, 10^{-6}M dexamethasone, 5mg/L transferrin; group B with M199 containing 5% FCS, 10^{-8}M insulin, 10^{-6}M dexamethasone, 5mg/L transferrin plus 10μg/L EGF. The medium was renewed every 24h thereafter.

Production of retroviral infected hepatocytes

PA317 cell line producing simutaneously the

recombinant retrovirus PGCEN/β-gal expressing β-galactosidase gene (LacZ) and neomycin-resistance gene (NeoR) was a gift from Prof. Cheng Shishu (Human Genetic Therapy Research Center, SSMU). These two genes were controlled by the same promotor. Its structure is shown in Figure 1. The producer PA317 was maintained in DMEM supplemented with 10% FCS. Virus-containing medium was harvested from the producers after 16 -20 hrs, filtered through a 0.45mm filter unit, and used for infecting the cultured hepatocytes. The viral titer ranged from 1 to 2×10^6 blue colony-forming unit (bcfu)/mL, when tested with NIH 3T3 cells.

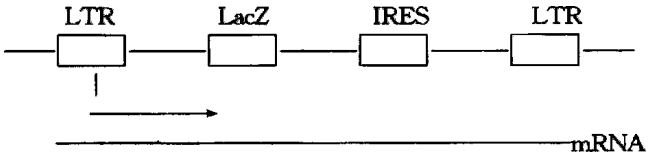

Figure 1 Structure of bicistronic retroviral vecto r PGCEN / β-gal. Arrow below vector indicates initiated site of transcription.

Hepatocytes in group B were grown for 1 to 5 days. Three dishes were selected randomly at 24, 48, 72, 96 and 120 hrs. The medium was removed and rinsed with PBS. The cells were incubated for 6 hrs with 1mL of viral supernatant plus 8μg of polybrene permL. Then the viral supernatant was cultured by replacing with fresh medium. Repeat the infection once a day for 24 to 96 hrs.

Detection of LacZ expression by X-Gal staining

Cells infected with PGCEN/ β-gal virus constitutively produ ced high levels of cytoplasmic β-galactosidase. In order to detect β-gal activity, infected hepatocytes were washed in phosphate-buffered saline (PBS) containing Ca^{2+}/Mg^{2+}, and fixed 5 min in 4% formaldehyde in PBS pH 7.4, rinsed again with PBS, then stained at 37℃ with X-Gal (1g/L) for 2 - 24 hrs, as previously described[16]. Blue precipitate in infected cells were seen under microscope. Areas of X-gal-stained rat primary hepatocyte culture dishes were quantitated for transducted cells using VIDAS computer-assisted image analysis. Three fields were randomly chosen and gene transductive efficiency was evaluated as follows:

Gene transduction efficiency(%)= Blue-stained cells areas / Total cells areas ×100%

Measurement of hepatocyte DNA synthesis

In this assay, cells were cultured in absence or in presence of EGF for various durations. DNA synthesis was measured by ^{3}H-thymidine incorporation. Cultures in 35 mm-dish were treated with ^{3}H-thymidine (1μCi/ mL) for 5 hrs before harvest. Cells were detached by trypsinization at 37℃, washed with PBS twice and counted. The cells were lysed with distill water. DNA were precipitated with 5% trichloroacetic acid and absolute ethanol, and collected on glass fiber-filters. DNA were then assayed for radioactivity in a liquid scintillation counter. ^{3}H-thymidine incorporation in DNA was expressed as cmp/10^5 cells.

Glucose-6-phosphatase activity by cytochemical procedure

Glucose-6-phosphatase activity was detected by the lead phosphate enzyme cytochemical procedure[17]. Characteristic brown/black cytoplasmic staining was seen if cultured hepatocytes expressed glucose-6-phosphatase.

Detection of NeoR expression by molecular method

DNA from cultured cells was isolated by proteinase K digestion in 10mM Tris-hydrochloric acid (pH 8.0) and 1% sodium dodecyl sulfate at 55℃ for 2 hrs, followed by phenol extraction and ethanol precipitation. According to the reference[10], the primer was synthesized by Bioengineering Research Center in Shanghai of Chinese Academy of Science. The nucleotide sequences of NeoR primers were as follows:

Sense 5'CAAGATGGATTGCACGCAGG 3',
antisense 5'CCCGCTCAGAAGAACTCGTC3'790bp.

The total 50μL PCR reaction system consisted of 10 × amplification buffer solution 5 μL, 2.5mmol/L DNTP 4μL, 25mmol/L $MgCl_2$ 3μL, 25μmol/L primer 2μL, reverse transcription product 10μL, and added ddH_2O up to 50μL mixing together, and added to *Taq* DNA polymerase 0.5μL after denatured for 5min. The amplification condition was predenatured at 94℃ for 1min, 60℃ for 1min, 72℃ for 1.5min altogethe for 30 cycles, finally, extension at 72℃ for 10 min.

Ten μL PCR product ran in agarose gel (1%, containing ethdium bromide 0.5mg/L) electrophoresis at 100 V for 40min and photographed under ultraviolet lamp.

RESULTS

Isolation and culture of hepatocytes

Each rat liver weighing 150-200 g was perfused by modified two-step collagenase via portal vein. The yield of hepatocytes was 1 - 2×10^8 cells. The viability was over 95%. The cells were seeded at densities of 3×10^4 cells/cm^2 on 35mm dish. They became attached to the dishes in 3 - 4 hrs. Hepatocytes became polygonal epithelium-like structure. The majority of cells were mononucleated; some were bi-or multi -nucleated. The membranes were clearly seen. Hepatocytes started to divide in aggregates a few hours after

attachment and became confluent within 3-4 days (Figure 2).

Influence of EGF on hepatocytes proliferation in vitro

In order to compare the DNA synthesis in hepatocytes, the level of ^{3}H-thymidine incorporation in the cell layer was measured in EGF stimulated and unstimulated primary hepatocyte cultures. After the addition of 10μg/L EGF to the cultures, the level of ^{3}H-thymidine incorpor ation began to increase at 48hr of culture, and reached the peak on the 5th day. Fifty-nine-folds increase of ^{3}H-TdR incorporation was found in EGF-treated cultures compared to conventional cultures. The differences between the two culture conditions were statistically significant at 48, 72, 96 and 120 hrs (P<0.01). The addition of 10μg/L EGF to the culture increased 50 times incorporation at 120 hrs as compared with that at 24 hrs. There was also significant difference between the two time points (Table 1).

Table 1 Effect of EGF on DNA synthesis of rat hepatocyte by ^{3}H-TdR incorporation [cpm/(10^{5} cell·h)]

Group	Time in culture (day)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Group A	34±3	29±6	25±6	31±4	22±3
Group B	26±3[a]	42±6[b]	263±27[b]	876±112[b]	1287±215[bc]

[a]P<0.05, *vs* Group A; [b]P<0.01, *vs* Group A; [c]P<0.01, *vs* Day 1 of Group B.

Retrovirus transduction in cultured hepatocytes and detection of LacZ expression

Triplicate cultures of infected hepatocytes were analyzed *in situ* for retrovirus transduction and expression by cytochemical staining for β-galactosidase. Cells that expressed viral-directed β-galactosidase was exhibited specifically by this procedure. Although the proliferation of rat primary hepatocyte was limited, the highest rate of infection was obtained by adding EGF. The rate of infection was gradually increased on the first 4 days, reached the peak on the 4th day of infection, but transduction efficiency dropped gradually in cultures on the 5th day of infection. The transduction efficiency in repeated infection group was about 22% (Figure 3).

Expression of hepatocyte function

We used a liver-specific cytochemical stain to detect the functional hepatocytes. The expression of glucose-6-phosphatase was analyzed in culture at different periods by cytochemical staining. Characteristic brown/ black cytoplasmic staining was seen in >85% of the cells at 48 hr of culture. The activity was still present at a slightly diminished level in >60% of cells on the 6th day.

PCR detection of NeoR gene

Transduction with PGCEN/β-gal was also assessed by PCR detection of NeoR gene. Analysis of PCR product showed that the amplified product with 790bp was visualized with ethidium bromide after electrophoresis in transduced rat hepatocytes, while this specific PCR product was absent in nontransduced primary rat hepatocytes (Figure 4).

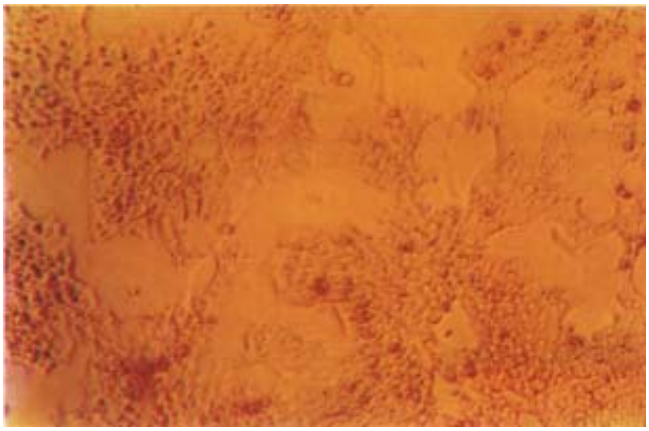

Figure 2 Morphology of hepatocytes in culture. ×100
Hepatocytes became polygonal epitheliumlike structure. The majority of cells were mononucleated; Some cells were bi-or multi-nucleated. The membranes were visible. Hepatocytes became confluent at 4 days postplating.

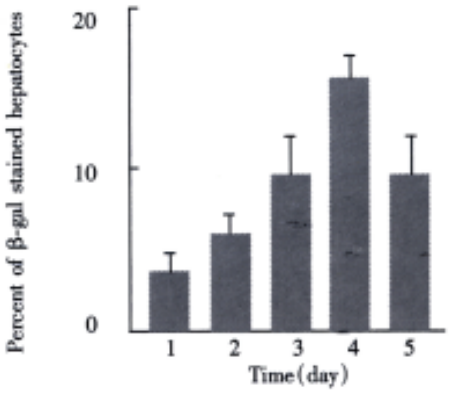

Figure 3 Transduction efficiency of hepatocytes by retroviral vector.

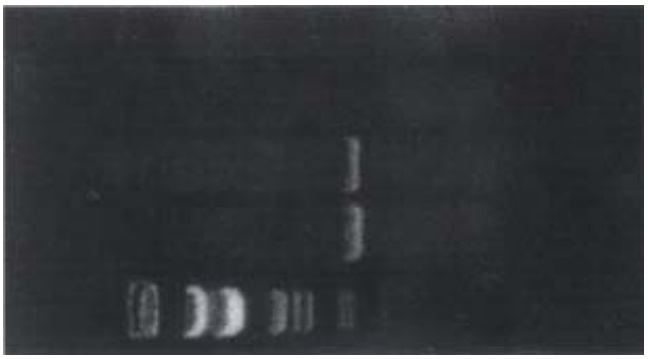

Figure 4 PCR detection of NeoR in primary rat hepato cytes transduced by PGCEN / β-gal.
A: Size markers (Lambda DNA / EcoR+Hind III Marker)
B: Positive template (MN45Li cell lines modified by NeoR gene)
C: Primary rat hepatocytes transduced with retroviral vector PGCEN / β-gal
D: Nontransduced primary rat hepatocytes
E: Water control

DISCUSSION

Two-step collagenase perfusion via portal vein is the conventional method for availability of hepatocytes, reports were available regarding cultured hepatocytes prepared by this method. In our present study a two-step collagenase perfusion in combination with 49.2% ficoll gradient centrifugation was used. This techinique provided a higher yield of viable rat hepatocyte with a minimal nonparenchymal cells. It was helpful to culture hepatocytes *in vitro* and to perform various studies of hepatocytes, such as establishment of cell bank and retrovirus-mediated gene transfer.

It is generally considered that efficient transduction of retroviral gene is dependent upon active proliferation of infected cells[9-13]. Although hepatocytes maintain growth potential *in vivo* and are capable of substantial regeneration following partial hepatectomy, the ability of adult hepatocytes to grow in culture without growth factors stimulation is limited[18-25]. In this experiment, with addition of 10μg/L EGF to the conventional culture, the cells retained their ability to proliferate, and showed excellent hepatocyte morphology. We were able to demonstrate that these cells could divide in short-term culture, and could be infected with recombinant retrovirus.

Liver cell transplantation can support the impaired liver. If the transplanted cells exhibit a growth preponderance and specific liver functions, they will rapidly replace the patient's hepatocytes. Glucose-6-phosphatase is a well-recognized specific enzyme expressed by the hepatocytes. It can be detected in viable hepatocytes by cytochemical staining. In our experimental system, hepatocytes could be isolated and cultured under conditions that maximized the division of parenchymal cells and prolonged the expression of glucose-6-phosphatase activity more than 10 days. The labelled donor hepatocytes via transducing these cells with a recombinant retroviral vector carrying a marker gene is used for evaluating the fate and function of the transplanted cell *in vivo*. Our data demonstrate that recombinant retroviruses are efficient tools to transfer marker gene into rat primary hepatocytes. The rat hepatocytes proliferated increasingly under EGF stimulation, 20% of the cells could be transduced. Our experiments of ^{3}H-TdR incorporation corrobrate the observation of Chenoufi *et al*[25]. We found that the cells had a high DNA synthetic rate that could be increased by adding EGF. In the first four days after plating, efficient transduction correlates positively with the state of proliferation (r = 5.427, P<0.05). Transduction rate decreased after the fifth day. Thus, our results indicate that the susceptibility to retroviral infection of hepatocytes varies with the ability of the cell proliferation, functional status of hepatocytes such as the level of receptor expression and many other factors[9-13].

retroviral vector was frequently used in gene therapy[26,27]. Recombinant retroviral vector PGCEN/β-gal used in the present study is an bicistronic retroviral vector expressing β-galactosidase gene and NeoR gene simutaneously, because these two genes were connected by the internal ribosome entry site of encephalomyocarditis virus and controlled by the same promotor[28]. Transduction efficiency was estimated by detection of β-gal gene expression by *in situ* staining, which was shown clearly and quan titatively. NeoR gene and target gene could be detected in the integrated cells from DNA level by PCR or southern blot[29-31]. PCR method increased the sensitivity of detection, which is helpful in tracing the life span of transplanted cell *in vivo*[30,31]. One study indicates that primary rat hepatocytes can be efficiently tranduced by a NeoR and β-gal-expressing recombinant retrovirus (PGCEN/β-gal). This approach is now being used to determine the most efficient way of cell transplantation and to investigate the location, life span and function of the transplanted hepatocytes.

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