

Expression of vascular endothelial growth factor gene in primary cultured rat hepatocytes

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BACKGROUND: It is the key point for vascular endothelial growth factor (VEGF₁₂₁) gene related therapy as to how to transfect and express the gene safely, effectively and repeatedly. This study was designed to investigate the VEGF₁₂₁ transfection and expression in primary cultured rat hepatocyte.

METHODS: After construction of vector internal ribosome entry site-enhanced yellow fluorescent protein (pIRES-EYFP)/VEGF₁₂₁, the transfection and expression of the exogenous VEGF₁₂₁ gene in primary cultured rat hepatocytes were observed through RT-PCR, Western blot and fluorescent microscopy.

RESULTS: pIRES-EYFP/VEGF₁₂₁ plasmid was constructed and transfected successfully into primary cultured rat hepatocytes, the transfection and expression of gene in primary cultured rat hepatocytes were examined by RT-PCR and Western blot, and yellow-green fluorescence was observed through a fluorescent microscope.

CONCLUSION: The successful transfection and expression of plasmid pIRES-EYFP/VEGF₁₂₁ in primary cultured rat hepatocytes provides a foundation for hepatocyte transplantation and gene therapy after modification of hepatocytes by the gene.

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KEY WORDS: vascular endothelial growth factor; cell, cultured; yellow fluorescent protein; gene therapy; transfection; hepatocyte transplantation

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Introduction

In recent years, hepatocyte transplantation has drawn much attention of surgeons in liver transplantation and has the trend to partly replace liver transplantation; but its long-term therapeutic effect is hindered by proliferation transplanted hepatocytes. The proliferation and expression of transplanted hepatocytes are considered to be related to blood vessel formation or microenvironment.^[1] The reconstitution of the liver is essential to life-long therapy. Vascular endothelial growth factor (VEGF₁₂₁) is able to enhance proliferation of vascular endothelial cells.^[2] In the presence of a basal lamina-type extracellular matrix, it specifically induces fenestrations in endothelial cells and increases vascular permeability.^[3] Exogenous administration of early after partial hepatectomy stimulates liver regeneration through the growth of new blood vessels.^[4] The expression of promotes colonization, vascularization, and growth of transplanted hepatic tissues in mouse, showing that *ex vivo* gene transfection into hepatocytes is a useful method for the induction of liver reconstitution *in vivo*.^[5] appears to have a vascular protective function by directly inhibiting the proliferation of vascular smooth muscle cells.^[6] VEGF₁₂₁ released from parenchymal cells may facilitate hepatocyte engraftment and endothelial reconstitution by the mechanisms involved in tumor cell metastasis.^[7] It is possible that the microvessel formation of transplanted tissues can affect the proliferation of transplanted cells and tissue formation.

In order to provide data for liver gene therapy, fusion eukaryotic gene expression vector pIRES-EYFP/VEGF₁₂₁ carrying the human-derived vascular endothelial growth factor gene and the enhanced yellow fluorescent protein gene was constructed and transfected into primarily cultured rat hepatocytes. The transfection and expression of the VEGF₁₂₁ gene in primarily cultured rat hepatocytes were observed through RT-PCR, Western blot and fluorescence microscopy.

Methods

Material

pcDNA3VEGF₁₂₁ was preserved in our laboratory. In-

ternal ribosome entry site-enhanced yellow fluorescent protein (pIRES-EYFP) and Clonfectin were purchased from Clontech Co., USA. Type I collagenase from Sigma Co., USA. Plasmid purification kit, TRIzol RNA kit, Titan™ one tube RT-PCR kit and prestained SDS-PAGE standards were bought from Gibco Brl Co., USA, dNTP from Sino-American Biotechnology Co., China, and electroporator from Bio-RAD, Co., USA. Mouse VEGF₁₂₁ monoclonal antibody and rhVEGF₁₂₁ standard reagent were purchased from Jingmei Co., Shenzhen, China. Reverse phase contrast microscope and reverse fluorescent microscope were purchased from Leica Co., Germany. Manhood SD rats weighed about 200 g were provided by the Medical Experimental Animal Center of Sun Yat-Sen University.

Experimental grouping

pIRES-EYFP/VEGF₁₂₁ vector transfection group was established as group C, pIRES-EYFP vector transfection group as control group B, and NAIR-1 culture medium prescribed by the National Advanced Interdisciplinary Research, Japan as control group A.

Eukaryotic vector pIRES-EYFP/VEGF₁₂₁

Construction and identification of pIRES-EYFP/VEGF₁₂₁ was constructed by cloning VEGF₁₂₁ cDNA sequence from pcDNA3VEGF₁₂₁ into pIRES-EYFP. Then the vector was amplified, purified, restriction enzyme digested, PCR amplified, and partial DNA sequence confirmed.^[8]

Isolation and primary culture of rat hepatocytes

Rat hepatocytes were isolated and purified using modified *in situ* collagenase perfusion method,^[9] and plated into NAIR-1 culture medium.

Transfection of VEGF₁₂₁ gene into primary culture rat hepatocytes

The manual of Clonfectin and the method of Watanabe^[10] were consulted. Vector DNA and Clonfectin were mixed and incubated at a ratio of 5 µg:15 µg, then was diluted with NAIR-1 culture medium. The mixture was added into a 6-well tissue culture plate, incubated for 2 hours at 37 °C in 5% CO₂ (V/V). The transfected hepatocytes were washed three times with phosphate-buffered saline (PBS) and cultured in the NAIR-1 culture medium.

RT-PCR for amplifying VEGF₁₂₁ mRNA

Total RNA in each group was extracted from the cells after 48-hour transfection and used as template after being examined by a violet spectrometer. Sense primer for β-actins mRNA was 5'-ATGTGGCACACCTTC-TACAATGAGCTGCG-3' and antisense primer mRNA

was 5'-CGTCATACTCCTGCTTGCTGATCCACA-TCTGC-3'. One pair of VEGF₁₂₁ primer was synthesized using one sequence of 258bp from 97bp to 354bp in VEGF₁₂₁ cDNA as template. Briefly, sense primer for VEGF₁₂₁ mRNA S1 was 5'-GAGGGCAGAATCATCACGAAGT-3', and S2 was 5'-TCCTATGTGCTGGCCTTGGTGA-3' as antisense primer of VEGF₁₂₁ mRNA. In 50 µl RT reaction mixture, VEGF₁₂₁ cDNA fragments were amplified by 35 rounds of PCR consisting of 30 seconds at 94 °C, 30 seconds at 60 °C, and 60 seconds at 68 °C. Then electrophoresis was performed with 10 µl RT-PCR supernatant.

Western blot analysis for VEGF₁₂₁ protein

The culture medium was collected after transfection for 48-72 hours, concentrated by lyophilization, and then put into boiling water and liquid nitrogen for three times repeatedly. After centrifugation for 10 minutes at 10000 g, 20 µl of the medium was loaded to run SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein in PAGE gel was transferred to nitrofibrotin membrane at 4 °C with current of 15-20 mA. The blot was then blocked for 2 hours at room temperature with solution, rocking on a rotating shaker. The primary antibody and secondary antibody (HRP-labeled goat-anti-mouse IgG) were diluted to 1:750 with the blocking solution and probed consecutively. Excessive secondary antibody was rinsed from the blot, which was subsequently stained with standard method of chemiluminescent detection using DAB and photographed.

Results

Sequence of pIRES-EYFP/VEGF₁₂₁

The sequence of plasmid pIRES-EYFP/VEGF₁₂₁ was identical to that of VEGF₁₂₁ cDNA in the GeneBank (Access number: AF214570). Hepatocytes obtained from each SD rat in each group were about 0.6×10^9 on average, and their survival rate was above 95%. These hepatocytes were added into NAIR-1 culture medium in a 6-well plate for primary culture. RNA in each group was extracted from the cells after 48-hour transfection for subsequent RT-PCR. Bands about 836bp of β-actin protein were seen in each group, and bands about 836bp and 258bp were seen in group C. It was indicated that the human VEGF₁₂₁ gene was expressed at the mRNA level of primarily cultured rat hepatocytes in group C (Fig 1).

VEGF₁₂₁ protein expression

The results of Western blot showed that a band in about 42 kDa in the cells of group C but not in groups A and B. The human VEGF₁₂₁ gene was expressed in

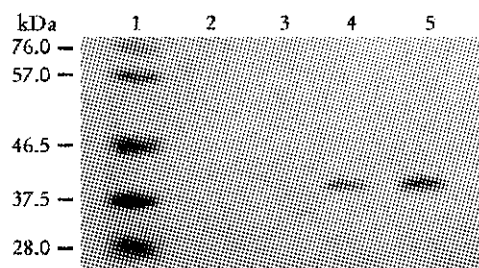


Fig. 1. Protein expression of VEGF₁₂₁ gene transfected hepatocyte (Western blot). 1; Prestained SDS-PAGE standards; 2; NAIR-1 (Group A); 3; pIRES-EYFP (Group B); 4; pIRES-EYFP/VEGF₁₂₁ (Group C); 5; standard.



Fig. 2. Fluorescent microscope observation in VEGF₁₂₁ gene transfected hepatocyte (original magnification×200).

primarily cultured rat hepatocytes in group C (Fig. 1).

Positively transfected cells and fluorescence transfection

Normal hepatocytes transfected by VEGF₁₂₁ were multiangular with two nuclei, with a high ratio of nucleus to cytoplasm under a fluorescent microscope. Yellow-green fluorescence was observed in hepatocytes transfected positively with plasmid pIRES-EYFP/VEGF₁₂₁ under a fluorescent microscope (Fig. 2).

Discussion

Liver gene therapy using hepatocytes as receptors has been popular in recent years.^[11] VEGF₁₂₁ is related to angiogenesis, organ development and growth of tumors.^[13-14] VEGF₁₂₁ is a 34-kDa to 46-kDa heparin-binding, diametric glycoprotein.^[2,15] The transfection and expression of the VEGF₁₂₁ gene can promote the growth of hepatocytes in the spleen, but it is hard to observe and real-time monitor the expression of the VEGF₁₂₁ gene transfected into primarily cultured rat hepatocytes.^[16]

The reporter gene is usually used to observe the destiny and status of the exogenous gene after transfection and expression in primarily cultured rat hepatocytes, but the observation of the exogenous gene can only be indirect and hard to examine the transfection rate and the expression level at one time. Enhanced green fluorescent protein (EGFP) and human VEGF₁₂₁ expression plasmids were used as model reporters and therapeutic genes to investigate the feasibility of non-viral gene delivery.^[17] Plasmid pIRES-EGFP is adapted to filtrate effectively mammalian cells that were transfected and expressed EGFP protein and protein of interest transiently via flowcytometry.^[18] Fourty-eight to 60 hours after transfection, yellow-green fluorescence from transfected cells can be observed at the gene using EGFP as a reporter protein through a fluorescent microscope with an emission length at 488 nm or 513 nm. Through flowcytometry it is conveniently and quickly real-time to detect and to localize intracellular expression of the interested gene.^[19] Enhanced yellow fluorescent variant (EYFP) may shift the emission from green to yellowish-green, and the fluorescence level of EYFP is roughly equivalent to that of EGFP.

The results of this study showed that the human VEGF₁₂₁ gene can be transfected and expressed in primarily cultured rat hepatocytes and yellow-green fluorescence from transfected cells can be observed under a fluorescent microscope. Hence it is easy to real-time monitor the transfection and expression of the exogenous VEGF₁₂₁ gene in primarily cultured rat hepatocytes. This provides a basis for the study of transplantation of exogenous VEGF₁₂₁ gene modified hepatocytes and liver gene therapy.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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