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ДОКЛИНИЧЕСКОЕ ИССЛЕДОВАНИЯ ЭФФЕКТИВНОСТИ ГЕННО-КЛЕТОЧНОЙ ТЕРАПИИ ИШЕМИИ СЕРДЦА

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Продемонстрированы многообещающие результаты использования мультипотентных мезенхимальных стромальных клеток жировой ткани, трансфицированных pWZ Blast-VEGF для лечения ишемической болезни сердца. В группе с введением pWZLBlast-VEGF трансфицированных клеток, по сравнению с контролем, в области моделирования ишемии выявлено значительное увеличение числа функционирующих капилляров (на 224,2%, $p<0,05$), плотности капиллярного русла (на 350 , 1% $p<0,01$) и обмена поверхности капиллярного русла(245,4%, $p<0,01$) и pO_2 (на 282% $p<0,01$). Кроме того, по сравнению с контролем, введение pWZLBlast-VEGF трансфицированных клеток сопровождалось значимым увеличением числа анастамозов между сосудистой сетью непораженной области миокарда и новообразующимися сосудоми в зоне ишемии.

GENE-CELL THERAPY OF LIVER FAILURE. EXPERIMENTAL PART I

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We report a method for systemic derivation of functional hepatocyte-like cells from human MSCs, which are readily accessible from adipose tissue.

In summary, our findings indicate that AT MSCs derived from adipose tissue can differentiate into functional hepatocyte-like cells in vitro, in addition to mesodermal and ectodermal lineages. The results of this study shows the perspective of the restricted differentiation potential of adult-derived stem cells. Most important of all, MSCs may serve as a cell source for tissue engineering or cell therapy of liver.

Introduction

One of a pathology for which organ transplantation is the only definitive therapy is a liver failure [9]. Developments of new methods to treat patients with hepatic diseases show the way to eliminate the need for liver transplantation. Identification of specific growth factors that promote liver regeneration has allowed the development and the use of recombinant growth factors, but the success of this strategy was hampered by the short half-life of these proteins and the need to administered them continuously [4, 10]. To overcome this problem, investigators successfully used gene technology to transfer the genes that encode these factors to liver to enhances its proliferation [11].

But the efficicncy and selectivity of in vivo transfection with the introduction of the vector into the main blood stream is very small. Vectors based on viruses show better results but they integrate into the genome, which create limitation of the cells application in clinic. Furthermore, they cause the immune response that severely limits of applicability in clinical practice

On the other hand it was shown that the introduction of mesenchymal stem cells (MSCs) accompanied with a good clinical effect. Moreover, human MSCs have been described as an attractive cellular vehicle for gene.

Secondly it has long been thought that the differentiation potential of adult stem cells is limited to their germ layer of origin, but recent studies have demonstrated that adult stem cells are more plastic than once believed [5].

However, subsequent work by several independent groups has clearly shown that hepatocyte replacement levels after MSCs transplantation are low (<0.01%), unless the MSCs have a selective growth advantage [3, 15]. Furthermore, in most of the cases, fusion with host hepatocytes, but not transdifferentiation of extrahepatic cells, has been described as the underlying principle of the therapeutic effect [1, 12, 14, 17, 18]. More over the failure of other research to show an impact of bone-marrow-derived MSCs on liver regeneration [3, 6, 15], clearly show that the use of bone-marrow-derived cells for the treatment of liver diseases is far from clinical application and that additional basic research on reprogramming is needed.

Adipose tissue MSC (AT-MSCs), like bone marrow, is a mesodermally derived organ that contains stem cells [19].

AT-MSCs share many of the characteristics of their bone marrow counterpart, including cell morphology, extensive proliferation potential, tumor tropism and the ability to undergo multilineage differentiation [2, 8, 15]. But, unlike bone marrow MSCs, AT-MSCs can be obtained by a less invasive method and in larger quantities (40-fold higher compared with that of bone marrow than bone marrow [7] and did not show any detectable chromosomal abnormalities or formation of tumors in the host's tissues.)

Thus the purpose of this study is to investigate the possibility of MSCs differentiation into hepatocytes as a result of cells transfection by using a vector that encodes a hepatocyte growth factor (HGF).

Vector construction

pBABE-puro was used as a ("Addgene plasmid 176). DNA was synthesized by a Reverse Transcription System Kit (Promega) following the protocols listed in instructions. The primers for HGP PCR were designed by using NCBI's site based on Human HGP-1 mRNA sequences from GenBank. The specific primers for HGF-1 mRNA were designed by using Primer 3 software.

Hepatocyte differentiation

MSCs were harvested from the culture bottles with 0.25% trypsin. Cultured cells at passage 3 were reseeded in 6-well cell culture clusters. After cells grew to 70% confluence, the control group was continuously cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. The hepatocyte differentiation group was transfected with pBABE-puro HGP and cultured in DMEM supplemented with 10% FBS, 20 ng/mL HGF, 10 ng/mL FGF-4, 100 U/mL penicillin and 100 U/mL streptomycin. Two millilitre medium was added to each well and changed every 4 days. The changed medium was stored at - 20°C for albumin (ALB), alpha-fetoprotein (AFP) and urea assay.

Reverse-transcription–polymerase chain reaction analysis showed the expression of alpha-fetoprotein and glucose 6-phosphatase by day 14, while tyrosineaminotransferase, a late marker gene of hepatocytes was detected by day 28. Expression of cytokeratin-18, albumin, and tryptophan 2,3-dioxygenase was detected at all time points and increased with time of differentiation, whereas undifferentiated cells did not express alpha-fetoprotein, tyrosine-aminotransferase, or glucose 6-phosphatase but did express low levels of albumin and cytokeratin-18, and tryptophan 2,3-dioxygenase.

The expression of cytochrome P450 was detectable by 3 weeks postinduction, and the expressionof hepatocyte nuclear factor-4, a transcription factor largely expressed in adult liver, was detected at 6 weeks postinduction.

Results

In Vitro Functional Characterization of MSC Derived Hepatocyte-Like Cells and Expression of Bile Canaliculi-Specific Antigen.

While undifferentiated MSCs did not exhibit the ability to uptake LDL, differentiated cells showed the ability to uptake significant levels of LDL.

At 6 weeks postinduction, differentiated cells demonstrated the ability to metabolize pentoxyresorufin in the absence of Phenobarbital, and a significant increase in fluorescence activity was observed in the presence of Phenobarbital. The presence of stored glycogen, as determined by PAS staining, was visualized at 6 weeks postinduction, (approximately 50% of differentiated cells stored glycogen). Undifferentiated cells did not show the ability to store glycogen. Urea production was detectable after 6 weeks postinduction and increased in a time-dependent manner with prolonged treatment in the presence of maturation medium, while undifferentiated MSCs, did not produce urea.

The monoclonal antibody 9B2 is a liver-specific antibody found to react with an antigen expressed on the bile canaliculi formed between adjacent hepatocytes. Analysis by flow cytometry revealed that differentiated hepatocyte-like cells were positive for the expression of antigen 9B2, and immunofluorescence assays further showed that the antibody was predominantly localized on the surface membrane bordering adjacent differentiated cells.

Conclusion

We report a method for systemic derivation of functional hepatocyte-like cells from human MSCs, which are readily accessible from adipose tissue.

The differentiation of MSCs into hepatocyte-like cells requires only a 1-step procedure without the need of additional surface coatings or treatments to culture vessels.

Differentiated hepatocyte-like cells can be sustained for 80 days or more, with in vitro functions, making it an ideal candidate for pharmacological and toxicological studies, as well as bioartificial liver studies.

Undifferentiated AT-MSCs can be expanded in vitro and, thus, large quantities of differentiated cells can be generated for cell therapy or tissue-engineering applications.

A magnitude of functional hepatocyte-like cells can be generated from a adipose tissue aspirate within a reasonably short period of time.

In summary, our findings indicate that AT MSCs derived from adipose tissue can differentiate into functional hepatocyte-like cells in vitro, in addition to mesodermal and ectodermal lineages. The results of this study shows the perspective of the restricted differentiation potential of adult-derived stem cells. Most important of all, MSCs may serve as a cell source for tissue engineering or cell therapy of liver.

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ГЕННО-КЛЕТОЧНАЯ ТЕРАПИЯ ПЕЧЕНОЧНОЙ НЕДОСТАТОЧНОСТИ. ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ 1

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Описан метод получения гепатоцитоподобных клеток из мультипотентных мезенхимальных стромальных клеток жировой ткани путем трансфекции последних вектором, несущим ген, кодирующий гепатоцитарный фактор роста.

Полученные гепатоцитоподобные клетки имеют морфологические и биохимические показатели, тождественные показателям гепатоцитов. Работа является первой частью исследований возможности генно-клеточной терапии тяжелых поражений печени.

INDUCED PLURIPOTENT STEM CELLS FROM ADULT HUMAN ADIPOSE STEM CELLS FREE OF VECTOR AND TRANSGENE SEQUENCES

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We show here a novel strategy to transfect human MMSC cells by using of the new type of episomal self-destructing vector system to produce induced pluripotent stem cells. The number iPS colonies and hence the number of reprogramming events was significantly higher for our new method (221.88+/-38.28 colonies, 2.24+/-0.77%.) when compared with the standard protocol (118.20 +/- 38.28 colonies, 1.14 +/- 0.77% p=0.97). MMSC iPS cells were demonstrated to express markers of pluripotency and could differentiate to the three germ layers, both in vitro and in vivo, to cells representative of the three germ lineages. Our findings confirm that MMSC are an ideal candidate as a readily accessible somatic cell type for high efficiency establishment of iPS cell lines, especially when using new "push pull" protocol.

Since the first publications, reporting the reprogramming of somatic cells to induced pluripotent stem cells (iPS cells) by the ectopic expression of the transcription factors, high expectations regarding their potential use for regenerative medicine have emerged. However, there are two major problems impeding the use of these cells in medicine. First of all, human iPS cell derivation required vectors that integrate into the genome, which can create mutations and limit the utility of the cells in both clinical and research applications. In addition, the effectiveness of the known methods of transfection are less than 0.5%.

Well-known attempts to use a nonintegrating episomal vector further reducing the efficiency of transfection, but remain the only alternative to vectors that integrate into the genome.

So further development of this direction requires the improvement of vectors, transfection methods and search for more suitable cells for reprogramming in the IPS/

To solve these problems we developed an approach assumes the derivation of human iPS cells from adipose stem cells which presumably have a greater potency for the reprogramming. After self-destruction of the new type of episomal vector, iPS cells are completely free of vector and transgene sequences and are similar to human embryonic stem (ES) cells in proliferative and developmental potential. These results removes one obstacle to the clinical application of human iPS cells. Furthermore, the transfection of multipotent mesenchymal stromal cells using a new developed "push-pull" method allowed to raise the efficiency of transfection up to 2%.

Materials and methods

Cell culture. Human MMS cells and iPS cells were maintained in DMEM/F12 culture medium supplemented with 20% knockout serum replacer, 0.1 mM non-essential amino acids (all from Sigma, USA), 1 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 100 ng/ml zebrafish basic fibroblast growth factor (zbFGF). The feeder-free culture on matrigel (BD Biosciences, MA) with conditioned medium was carried out as previously described [11] except with 100 ng/ml zbFGF.

Construction of expression vectors. The cDNAs for the open reading frames (ORFs) of human *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-Myc*, and *KLF4* genes were obtained by direct PCR of human ES cell cDNA. The cDNA for the SV40 large T (*SV40 LT*) antigen ORF was obtained from plasmid pBABE-puro SV40 LT (plasmid 13970, Addgene, Cambridge, MA), and the cDNA for the *hTERT* gene ORF was cloned from plasmid pBABE-hygro-hTERT (plasmid 1773, Addgene Cambridge, MA). The internal ribosome entry site 1 (IRES1) was derived from plasmid pIRESpuro3 (Clontech, Mountain View, CA), while IRES2 was obtained from plasmid pIRES2EGFP (Clontech).

The foot-and-mouth disease virus-derived F2A peptide coding sequence (5′ ggttccggaaaaattgtcgctcctgtcaaacaaactcttaactttgatttactcaaactggctggggatgtagaaagcaatccaggtcca 3′) was chemically synthesized and placed in frame with the upstream *OCT4* ORF (no stop codon) and the downstream *SOX2* ORF via PCR. Transgenes were cloned into a 1 modified lentiviral vector *(S5)* or the oriP/EBNA1-based pCEP4 episomal vector (Invitrogen) for reprogramming

Reprogramming human somatic cells. For reprogramming with oriP/EBNA1-based episomal vectors, episomal plasmids were cotransfected into cells in serum-free DMEM.