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An injectable spheroid system with genetic modification for cell transplantation therapy

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ABSTRACT

The new methodology to increase a therapeutic potential of cell transplantation was developed here by the use of three-dimensional spheroids of transplanting cells subsequent to the genetic modification with non-viral DNA vectors, polyplex nanomicelles. Particularly, spheroids in regulated size of 100-μm of primary hepatocytes transfected with luciferase gene were formed on the micropatterned culture plates coated with thermosensitive polymer, and were recovered in the form of injectable liquid suspension simply by cooling the plates. After subcutaneously transplanting these hepatocyte spheroids, efficient transgene expression was observed in host tissue for more than a month, whereas transplantation of a single-cell suspension from a monolayer culture resulted in an only transient expression. The spheroid system contributed to the preservation of innate functions of transplanted hepatocytes in the host tissue, such as albumin expression, thereby possessing high potential for expressing transgene. Intravital observation of transplanted cells showed that those from spheroid cultures had a tendency to localize in the vicinity of blood vessels, making a favorable microenvironment for preserving cell functionality. Furthermore, spheroids transfected with erythropoietin-expressing DNA showed a significantly higher hematopoietic effect than that of cell suspensions from monolayer cultures, demonstrating high potential of this genetically-modified spheroid transplantation system for therapeutic applications.

1. Introduction

Cell transplantation therapy has attracted considerable attention for the treatment of various intractable diseases. The therapeutic potential of cell transplantation is primarily dependent on the efficacy and longevity of bioactive factors secreted from the transplanted cells [1]. In this respect, genetic modification of transplanted cells by introducing transgene(s) using either viral or non-viral methods is a promising approach to modulate the secretion of bioactive factors [2]. In addition to endogenous factors, transgenes expressing functional proteins and peptides such as growth factors and coagulation factors can further enhance the therapeutic potential of transplanted cells [3–5]. Furthermore, a scheme to maintain transplanted cells in optimal long-acting conditions is a key for successful treatment. Although the survival rate of cells varies depending on cell type and source, therapeutic effects are likely to be limited by the death of transplanted cells or the loss of cell activity due to unfavorable microenvironments such as ischemia, hypoxia, or inflammation [6].

Three-dimensional (3D) spheroid cell culture is a promising technique to improve cell survival and function by preserving cell-to-cell interactions. Several groups including ours have reported that 3D spheroid cultures could increase the survival rate of the cells and enhance innate functions such as albumin secretion from primary hepatocytes and multilineage differentiation of mesenchymal stem cells (MSCs) [7–12]. Recently, we introduced a procedure in genetically-modified cell transplantation using a 3D spheroid culture system on micropatterned culture plates (Cell-

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able™ multi-well plates; Transparent, Chiba, Japan) combined with gene transfection by polyplex nanomicelles [13]. The polyplex nanomicelle is a non-viral gene carrier composed of plasmid DNA (pDNA) and poly(ethylene glycol) (PEG)–polycation block copolymers, which possesses core–shell structure consisting of PEG shell and inner core of pDNA in a condensed state [14–16]. For the polycation, we developed poly[*N*'-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide] [PAsp(DET)]. This polycation possesses two distinguished properties: the efficient capability of endosomal escape and rapid biodegradability in the cytoplasm, allowing safe and effective gene introduction into various cells [17–19]. Using this polyplex nanomicelle system, we achieved high and prolonged transgene expression for more than one month from the spheroids of rat primary hepatocytes cultured on micropatterned plates [13]. The nanomicelle-treated spheroids also exhibited sustained albumin secretion at a level comparable with that exhibited by untreated spheroids, suggesting that this system allows safe gene transfection without impairing the innate function of hepatocytes.

In this study, the hepatocyte spheroids gene-transfected by the polyplex nanomicelles were transplanted into mice to obtain insight into their application in cell therapy. For transplanting spheroids in their intact 3D form, we introduced thermosensitive property to the micropatterned culture plate, which allows the recovery of spheroids simply by lowering the temperature of the plate. Then, hepatocyte spheroids transfected with luciferase expressing pDNA were transplanted to subcutaneous tissue to evaluate the efficiency of transgene expression in host animal. The advantages of this system were analyzed in detail by intravital imaging of transplanted cells in the host tissue. Finally, to examine therapeutic potential, hepatocyte spheroids receiving transfection with erythropoietin-expressing pDNA were transplanted, followed by evaluation of the hematopoietic effect in the host mice.

2. Materials & methods

2.1. Materials

Collagenase, dimethylsulfoxide (DMSO), dexamethasone, insulin, and L-proline nicotinamide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hank's buffered salt and L-ascorbic acid 2-phosphate (Asc-2P) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin inhibitor, and Pen-Strep-Glut (PSQ) were purchased from GIBCO (Frederich, MD, USA). Human epidermal growth factor (hEGF) was purchased from Toyobo (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). For the construction of plasmid DNA (pDNA) expressing luciferase, the protein-expressing segment of pGL4.13 plasmid (Promega, Madison, WI, USA) was cloned into pCAG-GS plasmid (RIKEN, Tokyo, Japan) to obtain expression under CAG promoter/enhancer. For pDNA expressing Gaussia luciferase (Gluc), the protein-expressing segment of pCMV-Gluc control plasmid (New England BioLabs, Ipswich, MA, USA) was cloned into pCAG-GS. For pDNA expressing mouse erythropoietin (mEpo), protein expressing segments of pCMV-X4 plasmid (OriGene, Rockville, MD, USA) were cloned into pCAG-GS. These pDNAs were amplified in competent DH5α *Escherichia coli* and purified using a NucleoBond® Xtra Maxi Plus (Takara Bio, Shiga, Japan).

2.2. Animals

Balb/c nude mice (female; 7 weeks old) and Wistar rats (male; 5 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). Transgenic Sprague–Dawley (SD) rats (male; 5 weeks old) expressing EGFP in all tissues under the control of CAG promoter/enhancer (EGFP–SD rats) were purchased from Japan SLC (Shizuoka, Japan). All animal studies were conducted with the approval of the Animal Care and Use Committee of the University of Tokyo, Tokyo, Japan.

2.3. Isolation and culture of primary hepatocytes

Rat hepatocytes were isolated using a modified two-step collagenase digestion process as previously reported [20,21]. In brief, after the rat liver was perfused from the hepatic portal vein with a special solution described below, the collagenase solution was recirculated through the liver to obtain hepatocytes. The perfusion medium (pH 7.2) was composed of 8 g/L sodium chloride (NaCl), 400 mg/L potassium chloride (KCl), 78 mg/L sodium dihydrogen phosphate dehydrate ($NaH2PO4{\cdot}2H_2O$), 151 mg/L disodium hydrogen phosphate 12-water ($Na_2HPO_4{\cdot}12H_2O$), 2.38 g/L 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 190 mg/L ethylene glycol tetraacetic acid (EGTA), 350 mg/L sodium hydrogencarbonate ($NaHCO_3$), and 900 mg/L glucose. The collagenase solution (pH 7.2) was composed of 500 mg/L collagenase, 9.8 g/L Hank's buffered salt, 2.38 g/ml HEPES, 556 mg/ml calcium chloride hydrate ($CaCl_2{\cdot}H_2O$), 350 mg/L $NaHCO_3$, and 50 mg/L trypsin inhibitor. To preserve the function of hepatocytes under *in vitro* conditions, a special medium comprising DMEM supplemented with 10% FBS, 1% PSQ, 1% DMSO, 10^{-7} mol/L dexamethasone, 0.5 μg/ml insulin, 10 mmol/L nicotinamide, 0.2 mmol/L Asc-2P, and 10 ng/ml hEGF was used for cell culture [22].

2.4. Recovery and transplantation of the cells from spheroid and monolayer cultures

The micropatterned architecture was constructed on thermosensitive cell culture plates (Upcell™, CellSeed Inc., Tokyo, Japan) to prepare the thermosensitive micropatterned plates, in which cell adhesion sites of a 100-μm diameter are regularly arrayed surrounded by a non-adhesive area. Primary hepatocytes were seeded onto 12- or 96-well culture plates at densities of 4×10^5 cells/well or 4×10^4 cells/well, respectively. The spheroid cells were recovered as a suspension for transplantation studies by lowering the temperature without any damage to the structure of the spheroids. Cells on monolayer culture plates were recovered by trypsinization followed by centrifugation at 200× g for 3 min. The recovered suspension from a spheroid and monolayer culture was transplanted to the subcutaneous tissue of Balb/c nude mice by injection using 23-gauge needles. The number of cells to be transplanted per mouse was adjusted at the stage of seeding the cells onto the plate, to be 2×10^5, 4×10^5, and 1.2×10^6 for the transplantation to forelimb, abdomen, and earlobe, respectively. Because the number of recovered cells per well in 12-well plate was determined to be $(4.2 \pm 0.6) \times 10^5$, and $(4.8 \pm 0.7) \times 10^5$ (means ± SD) in spheroid and monolayer culture respectively, the number of transplanted cells per mouse were comparable between these two groups.

2.5. Gene introduction using polyplex nanomicelles

PEG–PAsp(DET) block copolymer and PAsp(DET) homopolymer were synthesized as previously reported [17]. PEG used in this study had a molecular weight (Mw) of 12,000, and the polymerization degree of the PAsp(DET) segment was determined to be 59 by ^{1}H-NMR. The polymerization degree of the PAsp(DET) homopolymer determined by ^{1}H-NMR was 55.

Polyplex nanomicelles were prepared as described in our previous report [13]. In brief, the nanomicelle was formed by mixing polymer and pDNA solutions in 10 mM Hepes buffer (pH 7.3). For preparing the polymer solution, we recently revealed that the combined use of two polymers, PEG–PAsp(DET) block copolymer and PAsp(DET) homopolymer, was advantageous to achieve both effective PEG shielding and functioning of PAsp(DET) to enhance endosomal escape [23]. Thus, in this study, nanomicelles were prepared by mixing pDNA solution with a premixed solution of the two polymers at the equal molar ratio of residual amino groups at the N/P ratio (residual molar ratio of total amino groups in the two polymers to phosphate groups in pDNA) of 10. The diameter of the resulting nanomicelles was determined to be approximately 70 nm by dynamic light scattering (DLS) [24]. In 12-well plate, a total of 10 μg of pDNA was added to 1 ml of culture medium for each well, and in 96-well plate, 1 μg of pDNA was added to 100 μl of culture medium.

2.6. In vivo and in vitro measurement of luciferase expression

In vivo luciferase expression after transplantation was measured using an IVIS™ Imaging System (Xenogen Corp., Alameda, CA, USA) after intravenous injection of D-luciferin (150 mg/kg, Sumisho Pharmaceuticals International, Tokyo, Japan).

In vitro analyses were performed using Gluc-expressing pDNA. Expressed Gluc is secreted into the culture medium and remains stable for more than a week [25]. In this study, to trace the real-time activity of transgene expression, the culture medium was replaced with fresh medium precisely 24 h before each indicated measuring point. 24 h after the replacement, the culture medium was collected to quantify Gluc secretion during the last 24 h, using a Renilla Luciferase Assay System (Promega) and GloMax® 96 Microplate Luminometer (Promega) following the manufacturer's protocol.

2.7. Quantification of transplanted cells and transgene and gene expression in host tissue

At 24 h after hepatocyte transplantation into the forelimb of mice, total DNA and mRNA in whole of the cutaneous and subcutaneous tissue in the forelimb were extracted from the transplantation site using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) and RNeasy Mini Kits (Qiagen), respectively, according to the manufacture's protocol. Using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA), quantitative real-time PCR (qRT-PCR) was performed. Because hepatocytes from male rats were transplanted to female mice, the number of transplanted cells in the host tissue was proportional to the copy number of SRY genes on Y chromosomes, which was amplified using the following primer pair: forward, CATCGAAGGGTTAAAGTGCCA; reverse, ATAGTGTGTAGGTTGTTGTCC, with standardization by quantifying pDNA copies of mouse β-actin (Mm00607939, Applied Biosystems). The number of transgenes (luciferase-expressing pDNA) in the host tissue was quantified using the following primer pair: forward,