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RNAi-mediated silencing of HLA A2 suppressed acute rejection against human fibroblast xenografts in the striatum of 6-OHDA lesioned rats

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ABSTRACT

Major histocompatibility complex class I (MHC I) molecules play a role in determining whether transplanted cells will be accepted or rejected, and masking of MHC I on donor cells has been found useful for immunoprotection of neural xenografts. In the present study, primary human embryonic lung fibroblasts (HELF), HELF treated with lentivirus-mediated small interfering RNAs (siRNAs) targeting human leukocyte antigen A2 (HLA A2, MHC I in humans) (siHELF), and rat embryonic lung fibroblasts (RELF) were stereotaxically grafted into the striatum of 6-hydroxydopamine lesioned rats to explore whether knockdown of HLA A2 could reduce host immune responses against xenografts. Before lentiviral infection, the cells were transduced with retroviruses harboring tyrosine hydroxylase cDNA. Knockdown of HLA A2 protein was examined by Western blotting. The immune responses (the number of CD4 and CD8 T-cells in the brain and peripheral blood), glial reaction, and survival of human fibroblasts were quantitatively evaluated by flow cytometry and immunohistochemistry at 4 d, 2 w, and 6 w post-graft. Animal behaviors were assessed by counting apomorphine-induced rotations pre- and post-grafts. It was shown that a lower level of HLA A2 was observed in siHELF grafts than in HELF grafts, and knockdown of HLA A2 decreased rat immune responses, as indicated by less remarkable increases in the number of CD8 and CD4 T-cells in the brain and the ratio of CD4:CD8 T-cells in the peripheral blood in rats grafted with siHELF. Rats grafted with siHELF exhibited a significant improvement in motor asymmetry post-transplantation and a better survival of human fibroblasts at 2 w. The increasing number of activated microglia and the decreasing number of astrocytes were found in three groups of rats post-implantation. These data suggested that RNAi-mediated knockdown of HLA A2 could suppress acute rejection against xenogeneic human cell transplants in the rat brain.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra and concomitant reduction of the neurotransmitter dopamine (DA) in the striatum. Transplantation of allogeneic fetal ventral mesencephalic tissue into the striatum of PD patients could restore striatal dopaminergic function and alleviate Parkinsonian signs and symptoms (Buttery and Barker, 2014; Ghosh et al., 2014; Lindvall, 2016). However, limited availability of donor tissues and ethical concerns are major barriers to the widespread use of human fetal mesencephalic transplantation (Buttery and Barker, 2014; Ghosh et al., 2014; Lindvall, 2016). The use of xenogeneic tissues has drawn considerable interest as a potential source of donor tissues for replacement treatment of PD patients (Piquet et al., 2012; Poncelet et al., 2009) and Parkinsonian motor symptoms have been shown to improve in 30% of the patients with PD after striatal xenotransplantation of porcine fetal neuronal cells (Poncelet et al., 2009).

One concern in the use of xenogeneic tissues is the possibility of rejection by host immune system. Xenografts give rise to more rigorous immune responses than allogeneic cells because xenogeneic tissues of another species offer more foreign antigens as targets for immune responses (Sykes et al., 2013). The donor antigens are recognized by recipient cytolytic T lymphocytes (CD8 T-cells) which interact with donor major histocompatibility complex (MHC) class I molecules and eventually destroy the target grafts (Sykes et al., 2013). A useful strategy, masking of MHC I, was put forward for immunoprotection of neural xenografts (Pakzaban et al., 1995). Porcine fetal striatal cells masked with F(ab′)2 fragments of antibody directed against porcine MHC I (Pakzaban et al., 1995) and genetically modified mouse dopamine neurons lacking MHC molecules (Duan et al., 2001) have been shown to survive grafting into the rat striatum. It was shown that permanent silencing of HLA antigen expression by lentivirus-mediated delivery of small interfering RNAs (siRNA) targeting HLA class I resulted in enhanced resistance to alloreactive T lymphocyte-mediated cytotoxicity

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(Figueiredo et al., 2006; Hacke et al., 2009; Haga et al., 2006) and to antibody-mediated cell lysis (Figueiredo et al., 2006).

The most common HLA antigen is A2, which is found in roughly 50% of individuals from populations around the world (Cecka et al., 2010). In the present study, primary human embryonic lung fibroblasts (HELF), HELF infected with lentiviruses encoding HLA A2 short hairpin RNAs (shRNA, precursors to siRNA) (siHELF), and rat embryonic lung fibroblasts (RELF) were grafted into the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats. The number of CD4 and CD8 T-cells in the brain and peripheral blood, and glial reaction were quantitatively evaluated post-graft to explore whether knockdown of HLA A2 expression could reduce host immune responses against the engrafted xenogeneic fibroblasts.

2. Materials and methods

2.1. siRNA design and lentivirus production

Three siRNA sequences targeting HLA A2 heavy chain were selected based on the literature (Figueiredo et al., 2006) and the web-based prediction algorithms (http://www.genelink.com/sirna/RNAicustom.asp): 5′-GGAGACACGGAATGTGAAG (si01), 5′-CACCATCCAGATAATG TAT (si02), and 5′-GCAGAGATACACCTGCCAT (si03) (location: 258 to 276, 351 to 369, and 834 to 852, respectively). The negative control (NC) sequence was 5′-TTCTCCGAACGTGTCACGT. The complete shRNA expression cassette was designed as a hairpin loop structure of shRNA-coding DNA oligonucleotide (oligo) containing a sense sequence, followed by a short spacer (TTCAAGAGA), the antisense sequence, and RNA Polymerase III terminator (TTTTTT). A *Xho* I 5′-overhang (CTCGA) was added at the 5′-end of the complementary oligo. The DNA oligos and their complementary strands were annealed by heating to 90 °C for 4 min, 70 °C for 10 min, then cooling slowly to room temperature. The double stranded oligos were cloned into *Hpa* I and *Xho* I restriction sites of lentiviral vector pSicoR (Addgene plasmid 11,579) to drive shRNA expression under U6 promoter (Ventura et al., 2004). The recombinant pSicoR clones were identified after transforming *Escherichia coli* DH5α by the polymerase chain reaction (PCR) amplification using pSicoR-specific primers (forward: 5′-GCCCCGGTTAATTTGC ATAT (2706–2725 bp), reverse: 5′-GTAATACGGTTATCCACGCG (2986–3005 bp)). The orientation and the accuracy of DNA inserts were verified by DNA sequencing.

Lentivirus preparations were produced with a standard second generation packaging system (Ventura et al., 2004). 6.0 μg of packaging plasmid psPAX2 (Addgene plasmid 12,260) and 3.0 μg of envelope plasmid pCMV-VSVG (Addgene plasmid 8454) were co-transfected along with 8.0 μg of the recombinant lentiviral vector pSicoR into HEK293T cells by calcium phosphate precipitation. The lentivirus-containing supernatants were harvested at 72 h post-transfection, filtered through 0.45 μm filter, and centrifuged at 25,000 rpm (Beckman V70ti) for 90 min at 4 °C. Virus titers were determined by infection of HEK293T cells with serial dilutions (from $1:10^2$ to 10^7) of the concentrated virus preparation.

2.2. siRNA selection

The silencing efficiency of the shRNAs against HLA A2 was assessed after transient transduction of HEK293T cells with a recombinant vector pEGFP-C1 (Clontech, Palo Alto, CA, USA) harboring Flag-tagged HLA A2 cDNA (a generous gift of Prof. Pierre van Bruggen, Ludwig Institute for Cancer Research, Brussels, Belgium) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 12 h later, Flag-HLA A2-transfected cells were infected with HLA A2 shRNA-expressing lentiviruses. Total cellular proteins were collected 4 d after infection, and specific lentiviral shRNA-mediated knockdown of HLA A2 was analyzed on the protein level by Western blotting.

2.3. Primary fibroblast culture and viral infection

HELFs were isolated from a 12-week-old aborted fetus (Beijing Jingbei Hospital, China). The pregnant woman and her other family members were informed of and agreeable to the Informed Consent Form. RELFs were isolated from embryonic day 14 SD rats. The fibroblasts were cultured in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) (Gibco) in a 37 °C incubator containing 5% CO_2 and split 1:2 in the first 3 to 5 passages and then passaged at a 1:3 or 1:4 ratio. The experiment was approved by the Research Ethics Committee at the Capital Medical University.

3×10^6 of HELF or RELF were plated into a 75 ml flask and cultured in a 37 °C incubator containing 5% CO_2. When the cells were 50%–60% confluent, 3 ml of fresh complete culture medium was added together with 10^6 of the recombinant retrovirus harboring rat TH (a rate-limiting enzyme in catalyzing the synthesis of DA) cDNA and polybrene (to a final concentration of 8 μg/ml) (Chemicon, Temecula, CA, USA). The retroviral construct with hygromycin resistance was previously constructed in our laboratory and the viruses were packaged from PT67 cells (Clontech, Palo Alto, CA, USA), collected, filtered and centrifuged at 4000 rpm for 45 min (4 °C). The cells were passaged 48 h after retrovirus infection, and hygromycin was added at an increased gradient of 50–100 μg/ml each day to a final concentration of 400 μg/ml.

The stably transduced fibroblasts were plated onto a 60 mm Petri dish at a density that gave 70% confluence on the second day of lentiviral infection. The dish was washed twice with FCS-free DMEM, and then added 3 ml of FCS-free DMEM containing the siRNA lentiviruses or NC lentiviruses (MOI: 20–25) and polybrene (to a final concentration of 4 μg/ml). After 8 h of culture in a 37 °C incubator containing 5% CO_2, half volume of the infection medium was removed and an equal volume of fresh DMEM medium containing 15% FCS was added. On the following day, the cells were washed and cultured in fresh complete culture medium. Transduction efficiency can be evaluated under a fluorescent microscope 72 to 96 h later.

2.4. 6-Hydroxydopamine lesion surgery

Adult female Sprague–Dawley (SD) rats weighing 180–210 g were obtained from the Laboratory Animal Center of the Capital Medical University (Animal Health Certificate: SCXK (Beijing) 2000–0012) and housed with free access to food and water on 12-h light/dark cycles. Rats were anesthetized by intraperitoneal injection of 6% chloral hydrate (6 ml/kg wt, Shenyang Chemical Reagent Factory, Shenyang, China) and positioned in a stereotaxic apparatus (Benchmark-900; David Kopf, Tujunga, CA, USA). The dopaminergic toxin 6-hydroxydopamine (6-OHDA) (Sigma, St. Louis, MO, USA) (3 μg/μl, dissolved in ascorbate-saline) was stereotactically injected (1 μl/min every 2 min) into the left striatum at the two sites (4.0 μl per site, in each case with the cannula being left in place for another 15 min before being withdrawn). The coordinates of the injections were as follows: (1) anteroposterior (AP): 4.0 mm (posterior to bregma), mediolateral (ML): 0.8 mm, dorsoventral (DV): 8.1 mm, with the tooth-bar set at 3.4 mm above the interaural line; and (2) AP: 4.4 mm, ML: 1.1 mm, DV: 7.9 mm, with the tooth-bar set at 2.4 mm below the interaural line.

The experimental procedures were evaluated and approved by the Animal Care and Use Committee of the Capital Medical University.

2.5. Rotation

The rats were tested for rotational behavior 1 w after the 6-OHDA lesion surgery. Striatal DA receptors become hypersensitive as a result of 6-OHDA lesion, and apomorphine induces contralateral turning behavior to the lesion side. Motor asymmetry was monitored in an automated rotation-measuring apparatus for the mean number of turns per 40 min after subcutaneous injection of a DA agonist apomorphine