Biomaterials 34 (2013) 5872–5882

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

An *ex-vivo* multiple sclerosis model of inflammatory demyelination using hyperbranched polymer

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ARTICLE INFO

Article history:
Received 13 March 2013
Accepted 4 April 2013
Available online 6 May 2013

Keywords:
Non-viral gene delivery
Organotypic brain slice
Multiple sclerosis
Demyelination
Inflammation

ABSTRACT

Multiple sclerosis (MS) is characterized by the presence of inflammatory demyelinating foci throughout the brain and spinal cord, accompanied by axonal and neuronal damage. Although inflammatory processes are thought to underlie the pathological changes, the individual mediators of this damage are unclear. In order to study the role of pro-inflammatory cytokines in demyelination in the central nervous system, we have utilized a hyperbranched poly(2-dimethyl-aminoethylmethacrylate) based non-viral gene transfection system to establish an inflammatory demyelinating model of MS in an *ex-vivo* environment. The synthesized non-viral gene transfection system was optimized for efficient transfection with minimal cytotoxicity. Organotypic brain slices were then successfully transfected with the TNF or IFNγ genes. TNF and IFNγ expression and release in cerebellar slices via non-viral gene delivery approach resulted in inflammation mediated myelin loss, thus making it a promising *ex-vivo* approach for studying the underlying mechanisms of demyelination in myelin-related diseases such as MS.

1. Introduction

Multiple sclerosis (MS) is the most common cause of neurological disability in young adults and is considered to be a chronic autoimmune, demyelinating disease of the central nervous system (CNS) [1]. MS is characterized, neuropathologically, by multiple foci of inflammation and demyelination in the brain and spinal cord, accompanied by a variable degree of axonal damage in the white matter and neuronal loss in the grey matter [2]. The presence of inflammatory processes in this pathology is well established but determination of the exact pathogenic mechanisms and identification of individual disease mediators continue to be a challenge.

Magnetic resonance imaging (MRI) studies and autopsy tissue analysis from patients have made significant contributions towards an understanding of MS pathogenesis, but to fully understand underlying mechanisms of damage, inevitably animal models of MS will be required. The most commonly used animal model of MS is experimental autoimmune encephalomyelitis (EAE) [3], which involves the immunization of genetically susceptible animals with a myelin protein. This results in a progressive clinical course characterized by an immune mediated demyelination, primarily in the spinal cord. Although EAE models have been widely used to study disease mechanisms and as a pre-clinical test-bed for new therapeutics, they often fail to predict clinical efficacy in MS patients. This is largely due to their inability to reproduce the location and characteristics of the pathology within the CNS [4,5]. Approaches involving the targeting of EAE lesions to particular anatomical structures and using specific inflammatory mediators are proving helpful in overcoming these limitations [6,7]. However, the complex interactions between the immune and nervous systems *in-vivo* and the relative expense of EAE models makes using an *ex-vivo* approach an attractive alternative for studying disease mechanisms and testing drug efficacy.

Organotypic slice culture models have been used previously to investigate demyelination and remyelination with myelin specific antibodies [8,9]. Recent studies have used slices cultured on semi-porous membranes to allow a greater ability to manipulate the molecular environment [10] and for testing remyelination enhancing therapeutic approaches [11]. Our recent studies of

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http://dx.doi.org/10.1016/j.biomaterials.2013.04.010

human post-mortem MS tissues have suggested an important role for the chronic inflammatory milieu in the CSF of the subarachnoid space that overlies the brain parenchyma [12,13]. In this study we sought to establish a system for testing the role of the chronic presence of pro-inflammatory cytokines in demyelination. To do this, we have used a non-viral gene delivery approach and organotypic slice cultures to transduce cells with genes encoding the pro-inflammatory cytokines TNF and IFNγ. Both TNF and IFNγ have been shown to induce demyelination in the rodent CNS [14,15].

Gene transfection of organotypic brain slices has previously been carried out using viral vectors [16,17] or by non-viral methods such as electroporation [18,19], biolistics [20,21], microprojectiles [22] and lipotransfection [18]. Although viral vectors provide a good transfection efficiency, their application is limited due to cytotoxicity and immunogenicity [23]. Electroporation can cause severe damage to the tissue and only transfects cells around the edges of a slice [18,24]. The use of liposomal and polymeric vectors has increased due to their large scale manufacture and lower immunogenicity. However, relatively few studies have used non-viral gene transfection via liposomes in an organotypic brain slice system. In this study we have optimized various parameters for maximum *ex-vivo* transfection using a hyperbranched PEGmethacrylate linear poly(2-dimethyl-aminoethylmethacrylate) (lpD-b-P/E) synthesized previously in our laboratory [25].

2. Materials and methods

All reagents including HPLC and analytical solvents, synthesis and cell culture reagents were obtained from Sigma–Aldrich (Ireland), unless otherwise stated.

2.1. Synthesis and characterization

Hyperbranched PEGmethacrylate linear poly(2-dimethyl-aminoethylmethacrylate) (lpD-b-P/E) was synthesized as previously described [25] with slight modifications: the molecular weight of lpD-b-P/E was restricted to 24,000 Da.

2.2. Plasmid preparation and amplification

Human TNF and IFNγ cDNAs were cloned from human cDNA clones NM_000594.2 and NM_000619.2, respectively (Origene, Rockville, USA). The homology of human TNF and IFNγ was matched with the homology of rat TNF and IFNγ. Briefly, cDNA clones were digested using Not-I (New England Lab, USA) before amplification of the gene of interest by long proof-read long amplification (Extensor Long Range PCR polymerase ReadyMix kit, Thermoscientific – Fisher, Ireland) according to the supplier's instructions (human IFNγ (Forward-TGT CCA ACG CAA AGC AAT AC; Reverse-ATC TGA CTC CTT TTT CGC TTC C), human TNFα (Forward-CCA GGC AGT CAG ATC ATC TTCTC; Reverse-AGC TGG TTA TCT CTC AGC TCC AC) and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forward-GTG CCA GCC TCG TCT CAT AGA CAA G; Reverse-GCC GTG GGT AGA GTC ATA CTG GA)). PCR products were then run on a 1% agarose gel, extracted and purified using the MiniElute® kit (Qiagen, Ireland). After purification the PCR product was cloned into a pCR™ 2.1-TOPO® vector (CMV promoter driven plasmid) following the manufacturer's instructions. The size of positive clones was checked by electrophoresis on a 1% agarose gel after digestion using Nco I (Promega, Ireland) and by sequencing (MWG-Operon, Germany). After validation, the plasmid was amplified and purified using the Qiagen Plasmid Mega Purification kit (Qiagen, UK). G-luciferase (GLuc) plasmid (CMV promoter driven plasmid) (New England Lab, USA) was used as an empty vector. In some transfection studies a green fluorescent protein (GFP) plasmid (CMV promoter driven plasmid; New England Lab, USA) was also used for transfection analysis. Amplification and purification of the plasmid were performed using the Qiagen Plasmid Mega Purification kit (Qiagen, UK).

2.3. Organotypic brain slice culture

All procedures were performed under a licence issued by the Irish Government Department of Health and were approved by University College Cork Ethics Review board. Postnatal day 15 (P15) Sprague–Dawley rats (Biological Services Unit, University College Cork, Ireland) were decapitated and the brain was dissected out into ice cold artificial cerebrospinal fluid (ACSF; prepared as described in Ref. [26]). The cerebellum and cerebral hemispheres were separated and 250–400 μm sections were cut in ice cold ACSF using a vibratome (VT1200, Leica Microsystems). The cerebellum was sectioned in the parasagittal plane to preserve the cerebellar circuitry and the cerebral hemispheres were sectioned in the coronal plane. Slices were positioned on Millicell®-CM Low Height Culture Plate inserts (PICM0RG50; Millipore, Germany) in 6-well plates containing 1 ml of culture medium per well (Basal Medium Eagle [Gibco, Ireland], supplemented with Hanks Balanced Salt Solution [25% *v*/*v*], D-glucose [4.5 mg/ml], penicillin [100 U/ml], streptomycin [100 U/ml], glutamine [0.58 mg/ml], and 10% heat inactivated foetal calf serum). Plates containing tissue slices were then placed in an incubator with 5% CO_2 at 37 °C.

2.4. Gene transfection

For optimization experiments, cerebellar and cortical slices were cultured for approximately 1 h before they were exposed to the polymer/plasmid DNA (pDNA) complex (polyplex). Each treatment group contained six wells containing three slices per well. The gene transfection efficiency of the polyplex was assessed via G luciferase activity (New England Labs, USA). GFP expression was used for qualitative studies.

The polyplex was added either on top of the slice or into the culture media. Briefly, 15 μl of GLuc plasmid (1 μg, 2.5 μg or 10 μg per slice) was slowly added to 15 μl of lpD-b-P/E solution (2:1, 8:1 or 15:1 polymer/pDNA *w*/*w* ratio). After brief vortexing, the mixture was then incubated for 30 min at room temperature to allow the formation of polyplex. 30 μl or 100 μl of polyplex were applied on top of each slice above the culture insert membrane and maintained at 37 °C in a humidified incubator with 5% CO_2 for 48 h. The polyplex added into the cell culture media was prepared as follows: 45 μl of GLuc plasmid (7.5 μg per well) was slowly added to 45 μl of lpD-b-P/E solution (8:1 polymer/pDNA *w*/*w* ratio). After brief vortexing, the mixture was then incubated for 30 min at room temperature to allow the formation of polyplex. 90 μl of polyplex was then diluted in 900 μl of culture media. Fresh culture medium below the culture insert was replaced with culture media containing polyplex. The culture was maintained at 37 °C in a humidified incubator with 5% CO_2 for 48 h. After 48 h the tissues were homogenized in the culture media. 50 μl medium from all the samples was collected for quantification of luciferase expression.

Control transfections were performed using commercial transfecting agents. All commercial transfection agents (Lipofectin® reagent (Invitrogen, Ireland), Lipofectamine 2000® reagent (Invitrogen, Ireland) and FuGene® reagent (Promega, Ireland))/pDNA complexes were prepared as per the manufacturer's protocol.

2.5. Assessment of cell death

Cell death was determined using the fluorescent viability indicator propidium iodide (PI). PI was added on top of the slice at a concentration of 2 μg/ml and was incubated at 37 °C in a humidified incubator with 5% CO_2 for 15 min. Slices were then thoroughly washed with PBS and fixed with 4% PFA. PI fluorescence emission was visualized using an OlympusBX51™ microscope. Image J software was used to analyse the PI density of the whole slice at a set threshold. Cell death was determined by dividing the PI density with the total area of the slice. A minimum of nine slices were used per treatment group.

2.6. Demyelination via gene transfection and LPC treatment

Cerebellum slices were cultured for approximately 48 h before they were exposed to polyplexes or lysophosphatidylcholine (lysolecithin, LPC). Each treatment group contained three wells consisting of three slices per well. 15 μl of TNF or IFNγ plasmid (2.5 μg per slice) was slowly added to 15 μl of lpD-b-P/E solution (8:1 polymer/pDNA *w*/*w* ratio). After vortexing, the mixture was then incubated for 30 min at room temperature to allow the formation of polymer/DNA complexes. 30 μl of lpD-b-P/E/pDNA complexes were applied on top of each slice above the culture insert membrane and maintained at 37 °C in a humidified incubator with 5% CO_2 for 2, 4, 7, 14 and 21 days. Alternatively, 0.5 mg/ml of LPC was added to the medium for 17 h, after which slices were returned to normal media. After each time point the slices and media were collected and either fixed with 4% PFA or stored at −80 °C until further use. lpD-b-P/E alone, lpD-b-P/E complexed with GLuc plasmid (i.e. an empty vector), and Lipofectamine 2000® reagent complexed with TNF and IFNγ was used as controls. Lipofectamine 2000® complexes were prepared as per manufacturer's instructions.

2.7. Gene expression

After 2, 4, 7, 14 and 21 days, total RNA was extracted using a variant of Trizol isolation. Briefly, TriReagent® (Invitrogen, Ireland) was added to the cerebellar slices. Slices were mechanically disrupted under an oscillation frequency at 50 Hz/s for 2 min using Tissue Lyser LT System™ (Qiagen, Germany). Phase separation was performed using chloroform, and total RNA was purified using RNeasy™ mini kit (Qiagen, UK) according to the supplier's protocol. Total RNA quantity and purity were determined using an ultraviolet spectrometer (NanoDrop 2000™ Spectrophotometer, Thermoscientific Inc., USA). Reverse transcription (RT) was performed using the reverse transcription system (Promega, UK) according to the manufacturer's protocol. Gene transcription was examined using real-time polymerase chain