Stable Transfection of pEGFP-N1-MOG Plasmid to Utilize in Multiple Sclerosis Gene Therapy

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ABSTRACT

Introduction: Multiple sclerosis (MS) is a disease of the immune system: it attacks the myelin around the axons and leaves them exposed. Destruction of myelin weakens the electrical conduction of ions and thus leads to a lack of communication in the nervous system.

Methods: In the present study, we constructed recombinant plasmid and then transformed to E. coli cell. The colonies containing plasmid were selected by Colony PCR. Enzyme digestion and sequencing were utilized to approve the accuracy of the extracted plasmid of these clones. Recombinant plasmid transfect in to mesanchymal stem cells.

Results: Plasmid was verified correctly. After transfection, the transcription of MOG gene and the expression of MOG protein were proved by RT-PCR, western blotting and Elisa.

Conclusion: Plasmid was constructed correctly and mesenchyme stem cells were successfully transfected by transfection and protein can be expressed well, setting a proper foundation for the future studies on the transplantation of gene modified mesanchymal stem cells in order to promote Multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease caused by a combined attack of T cells, B cells, and macrophages against myelin components of the CNS [1, 2]. Literature witnesses studies characterizing well the immune responses against the quantitatively major myelin proteins, specifically myelin basic protein (MBP) and proteolipid protein, both in animal models (experimental allergic encephalomyelitis) and in human MS [3]. MS can present in several forms and with new symptoms, sometime either occurring in isolated attacks (relapsing forms), and sometimes developing over time (progressive forms) [4]. Myelin Oligodendrocyte Glycoprotein (MOG) is a gly-

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coprotein significant in the myelination of nerves in the central nervous system (CNS). This protein, encoded by the MOG gene in humans [2, 3, 4], is known to serve as a necessary "adhesion molecule" so as to provide structural integrity to the myelin sheath and is known to develop late on the oligodendrocyte [5]. MOG is a minor myelin protein exclusively expressed in the CNS. MOG is located on the extracellular membrane of oligodendrocytes, their processes, and the outermost myelin lamellae [3–7]. MOG is highly encephalitogenic in several animal species, most notably non-human primates, and brings about a primary demyelinating disease that closely mimics human MS [8–14]. Characteristics of MOG-induced experimental allergic encephalomyelitis include large concentric areas of macrophage infiltration, autoantibody deposition, and vesicular demyelination [15, 16]. As for humans, it seems that autoimmunity against MOG, too, plays a causal role in the pathogenesis of MS. MOG specific autoantibodies have been detected in situ in actively demyelinating MS lesions [15].

MS is also recognized to be associated with high levels of reactivity of PBMC against MOG [17–19], high precursor frequencies of MOG-reactive T cells in serum and cerebrospinal fluid (CSF), and CSF autoantibodies [20-22]. In the current study, recombinant plasmid pEGFP-MOG and transfect in mesenchyme stem cells were constructed and then analyzed by RT-PCR, western blotting, and Elisa technique.

2. Materials & Methods

Sub-cloning

After amplifying the carrier vector, this plasmid was subjected to PCR amplification via two digested primer containing Dra III and Not1 restriction enzyme sites in the forward and reverse primers, in that order (Figure 1). Then, a low-melting-point agarose gel electrophoresis was utilized to separate the amplification product. The purified DNA fragment (MOG gene) was sub-cloned into the Dra III and Not1-digested PEGFP-N1 mammalian expression vector making use of T4 DNA ligase. This vector was then subjected to colony-PCR as well as DNA sequencing for the accurate assessment of the sub-cloning processes.

Ligation

After confirmation, the MOG gene was prepared to be ligated to expression vector. Clean up gene kit was used to clarify the gene particle, already proliferated via PCR. The expression vector was cultured at the same time so as to proliferate in L.B medium and was transformed to linear form using Hind III enzyme. The gene particle was then ligated to the vector using T4 DNA ligase. The ligation product was incubated in 4 degrees circumstance for one night and was then entered into the transformation process.

Colony PCR

In order to confirm the entrance of the gene into the destination vector, the bacteria were cultured on the plates containing antibiotic. The proliferated colonies entered the colony PCR stage. The proliferated colonied on the antibiotic-containing plates were picked via a sampler and along with revers and forward primers and Taq DNA polymerase were entered into a micro tube and were put inside the thermo cycler machine. The PCR product was run on agarose gel and the positive samples producing a distinct band were transferred to antibiotic-containing L.B. medium. The product was purified using high pure plasmide isolation kit on the same day. The recombinant plasmid was analyzed with two simultaneous enzymes with a common buffer. The action site of these enzymes was located at the two sides of the inserted particle on the plasmid. The samples were then incubated for one hour in 37 degrees and all were run on the gel.

Digestion and sequencing

For final confirmation of recombinant plasmid after extraction by kit, the plasmids that showed a correct pattern in enzymal analysis were sent to Seq lab company in China for sequencing via universal primers. The results of the sequencing were assessed using Blast program from www.ncbi.nlm.nih.gov/blast to be matched with those of the genes of the genetic bank.

Cell culture

The umbilical cord was obtained from Pasture Institute of Iran, Tehran. It was rinsed with PBS and isolated from Amniotic membrane. Then, the jelly fraction of the cord was cut into pieces and cultured. After two weeks, the tissues were discarded and the cells were fed with DMEM/F12 medium supplemented with 10% FBS and antibiotics. The cells were grown to 60% confluence and passaged by trypsinization.

Transfection

Mesenchyme stem cells were cultured in DMEM/F12 medium supplemented with 10% FBS. As for the next stage, the transfection method was utilized for importing gene into the cells. The designed gene was imported into the plasmid and then the plasmid was transferred to the mesenchyme

stem cells. The transfection was carried out in 6 chambered petries wherein the cells were cultured between 24-48 hours before. During transfection, the population of cells was about 2.5 8 10^5 in each petri. Transfection was performed via liposomes using a standard kit.

In the present study, lipofectamin ragin was used. One microgram of plasmid DNA was added to 350 microgram of a medium containing no serum and was left in room temperature for 30 minutes. Simultaneously, 10 micro grams of lipofectamin was added to 350 micrograms of medium and was left for 30 minutes in room temperature. Next, these two tubes were mixed up and the product was incubated in 37 degrees for 1 hour. The content of the micro tube was then thrown on the cells and they were incubated for 4 hours. Later, the medium and the serum were added to them and were incubated for 48 hours. The medium of cells exchanged one day after transfection. In the current study, we used SDS-page and Western Blotting test to trace the recombinant protein in eukaryotic systems.

RT-PCR

To evaluate the expression of MOG in transfected and not-transfected BMSCs, RT-PCR was implemented. The primers used in the study are presented in Table 1. To extract total RNA, PurelinkTM RNA mini kit (Invitrogen) was used according to the manufacturer's instructions. To obtain purified RNA, the total RNA was treated by DNase I (invitrogen) and then analyzed by spectroscopy and agarose gel electrophoresis methods. Next, as instructed by the manufacturer, 1000 ng extracted RNA was used to synthesize 20 µl first strand cDNA (Revertaid™ first strand cDNA synthesis, Fermentas) of cDNA (500 ng) for PCR (Master Mix, 2×, Fermentas) making use of a thermocycler (Bio RAD) for 35 cycles. cDNA was omitted from the reaction as a negative control. The product size of PCR was analyzed by 2% agarose gel electrophoresis.

Protein isolation and western blotting

Cell culture media were suctioned off and the cell surfaces were washed three times, each for 5 min with cold PBS. Protein extraction of the mentioned cells was performed on ice with lysis buffer (the Proteo Jet Mammalian Cell Lysis Reagent, Fermentas) and protease inhibitor cocktail (Fermentas) was immediately added to the samples and protein concentration was assessed by Bradford assay. Equivalent amounts of each cell lysate and the proportional amount of serum-free conditioned medium protein were diluted in the 4×sample buffer, boiled at 95°C for 10 min, and then subjected to SDS-PAGE making use of two separate gels with the gradient running from 4 to 12% gel. After electrophoresis, one of the gels was visualized by silver nitrate stain method. Another gel was transferred onto the nitrocellulose membrane (Millipore) by semi-dry method (BioRad) and, then, the membrane was blocked for 1 h at 37°C in TBST containing 5% blocking solution (Amersham).

ELISA analysis

Cell culture media were suctioned off and the cell surfaces were washed three times, each for 5 min using cold PBS. Lysis buffer (Proteo Jet Mammalian Cell Lysis Reagent, Fermentas) was utilized to perform protein extraction of the cited cells on ice and immediately protease inhibitor cock-

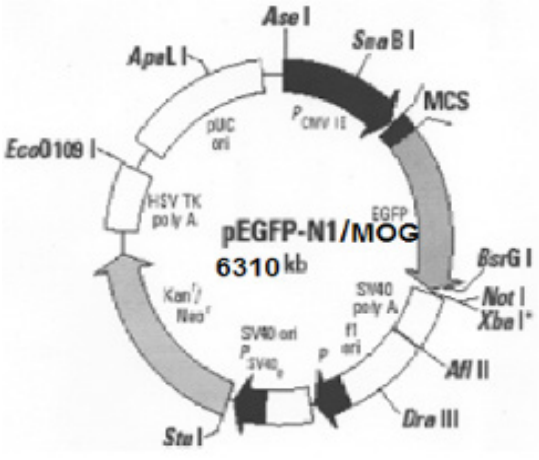

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Figure 1. The construct of MOG-overexpressing vector. MOG was subcloned into the expression vector after murine Ig-Kappa chain V-J2-C signal peptide in the PEGFP/N1 between the Dra III and Not1 restriction sites so as to construct PEGFP/N1- MOG vector.

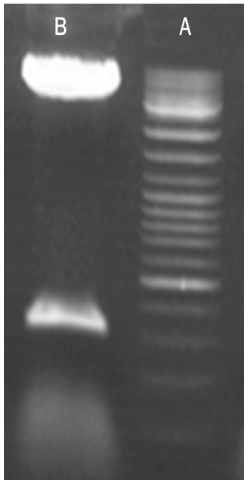

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Figure 2. Restrictive enzyme analysis of PEGFP/MOG by Dra III and Not1. The lanes are as follows: A: Ladder, B: MOG gene and PEGFP/N1.

tail (Fermentas) was added to the samples; protein concentration was assessed by Bradford assay. The supernatant of the conditioned media was collected after 48 hours of cells incubation and was used as the sample for ELISA analysis. The ELISA analysis was performed making use of a human MOG ELISA kit (abcam) according to the manufacturer´s instructions. A standard curve was established, as a reference for quantification, by a serial dilution of purified MOG protein (8pg/ml-2.0 ng/ml).

3. Results

MOG sub cloning

Sequencing of the data showed that the MOG gene subcloned precisely into the pEGFP/N1 to construct PEGFP-N1/MOG vector without any mutation and sequencing data analyses of the inserted MOG in the expression vector confirmed the validity of the sequence of MOG gene as published by Uniprot and Gene Bank bioinformatics databases.

Enzyme digestion

The cloned recombined plasmid of 6310 bp was named PEGFP-N1/MOG. In theory, by the restriction enzyme Dra III and Not1, PEGFP-N1/MOG should have 4700 bp and 1610 bp fragments which demonstrated the size of the recombinant plasmid and restriction fragments corresponded with the theoretical value indicating that MOG signal peptide combined successfully (Figure 2).

Stable transfection of MSCs with expression vector

Adding Neomycin at optimum concentration in the medium resulted in the appearance of the individual antibiotic resistant rat bone marrow stromal cell colonies. These cells were then subjected to further analysis.

RT-PCR

The results of RT-PCR indicate that the stable transfected MSCs transcribed MOG mRNA and that it is detectable (Figure 3).

ELISA analysis

As shown in Figure 5, the levels of MOG protein in the supernatant of the stable transfected mesanchymal stem cells was much higher (ng/ml±SEM) as compared with those of not-transfected cells (ng/ml±SEM) and the results of the present study revealed that the transfected cells secrete the MOG 24 fold (Figure 4).

SDS-PAGE and Western blot results

In the SDS-PAGE analysis of the stable transfected rat mesanchymal stem cells Cells, MOG expression was detected in their supernatant and lysate with approximately 27 kDa. Moreover, semi-quantitative Western-blot data analysis revealed that the MOG synthesis and secretion in the transfected rat mesenchyme stem cells can be expressed (Figure 5).

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Figure 3. RT-PCR results for mRNA expression in the transfected MSCs. Detection of gene expression were performed for transfected MSCs via RT-PCR technique to detect MOG mRNA. The lanes are as follows: Lane a, 100bp DNA ladder marker; lane b MOG amplicon in the transfected rat MSCs.

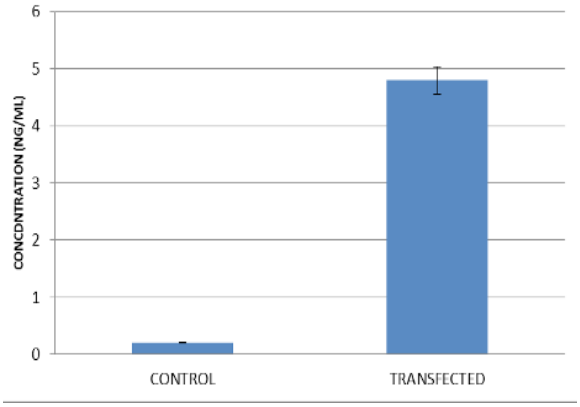

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Figure 4. ELISA results for MOG expression in the supernatant of not-transfected and transfected MSCs for MOG, in that order. The MOG protein level in the supernatant of the stable transfected rat MSCs was much higher (ng/ml±SEM) in comparison with not-transfected cells (ng/ml±SEM). In fact, it was found that the transfected MSCs secrete the MOG nearly 24 fold more in comparison to that by rat MOG.

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Figure 5. The Western-blot analysis of the stable transfected rat mesenchymal stem cells showed that the MOG synthesis and secretion in the transfected rat mesenchyme stem Cells can be expressed.

4. Discussion

In the present study, the recombinant vector containing fusion gene MOG was successfully constructed using the techniques of molecular biology. The findings will hopefully pave the way and provide the basis for further research in the field of gene therapy. MOG is a glycoprotein considered to be significant in the myelination of nerves in the CNS. This protein is encoded by the MOG gene in humans [2, 3, 4]. It is speculated that it serves as a required "adhesion molecule" to provide structural integrity to the myelin sheath and is known to develop late on the oligodendrocyte [5]. It is not still known what the main molecular function of MOG is; however, it may be that its likely role with the myelin sheath is either in sheath "completion and/or maintenance [4]. More specifically, MOG is speculated to be "necessary" as an "adhesion molecule" on the myelin sheath of the CNS to provide the structural integrity of the myelin sheath [5]. MOG's cDNA coding region in humans have been shown to be highly homologous to that in rats, mice, and bovine, and hence highly conserved. This suggests an important biological role for this protein [4].

Interest in MOG has centered on its role in demyelinating diseases many of which are not-inflammatory, like adrenoleukodystrophy, vanishing white matter disease, and Rubella-induced mental retardation [13]. MS studies have given MOG much of its laboratory attention. Some of these studies have shown a role for antibodies against MOG in the pathogenesis of MS [5]. Animal models of MS, EAE, have shown that "MOG-specific EAE models (of different animal strains) display/mirror human multiple sclerosis" [5], yet it basically explains the part involved in the optic neuritis [15]. These models with anti-MOG antibodies have extensively been and are considered the only antibodies with demyelinating capacity [5] but, again, EAE pathology is closer to NMO and ADEM compared with the confluent demyelination observed in MS. Literature witnesses many studies carried out on neuron growth factors and protective agents [8]. Multiple experimental and animal models have proven that neuronal growth factors have protective effects on diseases related to central nervous system like strokes, traumatic lesions, and neurodegenerative problems [9].

Some of these neuronal growth factors have already been implemented in certain clinical applications, e.g. Neurotrophins [10]. It has been shown that these factors have trophic effects on nervous system growth; however, it is not known how the protective effects of these factors work exactly. These studies differentiated rat emberional stem cells to neural and glial progenitors first and then by cloned and transfected MOG gene to this cells and then injected them into the injured site of the animal via CCI technique. It seems that MOG overexpression can have both neuroprotective and recovery effects [11]. Data obtained in the current study showed that MOG is the best choice to myelin regeneration at least in specific anatomical situations under in vivo conditions [12]. The observation that cultured neurons are unable to extend neurites into optic nerve, explaining the presence of optimal trophic factor conditions, have resulted in the recent understanding that adult mammalin CNS neurons are unable to regenerate; this is also due to the predominance of neurite growth-inhibiting molecules [13]. The most prominent members of this group are the myelin and oligodendrocyte-associated inhibitor of neurite growth, the myelin oligodendrocyte glycoprotein, the glycoprotein tenascin-C, and several proteoglycans such as chondroitin sulphate, heparan sulphate, and keratan sulphate proteoglycan. It appears that the balance between neurite regrowth-inhibiting molecules and neurite regrowth-inducing molecules influences the axonal fate and in our case we believe that, by transplanting cells capable of neurotrophin secretion, the balance tilted toward triggering the regeneration processes.

As demonstrated here, the not-transfected MSCS low level expressed MOG in comparison with transfected ones and also these transplanted cells could be effective at the site of injury. On the other hand, complete axonal re-arborization may need more sophisticated methods and strategies as important extrinsic constraint that could limit regrowth of lesioned-axons in the adult. CNS is the formation of a prominent glial scar in the affected area.

Although neurotrophic therapy is a promising approach to tilt the balance toward treatment of MS, even if at optimum secretion levels, combine therapy seems to be a more realistic approach to overcome these problems [15]. On the other hand, it is postulated that, in order to elicit a desired response, the ideal neurotrophin delivery into the nervous system should be target specific, regionally restricted, well-tolerated, and of enough concentration [16]. The most often used technique for gene transfer is the ex vivo approach, wherein genetically modified cultured cells are transplanted into the CNS [17]. Numerous studies have made use of fibroblasts and viral vectors for in vitro gene transfer of neurotrophins and have been proven to be effective in CNS, including those derived from herpes viruses, adenoviruses, adeno-associated viruses, and human immunodeficiency viruses [18]. However, the injected viruses have been exerted immune responses indicating the cytotoxicity of the current viral generations. This risk and the occurrence of replication competent viral particles in viral vector stocks are severe problems [19]. Thus, in the present study, we used non-viral gene therapy method under ex vivo conditions.

In conclusion, we made an effort to transfer non-viral MOG gene to MSCS and subsequently to stabilize its expression. Transplantation of these modified cells in the rat MS to explore functional recovery after induction revealed that these modified cells can be promising in MS therapy.

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