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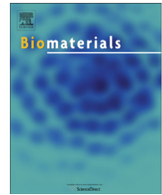

Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model

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ABSTRACT

Spinal Cord Injury (SCI) is a highly incapacitating condition for which there is still no cure. Current clinical approaches are mainly based on palliative care, so there is a need to find possible treatments to SCI. Cellular transplantation is regarded with great expectation due to the therapeutic potential of cells such as Adipose tissue-derived Stromal/Stem Cells (ASCs) or Olfactory Ensheathing Cells (OECs). Both are accessible sources and present positive paracrine and cell-to-cell interactions, previously reported by our group. Additionally, biomaterials such as hydrogels have been applied in SCI repair with promising results. We propose to combine a GRGDS-modified gellan gum hydrogel with ASCs and OECs in order to promote SCI regeneration. *In vitro*, ASCs and OECs could be co-cultured within GG-GRGDS hydrogels inducing a more robust neurite outgrowth when compared to controls. *In vivo* experiments in a hemisection SCI rat model revealed that the administration of ASCs and OECs encapsulated in a GG-GRGDS hydrogel led to significant motor improvements when compared to both control (SCI) and hydrogel alone (GG-GRGDS) groups. This was accompanied by a decreased infiltration of inflammatory cells and astrocytes, and by an increased intensity of neurofilament. These results suggest evident gains induced by the encapsulation of ASCs and OECs in GG-GRGDS based hydrogels.

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1. Introduction

Spinal Cord Injury (SCI) is a highly debilitating condition for which there is still no cure. SCI individuals usually have life-long loss of function and reduced quality of life. Its incidence internationally varies from as low as 2.3 to as high as 83 per million inhabitants every year [1]. The secondary events occurring after the primary injury increase the complexity of the disease, which hinders SCI treatment [2]. The current medical approaches after a spinal cord trauma are limited, consisting of the stabilization of the spine, decompression of the cord and eventually the administration of anti-inflammatory drugs [2]. Therefore, the development of novel therapeutic strategies targeting this condition is imperative.

Amongst the different approaches suggested so far, cellular based therapies have been one of the most frequently explored. From the different sources of cells currently being tested, Adipose tissue-derived Stromal/Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) have shown promising results [3,4]. For instance, intraspinal transplantation of murine ASCs in a SCI animal model, one week after injury, promoted the protection of denuded axons probably by preventing oligodendrocytes' degeneration and by participating in the regeneration of the myelin sheath [5]. In addition, ASCs transplantation also induced evident gains in motor performance [5]. These beneficial outcomes have been mostly related with the nature of the ASC's secretome, that is, the panel of molecules secreted by these cells to the extracellular

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milieu. In fact several reports have shown that ASC's secretome contains important neuroregulatory molecules such as Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Glial cell line-derived Neurotrophic Factor (GDNF), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), basic Fibroblast Growth Factor (bFGF), Insulin-like Growth Factor 1 (IGF1), Transforming Growth Factor Beta 1 (TGF-β1), among others, that are able to modulate neuronal and glial survival and differentiation [6–8]. Moreover, the molecules secreted by ASCs have also been shown to modulate the response of the immune system [7,9]. Alternatively, OECs are mainly characterized by participating in the growth and guidance of primary olfactory neurons. Their common origin with Schwann cells may explain some similarities observed between these two cell types, namely the capacity of OECs to surround olfactory axons, form fascicular processes and synthesize peripheral-like myelin [10]. The potential OEC transplantation as a therapy for CNS damage has already been explored *in vivo*. About two thirds of the experimental studies using these cells reported improvements in behavior outcome [11]. For instance, murine OECs were able to remyelinate axons in spinal cord injured rats [12,13], leading also to functional improvement of electric conduction in previously demyelinated axons [13]. Therefore, it is considered that OECs can create a permissive environment for axonal regeneration, in the usually hostile milieu of the damaged CNS [14]. For all these reasons, autologous transplantation of OECs in SCI patients has already been performed. In one clinical trial, results showed that autologous OECs are safe after three years post-transplantation [15,16].

ASCs and OECs present themselves as promising candidates for SCI cell therapy, mostly because they are easily accessible (ASCs can be obtained in large quantities from lipoaspirates while OECs can be safely isolated from nasal biopsies) and can be applied in an autologous manner, avoiding ethical concerns and the need for immunosuppression. By combining both, we envision taking advantage of the beneficial properties of each cell type simultaneously; namely, the neuronal regeneration guided and supported by OECs, which can be boosted by the paracrine effects of ASCs. Furthermore, previous work from our group showed that the secretome of rat-derived OECs has a positive effect on MSCs from different sources, but more evidently on human ASCs by increasing their proliferation and metabolic activity. Similarly, the secretome of ASCs also proved to be particularly beneficial for OECs in the same parameters [17]. These results reinforce the potential benefits of the combined use of these cells.

Therefore, the objective of the present work was to assess if the combined delivery of human ASCs and murine OECs into the injury site of a rat lumbar hemisection model, was able to induce motor and histological improvements in the injured rats. For this purpose and in order to efficiently deliver both cell populations, a hydrogel-based biomaterial (gellan gum, chemically grafted with a fibronectin-mimetic GRGDS peptide – GG-GRGDS) was used. Hydrogels are particularly appealing to be used as vehicles for cell transplantation because not only can they enhance cell survival, but they can also be designed to match the mechanical properties and water content of the CNS [18–21]. Our proposed chemically-modified gellan gum hydrogel is a biocompatible and biodegradable natural polysaccharide composed of repeating units of glucose, glucuronic acid and rhamnose [22,23] and is FDA approved [24]. Its physical properties allow the injection *in situ* and the filling of cavities provoked by the injury, in a minimally invasive manner. Moreover when chemically grafted with the fibronectin-mimetic peptide GRGDS (GG-GRGDS), this biomaterial was shown to have enhanced cell adhesion and proliferation [25,26], which lead to significant improvements of the metabolic activity and secretome of encapsulated cells [25,26].

2. Materials and methods

2.1. Cell isolation and culture

Human Adipose tissue-derived Stromal/Stem Cells (ASCs) were isolated according to the protocol described by Dubois et al. [27] from human lipoaspirates obtained from consenting donors under an institutional review board approved protocol at LaCell LLC. These cells were cultured and maintained in α-MEM (Invitrogen, USA), with 10% Fetal Bovine Serum (FBS, Biochrom AG, Germany) and 1% antibiotic-antimycotic solution – penicillin-streptomycin (Invitrogen, USA) at 37 °C and 5% CO_2 (v/v).

The animal care committee of the research institute approved all the animal protocols in accordance with standardized animal care guidelines [28]. Olfactory Ensheathing Cells (OECs) were harvested from olfactory bulbs of neonatal (P5-P7) Wistar-Han rats, according to the protocol previously described [29]. Briefly, upon dissection, the meninges and blood vessels were removed and the tissue was digested with collagenase type I (2.5 mg/ml, Sigma, USA) for 30 min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5 ml pipette and centrifuged at 1000 rpm for 5 min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation, cells were resuspended and seeded on uncoated plates for two consecutive periods of 24 h. It is expected that most of the fibroblasts and astrocytes attach in the first and second periods, respectively. After this purification step, the remaining cells were seeded on fibronectin coated surfaces (for 2D direct co-cultures) or encapsulated in GG-GRGDS hydrogel (for 3D co-cultures). Coating was done overnight with a 1 mg/ml fibronectin solution (Sigma, USA). Cells were cultured in DMEM/F12 (Invitrogen, USA) with 10% FBS and 1% antibiotic-antimycotic solution at 37 °C and 5% CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5.36 μg/ml, Invitrogen, USA) and Forskolin (1.4 μg/ml, Sigma, USA).

2.2. 2D direct co-cultures

In order to assess the potential positive or negative interactions between OECs and ASCs, a direct co-culture system with these two cell types was used. After isolation and purification (described in section **2.1**), OECs were seeded (60 000 cells/cm^2) on fibronectin coated coverslips. 24 h later, ASCs were seeded (10 000 cells/cm^2) over the OECs culture. Cells were allowed to grow in OECs culture medium (DMEM/F12 with supplements) since previous experiments showed its suitability for the culture of ASCs (data not shown). Medium was changed once, after three days of culture. Following one and seven days of incubation both cell growth and morphology was assessed by immunocytochemistry (ICC). OECs (60 000 cells/cm^2) and ASCs (10 000 cells/cm^2) monocultures were used as controls. Cell counts were performed by taking ten representative micrographs per sample, and the mean number of cells per field was determined for each sample of each group ($n = 3$).

2.3. Synthesis of GG-GRGDS hydrogel

The synthesis of GG-GRGDS hydrogel was performed according to the protocols described by Silva et al. [25]. Briefly, gellan gum (Sigma, USA) was first dissolved in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5, Sigma, USA) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Sigma, USA) and furfurylamine (Acros Organics, Belgium) were then added in a 4:1 M ratio (of each reagent relative to the –COOH groups in gellan gum) and stirred at 37 °C for 48 h. The solution was then dialyzed (Mw cutoff 12–14 kDa, Spectrum

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