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Research Paper

Transgenic human embryonic stem cells overexpressing FGF2 stimulate neuroprotection following spinal cord ventral root avulsion

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ABSTRACT

Ventral root avulsion (VRA) triggers a strong glial reaction which contributes to neuronal loss, as well as to synaptic detachment. To overcome the degenerative effects of VRA, treatments with neurotrophic factors and stem cells have been proposed. Thus, we investigated neuroprotection elicited by human embryonic stem cells (hESC), modified to overexpress a human fibroblast growth factor 2 (FGF-2), on motoneurons subjected to VRA. Lewis rats were submitted to VRA (L4-L6) and hESC/FGF-2 were applied to the injury site using a fibrin scaffold. The spinal cords were processed to evaluate neuronal survival, synaptic stability, and glial reactivity two weeks *post lesion*. Then, qRT-PCR was used to assess gene expression of β2-microglobulin (β2m), TNFα, IL1β, IL6 and IL10 in the spinal cord *in vivo* and FGF2 mRNA levels in hESC *in vitro*. The results indicate that hESC overexpressing FGF2 significantly rescued avulsed motoneurons, preserving synaptic covering and reducing astroglial reactivity. The cells were also shown to express BDNF and GDNF at the site of injury. Additionally, engraftment of hESC led to a significant reduction in mRNA levels of TNFα at the spinal cord ventral horn, indicating their immunomodulatory properties. Overall, the present data suggest that hESC overexpressing FGF2 are neuroprotective and can shift gene expression towards an anti-inflammatory environment.

1. Introduction

Lesions to the interface between the central and peripheral nervous systems cause extensive degeneration of spinal motoneurons (Kishino et al., 1997; Oliveira and Langone, 2000), resulting in permanent paralysis. Such injuries show substantial wasting traits and still lack effective treatments. Therefore, development of therapies, which limit the damage to the central nervous system (CNS) and increase the regenerative potential of injured neurons, is in the focus of global research.

Spinal root avulsion occurs as a result of traction forces that act on the region between the spinal cord and the rootlets. This results in denervation and interruption of retrograde flow of the neurotrophic factors produced by the target organ (Livesey and Fraher, 1992). Root avulsion in adults often follows high-energy traumas, as seen in motorcycle accidents, although it may also happen *e.g.* during complicated child delivery (Carlstedt, 2008; Malessy et al., 2009). Ventral root avulsion exclusively affects motor fibers leading to the retrograde degeneration of axotomized spinal motoneurons. Experimental VRA is an efficient model for studying degenerative changes of motoneurons and their underlying mechanisms (He et al., 2000; Koliatsos et al., 1994); as well as a reliable model for testing various therapeutic agents, including trophic factors (Koliatsos et al., 1994; Watabe et al., 2005).

In addition to the general loss of cells, any surviving motoneurons suffer a substantial decrease of presynaptic terminals, which further reduces or even abolishes synaptic transmission (Carlstedt, 2009). In this scenario, the microglial cells and astrocytes become reactive, increasing the degree of synapse elimination (Novikov et al., 2000; Ohlsson and Havton, 2006).

It has become clear that multifaceted strategies are necessary to successfully recover appropriate motor functions following VRA. In this regard, the priority *post lesion* is to preserve as many axotomized motoneurons as possible. Thus, recent studies have proposed different approaches, including cell therapy (Rodrigues Hell et al., 2009; Spejo et al., 2013) and therapy with neurotrophic factors (Liu et al., 2011; Romero et al., 2001). However, the use of embryonic stem cells has not yet been tested.

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Embryonic stem cells (ESC) are pluripotent cells isolated from inner cell mass of blastocysts (Evans and Kaufman, 1981; Thomson et al., 1998). Because of their pluripotency and ability for self-renewal, these cells were considered as an ideal source for cell replacement therapies (Ovchinnikov et al., 2012; Weissman, 2000). The ESCs, under appropriate culture protocols, maintain a regular karyotype and appear not to be susceptible to mitochondrial or epigenetic changes (Zeng and Rao, 2007). Importantly, the cell lines of hESC can be genetically manipulated (Robertson, 1987). Thus, several technologies have been developed to generate stably transfected hESC clones (Moore et al., 2010).

Among the variety of trophic factors, FGF2 is particularly conspicuous. Its receptors (FGFRs) are expressed in various cell types, including neurons (Eckenstein, 1994; Gómez-Pinilla et al., 1992), normal and reactive astrocytes (Follesa et al., 1994; Leme and Chadi, 2001; Liesi and Kauppila, 2002; Reilly et al., 1998) and microglia (Liu et al., 1998). FGF2 promotes proliferation of multipotent neural stem cells (Shihabuddin et al., 1997) and neural progenitors (Martens et al., 2002) in the spinal cord of the adults following lesions to the nervous system (Dono, 2003; Zai et al., 2005). *In vitro*, FGF2 increases neuronal survival, protects neurons against excitotoxicity, decreases apoptotic cell death and promotes myelination (Mattson and Scheff, 1994; Walicke, 1988). Moreover, FGF2 is also capable of stimulating synaptic plasticity (Ishiyama et al., 1991; Terlau and Seifert, 1990) and plays a major role in acute axonal regeneration after spinal cord injury (Zai et al., 2005).

Based on its robust neuroprotective properties, the FGF2 has been attempted as a therapeutic agent and showed mostly positive results (Fawcett and Asher, 1999; Rabchevsky et al., 2000; Tsai et al., 2008). In this sense, the present work aimed at improving motoneuron survival after VRA *via* application of transgenic hESCs that overexpress FGF2 in an inducible mode. hESCs were introduced to the lesion site by using a fibrin sealant scaffold. The results obtained can shed light on mechanisms of neuroprotection and can contribute to development of therapeutic strategies after VRA.

2. Material and methods

2.1. Experimental animals

Adult female Lewis rats 9–12 weeks old with body weight about 200 g were used for this study. All procedures were done in accordance with the ethical principles regulated by the National Council of Animal Experimentation (CONCEA) and with the approval of the Ethics Committee on Animal Experimentation of University of Campinas (CEUA/UNICAMP, protocol no 3373-1). Doxycycline (DOX) was used as an inducer of expression of FGF2 in the transgenic hESC. To induce overexpression of FGF2 in the hESCs *in vivo*, the inducer was combined with the pelleted food and given to animals *ad libitum*, for the whole duration of the experiment (2 weeks), at a concentration of 625 mg of DOX per kg of pelleted food, as described (Cawthorne et al., 2007). Recombinant FGF2 (rFGF2) was applied directly to the site of injury in a select control group. The following experimental groups ($n = 10$ in each group) were established: I, Avulsion with fibrin sealant (AV + FS); II, AV + FS + hESC + DOX (*cells on*); III, AV + FS + DOX; IV, AV + FS + hESC (*cells off*); V, AV + FS + rFGF2. The tissue samples from the contralateral side of the spinal cord, relative to the lesion side, were used as controls. For RT-qPCR experiments, samples from animals without injury ($n = 5$) were used as controls.

2.2. Ventral root avulsion

The animals were anesthetized with a combination of xylazine (Anasedan®, 10 mg/kg, Sespo Indústria e Comércio Ltda, Paulinia, SP, Brazil) and ketamine (Dopalen®, 90 mg/kg, Sespo Indústria e Comércio Ltda, Paulinia, SP, Brazil). A dorsal incision, parallel to the spine, was performed in the upper lumbar/thoracic region. The paravertebral musculature of the spine was removed to expose the lower thoracic and upper lumbar vertebrae. Laminectomy of approximately three vertebrae was performed to expose the lumbar intumescence. The dural sac was opened through a longitudinal incision and, after dissection of the denticulate ligament, the ventral roots were moved and followed carefully until the respective rootlets could be detected and avulsed. The unilateral avulsion was performed by pulling out the rootlets at the L4, L5 and L6 spinal segments, with a fine forceps (No.4). After the surgical procedures, the moved roots were replaced in their original position, after which the musculature, fascia and skin were sutured in layers, and the animals were kept in the Laboratory of Nerve Regeneration animal house for two weeks.

2.3. Vector construction

The Phusion High Fidelity DNA Polymerase (Finnzymes/Thermo Fisher Scientific, Vantaa, Finland) was used to amplify DNA fragments intended for cloning and/or transfections. For constructing the hFGF2 bacterial overexpressing vector, a PCR fragment 475 bp in length was obtained using primers FGF2-cds-2C, 5′-gaacatatggc agccgggagcatcac-3′ (with NdeI recognition site, underlined), and FGF2-cds-2D, 5′-atccctcgagctagcttagcagacattgg-3′ (with XhoI recognition site, underlined); and a template DNA from the ORFeome clone OCABo5050E0522D obtained from Source BioScience (Nottingham, United Kingdom). Its NdeI-XhoI subfragment 470 bp in length was then cloned into pET28b (Novagen/Merck Millipore, Darmstadt, Germany) using appropriate restriction endonucleases. The resulting vector pTe133 (GenBank accession number KX834270) was induced with IPTG in *E. coli* BL21(DE3) using EnPresso growth system from BioSilta Oy (Oulu, Finland) based on enzyme-controlled glucose autodelivery. The soluble recombinant protein was purified using Ni affinity column. The (His)x6 tag was cleaved off by thrombin, and the resulting rFGF2 was further purified using heparin affinity column. The biological activity of the purified rFGF2 was verified using 2 methods: *via* cell proliferation assay in hESC; and then *via* the Erk phosphorylation assay in hESC.

To obtain a stable hES cell line overexpressing FGF2-GFP fusion in an inducible mode, a Tet-On system (Clontech/Takara Bio, Mountain View, CA, USA) was used. For constructing a vector for inducible overexpression of hFGF2, a PCR fragments obtained with primers FGF2-cds-G, 5′-cagaattcatggcagccgggagcatc-3′ (with EcoRI recognition site, underlined) and FGF2-cds-H, 5′-tgggtaccaagctctta gcagacattgg-3 (with KpnI recognition site, underlined), containing ORF of hFGF2, was cloned (477 kb) into respective sites of pEGFP-N1 (Clontech), giving rise to an intermediate vector pTe104, overexpressing hFGF2-GFP fusion in a constitutive (non-inducible) mode. Then, the pTe104 was used as a template to amplify the fragment containing hFGF2-GFP fusion ORF with the primer FGF2-cds-G and a primer cdsGFP-B, 5′-cgtctagattacttgtacagctcgtccatg-3′ (with XbaI recognition site, underlined). This fragment after digestion (1227 bp in length) was cloned into respective sites of pTRE-Tight (Clontech), giving rise to a vector pTe106 (GenBank accession number KX844812). For stable cell transfections, the DNA of the pTe106 vector was used as a template for amplifying a linear transfection fragment 2275 bp in length containing essential parts of the vector and omitting the unnecessary bacterial sequences, with primers TRE-Lin-FWD, 5′-gaagcatttatcagggttattgtctc-3′ and TRE-Lin-REV, 5′-agggagaaaggcgga caggtatc-3′.

2.4. Preparation and administration of the fibrin sealant

The fibrin sealant was manufactured using snake venom from rattlesnake *Crotalus durissus terrificus* and was kindly provided by the Center for Study of Venoms and Venomous Animals (CEVAP – Unesp, Brazil); its components and application formula are stated in the respective patents (record numbers BR1020140114327 and BR1020140114360). At the moment of application, immediately after the avulsion, the fibrin sealant was prepared by mixing its three components at the ventral surface of the lumbar intumescence: fibrinogen derived from buffalo blood