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FIBROBLAST GROWTH FACTOR AND ENDOTHELIN-1 RECEPTORS MEDIATE THE RESPONSE OF HUMAN STRIATAL PRECURSOR CELLS TO HYPOXIA

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Abstract—Fetal striatal transplantation has emerged as a new therapeutic strategy in Huntington's disease (HD). Hypoxia is one of the microenvironmental stress conditions to which fetal tissue is exposed as soon as it is isolated and transplanted into the diseased host brain. Mechanisms that support neuroblast survival and replenishment of damaged cells within the HD brain in the hypoxic condition have yet to be fully elucidated. This study is aimed at investigating the molecular pathways associated with the hypoxic condition in human fetal striatal neuroblasts (human striatal precursor (HSP) cells), using the hypoxia-mimetic agent cobalt chloride ($CoCl_2$). We analyzed the effect of $CoCl_2$ on HSP cell proliferation and on the expression of hypoxia-related proteins, such as hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF). Moreover, we evaluated fibroblast growth factor 2 (FGF2; 50 ng/ml) and endothelin-1 (ET-1; 100 nM) proliferative/survival effects in HSP cells in normoxic and hypoxic conditions. Dose–response experiments using increasing concentrations of $CoCl_2$ (50–750 μM) showed that the HSP cell growth was unaffected after 24 h, while it increased at 48 h, with the maximal effect observed at 400 μM. In contrast, cell survival was impaired at 72 h. Hypoxic conditions determined HIF-1α protein accumulation and increased gene and protein expression of VEGF, while FGF2 and ET-1 significantly stimulated HSP cell proliferation both in normoxic and hypoxic conditions, thus counteracting the apoptotic $CoCl_2$ effect at 72 h. The incubation with selective receptor (FGFR1, endothelin receptor A (ETA) and endothelin receptor B (ETB)) inhibitors abolished the FGF2 and ET-1 neuroprotective effect. In particular, ET-1 stimulated HSP cell survival through ETA in normoxic conditions and through ETB during hypoxia. Accordingly, ETA expression was down-regulated, while ETB expression was up-regulated by $CoCl_2$ treatment. Overall, our results support the idea that HSP cells possess the machinery for their adaptation to hypoxic conditions and that neurotrophic factors, such as FGF2 and ET-1, may sustain neurogenesis and long-term survival through complex receptor-mediated mechanisms.
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Key words: $CoCl_2$, human striatal neuroblasts, FGF2, ETA, ETB, Huntington's disease.

INTRODUCTION

Transplantation of human nervous primordia is being explored as a reparative strategy in the treatment of neurodegenerative diseases, such as Huntington's disease (HD; Peschanski et al., 1995; Thompson et al., 1999; Björklund and Lindvall, 2000). The challenge is for grafted cells to proliferate, differentiate, and restore impaired circuitries (Tuszynski, 2007). However, a variety of insults, including mechanical trauma, hypoxia, free radical production, growth factor deprivation and amino acid excitotoxicity (Cicchetti et al., 2009; Watmuff et al., 2012), may hamper graft survival. To help understand how primordium cells could face those acute and chronic stressors, *in vitro* modeling is warranted.

Hypoxia is one of the microenvironmental stress conditions to which fetal tissue is exposed as soon as it is isolated and transplanted into the diseased host brain, where the loss of neurons is also accompanied by reduced trophic support due to both astrocyte and blood vessel atrophy (Cisbani et al., 2013). Because oxygen insufficiency can lead to cell death, hypoxia has traditionally been regarded as a negative condition. However, it is known that hypoxia can also act as a potent regulator of cell behaviors not only during normal development, but also in adaptation to stress and tissue regeneration. In particular, in the mammalian central nervous system oxygen tension plays a critical role in regulating the growth and differentiation state of neural stem cells (NSCs), as observed in both experimental and clinical settings (Panchision, 2009; Zhang et al., 2011). Mild hypoxia (2.5–5.0% O_2) promotes NSC self-renewal and also

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Abbreviations: ECE1, endothelin-converting enzyme 1; ETA, endothelin receptor A; ETB, endothelin receptor B; ET-1, endothelin-1; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; HSP, human striatal precursor; HD, Huntington's disease; HIF, hypoxia-inducible factor; IgG, immunoglobuline G; NSC, neural stem cell; PBS, phosphate buffered saline; qRT-PCR, Quantitative real-time polymerase chain reaction; VEGF, vascular endothelial growth factor.

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favors the success of engraftment when *in vitro*-expanded NSCs are transplanted into the brain of experimental animals (De Filippis and Delia, 2011). The cellular and molecular mechanisms of O_2-sensing within the brain have yet to be fully elucidated. However, it is well established that the cellular responses to hypoxia are mediated through specific changes in gene expression regulated by hypoxia-inducible factors (HIFs), mainly represented by HIF-1α protein (Mohyeldin et al., 2010). Under normoxic conditions HIF-1α protein is continuously degraded by the action of prolyl hydroxylase enzymes. Hypoxic stimulus determines the stabilization of HIF-1α, which translocates to the nucleus and, upon binding to HIF-1β subunit, forms an active transcription factor for the expression of target genes facilitating cellular adaptation to hypoxia, such as vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1) (Keith and Simon, 2007).

ET-1 is a vasoactive peptide released from endothelial cells under hypoxic conditions (Kourembanas et al., 1991) that can bind to two G-protein-coupled receptors: endothelin receptor A (ETA) or endothelin receptor B (ETB) (Schinelli, 2006). In the brain, ET-1 is present not only in endothelial cells (Yoshimoto et al., 1990), but also in neurons (Fuxe et al., 1991) and astrocytes (MacCumber et al., 1990), and its secretion increases in several pathologies, such as cerebral ischemia (Yamashita et al., 2000).

We have recently reported the characterization of human striatal precursor (HSP) cells isolated from striatal primordium in 9–12-week-old human fetuses (Sarchielli et al., 2014), which has proven to be an optimal cell source in HD neural transplantation protocols (Gallina et al., 2008, 2010; Paganini et al., 2013). HSP cells showed features of a mixed cell population of immature elements, neuronal/glial-restricted progenitors and striatal neurons, indicative of a plastic phenotype already committed to become striatum. Interestingly, these cells also express neurotrophins, such as brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor 2 (FGF2), along with the cognate receptors TRKB and FGFR1, respectively. When exogenously administered, both neurotrophins have shown the ability to induce neurogenesis, migration and survival of HSP cells (Sarchielli et al., 2014), all basic requirements for graft development.

The present study was aimed at investigating the adaptive response of HSP cells to *in vitro* hypoxia using cobalt chloride ($CoCl_2$), an hypoxia-mimetic agent able to stabilize HIF-1α by inhibiting prolyl hydroxylase enzymes (Ho and Bunn, 1996), and we demonstrated that FGF2 and ET-1 sustain HSP cell proliferation and survival both in normoxic and hypoxic conditions, through receptor-mediated mechanisms.

EXPERIMENTAL PROCEDURES

Cell culture

The primary HSP cell culture was established and propagated *in vitro* from human striatal primordium, as previously described (Sarchielli et al., 2014) and used within the 11th passage. HSP cells were cultured at 37 °C 5% CO_2 in Coon's modified Ham's F12 medium (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Hyclone Logan, UT, USA) and antibiotic/antimycotic solution.

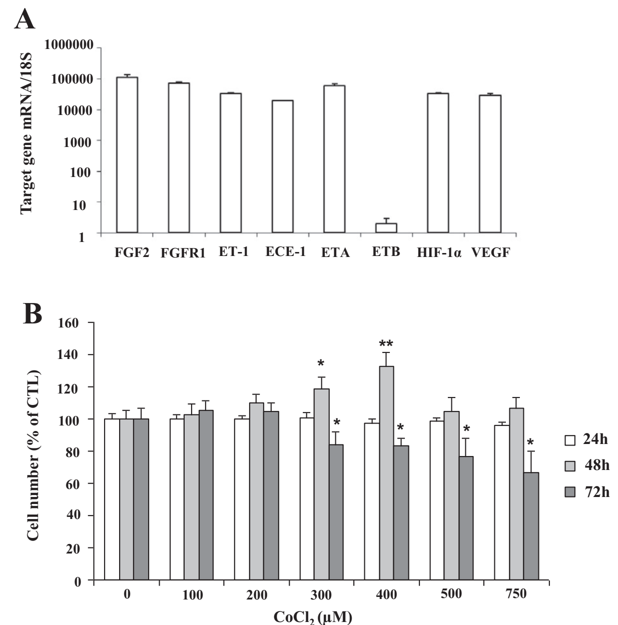

Fig. 1. Constitutive gene expression profile in HSP cells and effect of $CoCl_2$ on cell growth. (A) Quantitative mRNA expression analysis by qRT-PCR in HSP cells under normoxic conditions. Data are normalized over 18S ribosomal subunit taken as the reference gene, and are reported as mean ± SD (n = 4). (B) MTT assay of HSP cells untreated (CTL) or treated with increasing doses of $CoCl_2$ (100–750 μM) for 24, 48 or 72 h; data are expressed as a percentage of the respective CTL at each time point, and are reported as mean ± SD (n = 3); $^*p$ < 0.05, $^{**}p$ < 0.01 vs. the respective CTL.

RNA extraction and quantitative real-time RT-PCR

Isolation of total RNA from cells was performed using the "RNeasy Micro kit" (Qiagen, Milan, Italy) according to the manufacturers' instructions. cDNA synthesis was carried out using a kit based on the random primers technique (Taqman Reverse Transcription Reagents kit; Applied Biosystems, Forster City, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to the fluorescent TaqMan methodology, as previously described (Gallina et al., 2010; Sarchielli et al., 2014). PCR primers and probes for target genes were from Applied Biosystems, as follows: FGF2, Hs00266645_m1; FGFR1, Hs00241111_m1; ET-1, Hs00174961_m1; ECE-1, Hs01043735_m1; ETA, Hs00609865_m1; ETB, Hs00240747_m1; HIF1, Hs00153153_m1; VEGF, Hs00173626_m1. Gene 18S Hs99999901_s1 was chosen as the housekeeping gene and used for relative quantification of the target genes. Data analysis was based on the comparative threshold cycle (Ct) using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001). Amplification and detection were performed using an ABI Prism 7700 Sequence Detection system (Applied Biosystems).

MTT assay

Cell viability was determined by MTT assay (Sigma–Aldrich Corp., St. Louis, MO, USA). Briefly, 8×10^3 HSP

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