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Unilateral induction of progenitors in the spinal cord of hSOD1^{G93A} transgenic rats correlates with an asymmetrical hind limb paralysis

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Abstract

Transgenic rats expressing a mutated form of the human Cu/Zn superoxide dismutase (hSOD1^{G93A}) develop an amyotrophic lateral sclerosis (ALS)-like phenotype, including motor neurone degeneration and reactive gliosis in the spinal cord. This study aimed at examining the presence of endogenous neural progenitors in the lumbar spinal cord of these rats at the end-stage of the disease. Immunohistochemical data clearly demonstrated the induced expression of the stem cell factor reported as a chemoattractant and survival factor for neural stem cells as well as nestin (neuro-epithelial stem cell intermediate filament) in the spinal cord sections. While the stem cell factor immunolabelling appeared diffuse throughout the gray matter, nestin labelling was restricted to clusters within the ventral horn. Interestingly, as paralysis regularly develops asymmetrically, induction of nestin was only detected on the ipsilateral side of the predominant symptoms. Finally, immunohistochemical detection of the stem cell factor receptor (c-Kit) revealed its specific induction which coincided with nestin immunolabelling. Together, these results are indicative of endogenous recruitment of neural progenitors within lesioned tissues and could support the development of treatments involving endogenous or exogenous stem cells.

Keywords: Amyotrophic lateral sclerosis; hSOD1^{G93A}; Stem cell factor; Nestin; c-Kit

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disorder characterised by progressive motor dysfunction leading to paralysis, muscular atrophy and death. The pathology results from the death of large motor neurones in the spinal cord and brainstem [15]. ALS occurs in both sporadic and familial forms [23], the latter representing 5–10% of reported cases. Approximately, 15–20% of familial ALS cases have been linked to autosomal dominant inheritance of mutated forms of Cu/Zn superoxide dismutase 1 (SOD1) [5,22]. While oxidative stress and disturbed control of the glutamatergic neurotransmitter system have been implicated, the precise cellular mechanism underlying the anatomically selective neurodegeneration in this disease has still to be identified [4].

Recently, transgenic rats carrying the human SOD1^{G93A} (hSOD1^{G93A}) gene have been generated [18] and constitute a dedicated experimental animal model of ALS. At the end-stage of the disease, they show motor neurone degeneration in the spinal cord with decreased expression of MAP2 and tissue vacuolisation. Simultaneously, a pronounced gliosis is observed in the spinal cord and the brainstem [6,11]. Gliosis is a prominent feature of several diseases of the CNS [21] in which reactive astrocytes invade lesioned areas and participate in both neurone replacement as well as neuroprotection, thanks to their ability to take up excitotoxic glutamate and to release neurotrophic factors [2,3,19].

In this context, the present study aimed at examining presence of endogenous neural progenitors in the lesioned tissues

Abbreviations: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; CSF, cerebrospinal fluid; Cy3, cyanin3; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride hydrate; FGF_2, fibroblast growth factor 2; FITC, fluoroscein isothiocyanate; GFAP, glial fibrillary acidic protein; GLT-1, glutamate transporter-1; MAP2, microtubule-associated protein 2; nestin, neuro-epithelial stem cell intermediate filament; NSC, neural stem cell; PBS, phosphate buffered saline; SCF, stem cell factor; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor

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of hSOD1^{G93A} which could indicate the emergence of repair processes during the progression of the pathology. We investigated the expression of the stem cell factor (SCF). Initially characterised as a key factor in the regulation of haematopoiesis [1], SCF acts as a chemoattractant and survival factor for neural stem cells (NSC) [8]. Hence, its tyrosine kinase receptor (c-Kit) is present on NSCs and its activation triggers cell migration both in vivo and in vitro [8,25]. Finally, the expression of nestin (neuro-epithelial stem cell intermediate filament) was also examined. This intermediate filament protein expressed by proliferating neuro-epithelial cells during the development of the CNS is also expressed by NSCs in the adult mammals and classically used to identify adult neural progenitors in culture [17]. The results of this immunohistochemical study reveal modulated expression of these markers in the spinal cord of end-stage transgenic animals. We suggest that these changes figure an adaptation of the organism in response to the degenerative process and we propose that it could provide a biological support for treatments involving endogenous or exogenous stem cells.

Transgenic Sprague–Dawley rats harbouring a 12-kb *Eco*RI/*Bam*HI restriction fragment of the human SOD1 gene with the G93A mutation (hSOD1^{G93A}) were previously described [18] and obtained from Taconic Farms Inc., NY. Animals housed in plastic cages were kept at constant temperature ($23 \pm 1\,^{\circ}C$) and relative humidity (40–60%) on a 12 h light, 12 h dark cycle with access ad libitum to both food and water. All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC). Newborn rats were analyzed for the presence of the transgene by PCR performed on genomic DNA extracted from tails biopsy obtained a few hours after birth. Tissue from each animal was incubated with proteinase K (100 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) at 56 °C overnight and after centrifugation, precipitation and washes, purified DNA was quantified. Samples of genomic DNA were subjected to PCR using specific primers SOD-i3f (5′-GTGGCATCAGCCCTAATCCA-3′) and SOD-E4r (5′-CACCAGTGTGCGGCCAATGA-3′) and the program consisted in 30 cycles of denaturation (95 °C/45 s), hybridisation (60 °C/45 s) and extension (72 °C/45 s). Twenty microlitres of each sample were electrophoresed on a 1% agarose gel and visualised using ethidium bromide. Transgenic animals were diagnosed by the presence of a 160 bp amplification product.

The behaviour of each animal was routinely monitored. The onset of the disease was revealed by observation of the animal when walking on a flat surface. Animals showing unilateral symptoms use the preserved hindlimb for locomotion while the paralysed one is dragged. The progression of the muscular weakness was confirmed by the loss of the extension reflex. In comparison with healthy animals, paralysed animals do not extend their hindlimbs when suspended by the tail. This test allowed to validate unilateral symptoms as extension is then preserved for one limb while the other remains retracted close to the body.

As the animal's condition dramatically deteriorated, rats were anaesthetised with CO_2, their spinal cord was immediately dissected and fixed in formol 10% for 48–72 h. Tissues from age-matched wild-type littermates were processed simultaneously for control. After rinsing in 0.1 M phosphate buffered saline (PBS, NaCl 0.137 M, KCl 2.68 mM, KH_2PO_4 1.5 mM, Na_2HPO_4 8 mM, pH 7.4) and progressive dehydratation in ethanol 70, 90 and 100° (24 h each), tissues were immersed in toluol (16 h) and embedded in paraffin. Five-micrometer sections of tissues were cut in a microtome and apposed on coated glass microscope slides. Sections were deparaffinized with toluol, rehydrated in alcohol and rinsed in distilled water. Non-specific labelling was blocked by incubating the sections in PBS containing 1% bovine serum albumin for 1 h at room temperature. Sections were then incubated during 1 h with primary antibodies, i.e. a mouse anti-nestin antibody (Chemicon, Hampshire, UK – 1:200), a rabbit anti-glial fibrillary acidic protein (GFAP) antibody (DAKO, Heule, Belgium – 1:400), a guinea pig anti-glutamate transporter-1 (GLT-1) antibody (Chemicon, Hampshire, UK – 1:300), a mouse anti-microtubule associated protein (MAP2) antibody (Sigma, Bornem, Belgium – 1:200), a rabbit anti-SCF antibody (Chemicon – 1:250) and a rabbit anti-c-Kit antibody (Sigma, Bornem, Belgium – 1:100). Secondary antibodies (all from Jackson Immunoresearch Laboratory, De Pinte, Belgium), applied for 1 h at room temperature were fluoroscein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (1:200) for nestin and MAP2, FITC-conjugated anti-rabbit IgG antibody (1:200) for GFAP, SCF and c-Kit, cyanin3 (Cy3)-conjugated anti-mouse IgG antibody (1:200) for nestin and Cy3-conjugated anti-guinea pig IgG antibody (1:200) for GLT-1. The Tyramide Signal Amplification Kit (Invitrogen-Molecular Probes, Merelbeke, Belgium) was used for SCF and c-Kit detection following manufacturer's advice, in order to enhance specific signals. Cell nuclei were stained during 15 min with the nuclear dye 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1:5000). After three rinses with PBS, the preparations were mounted in Fluoprep and examined using an Olympus IX70 inverted fluorescent microscope coupled to a CCD camera (T.I.L.L. photonics, Martinsried, Germany). Excitation light (485, 540 and 400 nm wavelength for FITC, Cy3 and DAPI, respectively) was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. photonics, Martinsried, Germany). Digital images were acquired using appropriate filters and combined using the TILLvisION software (T.I.L.L. photonics, Martinsried, Germany).

Each litter contained both wild-type and transgenic pups and therefore, each animal has been genotyped for the presence of the hSOD1^{G93A} gene by PCR on genomic DNA. The behaviour of each animal was routinely monitored and the apparition and progression of the symptoms were recorded. As documented in previous studies [18,28], the majority of the transgenic animals tested showed primarily a unilateral weakness of one hindlimb (Fig. 1a and Table 1). Thereafter, animal's condition rapidly declined and complete paralysis was observed within 2 weeks after detection of the first symptoms. At that stage, animals were sacrificed and tissues were immediately processed for immunohistological analysis.