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Transplantation of mesenchymal stem cells in ALS

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating incurable neurodegenerative disease that targets motor neurons (MNs) in the primary motor cortex, brainstem and spinal cord, leading to muscle atrophy, paralysis and death due to respiratory failure within 2-5 years. Currently, there is no cure for ALS. The development of a therapy that can support or restore MN function and attenuate toxicity in the spinal cord provides the most comprehensive approach for treating ALS. Mesenchymal stem cells might be suitable for cell therapy in ALS because of their immunomodulatory and protective properties. In this review the authors discuss the major challenges to the translation of *in vitro* and animal studies of MSCs therapy in the clinical setting.

Key words: mesenchymal stem cells, neurodegeneration, neuroprotection, amyotrophic lateral sclerosis, transplantation, bone marrow

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating incurable neurodegenerative disease that targets motor neurons (MNs) in the primary motor cortex, brainstem, and spinal cord leading to muscle atrophy, paralysis and death due to respiratory failure within 2-5 years. The incidence is 2-3 cases per 100,000 general population, and the prevalence is around four to six per 100,000 (Chiò et al., 2009). Approximately 90% of all cases are classified as sporadic (SALS), defined as having no family history of the disease. The remaining cases are dominantly inherited, i.e. familial (FALS). Until very recently, only 30% of FALS cases had been accounted for known genetic mutations. The recent identification of the C9orf72 hexanucleotide repeat on chromosome 9p21 appears responsible for 30% to 60% FALS cases, and about 8% of apparently sporadic cases (Dejesus-Hernandez et al., 2011; Renton et al., 2011). Currently, there is no cure for ALS. Riluzole is the only drug approved by the Food and Drug Administration for ALS treatment but its outcome is very limited, since it increases survival by only 2–3 months compared with placebo (Bensimon et al., 1994).

ALS was traditionally considered a pure motor disorder. However, recent findings suggest that ALS is a multisystem disorder in which the MNs tend to be affected the earliest and the most severely (Ince et al., 2008). The pathogenic processes underlying ALS are multifactorial and, at present, not fully determined (Ferraiuolo et al., 2011). Recent data have implicated the microenvironment of the MNs, rather than the MN itself, as a primary target. ALS therefore has emerged as a non-cell-autonomous neurodegenerative disease, i.e., not independent of mutant damage accumulated within other cell types that interact with the affected neurons. Putative mechanisms of toxicity targeting MNs include oxidative damage, accumulation of intracellular aggregates, mitochondrial dysfunction, defects in axonal transport, defective growth factor trophic support, altered glial function, aberrant RNA metabolism and glutamate excitotoxicity. Each of these mechanisms represents a potential therapeutic target and many clinical trials have been developed, even though currently none of the candidate compounds has been demonstrated effective. A cell-based therapy may have the advantage of exerting multiple therapeutic effects (Lepore et al., 2008; Suzuki et al., 2008) at various sites and times within the lesion, as the cells respond to a particular pathological microenvironment (Liu and Martin, 2006) by protecting existing MNs from ongoing degeneration.

The development of a therapy that can support or restore MN function and attenuate toxicity in the spinal cord provides the most comprehensive approach for treating ALS.

Stem cell transplantation as a therapeutic strategy.

Cell therapy uses cell or tissue grafts to treat diseases or injury. Treatment focuses on cell replacement or providing environmental enrichment.

Lower MNs can be generated *in vitro* from stem cells of various sources. The newly transplanted neurons may then integrate, receive and make synapses, and recapitulate a neural network after transplantation into adult rats (Kerr et al., 2003; Yan et al., 2007; Bohl et al., 2008; Xu et al., 2009). However, practical issues might limit the clinical translation of direct MN replacement to humans. Grafted neurons must receive functional synapses, send axons through inhibitory white matter, and direct axons over long distances to the target muscles in order to retain neuromuscular function. Given these limitations neuronal replacement in ALS patients seems a distant goal.

Several studies have recently clarified the role of glia in ALS pathogenesis (Boillé et al., 2006; Ilieva et al., 2009). Neuron-astrocyte interactions are essential for the regulation of glutamate transmission. Without astrocytes, neurons become increasingly vulnerable to excitotoxicity. The disruption in astrocytic function can markedly promote neurodegeneration. The contribution of non-neuronal cells to the pathogenesis of MN degeneration has been studied in mutant SOD1 mice, in which the transgene was excised in specific cell types. Primary astrocyte and microglial cultures derived from the superoxide dismutase-mutant mouse model of ALS produce neurotoxic mediators in conditioned media that kill wild-type MNs (Nagai et al., 2007). Haidet-Phillips et al. (2011) showed that astrocytes derived from post-mortem tissue from both FALS and SALS patients are similarly toxic to MNs. We might hypothesize that replacement or enrichment with healthy astrocytes could be a therapeutic approach for slowing or blocking the disease course. Studies with chimeric mice showed that delivering wild type glial cells in the ALS model can improve the disease phenotype (Beers et al., 2006; Clement et al., 2003).

Alternatively, astrocyte replacement has also been proposed as a potential therapy for slowing disease progression in ALS. Rodent glial-restricted precursors (GRP) transplanted into the spinal cords of mutant SOD1 rats differentiate into astrocytes, restore the levels of astrocyte glutamate transporter, and extend survival of these rats (Lepore et al., 2008). Human GRP transplants robustly survive and migrate in both gray and white matter and differentiate into astrocytes in SOD1G93A mouse spinal cord, despite ongoing disease progression. However, cervical spinal cord transplants did not result in MN protection or any therapeutic benefits on tests of functional outcome (Lepore, 2011).

Growth factors and ALS

Growth factors are naturally occurring proteins essential for neuronal survival: their deficiency could induce MN death in ALS patients (Henriques et al., 2010). Several growth factors were therapeutic in animal models but not in humans: clinical trials using brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, and insulin-like growth factor demonstrated no significant survival benefits (The BDNF Study Group, 1999; Bongioanni et al., 2004; Sorenson et al., 2008). The reasons for these failures might be related to an inadequate route of administration. Penetration of large peptides, such as growth factors, into the CNS, in fact, is limited by the blood-brain barrier (BBB). On the other hand, stem cells transplanted into the nervous system produce and deliver neurotrophic and growth factors and their efficacy could be improved by genetic modification to secrete molecules that promote MN survival (Klein et al., 2005; Ericson et al., 2005). As such, stem cells might be able to both detoxify the local environment around dying MNs by generating glial cells and delivering trophic factors after transplantation (Suzuki and Svendsen, 2008; Gowing, and Svendsen, 2011).

The beneficial effects of glial cell replacement and enhancement of neurotrophic support in ALS provide the basis for the use of stem cells to treat this disease (Lunn et al., 2011).

Mesenchymal stem cells

Stem-cell-based therapies represent a new possible scenario for neurodegenerative diseases.

Multipotent mesenchymal stem cells (MSCs) are bone marrow cells that can be expanded *ex vivo* and will readily differentiate into mesodermal cell derivatives (Pittenger et al., 1999).

MSCs represent a small fraction (0.001-0.01%) of the bone marrow (BM) cell population; therefore, to obtain a sufficient number of cells, they must be extensively expanded *ex vivo*. Although BM is the best characterized source of MSCs, umbilical cord blood (UCB), Wharton's jelly, placenta, adipose tissue (AT), and many others represent promising alternatives (Otto and Wright, 2011). Human amniotic fluid (hAF), is also considered an attractive source of MSCs for therapeutic transplantation (In'tAnker et al., 2003) and recently new reports confirm the importance of stem cells isolated from hAF for banking and multiple clinical applications (De Coppi et al., 2007; Sessarego et al., 2008; Mareschi et al., 2009).

MSCs show their ability to differentiate into mature neural cell types. They have been suggested to adopt "astrocytic" and "neuronal like" cell fates (Wislet-Gendebien et al., 2005). When cultured in neural progenitor maintenance medium (NPMM), hMSCs acquire new morphological

characteristics, neural markers, and electrophysiological properties, which are suggestive of neural differentiation (Mareschi et al., 2006).

MSCs promote “bystander” immunomodulation, as they can release soluble molecules such as cytokines and chemokines and express immuno-relevant receptors such as chemokine receptors and cell-adhesion molecules (Uccelli et al., 2008), and they can control drug-resistant graft-versus-host disease in humans. In animals, they also seem to limit damage to, or mediate repair of, CNS tissue via mechanisms other than cell replacement or trans-differentiation, probably via their paracrine function (Uccelli et al., 2011).

MSCs can be successfully isolated and expanded for clinical application without significant adverse effects, and could represent a new possible scenario for neurodegenerative diseases. We have demonstrated that BM-MSCs from ALS patients maintain their peculiar characteristics and when expanded *in vitro* do not display chromosomal alterations or cellular senescence. Moreover, they acquire, under specific conditions, new morphological characteristics and neural markers which are suggestive of neural differentiation as well as those obtained in the healthy donors (Ferrero et al., 2008).

Recent studies have revealed that MSC therapeutic action is related to the release of protective factors, even far from the site of injection, rather than to replacement of degenerating neurons. Such a therapeutic effect may be provided by different classes of molecules, including trophic factors, anti-inflammatory cytokines and immuno-modulatory chemokines released from transplanted cells.

MSC therapy might indeed represent a promising strategy for the development of a new therapeutic strategy for neurodegenerative diseases, such cell therapy in animal models.

Due to the multifarious and elusive pathogenesis of ALS, several different *in vivo* models of ALS have been identified, each of which displays some genetic and/or pathological hallmarks of the disease. The detailed description of the different animal models is not the aim of this review, but the reader can refer to Berthod and Gros-Louis (2012) and Boido et al. (2012). In brief, the most widely used model consists in the insertion of the human copper/zinc superoxide dismutase 1 (SOD1) gene bearing a mutation in G93A (a mutation at position 93 from glycine to alanine), which has been found in FALS (Gurney et al., 1994). This characteristic dominant “gain-of-function” mutation leads to a neurologic syndrome in mice which is similar to the human disease. The same mutation together with H46R has been introduced in rats to provide a more severe animal model for experimental manipulation (Nagai et al., 2001).

For the characteristics of their neurological syndrome, and for being the first model introduced in ALS research, mostly SOD1 rodents have been used to test the effects of stem cell therapy. MSCs have been transplanted in mice either by intraparenchymal, intracerebroventricular (i.c.v.), intraperitoneal (i.p.) or intravenous (i.v.) injections. The issue of the administration route will be discussed in details below, as well as that of immunosuppression, which in general is not needed (Vercelli et al., 2008).

A relevant issue relative to MSC transplantation is the donor species: on the one hand, when cell-to-cell interactions must be studied, the donor and the host should be of the same species. On the other hand, in order to use hMSCs as medicinal products (see below) and exclude harmful side effects, xenogeneic transplants are performed.

Following treatment, the effects of cell therapy are evaluated in terms of cell fate/integration, functional outcome (motor behavioral tests), survival of the animal and histopathological outcome (i.e. survival of MNs, astrogliosis and microglial activation). In the first studies, a great interest has been raised on the potential for transdifferentiation of MSCs into neurons and glial cells. In particular, neuronal differentiation has been hypothesized as a mean for cell replacement. Even though MSCs, isolated from BM, can transdifferentiate into neurons (Phinney and Isakova, 2005), it is unrealistic to expect that the transplanted stem cells or stem cell-derived MNs in ALS patients either in a clinical setting or in the experimental animal replace lost neurons, integrate into existing neural circuitry, and restore motor function. Rather, preventing cell death in host MNs via provision of neurotrophic, anti-inflammatory and immunomodulatory factors by transplanted stem cells or stem cell-derived MNs is a more realistic and achievable approach.

MSCs in fact can deliver neurotrophic, anti-inflammatory and immunomodulatory molecules (Caplan, 2009) to delay disease progression. Their interaction with the nervous system results in a reciprocal influence, which can modify the environment of MNs in ALS, delaying their degeneration and supporting their survival. MSCs act as minipumps which can be directed to express and produce molecules extending the survival of MNs (Sadan et al., 2009). Therefore, more recently, attention has been drawn to the effects of the diseased brain on MSCs in terms of production and delivery of immunomodulatory molecules and neurotrophic factors. Cultures of rat MSCs display an increased production of NGF and BDNF after stimulation with SOD1 rat brain extracts (Nicaise et al., 2011). Also, MSCs themselves can modify the local environment and influence the delivery of trophic factors and immunomodulatory molecules by the host cells

We have shown that intraparenchymal injection of donor hMSCs can improve motor behavior, delay motor neuron death and reduce neuroinflammation (Vercelli et al., 2008) (Figure 1).

Other authors reported similar results with i.v. injection of donor hMSCs (Zhao et al., 2007). I.v. administrated MSCs interact specifically with several mechanisms involved in MN death (Uccelli et al., 2012). Intrathecal administration of murine MSCs into SOD1 mice lead to increased lifespan and decreased neuroinflammation, and grafted cells were found to transdifferentiate into astrocytes (Boucherie et al., 2009). In contrast to this, when hMSCs from patients affected by ALS were transplanted in the cisterna magna into SOD1 mice, a dose-dependent efficacy was reported (Kim et al., 2010). A study on the effects of i.c.v. administration of hMSCs gave contrasting results in SOD1 mice, such as prolonged lifespan, but only in females, not significant increase in surviving MNs and no effects on neuroinflammation (Morita et al., 2008). Combined intraparenchymal and i.v. engraftment of MSCs increased the lifespan and improved motor function in SOD1 rats (Forostyak et al., 2011).

Sadan and colleagues recently developed a two-step medium-based differentiation protocol for inducing MSCs into neurotrophic factor secreting cells (NTF+). The induced cells produce and release high amounts of NTFs, such as BDNF and GDNF (Sadan et al., 2008; Dadon-Nachum et al., 2011) which protect cultured MNs. Also MSC-NTF derived xeno-free growth media, a technology licensed to Brainstorm, markedly increase the expansion potential of MSCs (Chaddah et al., 2011). Transplantation studies in mice and rats thus far have also proved successful. The mouse MSC-NTFs transplanted into SOD1 (G93A) mice improved motor functions and survival. The i.m. transplantation of rat MSC-NTFs into a rat model for sciatic motor nerve injury preserves motor function, protects neuromuscular junctions and accelerates regeneration (Dadon-Nachum et al., 2011).

Wild type, and not SOD1, murine MSCs can be expanded in culture and exposed to growth factors which increase transcription and expression of the high-affinity glutamate transporter GLT-1 and make the cells responsive to riluzole triggering an up-regulation of the GDNF production (Boucherie et al., 2008).

As reported, MSCs can be used as Trojan horses to deliver trophic factor into the diseased spinal cord, as shown by injecting i.m. MSCs transduced with a lentivirus encoding GDNF to express GDNF at high levels (see below) (Suzuki et al., 2008). This protocol is effective in asymptomatic animals, and greater effect is observed in animals with slow progression ALS.

Their biological properties independent of differentiation suggest that MSCs could have a therapeutic role in ALS through mechanisms other than tissue replacement.

Translation into the clinic

Many promising results obtained in animal models have been lost in translation to the clinic. This might happen for stem cell therapy as well. In fact, transposition of data obtained in animal models to patients has some limitations. First of all, ALS lacks animal models that closely mimic human disease. Recently, a large animal model with human like physiology, size and genetic lesions that gives an ALS type phenotype has been proposed. The transgenic pig is potentially a good model, but rejection of stem cells and the need for immunosuppression are major drawbacks, and for efficacy studies animals would need to be monitored for a long duration. A canine model displaying degenerative myelopathy, an adult onset neurodegenerative disease caused by a mutation in SOD1, shares many features with human ALS (Awano et al., 2009). This model could be extremely valuable for testing cell therapy strategies.

Animal models may be predictive, but often are not sufficiently reliable (Scott et al., 2008). First of all, animal models usually are conducted in a very homogeneous population, where most, if not all, cases become symptomatic and die at the same time. This is not the case for human patients. Also, humans quite often are affected by other diseases, which add to ALS. Moreover, many studies have been conducted in pre-symptomatic animals, which enhances the chances to change the disease course; nevertheless, in the human setting no pre-symptomatic diagnostic tests are available and experimental therapies can be employed only in already symptomatic patients. Finally, the most common animal model of ALS, the SOD1G93A mouse, is a quite unstable model, since the onset and the progression of the disease strongly depend on the number of the copies of the human gene, and also on the levels of the gene expression; therefore, the researcher must continuously check his colony, and also use a battery of behavioural tests to detect the exact time of onset of the disease in the individual mouse, in order to start treatment (Vercelli et al., 2010).

Allogenic vs autologous MSCs for transplantation

Bone marrow MSCs are, to date, the most common source of stem cell in the treatment of haematopoietic diseases and thus the protocols for their isolation and application are well established. MSCs may be collected from the patients themselves, thus allowing autologous transplantation, which may obviate the need for immunosuppression and also may facilitate the authorization of clinical studies. However, autologous cells might be more vulnerable to the disease and the current extensive requirements for cell manufacture and testing may render such approaches very expensive.

Ferrero et al. (2008) analyzed expanded MSCs from sporadic ALS patients and healthy donors, which did not show any evident difference in immunophenotypic and functional characteristics. However, the expansion potential varied in correlation with the age of patients. It is unclear whether these data may significantly affect the efficacy and the outcome of the treatment. Some recent studies (Koh et al., 2012), however, show that a reduction in the pluripotency and capacity to secrete trophic factors of the BM-MSCs of ALS patients correlates with poorer prognosis.

Healthy allogeneic BM-MSCs might be also considered for cell therapy in ALS patients. Mareschi et al. (2009) demonstrated that MSCs collected from amniotic fluid (AF-MSCs) have more advantageous immunophenotypic and functional characteristics compared to the BM-derived MSCs; hence they could be proposed in clinical use.

Characterization and manufacture of cell product for transplantation

The very low density of MSCs in BM requires isolation and expansion steps before their clinical use. MSCs can easily be isolated from BM thanks to their capacity to adhere, proliferate and expand in culture while maintaining their immunophenotypic characteristics and functions as multipotent cells. The International Society for Cellular Therapy proposed three minimal criteria to identify MSCs: the adherence to plastic, the specific surface antigen expression (positivity for CD105, CD73, CD90 and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II), and the multipotent capacity to differentiate into osteoblasts, adipocytes and chondroblasts under standard conditions for *in vitro* differentiation (Dominici et al., 2006). MSCs also express variable levels of CD44, stromal antigen-1, and a group of other adhesion molecules and receptors including CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule), and CD49 (very late antigen). The variability in the expression of adult MSC surface markers could be related to the different stages of culture (Mafi et al., 2011).

Many other variables such as culture media and additives, plating density, and passaging may influence MSC culture expansion. The use of MSCs as cell therapy products requires alternative expansion methods, avoiding the use of reagent of animal origin such as fetal bovine serum (FBS). The human platelet lysate (HPL) represents a valuable and promising alternative to FBS (Bernardo et al., 2007). HPL-cultured MSCs have comparable immunomodulatory capacities to their FCS-cultured counterparts making these cells an attractive cell therapeutic tool (Flemming et al., 2011). In addition to culture media and supplements, some authors suggested that low seeding densities result in faster proliferation and a larger fraction of multipotent adult stem cells (MAPCs) (Jiang et al., 2002), while others indicated that higher densities might even be

more effective for expansion of MSCs (Neuhuber et al., 2008). In accordance with other authors' data, Mareschi et al. (2012) confirmed that a low plating density results in higher yields and a faster expansion of MSCs, and may represent a good procedure for clinical use.

MSCs are considered to be advanced therapy medicinal products (ATMPs), as defined by the European Regulation N. 1394/2007 of the European Parliament on advanced therapy medicinal products, amending the 2001/83/EC Directive, that completes the regulatory setting on advanced therapies to be used in Member States. The quality and safety of ATMPs such as MSCs must be maintained throughout their production, ensuring their final use in the patient. MSCs are considered somatic-cell therapy products or tissue-engineered products depending on the source, manufacturing process and proposed indications. The regulation N. 1394/2007 refers to the European GMP rules, and is also in compliance with the 2004/23/EC Directive on donation, obtaining and testing of human cells and tissues. GMP ensures that products are consistently produced and controlled to the quality standards required to their intended use, from collection and manipulation of raw materials to the processing of intermediate products, the quality controls, the storage, labelling and packaging, and release.

The translation of research-based protocols into GMP-compliant procedures for large-scale production of clinical-grade MSCs requires careful analysis of all the risks and benefits to identify and control all critical aspects: source of MSCs and raw materials, culture media, supplements and disposable devices, quality control tests (Sensebé et al., 2011). Facilities, isolation methods, seeding density, growth factors and chemicals, can all influence the expansion potential and functional properties of MSCs, and should be considered throughout the production process.

The quality and safety of the cell preparations should be ensured by the implementation of a quality system that guarantees the certification and traceability of every batch of material and supply utilized for the procedures, the correct utilization and cleaning of instruments, and the locations necessary for stem cell manipulation. Furthermore, the organization structure, qualification of staff with high levels of expertise, and the appropriate equipment, must be implemented in dedicated clean-rooms, currently named "Cell Factories", in compliance with current GMP standards. The application of GMPs for aseptic production ensures the safety of the final cell therapy product.

How to get cells where they are needed?

A crucial point in transplantation is the route of delivery of the cells. A number of approaches have evolved for clinical translation of cell-based neurological therapy. Transplantation has been practised into the CNS via different routes, i.m., i.v. or i.c.v.. Also mobilization of endogenous progenitor cells has been proposed. Since ALS is a disseminated disease, the most rationale approach is to deliver cells intravenously. However, an apparent obstacle to the therapeutic plasticity of MSCs is the observation that following i.v. injection MSCs remain mostly trapped in lungs (Prockop, 2009), where they are rapidly degraded so that only small numbers enter the systemic circulation. After i.v. infusion of human MSCs into injured mice, only a small number of the infused cells (0.01%) were detected 96 hours later in peripheral organs including the brain (Lee et al., 2009). However Uccelli et al. (2012) demonstrate that i.v. injection of MSCs after disease onset in mutant SOD1 mice results in a significant delay of symptoms and death, and is associated with an improvement of several histological and biochemical parameters.

Intrathecal and intravenous transfer of autologous MSCs in patients with ALS seems safe (Karussis et al., 2010), but their therapeutic use requires an easy access to the target tissue to exert their therapeutic effect, since they respond to a particular pathological microenvironment (Liu and Martin, 2006). *In situ* administration can directly achieve this goal. The proximity of grafted cells favours the diffusion of trophic and immunomodulatory factors to MNs and surrounding glia. Most of the successful clinical outcomes with stem cells, whether neuronal or not, in ALS animal models have been achieved by direct intraspinal implantation (Garbuzova-Davis et al., 2001, 2002; Hemendinger et al., 2005; Deshpande et al., 2006; Corti et al., 2007; Lepore et al., 2008; Vercelli et al., 2008).

Although there is some evidence from preclinical studies that MSCs can firmly engraft in the CNS, robust techniques will need to be developed to track the homing of infused MSCs into the CNS (Karp et al., 2009). Many of the unsolved questions about the therapeutic effects of MSCs revolve around the question of how effectively the cells home into injured tissues. However, there have been several frustrating problems in tracking the cells *in vivo* (Prockop et al., 2010, Karp et al., 2009), such as the lack of reliable labels to identify the cells after engraftment and of definitive markers.

Intraparenchymal delivery

Local injections of stem cells, close to the anterior horn of the spinal cord, have the obvious advantage of placing the cells close to their therapeutic target. Transplantation in critical regions

of the spinal cord involved in crucial functions such as the respiratory capacity or the control of limb movements might offer the most significant clinical benefit. Respiratory failure due to phrenic motor neuron loss is the ultimate cause of death in ALS patients (Kaplan and Hollander, 1994); hence, an efficacious strategy on respiratory function could significantly modify their prognosis. Furthermore, increasing the number of transplantation sites for achieving delivery of cells to additional regions of the spinal cord may result in improved efficacy (Xu et al., 2011). A detailed protocol for multi-segmental, intraspinal transplantation of NPCs into the cervical spinal cord ventral gray matter of neurodegenerative models such as SOD1G93A mouse had been presented by Lepore (2011). Mazzini et al. (2003, 2006, 2008, 2012) have published the results of two open-label pilot study in which mesenchymal stem cells were injected with a surgical procedure into different levels of the thoracic spinal cord (T4-T5; T5-T6), of nineteen ALS patients. The cells were injected using a Hamilton syringe mounted on a table fixed arm with a micrometric system which permitted a constant flow of cellular suspension. In these studies the thoracic level was chosen i) because the risk of inducing a iatrogenic neuronal spinal cord injury is lower than at the more rostral level; and ii) because of the shorter distance between spinal cord and muscles. One mL of cell suspension was injected into the spinal cord in a pattern of three rows 3 mm apart, with no severe adverse events. 70% patients manifested non-severe events which resolved in a few weeks. No serious adverse events were seen also in the long-term (9 years follow-up) (Mazzini et al., 2012). Post-surgical MR scans revealed no pathologic intradural fluid collection, and confirmed the existence of an area of hyperintensity on T2w images at the graft site in all patients, which was probably due to the MSC suspension. In the long term, T2 relaxivity relative values measured in regions of interest showed a progressive reduction of spinal cord hyperintensity. In the long term (9 years) no evidence of new masses at the injection site or anywhere else in the neuraxis were visible in any of the MRI images of the whole follow up. There was neither syringomyelia nor pseudomeningocele in any of the patients. DTI tractography did not detect any structural changes in the corticospinal tracts (Mazzini et al., 2012).

In another clinical study, intraspinal injection of autologous bone marrow mononuclear cells at thoracic level (T3-T4) was performed in 11 patients (Blanquer et al., 2010, Blanquer Blanquer et al. 2012). The authors chose this injection site due to its low iatrogenic risk of surgery, to maintain the spinal column stability. Moreover, the intermediate intercostal nerves (approximately T3-T6) innervate the intercostal muscles with the most balanced inspiratory-expiratory function (De Troyer et al., 2005). The injection was made using a syringe attached to a conventional LP 22G needle which was installed on a specially designed microtargeting micromanipulator. 2 mL mononucleated cells were injected in 2 injection sites 1 cm apart to

each other. The MRI studies performed 7 days after surgery showed a transient extradural hematoma-seroma. In the follow-up studies no signs of tumour growth or posttraumatic syringomyelia were detected (Blanquer et al., 2010). The authors did not observe any severe transplant-related adverse events, but there were 43 non-severe events. Twenty-two (51%) resolved in ≤ 2 weeks and only hypoesthesia and constipation were still present at the end of follow-up (Blanquer et al., 2012). The necropsy samples did not show any damage due to surgery. CD90+ cells were found around the MNs in the transplanted segments of the spinal cord. This suggests that the cells grafted into the anterior horns of the spinal cord survive a long time (Blanquer et al., 2012).

In another study, bone marrow (BM)-derived hematopoietic progenitor stem cells were injected directly into the brainstem and in the upper spinal cord of 13 ALS patients with severe bulbar involvement. 0.1 mL stem cells were injected using a 21-gauge needle attached to a 1-mL syringe into multiple different areas in the spinal cord. No severe adverse events and some benefits in 9 patients are reported (Deda et al., 2009).

Improvement of the surgical procedures with evidence of technology capable of a safe, targeted, localized administration in humans has been provided by Riley and colleagues (Riley et al., 2011). This neurosurgical team in Atlanta developed a spinal cord stabilizer and an associated injection device. The device is anchored to the patient's vertebrae, and the stem cell preparation is injected into the spinal cord using a controlled pump to maintain cell viability and reproducible delivery rates. Accurate targeting to the ventral horn is determined for each patient by use of MRI. The system had been validated in a large animal, the minipig (Riley et al., 2011) and the FDA approved a clinical trial of Human Spinal Stem Cells injections into the spinal cord for ALS treatment. The study is currently underway. Twelve patients received either 5 unilateral or 5 bilateral (10 total) injections into the lumbar spinal cord at a dose of 100,000 cells/injection. All patients tolerated the treatment without any long-term complications related to either the surgical procedure or the implantation of stem cells (Glass et al., 2012). Next patients will be transplanted in the cervical spinal cord.

The results of these phase 1 clinical trials suggest that the delivery of cellular therapies to the spinal cord of ALS patients with a surgical approach can be proposed without significant adverse events at the thoracic and lumbar levels. Although Deda et al. (2009) report no adverse events with stem cells injection directly into the brain stem and upper spinal cord, transplantation in the cervical spinal cord need further phase 1 studies to ascertain the safety and feasibility.

Another critical point is the volume of cell suspension that could be injected into the living human spinal cord without adverse effects (Guest et al., 2011). From the analysis of these pilot studies we can conclude that at least up to 2 ml in 2 injection sites can be injected.

Intramuscular grafting

Another interesting approach consists in intramuscular grafting of MSCs expressing growth factors. In the SOD1G93A transgenic mice model the intramuscular transplantation with hMSCs engineered to secrete glial cell line–derived neurotrophic factor (hMSC-GDNF) resulted in muscle hyperinnervation by MNs and significant increase of the number of neuromuscular connections and motor neuron cell bodies in the spinal cord. Moreover, it delays disease onset, improves locomotor performance, and increases their lifespan (Suzuki et al., 2007).

Brainstorm recently gained approval for a phase I clinical trial in ALS patients using a new technology. The strategy will be to perform injections of MSC-NTF cells intramuscularly in early stage patients and intrathecally (via a standard lumbar puncture) in progressive ALS subjects (www.clinicaltrial.gov).

MSCs as immunomodulatory agents: intravenous and intrathecal delivery

It should be emphasized that many reports support a ‘touch and go’ mechanism for the therapeutic effects of MSCs that does not require long-term engraftment into the CNS or other tissues (Uccelli et al., 2008). MSCs have been shown to possess immunomodulating properties (Uccelli et al., 2008). They inhibit T cell proliferation both *in vitro* (Di Nicola et al., 2002) and *in vivo* (Bartholomew et al., 2002). In experimental autoimmune encephalomyelitis, i.v. MSCs ameliorate clinical course and decrease demyelination, immune infiltrates, and axonal loss (Zappia et al., 2005). Surprisingly, these effects do not require full CNS engraftment by MSCs, but rely on the property of MSCs to inhibit pathogenic immune responses and release neuroprotective and pro-oligodendrogenic molecules favouring tissue repair. Thus, it is reasonable that the main immunomodulatory activity of MSCs is exerted in the secondary lymphoid organs where MSCs migrate following i.v. administration and inhibit T cells homing in the CNS (Zappia et al., 2005; Kassis et al., 2008); systemic effects are observed after i.v. infusion of as little as 1 million MSCs that are largely trapped in the afferent vessels of the lung and apparently degraded there (Lee et al., 2009).

An immediate immunomodulatory effect induced by i.v. administration of MSCs has been shown in 5 ALS patients by Karussis et al. (2010). These included an increase in CD4(+)CD25(+) regulatory cells and a reduction in the proportion of activated dendritic cells and lymphocytes and of lymphocyte proliferation. The same results were obtained in a group of patients affected by Multiple Sclerosis. In this phase 1/2 clinical trial, the patients received both an intrathecal and an i.v. injection of autologous MSCs. The 6 to 25 months of follow-up did not reveal any significant immediate or late adverse effects and the authors concluded that the procedure is feasible and safe. Moreover the author conclude with a possible clinical benefit in terms of stabilization of the disease. However the small sample size and the variability of the disease does not allow to draw any conclusions on the efficacy.

How many cells need to be injected?

Future studies aimed at clinical translation should address the question of the number of cells to be transplanted in order to calculate a therapeutic and also a maximal tolerated “dose” of cells before toxicity becomes a limiting factor. Which is the suitable MSC dose in humans? One can hardly infer the cell dose from experimentation in animal models. One should aim to implant the largest possible number of viable therapeutic substrates (cells), so that the greatest local beneficial effect can be achieved. Also, a single dose might miss adverse events that might emerge in later trials or large effect. Mazzini et al. (2010,2012) found no correlation between the number of transplanted cells and the incidence and severity of the side effects or the outcome.

Another controversy in stem cell therapy concerns cell aging, given that there is little evidence regarding the lifespan of transplanted cells. In pre-clinical experiments (Vercelli et al., 2008) hMSCs transplanted into the lumbar spinal cord survive for long periods (more than 10 weeks), without immunosuppression (in agreement with Liu and Martin, 2006). Allotransplantation seems to improve MSC survival even in the absence of immunosuppression (Xu et al., 2009). However, in the case of MSC transplantation, that issue is not as critical because the delivery of trophic factors and the immunomodulatory activity are the main outcome, independently of the longevity of the cells. Moreover the capacity of MSCs to trigger proliferation, migration, and differentiation of endogenous NPs could explain some of the effects recorded in diseases in which few cells engrafted (Munoz et al., 2005).

Karussis et al. (2010) provided some evidence of immunomodulatory effects of MSCs within 24 hours of intrathecal injection but claims of iron oxide-labeled MSCs persisting after three to six months was less persuasive. Blanquer et al (2012) demonstrated by histopathological

analysis that autologous bone marrow mononuclear cells (BMNC) grafted into the anterior horns of ALS spinal cord, survive a long time as perineuronal nets.

Clinical trial design

One of the important hurdles in clinical study design for cell therapy trials is defining endpoints, as this is the measure of the trial's failure or success. This is particularly challenging given the degenerative nature of ALS and the complexity posed by the rate of progression and lack of validated surrogate markers of disease.

In 2008, the International Society for Stem Cell Research released a set of recommended guidelines for the development of stem cell-based treatments (Hyun et al., 2008). These recommendations include the use of experts in stem cell biology for peer review of research ranging from preclinical to clinical, emphasizing risks involved with stem cell-based therapies within the voluntary informed consent, new oversight criteria for medical innovative care that falls outside of the realm of a clinical trial, and the equality of benefits of stem cell treatments.

Clinical trial designs need to be debated focusing on critical questions. What do we expect the stem cells to do, and what outcomes are predicted? How do we anticipate patients early and later in the disease course will respond to treatments?

The clinical protocol of the studies in humans using stem cells should be carefully designed so as to minimize unexpected patient-related factors that may have a negative impact on post-transplantation outcome.

The successful administration of stem cells will critically depend on their transplantation at the optimal stage of the disease course. Other characteristics should be carefully considered including age at the time of the procedure, disease duration, disease severity. There is a tendency to enrol, in phase I trials with cell therapy, patients in the advanced phase of disease, in absence of any other viable options, because they may be more motivated and have a more acceptable risk/ benefit profile than patients early in the disease course. (Table 1). However, the late stages of ALS are associated with significant MN damage that might create an inhospitable environment for cell therapy. Moreover patients in the late stages of disease are more susceptible to surgical complications due to disease co-morbidities. Considering the possible negative influence of aging on the spinal cord microenvironment, the survival and trophic activity of transplanted stem cells might be affected. Hence we can speculate that younger patients might benefit most from stem cell transplantation. Furthermore in the case of MSCs the

outcome can depend on the route of administration.. The course of ALS and the clinical characteristics are extremely variable among patients; hence, emphasis should be placed on patient selection and stringent inclusion and exclusion criteria should be established, based on lessons from the pharmacological clinical trials.

The profile of the ideal candidates should be separately defined for phase I to phase III studies, as criteria may change from one study type to another. Stem cell translational research by its nature entails a high degree of risk: the risk/benefit ratio of stem cells trials clearly seems to require that a phase I study is a mixed design examining safety and efficacy simultaneously. Moreover, in the field of cell therapy, contrary to drug phase I trials, pilot trials cannot be performed on normal, volunteers. This means that the team members should take serious measures to collect data regarding several kinds of side effects and data for general, unexpected adverse events have to be properly acquired in an effective manner, using, e.g., the World Health Organization (WHO) severity scale.

At present, there is no clear consensus on how this studies should be performed in ALS (Table 1)

A “risk escalation” paradigm has been adopted for the recruitment of patients in the ongoing Neuralstem phase I trial (Lunn, 2011). Under this paradigm, risk to patients receiving human spinal cord stem cell transplants escalates across the different cohorts (designated A–E, with cohort A being the lowest-risk and cohort E being the highest-risk group) according to disease severity and the number and placement of injections.

Intrathecal and intravenous transfer of autologous MSCs in patients with ALS was tested by Karussis et al. (2010) in a small phase 1/2 pilot study. No patients manifested severe adverse events.

Cell transplants may survive for several years in patients, or their effects may be irreversible. Therefore, stem cell therapy requires careful patient monitoring and extended follow-up. Long-term follow-up must consider the possibility of the development of a tumor, cyst or syrinx at the site of transplantation. Advanced MRI, in particular diffusion tensor imaging (DTI), represents an important monitoring mean because it allows a satisfactory quantification of the iatrogenic damage. Mazzini et al.(2012) produced the first report of a very long term follow up (up to 9 years) of intraparenchymal transplantation of MSCs into the human CNS. The most relevant finding of this study consists in the complete absence of tumor formations or abnormal cell growth in neuroradiological scans. Assessment of the integrity and survival of the grafted cells at autopsy would be fundamental, but consent represents an ongoing challenge, since it depends on legal and cultural aspects which vary in different countries (Hyun et al., 2008).

Both studies with intraparenchymal (Mazzini et al., 2012) and systemic (Karussis et al., 2010) MSC delivery have shown a slight trend toward a delay in the decline of the performance in the functional rating scales. However, in the light of the small sample sizes, heterogeneity of disease severity within the cohorts and lack of adequate control groups in these pilot studies, the results must be interpreted with caution. Even though some indication about therapeutic efficacy could be derived from phase I trials, this is not a prerequisite for the onset of phase II studies, which specifically aim to evaluate efficacy.

Some open questions include the number of patients needed for phase II trials, how to quantify a response over a short time frame. Clinical trial designs need to be debated owing to the importance of ethical challenges, including sham control groups to assess the efficacy of invasive therapy. Ideally a transplanted group should be compared with one undergoing the same surgery but receiving the vehicle. Such a study is unlikely to be approved by institutional review boards in most European Countries. Although a randomized and blinded trial design is always preferable and should be undertaken wherever possible, an alternative approach is to carefully document the natural history of the disease and compare it with the outcome in transplanted patients in an open-label clinical trial. This trial design has been adopted in most pilot clinical trials conducted to date (Mazzini et al., 2012; Blanquer Blanquer, 2012; Glass et al., 2012).

The chances of confirming or convincingly denying the presence of a beneficial effect of treatment are increased by studying patient populations with comparable disease severity; however, they may fail to identify susceptibility to adverse effects or alternatively to detect benefit in particular patient subpopulations not represented in the initial studies. Thus, the presence of some variation in participants in Phase I–II stem cells trials may be appropriate.

The focus of phase II studies should include quantifiable clinical outcomes that result in a benefit for the patient. Examples of primary objectives for phase II studies include assessment of response rate (for example, defined as improvement in neurologic function), time to disease progression and overall survival. Unfortunately, the applicability and value of such endpoints may only be evident after an expensive and time-consuming trial is completed, with a long follow-up.

The cost-benefit analysis of the results must take into account as a major endpoint the quality of life of the patients and ethical issues should be addressed properly. Stable psychological status and social situation should be considered as a prerequisite in the choice of participants. Given the intense public interest and controversy surrounding stem cell ‘cures’, it is essential to ensure that the participants are able to fully comprehend the potential benefits or the lack of benefit

and risks associated with the procedure, and that the family and social supports are able to assist them.

Conclusions

MSC research and application is opening great opportunities in ALS treatment. Many trials of MSC treatment for ALS have been recently announced or started. This survey shows that preclinical and initial clinical data support the therapeutic potential of MSCs for ALS. But, while safety seems to be reliably demonstrated, particularly with autologous transplants, sustained curative benefit has not been consistently obtained. The scientific community and patients urgently need safety and efficacy to be addressed properly in the framework of rigorous controlled clinical trials. The distinction between stem cell trials approved by academic research ethic boards and the commercial delivery of stem cells is crucial for patients. Because MSCs are easily isolated and expanded and their safety consistently demonstrated, hundreds of patients with severe incurable neurological diseases such as ALS have been treated in uncontrolled conditions, encouraging so-called medical tourism. Numerous clinics around the world are exploiting patients' hopes by offering expensive, new stem cell therapies for ALS patients, which are claimed to be effective, but without credible scientific rationale, transparency, oversight, or patient protection. Recruitment and selection of appropriate patients for larger trials will be a challenge and will require national and/or international multi-center collaboration with multidisciplinary groups.

Nevertheless, encouraged by current progress, and especially by the terrific strides being made in preclinical studies, we envision a much more concerted effort toward translation that would make the process more accessible, integrated into academic and industry settings, and efficient, therefore improving the chance that the health benefits of research reach patients. Translation, by which we mean advancing scientific discoveries from the laboratory into practical applications for patient benefit, i.e., "bench to bedside," requires a comprehensive collaborative team approach: research scientists and clinicians must work closely with regulatory agencies, patient advocacy groups, ethic bodies, cell manufacturing facilities, and industry to achieve the quality of studies and necessary funding to ensure success (Aboody et al., 2011).

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Figure Legends

Figure 1. Effects of intraparenchymal transplantation of hMSCs in SOD1G93A mice (Vercelli et al., 2008). (a) Injection site in the lumbar spinal cord showing Bisbenzimidazole-stained hMSCs, after intraparenchymal transplantation in an ALS murine model. (b) At higher magnification, blue hMSCs (arrows) localized close to motor neurons (labeled in green by the neuronal antibody, anti-MAP2). (c-d) The number of motor neurons (arrowheads) in the lumbar spinal cord is significantly reduced in control mice (c) compared with transplanted ones (d). (e-f) Similarly, astrogliosis (anti-GFAP-immunostaining; arrowheads) results modulated in the grafted mice (f) in comparison with untreated ones (e). Scale bars: 200 μm in a, c-d; 20 μm in b; 50 μm in e-f.

Table 1. Clinical trials with adult stem cells in ALS patients

Stem cells	Study phase	Regulatory Oversight	Route of delivery	Immuno-suppression	Number of cells	Number of patients	Inclusion criteria	Outcome	Reference
Autologous BM- MSCs	I	Regional and Local Ethic Committees	Intraparenchymal transplantation (T4-T6)	no	Mean: 57×10 ⁶ Range: 7.0 – 152 ×10 ⁶	9	Age: 23-75 FVC>50% Severe Functional impairment Duration of the disease:8-80 mos	Safe and well-tolerated even in long-term (9 yrs)	Mazzini et al., 2003, 2006, 2008, 2012
Autologous BM - MSCs	I	National Institute of Health Regional and Local Ethic Committees	Intraparenchymal transplantation (T7-T9)	no	Median: 75×10 ⁶ range: 11–120 ×10 ⁶	10	Age: 20-61 Spinal onset Duration of the disease: < 3 yrs FVC> 50%	No serious transplant-related adverse events. MRI showed no structural changes (including tumour formation)	Mazzini et al., 2010, 2012
Fetal olfactory ensheathing cells	Controlled pilot study	in accordance with guidelines issued by the Chinese Ministry of Health	Bilateral corona radiata	no	2 ×10 ⁶	15	Age: 20-70	Safe The mean ALSFRS score remained stable in the first 4 mos	Huang et al., 2008
Autologous HCSs	Single-center pilot trial	Institutional review board	Intravenous infusion following total body irradiation; immuno-suppression	Tacrolimus methotrexate	=	6	Age: 35-69 FVC>60% Duration of the disease: 5-30 mos	Tolerated. No clinical benefits. HSCTs enter the human CNS at sites of motor neuron and engraft as immunomodulatory cells.	Appel et al., 2008
Autologous blood purified CD133(+)	Single-center pilot trial	Ethic and Research committees of the hospital.	Frontal motor cortex	no	2.5 - 7.5 ×10 ⁵	10	Age 38-62 Duration of the disease: 18-42 mos	Safe and well-tolerated (1 year follow-up) Patients survival significantly higher than control group. ALS-FRS improvement	Martinez et al., 2009
Autologous Bone marrow (BM)-derived hematopoietic progenitors	Single-center pilot trial	Regional Ethics board Ministry of Health Institutional Review Board	Intraparenchymal transplantation (C3-C4level) CSF IV	no	4 ×10 ⁶ 15 ×10 ⁶ 5 ×10 ⁶	13	Age 34-71 Duration of the disease: 5yrs Moderate-severe functional impairment	No complications Some degree of decline 1 year after transplantation	Deda et al., 2009
Autologous Bone marrow (BM)-derived hematopoietic progenitors	Open single arm phase I trial	Clinical Trials Ethics Committee of the University. Agencia Española de Medicamentos y Productos Sanitarios.	Intraparenchymal Transplantation (T3-T4)	no	462 ×10 ⁶ range 138 - 603×10 ⁶	11	Age:33-61 FVC>50% Spinal onset	Safe and well tolerated (2yrs follow-up) Histopathological exams showed a greater number of motoneurons in the treated segments and no degenerative ubiquitin deposits.	Blanquer Blanquer et al., 2012
Autologous BM- MSCS	Phase 1/2 open-safety clinical trial	Ethics committees of the hospitals. Registered in the National Institutes of Health database	Intrathecal and intravenously	no	54.710 ⁶ CSF 24.510 ⁶ iv	19	Age 25-65	Feasible and safe . Immediate immunomodulatory effects. The mean ALSFRS score remained stable during the first 6 mos	Karussis et al., 2010
Human spinal cord-derived stem cells (HSSC)	Phase I trial	University Institutional Review Board	Intraparenchymal Transplantation (lumbar spinal cord)	basiliximab prednisolone tacrolimus mycophenolate	5-10 injections (100,000 cells/ injection)	12	Age >18 yrs ALSFRS-R lower extremity subscore < 1 (Group A) and >2 (Groups B,C) FVC > 60%	Safe and well tolerated. One patient has shown improvement in his clinical status	Glass et al., 2012

Abbreviations: BM, Bone Marrow; HCSs., Hematopoietic Stem; HSSCs, Human spinal cord-derived stem cells. FVC, Forced Vital Capacity (Percentage of predicted); MSCs, Mesenchymal Stem Cells; PBSCs, Peripheral Blood Stem Cells;