Autologous mesenchymal stem cells: clinical applications in amyotrophic lateral sclerosis

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Objectives: *Our study was aimed to evaluate the feasibility and safety of intraspinal cord implantation of autologous mesenchymal stem cells (MSCs) in a few well-monitored amyotrophic lateral sclerosis (ALS) patients.*
Methods: *Seven patients affected by definite ALS were enrolled in the study and two patients were treated for compassionate use and monitored for at least 3 years. Bone marrow was collected from the posterior iliac crest according to the standard procedure and MSCs were expanded* ex vivo *according to Pittenger's protocol. The cells were suspended in 2 ml autologous cerebrospinal fluid and transplanted into the spinal cord by a micrometric pump injector.*
Results: *The* in vitro *expanded MSCs did not show any bacterial o fungal contamination, hemopoietic cell contamination, chromosomic alterations and early cellular senescence. No patient manifested major adverse events such as respiratory failure or death. Minor adverse events were intercostal pain irradiation and leg sensory dysesthesia, both reversible after a mean period of 6 weeks. No modification of the spinal cord volume or other signs of abnormal cell proliferation were observed. A significant slowing down of the linear decline of the forced vital capacity was evident in four patients 36 months after MSCs transplantation.*
Conclusions: *Our results demonstrate that direct injection of autologous expanded MSCs into the spinal cord of ALS patients is safe, with no significant acute or late toxicity, and well tolerated. The clinical results seem to be encouraging.* [Neurol Res 2006; **28**: 523–526]

Keywords: Amyotrophic lateral sclerosis; in vitro *expansion; mesenchymal stem cells; safety; transplantation*

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapid and progressive neurodegenerative disease that targets motor neurons in spinal cord, cortex and brain stem. The selective degeneration of motor neurons manifests as a linear decline in muscular function eventually resulting in paralysis, speech deficits and dysphagia. Within 2 to 5 years of clinical onset death, owing to respiratory failure, it occurs. There are no therapies available today.

Stem cells therapy holds potential for treating ALS by different mechanisms.Great interest is focused on inflammatory processes and microglia activation in the pathogenesis of ALS. Cells that surround motor neurons and are not nerve cells can play a major role in advancing or limiting the disease[1]. Recent research in superoxide dismutase (SOD1) mice has shown that healthy astrocyte and microglia can maintain the health of neighboring diseases motoneurons and greatly extend survival[2,3]. Neurodegeneration itself seems to promote proliferation, migration and transdifferentiation of autologous stem cells[4]. Production of neurotrophic and growth factors and stimulation of the regenerative processes by stem cells have been demonstrated in neurodegenerative diseases[5]. Moreover, stem cells might become eventually carriers of pharmacological treatments. Rats with the mutation responsible for some forms of inherited ALS that have received stem cells engineered to express a supportive factor called glial derived neurotrophic factor (GDNF) did produce encouraging evidence that stem cell implants can live, make connections and secrete GDNF as instructed[6].

Mesenchymal stem cells (MSCs) from bone marrow (BM) are pluripotent cells that contribute to regeneration of several tissues including the nervous system[7] through transdifferentiation and cell fusion with the damaged neurons[8]. MSCs have displayed unorthodox plasticity in

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their ability to trans-differentiate into non-mesenchymal lineages including astrocytes[9] and myelinating cells of the peripheral nervous system[10] and spinal cord[11]. *In vitro*, MSCs differentiate into cells expressing neuronal cell markers when exposed to mitogens such as brain-derived neurotrophic factor and nerve growth factor neurotrophins and retinoic acid, demethylated agents, physiologic neural inducers, antioxidants and compounds which increase intracellular cyclic adenosine monophosphate (AMP)[12,13]. MSCs have been transplanted in different animal models of central nervous systems diseases with evidences of their capability to survive, proliferate and migrate into the damaged tissue with positive functional effects[14]. Recently, it has been demonstrated that BM stromal cells reduce cell death and apoptosis and increase the DNA proliferation rate in astrocytes post-ischemia[15]. Moreover, they can be used as vectors of cytokines and trophic factors preventing cell death, tissue inflammation and damage[16].

All these evidences highlight the potential use for therapeutic strategies of MSCs in ALS.

This study was aimed to evaluate the feasibility and safety of intraspinal cord implantation of autologous MSCs in a few well-monitored ALS patients.

PATIENTS AND METHODS

The study was approved by the Ethical Committee of the Piedmont Region. Seven patients (four females and three males) were consecutively enrolled from October 2001 to May 2002. Two patients (one male and one female) were treated for compassionate use after approval by the local ethic committee. All patients gave their informed consent. Patients were included if they had definite ALS with spinal onset without signs of respiratory failure. *Table 1* reports the main clinical features of patients at entry. Standard therapies were used throughout the study. The patients, in order to estimate disease progression rate before transplantation, had a 6 month period of natural history observation. They were monitored every 3 months by clinical evaluation which included ALS-functional rating scale (FRS), Norris score, bulbar score and Medical Research Council (MRC) strength scale. Respiratory assessment included pulmonary function tests and nocturnal cardio-respiratory monitoring. Neurophysiologic monitoring included electromyography (EMG) and somatosensory evoked potentials. The neuroradiological assessment consisted of magnetic resonance imaging (MRI) of brain and spinal cord before and after gadolinium diethylenetriaminepentaacetic acid (DTPA) infusion. A clinical psychologist assessed quality of life by clinical interview and psychologic tests. After MSC implantation, the patients were monitored for at least 36 months by the same assessment.

Table 1: Clinical characteristics of patients at entry

Patient	Age	Sex	Norris score	Bulbar score	ALS-FRS score	FVC (%)
1	75	F	70	7	25	85
2	64	M	75	7	34	100
3	46	F	75	7	33	108
4	32	M	44	6	22	81
5	48	F	65	7	30	82
6	34	M	28	3	13	25
7	23	F	39	7	16	50
8*	49	M	67	6	32	100
9*	46	F	62	7	26	53

*Compassionate use; FVC=forced vital capacity; F=female; M=male.

Experimental procedures

BM was collected in epidural anesthesia according to the standard procedure. BM cells were layered on a Percoll gradient (density: 1.073 g/ml) and centrifuged at 1100 g for 30 minutes as previously reported[17]. Mononuclear cells at the interface were recovered, washed twice with phosphate buffer saline (PBS) (1X) (Cambrex, Walkersville, MD, USA) at 200 g for 10 minutes, seeded at a density of 800,000/cm^2 in MSC medium (Cambrex) in 150 cm^2 T-flasks and maintained at 37°C with an atmosphere of 5% CO_2. After 3 days, non-adherent cells were removed and the adherent cells were re-fed every 3–4 days. In order to expand the isolated cells, the adherent monolayer was detached with trypsin/ethylene diamine tetraacetic acid (EDTA) (Cambrex) for 5 minutes at 37°C, after 15 days for the first passage and every 7 days for the successive passages. The cells were seeded at a density of 8000/cm^2 and expanded for the passages at the latest. At each passage and before implantation MSCs were analysed for viability, sterility, mycoplasma detection and cytogenetic and telomeric analysis according to the guidelines of the Italian Institute of Health and showed by Mareschi *et al.*[18]. Moreover, the surface antigens, CD45, CD14 (Becton Dickinson, San Jose, CA, USA), CD90, CD106, CD29, CD44, CD105 and CD166 (Caltag Laboratories, Burlingame, CA, USA) were analysed on an Epics XL cytometer (Beckman Coulter, CA, USA) with the XL2 software program.

Before implantation the cells were maintained for at least 3 hours in basal MSC medium (Cambrex) without serum, detached and washed there times with PBS (1X) containing 1% human albumin and one time with autologous cerebrospinal fluid. The cells were suspended in ~1 ml autologous cerebrospinal fluid and directly transplanted into the surgically-exposed spinal cord at different thoracic levels. A laminectomy was performed and the dura was opened along the median line under microscopic vision. After a median mielotomy, the cells were injected in the most central part of the spinal cord by means of the Hamilton syringe previously mounted in an injection system with a micrometric pump injector supported by a table-fixed arm. At the end of the procedure, the dura was closed in a tight-water faction. The procedure was performed in general anesthesia using short-acting drugs.

The choice to transplant MSCs directly into the spinal cord was made given the impediment of stem cells to cross the blood–brain barrier which is intact in ALS.

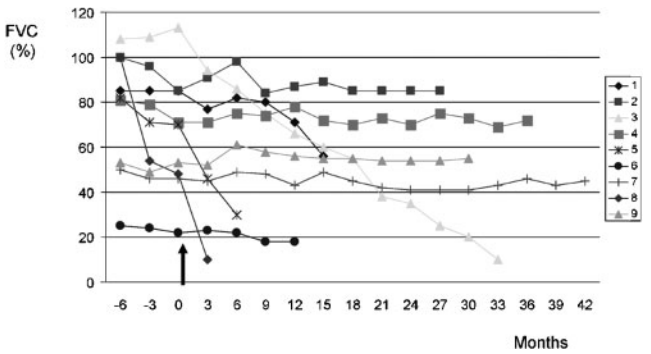

Figure 1: Clinical evaluation of patients before and after transplantation. Changes of the FVC in the 6 month period of natural history observation and after transplantation. The arrow indicates the time of MSCs transplantation

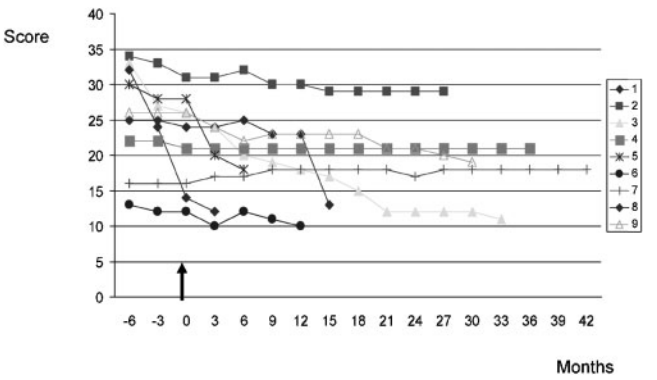

Figure 2: Clinical evaluation of patients before and after transplantation. Changes of the ALS-FRS in the 6 month period of natural history observation and after transplantation. The arrow indicates the time of MSCs transplantation

RESULTS

BM collection

No side effects (including pain at the posterior iliac crest lasting for 48 hours and infection episodes) were recorded after BM collection.

The median BM collection (without anticoagulant agents) was 607 ml (range: 238–732ml) and leucocyte collection was 104.4 $\times$ 10^8 (range: 33.3–144.5 $\times$ 10^8). After Percoll separation, there was a median of 11.32 $\times$ 10^8 (range: 4.31–26.79 $\times$ 10^8) counted and seeded cells.

Isolation and implantation of MSCs

The cells used for the implantation were at the second or third passage after a median of 32 culture days (range: 27–34 days). Each harvest revealed a homogenous population of cells, positive for CD29, CD44, CD105, CD166 and CD90 antigens (>90% of cells) by flow cytometry and negative for hemopoietic antigens CD45 or CD14. All bacteriologic tests performed on the cells at each passage were negative. Detached MSCs showed greater than 95% cell viability before implantation. Cytogenetic analysis did not show any karyotype alterations. No significant shortening related to a cellular senescence was evidenced by the telomere length analysis.

A median of 32 $\times$ 10^6 cells (range: 7.0–152 $\times$ 10^6) was implanted. *Table 2* shows the number of MSCs obtained at each passage in relation to the patient's age.

There were no anesthetic complications. No patients manifested severe adverse events defined as respiratory failure, death and neurological symptoms which persisted for more than 6 weeks after MSC implantation. Minor adverse events were intercostal pain irradiation (four patients) and leg sensory dysesthesia (six patients). No patients manifested bladder and/or bowel dysfunction or leg motor deficit. Serial MRI showed no evidence of structural changes of the spinal cord or signs of abnormal cell proliferation when compared with the baseline also in the long term (3 years after surgery).

Two patients died from the progression of the disease 9 months and 2 years respectively after MSCs transplantation, one patient underwent tracheostomy for respiratory complications owing to ab ingestis pneumonia. A significant slowing down of the linear decline of the FVC (*Figure 1*) and the ALS-FRS (*Figure 2*) was observed after transplantation of MSCs in five patients.

DISCUSSION

MSCs were easily isolated from BM in ALS patients. The *in vitro* cell population was homogeneous with a fibroblastic like morphology and no hemopoietic cell contamination. No cytogenetic alteration or early cellular senescence was observed in the expanded *in vitro* MSCs used for the implantation. The results of the long-term follow-up appear to confirm that the procedures of *ex vivo* expansion of autologous MSCs and

Table 2: MSCs manipulation and growth in culture

Patient	Age	Sex	After Percoll ($\times 10^8$)	The first passage ($\times 10^6$)	The second passage ($\times 10^6$)	The third passage ($\times 10^6$)
1	75	F	13	16	7	7
2	64	M	21	40	17	24
3	46	F	10	31	10	30
4	32	M	4	15	30	40
5	48	F	13	26	53	60
6	34	M	27	34	61	150
7	23	F	14	30	91	152
8*	49	M	4	15	16	24
9*	46	F	0.8	9	23	32

*Compassionate use.

transplantation in the spinal cord of humans are safe and well tolerated by ALS patients as previously reported[19,20].

The long-term follow-up of the patients shows also a significant slowing down of the decline of the FVC and of the ALS-FRS after transplantation in ~50% of them. Given the progressive course of the disease, these changes are therefore likely to be due to the positive effect of MSC transplantation. However, given the interindividual variability of the clinical course of the disease and the small number of the treated patients, we cannot speculate about the effects of the MSC intraspinal cord implantation on the natural history of the disease and survival.

Further well-designed clinical studies are warranted to determine its activity in this fatal, incurable disease.

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