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2. 実用新案登録　なし。

3.その他　なし。

研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表
（平成 21 年度～23 年度）

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研究成果の刊行物・別刷り

Chapter 25

Cellular Replacement Therapy in Neurodegenerative Diseases Using Induced Pluripotent Stem Cells

Takayuki Kondo, Ryosuke Takahashi, and Haruhisa Inoue

Abstract Neurodegenerative disorders are characterized by progressive neuronal loss, resulting in clinical deficit. Several drugs can improve neural symptoms transiently but cannot halt progression or recover deficits. Stem cell transplantation is focused as upcoming regenerative treatment. After recent advances in embryonic stem cells and induced pluripotent stem cells, research is accelerating their application to neurodegerative disorders, including amyotrophic lateral sclerosis (ALS). We review the recent progress and present our future vision concerning cell replacement therapy for ALS, and we also emphasize the hurdles to be overcome before clinical trials can be begun. Basic research focusing on the safety of transplantation, besides therapeutic experiments, should lead to a beneficial outcome.

Keywords Cellular replacement therapy · Embryonic stem cells · Parkinson's disease · Huntington's disease · Amyotrophic lateral sclerosis · Central nervous system

Introduction

Resolve Limitations in Cellular Replacement Therapy

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), mainly attack the central nervous system (CNS) and result in neuronal loss. A condition of decreasing numbers of neurons leads to dysfunction of the neural network, resulting in disorders such as memory loss, bradykinesia, involuntary movement or limb weakness. In the past several decades, a number of drugs have been developed to compensate at least partially for these disabilities. However, none of the drugs has been able to halt disease progression or replace neuronal loss.

To overcome these limitations of conventional drug therapy, cell replacement therapies are being prepared, step-by-step, toward clinical trials. Clinical trials using stem cells have been described for Huntington's disease, Parkinson's disease, spinal cord injury, and stroke. Several clinical trials have achieved successful improvement in neurological deficits in patients. However, most of these clinical trials were based on somatic stem cells, including fetal neural tissue, nasal mucosa progenitors, or mesenchymal stem cells. Nonetheless, the resource limitation of somatic stem cells remains a major hurdle for the universal application for cell replacement therapy.

Establishment of ESC and iPSC

In 1998 human embryonic stem cells (ESC) were first generated from the inner cell mass of the mammalian blastocyst (Thomson et al., 1998). ESC can proliferate almost indefinitely and differentiate into multiple cell-types of all three germ-layers in vivo. Additionally the molecular basis of reprogramming has been revealed by the exogenous expression of combinations of transcription factors. Four factors,

H. Inoue (✉)
Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan
e-mail: haruhisa@cira.kyoto-u.ac.jp

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Oct3/4, Sox2, Klf4, and c-Myc, which are important for self-renewal of ESC, have been shown to reprogram both mouseand human somatic cells into ESC-like pluripotent cells, calledinduced pluripotent stem cells (iPSC) (Takahashi et al., 2007).

ESC and iPSC theoretically can differentiate into various cell-types of neural lineage, including dopaminergic neuron, neural crest, retina, cerebral cortex, and spinal motor neuron (Li et al., 2008). Using these differentiation methods ESC or iPSC derived neurons were applied to disease modeling (Li et al., 2008; Ebert et al., 2009; Lee et al., 2009).

Clinical Trials Using ESC-Derived Cells

The development of ESC and iPSC is attractive not only for disease mechanism research but also for cellular replacement therapy. Several clinical trials with ESC are ongoing (Table 25.1). In 2009, the Food and Drug Administration (FDA) gave the first approval for an ESC-based clinical trial, conducted by Geron Corporation. Geron planned to apply human ESC-derived oligodendrocyte progenitor cells (OPCs) to the treatment of spinal cord injury patients. In a rat model of spinal cord injury, transplantation of ESC-derived OPCs dramatically improved disability to almost a normal level (Sharp et al., 2010). The mechanism of the improvement was well assessed, demonstrating secreting nerve growth factor and remyelination. The safety of the process, especially in terms of tumorigenicity, was also evaluated. However, the FDA placed a hold on this trial because of cystic structure in animal model after transplantation. After additional safety-proving data, phase I clinical trials of ESC-derived OPCs were released in 2010 (Strauss, 2010).

Furthermore, Advanced Cell Technology, Inc. (ACT) received FDA clearance for a run of second clinical trials using ESC of age-related macular degeneration (AMD) in 2010. AMD is the most common form of macular degeneration and is intractable by existing medicine. ACT plans to transplant ESC-derived retinal pigment epithelial (RPE) cells to AMD patients and perform safety evaluation as a phase I clinical trial (Zhu et al., 2010). ACT is also approved for Stargardt's macular degeneration by transplanting ESC-derived RPE cells in 2010 November.

Regenerative Therapy for ALS

Cell replacement therapy promises to be powerful and attractive especially for CNS disorders, which are impossible to cure by conventional therapy. One of the most intractable CNS disorders is ALS.

ALS is a fatal neurodegenerative disease with late-middle age onset. ALS is clinically characterized by dysfunction of both upper and lower motor neurons, resulting in rapid progression of weakness and respiratory failure. Median survival of ALS patients without ventilators is only a few years. Numerous drugs have been attempted in clinical trials, but almost all failed to achieve even disease modification. The solely successful drug, riluzole, can only slow disease progression slightly.

The neuropathological hallmark of ALS is a massive loss of motor neurons in both the primary motor area and the anterior horn of the spinal cord. A recent study revealed that TAR DNA binding protein of 43 kDa (TDP-43) aggregates are observed not only in degenerative neurons but also in glial cells (Arai et al., 2006; Neumann et al., 2006). Unveiling the role of TDP-43 and relevant molecules would accelerate ALS research, but would still not be sufficient for a complete cure. Therefore, replacement therapy using stem cells is expected to be a potent candidate for modifying or recovering from the disease state. Here we review the recent progress and present future vision for cell replacement therapy for ALS, also emphasizing the hurdles to overcome before clinical trials can commence.

Table 25.1 Clinical trials using ESC in neurological disorders

Company	Disease	Cell type	Progress
Geron	Spinal cord injury	OPCs	Phase I 2010 October
ACT	Stargardt's macular degeneration	RPE	Phase I 2010 November
ACT	AMD	RPE	Phase I 2010 November
California stem cell	SMA type I	Motor neuron	Hold 2011 February

Transplantation Research for ALS

Before clinical trials can be initiated, basic research using animal models is necessary for evaluating their safety and efficacy. Almost all transplantation research was established using human superoxide dismutase 1 (SOD1), with G93A mutation, transgenic rats or mice. SOD1 is the most common causative gene of familial ALS, and SOD1 transgenic animals represent lower motor neuronal loss, mimicking symptoms of ALS patients (Gurney et al., 1994).

For cell resources, various kinds of stem cells are used for transplantation, e.g., rodent bone marrow cells, human mesenchymal stem cells (hMSC), neurotrophic-factor-secreting hMSC, human umbilical cord blood cells (hUCBC), neural progenitor cells (NPC), and ESC-derived glial precursors (Lepore et al., 2008; Thonhoff et al., 2009).

Transplantation routes also vary, e.g., direct injection into the spinal cord, intraperitoneal, intracerebroventricular, and intravenous injection. Direct injection into the spinal cord can localize transplanted cells and provide high survival efficacy. In contrast, intravenous injection can deliver transplanted cells widely, but it has lower efficacy of engraftment or can result in pulmonary embolism. Outcome results vary from research to research, and graft-modifying technique (e.g., molecular modification to secrete neurotrophic factors) can enhance the efficacy of engraftment (Thonhoff et al., 2009). To evaluate the optimal delivery efficacy and survival rate, Takahashi et al., (2010) compared each transplantation route (including lesion-direct, intrathecal and intravenous injection) with spinal cord injury mice model and luciferase imaging. They concluded that direct injection achieved the highest delivery efficacy and graft survival 6 weeks after injection.

Clinical Trials of Cell Transplantation for ALS

Recently, several clinical trials using somatic stem cell transplantation for ALS have been conducted. All of the published clinical trials were based on autologous MSC from bone marrow (Table 25.2). According to the published data, they successfully achieved a safety endpoint, but spinal cord swelling at the transplanted site is noted in some cases (Karussis et al., 2010). Therefore, the risk of tumorigenicity has not been excluded even in adult somatic stem cells. Emory University and Neural Stem Inc., received FDA approval in 2009, and they have already started an ALS phase I clinical trial by transplantation of fetal neural stem cells.

Hurdles in Transplantation Therapy

Basic research using animal models will help to shed light on problems needing to be overcome before clinical trials.

Ethical Issues

To obtain ESC culture, it is necessary to manipulate embryos for scientific use. However, among various moral and ethical issues involved, the catholic church identifies embryos at this stage as having the same rights as a developing human being.

Robust Supply

As described above (CLINICAL TRIALS), MCS are widely applied and are clinically easy to access from general hospitals. However, because they are not of neural lineage, their effectiveness as cell replacement is limited. Up-coming clinical trials with neural stem cells (NSC) of fetal spinal are expected to prove them as a suitable cell resource for neural replacement. However, the graft cell resource will depend on the fetal spinal cord, limiting the number of graft cells.

Somatic cells and MSC have a finite replicative lifespan, beyond which senescence will prevent division. In contrast, ESC or iPSC can proliferate indefinitely and make robust stable freeze stocks. Furthermore, transplant of ESC- or iPSC-derived NSC can provide both neuronal replacement and protective glial cells, modifying the ALS environment around remaining neurons.

Table 25.2 Clinical trials of cell transplantation in ALS

Country	Company/Center	Date	Cell source	Cell type	Route	No. enrolled	Trial	Results
Italy	Eastern Piedmont Univ.	(2010 publish)	Auto, BM	MSC	Upper Th	10	Phase I	Safe
Turkey	Akay Hospital	(2009 publish)	Auto, BM	MSC	C1-2	13?	Phase I	Safe, improved
Spain	Hospital Universitario Virgen de la Arrixaca	2007 Feb–2010 Feb	Auto, BM	MSC	Th5-6	11	Phase I	
Spain	Hospital Universitario Virgen de la Arrixaca	2010 Oct~	Auto, BM	MSC	Th5-6	63	Phase II	(Currently recruiting)
Spain	Autonomous University of Barcelona	(2010 publish)	Auto, nose/BM	OEC/MSC		20	N.A.	Safe, no effect
Israel	Hadassah Medical Organization	2010 Jan~	Auto, BM	MSC-NTF	muscle	12	Phase I	(Currently recruiting)
Israel	Hadassah Medical Organization	2010 Jan~	Auto, BM	MSC-NTF	CSF (lumbar puncture)	12	Phase II	(Currently recruiting)
U.S.A.	TCA Cellular Therapy	2010~	Auto, BM	MSC	CSF (lumbar puncture)	6	Phase I	(Currently recruiting)
U.S.A.	Emory University	2009~	Fetal Spinal Cord	NSC	Cervical/ Lumbar	12	Phase I	N.A.
U.S.A.	Unknown	in plannning	hESC	Glial cells (astro-cytes)	–	–	–	–

Safety

Self-renewal and plasticity features of ESC and iPSC are also characteristics of cancer cells. Sometimes graft stem cells can lose control of appropriate proliferation and develop tumor as an unacceptable side-effect. We can decrease tumorigenicity risk by (1) using well-maturated cells for transplantation or (2) characterizing and selecting ESC or iPSC that have low tumorigenicity. To achieve (1), we have to enhance the sophistication of the differentiation and purification techniques of target cells. The tumorigenic potential of ESC will be reduced after maturation. For investigating (2), Miura et al., (2009) clarified that ES and iPSC have different tendencies to form neural tumor or teratoma from clone to clone. To decrease tumorigenicity, a novel iPSC reprogramming technique, using L-Myc instead of c-Myc, was reported (Nakagawa et al., 2010). By mixing and balancing these evaluation techniques, we will be able to avoid or decrease tumorigenicity in the future.

Before transplantation, ESC and iPSC need to pass through many steps to reach an appropriate state for use. Throughout, we must prevent contamination risk by harmful components and meet the standard of "Good Manufacturing Practice". In detail, adequate screening of donor material for infectious diseases as well as possible genetic testing will be necessary. In addition, avoiding the use of nonhuman animal components (a potential source of unknown infection) will also be important.

Functional Efficacy

Even if appropriate numbers of graft-cells can survive, it is difficult to make a neural network with the remaining neurons. For example, of fetus mid-brain transplantation in PD, cell therapy could improve motor symptoms and decrease drug dosage. However, therapy cannot improve dyskinesia (inappropriate secretion of

neuronal transmitter) (Barker and Kuan, 2010). This phenomenon is explained by failure to make a synchronized network with the remaining neurons around engrafting sites (Carlsson et al., 2006) or by contamination of serotonergic neurons in graft (Barker and Kuan, 2010). In the case of ALS, engrafted cells have to expand their axons to muscle (target site), far away from the spinal cord. Several researchers successfully overcame this difficulty and observed that transplanted cells (human neural stem cells) innervated host animal muscle (Gao et al., 2005; Deshpande et al., 2006).

For choosing cell type for the optimal state of ALS transplantation therapy, we can mainly list neural precursors, motor neurons, astrocytes, oligodendrocytes, and microglial cells. Simply stated, ESC- or iPSC-derived motor neurons would be a most suitable candidate for treating motor neuron disease. However, when generating or purifying motor neurons from ESC or iPSC, it is difficult to maintain moderate differentiation efficiency. Neural precursors, including various subtypes of neurons, are expected to have the most powerful ability to regenerate or protect damaged tissue. On the other hand, neural precursors generally contain immature cells and can have high tumorigenicity. In contrast to the regenerative effect of neural transplantation, glial cell transplantation can exert a neuroprotective effect via secreting neurotrophic factors and improving inflammatory damage of ALS. Then, for maintaining an all-around sufficient efficacy and safety level, matured glial cells would also be favorable candidates for clinical trials. More preclinical research will be required to approach solutions to this problem (Papadeas and Maragakis, 2009).

We utilize animal models for the evaluation of transplantation efficacy, and rodent is usually employed as disease model and recipient. However, rodent is a distinctively different species from humans. As an example of a spinal cord injury model, mice recover their motor function only a few days after injury, without any treatment. Natural recovery (or compensation), commonly observed in rodent, is never seen in humans. This difference is ascribed to the difference in upper motor neural tract between rodent and humans. Then, we have to investigate other species that are closer to humans, such as canine ALS models (Awano et al., 2009).

Difficulty of Efficient Cell-Delivery into ALS Lesions

The pathological changes of PD patients are mostly localized in midbrain or basal ganglia (striatum), and it is easy to apply direct injection of stem cells. However, pathological changes, based on TDP-43 immunostaining, are widely observed in the whole CNS (Liscic et al., 2008). ALS is contemporarily recognized as a multisystem neurodegenerative disorder. To overcome difficulties regarding the "wide-spread lesions of ALS" and the blood-brain barrier, transplantation via cerebral ventricle might be a solution (Morita et al., 2008).

For Future Clinical Trials

From the series of long discussions between the FDA and Geron, we can understand that safety is considered to be critically important for successful clinical trials. The International Society for Stem Cell Research (ISSCR) recently issued guidelines regarding threshold safety and ethical criteria for clinical transplantation therapy (Hyun et al., 2008). The ISSCR guidelines deal not only with not just ESC research but also with other pluripotent stem cells, including iPSC. ISSCR guidelines also point out the need to assess the risks of tumorigenicity. Abiding by the guidelines, stem cell transplantation is expected to win approval smoothly and to maintain a constant level of quality.

Cell Resource

We have two pluripotent stem cells, ESC and iPSC, as graft resource. Today, a few clinical or preclinical trials are mostly based on ESC or ESC-derived precursor cells, but not on iPSC. iPSC have the ability of proliferation and differentiation like ESC, and they are considered to have a similar character to that of ESC. However, recent study revealed a difference between them at the level of genome methylation or gene expression (Bock et al., 2011). We can characterize and select iPSC clones that are epigenetically

identical to ESC. Then, the transplantation management of ESC is applicable to iPSC. Here we list the pros and cons of choosing ESC or iPSC in future clinical applications.

Generally, differentiation and transplantation research using ESC has a decade of history, and both usability and safety information have already accumulated to some extent. This information is a great advantage for transplantation therapy, which requires a very strict safety level (but not enough). In contrast, iPSC technology is in its nascent stages, but recent rapid advances in the field are expected to bridge the gap.

To prevent GVHD risk, recipients must continue immunosuppressant drugs after ESC transplantation. Moreover, GVHD reaction has been pointed out as raising focal inflammation at the transplant site and exacerbating degenerative progression (Kordower et al., 2008). Besides the GVHD harmful events, there is also an interesting discussion regarding GVHD possibly acting as a safety-lock against tumorigenicity, which is an intolerable side-effect. In other words, reactivated GVHD reaction, by discontinuing immunosuppressants, could eliminate "tumor" derived from ESC-graft. We need to evaluate carefully this two-sided character of GVHD mechanisms through animal model research.

In contrast, we can generate iPSC from adult somatic cells of a patient (transplantation donor) and return patient-derived iPSC-graft back to the same patient (transplantation recipient), without GHVD risk. In the future, if a quick, safe, and low-cost method for iPSC generation is developed, every patient will be able to receive his/her own iPSC-derived cells, as ultimately customized transplantation therapy. However, the reprogramming state of iPSC is known to differ from clone to clone (Miura et al., 2009). The selection of safe iPSC will be critical for safe transplantation. To overcome the described hurdles of both ESC- and-iPSC based research, HLA type characterized iPSC bank will enable us to minimize the risk for GVHD and lower the dosage of immunosuppressant drugs (Nakatsuji et al., 2008). Furthermore, by using iPSC bank, we can also circumvent the ethical issue of using ESC.

In conclusion, innovations in stem cell manipulation will accelerate transplantation therapy using stem cells. Basic research focusing on the safety of transplantation, in addition to therapeutic experiments, can lead to beneficial outcome in practical use.

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Commentary

Edaravone in ALS

Ryosuke Takahashi*

Department of Neurology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawaharacho, Sakyoku, Kyoto 606-8507, Japan

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive and relatively selective degeneration of upper and lower motor neurons. Patients suffer from atrophy and paralysis of systemic voluntary muscles including respiratory muscles, leading to respiratory failure and subsequent death 3–5 years after the disease onset. Effective therapy for ALS that ameliorates its clinical course is still not known (Mitchell and Borasio, 2007).

Although ALS usually develops sporadically, 5 to 10% of cases are familial and hereditary. Twenty percent of familial ALS (FALS) are caused by mutations in the *copper and zinc-dependent superoxide dismutase* (*SOD1*) gene, which was first reported in 1993 (Rosen et al., 1993). Mutant SOD1 brought a breakthrough to this field, since mutant SOD1 transgenic mice recapitulate the clinical symptoms and pathological findings of human FALS (Gurney et al., 1994). Mutant SOD1 transgenic mouse models provided invaluable tools for testing effective drugs which extend their lifespan. Up to now, more than 20 drugs have been claimed to be effective in the therapy of mouse ALS.

A big problem, however, is arising: none of these drugs have yet to be shown to be effective as well in human sporadic ALS (SALS) patients (Benatar, 2007). Why? A couple of explanations are conceivable. First, mutant SOD1 transgenic mice may not be a good model for human sporadic ALS cases despite their apparent similarities. Indeed, mutant SOD1 associated FALS and SALS exhibit different microscopic neuropathology. The former is characterized by Lewy body-like inclusion containing mutant SOD1, whereas for the latter skein-like or round inclusions containing TDP-43. Since TDP-43 is implicated in the pathogenesis of SALS as well as in a subgroup of FALS, developing a new ALS mouse model based on TDP-43 could solve these problems in the future (Neumann et al., 2007). A second possible explanation is that the most therapies in mouse models are initiated prior to disease onset, which is impossible in human patients until presymptomatic diagnosis for ALS becomes available. Thirdly, whether drug dosage and bioavailablity comparable to mouse experiments are replicated in human trials remains unclear.

An alternative explanation is the difference in the design of mouse experimental therapies and human clinical trials. Randomized controlled trials, which are designed to eliminate numerous confounding factors including observation biases, are standard in human clinical trials. In contrast, mouse experiments are generally not performed as rigorously as human trials, increasing risks of producing "false positive" results (Benatar, 2007).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger that has been approved in Japan since 2001 as a therapeutic agent to reduce neuronal damage caused by acute ischemic stroke (Yoshida et al., 2006). Edaravone eliminates lipid peroxide and hydroxyl radicals by transferring an electron to the radical, thereby ameliorating the ischemic neuronal damage. Oxidative stress is implicated as one of the pathogenetic mechanisms for ALS (Barber et al., 2006). Moreover, a small-sized open trial of edaravone suggested that edaravone is safe and may delay the progression of functional motor disturbances in ALS patients (Yoshino and Kimura, 2006). Thus. edaravone is a promising therapeutic agent for human motor neuron diseases including ALS.

In a previous issue, Ito et al. reported an experimental therapy of a mutant SOD1 mouse model using edaravone (Ito et al., 2008). Taking the problems associated with the therapeutic experimental design in mouse experiments, they carefully optimized the dosage of edaravone so that the pharmacokinetic profile after intraperitoneal injection became comparable to that in human patients. Moreover, they started treatment only after the disease onset, similar to human ALS treatment. Furthermore, they used only female mice for analysis considering the gender difference in lifespan and randomized blind analyses were adopted for all the behavioral as well as pathological observations. This methodological rigorousness has never been considered seriously in previous experimental therapies of mutant SOD1 ALS mouse models, most of which have failed to be replicated in human patients.

Edaravone significantly slowed the motor function decline as assessed by multiple behavioral tests such as rotarod tests. However, the lifespan of edaravone-treated mice were not significantly higher

* Fax: +81 75 761 9780.
E-mail address: ryosuket@kuhp.kyoto-u.ac.jp.

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than those of control mice, suggesting that edaravone may improve the motor function of the ALS mice without apparent lifespan expanding effects (Ito et al., 2008). This uncoupling in the mechanisms underlying motor function and lifespan further implies that pathways causing motor function decline are not necessarily the ones causing eventual death, usually by respiratory muscle failure. That said, it would be possible to identify drugs that can improve the quality of life in ALS patients without affecting lifespan, which seems to be an easier goal compared with identifying lifespan-extending drugs for ALS. Moreover, it was clinically important that edaravone was effective even when administered after the disease onset. On the other hand, it would be intriguing to administer edaravone to ALS mice at their presymptomatic stage to understand how the point at which edaravone is used during the course of disease affects its outcome.

It is noted that high-dose edaravone treatment leads to a decrease of mutant SOD1 accumulation in the spinal cord. Since administration of edaravone resulted in a marked decrease of 3-nitrotyrosine/tyrosine ratio, a marker of oxidative stress, suppression of oxidative stress is likely to be upstream of the inhibition of aggregate formation (Kabashi and Durham, 2006; Valentine and Hart, 2003). It has long been debated how oxidative stress is induced by SOD1 mutations (Barber et al., 2006). Reduced enzymatic activity of SOD1 and generation of peroxynitrite due to aberrant copper chemistry have been proposed as plausible mechanism explaining "gain of toxic function" of mutant SOD1 (Beckman et al., 1993; Deng et al., 1993; Robberecht et al., 1994). However, the fact that a subgroup of SOD1 mutants retains full enzymatic activity and that H46R and H48Q mutants which completely lose binding sites for copper still cause ALS suggests that mechanisms unrelated to SOD1 activity may also be involved (Borchelt et al., 1994; Valentine et al., 2005; Wang et al., 2003). It has been shown that mutant SOD1 overexpression in a neuronal cell line leads to transcriptional repression of antioxidant proteins by reducing the level of transcriptional factor NRF2 (Kirby et al., 2005). It would be intriguing to investigate whether edaravone affects the level of NRF2 when administered to ALS mice.

Another interesting unresolved question is which cells are the targets of edaravone. Recently, it has been shown that motor neuron death in mutant SOD1 ALS mouse models is non-cell autonomous (Boillee et al., 2006; Yamanaka et al., 2008). In other words, mutant SOD1-expressing astroglial or miroglial cells promote motor neuron death. In this context, edarovone may decrease the aggregates in non-neuronal glial cells, resulting in amelioration of neurodegeneration. These questions should be addressed in further analysis in the future.

A recent systematic review of randomized controlled trials of antioxidant therapies against ALS including vitamin E and acetylcysteine has shown that there is no substantial evidence to support their clinical use (Orrell et al., 2008). However, the evidence for the beneficial effects of edaravone on human ALS patients awaits the publication of the results of a phase III clinical trial of ALS, currently ongoing in Japan (http://www.als.net/research/studies/tdfAnimalStudyList.asp).

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