STEM - CELL - FREE - THERAPY FOR ALS
- Experimental and Clinical Studies of Conditioned Medium -

Professor Emeritus, Nagoya University
Minoru Ueda

INTRODUCTION

1. Etiology

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that mainly develops after middle age and causes selective and systematic damage to upper and lower motor neurons. The course of the disease varies from case to case, but it begins with muscle atrophy in one upper limb, progresses to the contralateral upper limb and then to both lower limbs, during which time speech impairment, dysphagia and other symptoms of ball paralysis and respiratory muscle paralysis are added. Without respiratory management using a ventilator, death often occurs within 2-5 years of onset due to respiratory failure, making ALS the most severe of all neurological diseases.

ALS is one of the country's designated incurable diseases, and in 2018, 9805 patients were authorised to receive specific medical expenses in Japan. Approximately 90% of patients are classified as 'solitary' with no known cause, while the remaining less than 10% are classified as 'familial' with a possible genetic involvement. The disease is progressive and cannot be stopped once it has developed.

Although the number of people affected in Japan is not high, at 1 to 2.5 per 100,000 people (1), there have been reports of celebrities who have been diagnosed with the disease and have died, as well as the global outcry that began in the United States in 2014.

The difficulty of treating the disease has been recognised by society through events such as "The Ice Bucket Challenge" (*), which began in the United States in 2014 and has had a global impact.

(*) Participants cover their heads with ice water, and the videos they take are made public. The research grant of 90 million US dollars (approximately 10 billion Japanese yen) was carried out worldwide over a five-year period from 2014 to 2018.

The causes of ALS are still largely unknown, but factors related to the onset of the disease

are gradually becoming clearer. Among these, the mutation of the SOD1 gene, which is involved in the detoxification of reactive oxygen species in familial ALS, and the theory of excess glutamate are considered to be the most promising theories.
However, these are only strong hypotheses and the true cause of the disease and its mechanism are unknown. Therefore, there is no effective treatment for ALS.
In January 2021, we started a clinical study using stem cell form human foliated decidoues teeth derived conditioned medium (SHEDCM) to treat this neurodegenerative disease of unknown cause (2).

In this paper, we would like to clarify the position of conditioned medium therapy by reviewing past or ongoing therapies for ALS and contrasting them with these. The paper also looks at the future of stem cell-free therapies, including conditioned medium therapies.

2. Past efforts to treat ALS

The treatment of ALS includes drug therapy and stem cell therapy, and clinical research has been in full swing since the late 2000s.
In the area of drug therapy, currently the only approved drugs for ALS are Rilutek® (glutamate antagonist) orally and Radicat® (free radical scavenger) intravenously. These were developed based on the glutamate and reactive oxygen species hypotheses mentioned earlier, but neither of these can stop the progression of the disease.
Drugs being researched and developed based on the same series of hypotheses include hepatocyte growth factor (HGF) in Japan (under clinical trials / Tohoku University) , tofersen for hereditary ALS overseas (clinical trials completed / Biogen, USA / October 2021 / no efficacy and AMX0035 for solitary ALS (clinical trial completed /Amylyx, USA/March 2022/approval pending).On the other hand, clinical trials are underway for the anticancer drug bosnitib (under clinical trial/Kyoto University) and the dopamine agonist ropinirole (clinical trial completed/confirmed progression suppression/Keio University), which were found to be effective for ALS using an iPS cell screening method using patient cells. Clinical trials are underway. similarly, the inflammation suppressant MN-166 (under clinical trials/Medicine Nova) and the HIV drug OBP-601 (under clinical trials/Oricos Biopharma) are also being investigated. The end point of all of these therapies is not to cure or improve ALS, but to slow the progression of the disease.

Stem cell-based therapies for ALS are discussed next. The main reports are as follows.
Haematopoietic stem cells (clinical study/validation/ Deda, H. et al.: Cytotherapy, 2009(3))

Bone marrow-derived mesenchymal stem cells (NeuroNata-R) (clinical trial completed/confirmed progression inhibition/ approved in South Korea 2015)
Bone marrow-derived mesenchymal stem cells (Clinical trial/fuda medical university) (4)
Adipose-derived mesenchymal stem cells (1 case report/ Shigematu,K.et al : Eur.Rev.Phamacol.2021) (5)
Muse cells (under clinical trial/ LSII, 2021)
Neurotrophic factor-secreting mesenchymal stem cells (NurOwn) (clinical trial completed/BrainStrom, 2021/no efficacy (6))

As an interesting report the Cochrane Library in 2020 analysed 151 stem cell therapies for ALS in terms of efficacy, safety and feasibility. It concluded that it does not support the use of bone marrow-derived mesenchymal stem cells as a treatment for ALS (7). (C.M., Gabriella/NeuroRrhabilitation, 2020).

Under these circumstances, various bioactive substances secreted by stem cells (secretome) have attracted attention. The secretome is a general term for bioactive molecules secreted by stem cells, including cytokines, growth factors, chemokines and extracellular vesicles (exosomes). These are present in the conditioned medium of stem cell cultures after removal of cellular components and metabolic precipitates.

It is still unclear which components of the secretome have a specific effect on ALS, but several bioactive substances have been identified as active components, including BNDF, GDNF, IGF-1, VEGF, HGF and miRNA (8).

It is currently unclear whether all bioactive substances in the conditioned medium are required or whether specific substances alone are effective against ALS. However, the common effects may include the following

1 - Neuroprotective action.
2 - Inhibition of apoptosis.
3・Promoting secretion of neurotrophic factors from glial cells
4・Neutralising action of oxidative stress
5・M2 polarity-converting action of macrophages (release of anti-inflammatory substances).

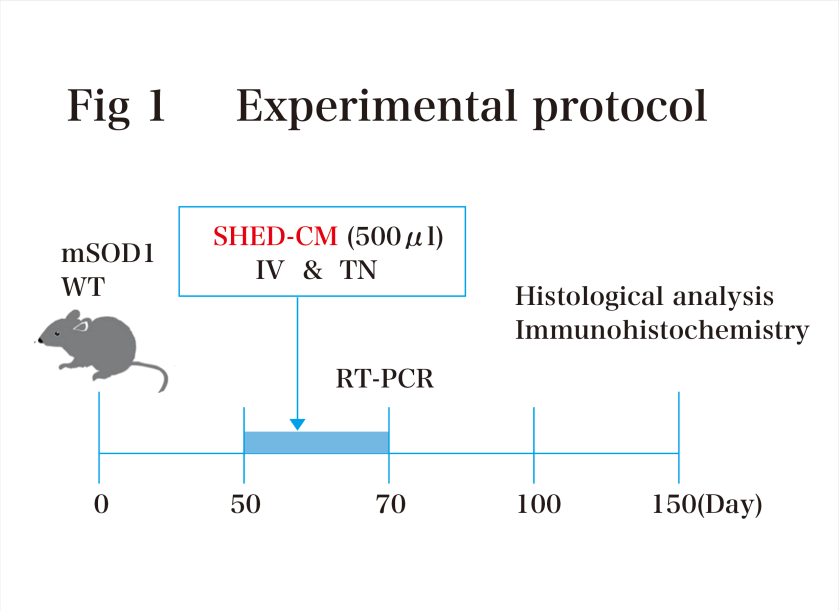
The secretome in conditioned medium contains all the components that induce the above effects.Therapy with conditioned medium may facilitate the development of future Stem - Cell - Free Therapy.

EXPERIMENTAL STUDIES USING MOUSE MODELS OF ALS

We conducted the following animal experiments to verify the effect of conditioned medium on ALS (9).

1. Materials and methods

The ALS model mice used in the experiment (mSOD1, Charles River) are recombinant G93A mutant SOD1 mice, which develop at around 70 days of age (P70) and die by P150. In this experiment, P50 to P150 were used for observation. Three groups were established in the experiment(Fig1)



Control group 1: WT (healthy mice) n=5

Control group 2: mSOD1 (ALS mice) n= 5

Experimental group: mSOD1 (ALS mice) treated with SHEDCM) n= 5

＊ SHED: Stem Cell form Human exfoliated Deciduous teeth

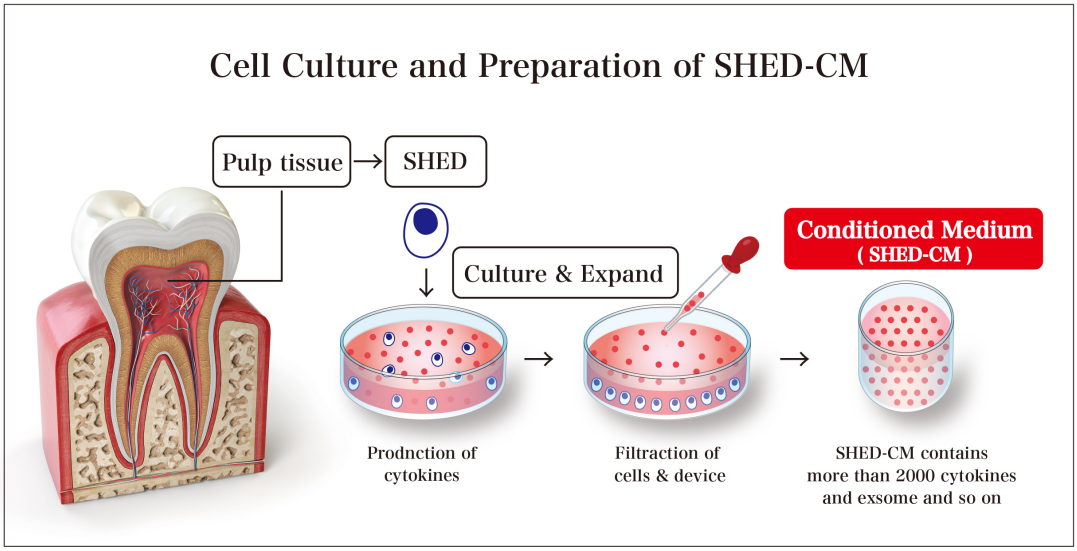
＊ CM: Conditioned Medium

The experimental group included ALS mice (mSOD1) from P70 (pre-onset) to P90 (post-onset).SHEDCM stock solution was administered via the tail vein at a daily dose of 0.5 ml for 20 consecutive days.

The control group (WT and mSOD1) received the same volume of saline.

Survival was observed up to P150, and at P50, P70, P90 and P110, a needle myopotential test was performed to determine the compound muscle action potentials (CMPA) of the lower limb.CMAP of the lower limb was measured as described in (10).

Fig. 2.



1) Preparation of conditioned medium (Fig. 2)

Conditioned medium derived from deciduous tooth stem cells (SHEDCM: Stem Cells from Human Exfoliated Deciduous teeth or milk teeth pulp stem cell derived Conditioned Medium) was prepared by the following procedure (11-13).

Collection of SHED :

Select SHEDs as adherent cells from dental pulp tissue collected from human deciduous deciduous teeth that have been defoliated.

Spontaneously deciduous teeth are disinfected with a chlorohexidine solution, the crown is divided and the dental the pulp tissue is recovered with a reamer.

Enzymatic treatment :

The collected pulp tissue is suspended in basic medium (Dulbecco's Modified Eagles Medium with 10% bovine serum and antibiotics (hereinafter referred to as 'DMEM') and treated with 2 mg/m1 of collanase and dispase at 3°C for 1 hour. The pulp tissue and pulp cells after enzyme treatment were collected by centrifugation (600-500 x g for 5 min).

Cell culture :

The pulp tissue and pulp cells collected as described above were suspended in 4 cc of DMEM or mesenchymal stem cell medium containing 5% to 15% bovine serum and 50 to 150

conit/ml of antibiotics and seeded into adherent cell culture dishes, 6 wells.
This is incubated in an incubator adjusted to approximately 37°C in an atmosphere of 5% CO2. When subconfluent (indicating that cells occupy approximately 70% of the surface area of the culture vessel) is reached, cells are treated with 0.05% trypsin-EDTA for 5 min at 37°C. Dental pulp-derived stem cells detached from the dish are seeded into 10 cm diameter adherent cell culture dishes and expanded cultures are made.
Passage cultures are performed 1 to 15 times to grow to the required cell number (approximately 1 x 10^7 cells/m1). After the above culture, the cells are collected and stored.
Cell collection：
After detaching the cells from the culture vessel by trypsin treatment, the cells (adherent cells) are collected by centrifugation to recover SHED. The centrifugation conditions are 700 and 5000 x g.
Preparation of pulp stem cell derived conditioned medium (SHEDCM)：

The SHED obtained by the method described above are placed in a culture vessel and DMEM with animal serum such as 10% serum FBS added to the basic medium. The cells are then cultured under 5% CO 2 at 37° C for 24-48 hours. The culture medium is then replaced with serum-free DMEM and incubated for another 24 to 72 hours, after which the conditioned medium is collected.

To completely remove the SHED from the collected culture supernatant, the collected is centrifuged at 600 and 5000 x g for 7 min to obtain treated SHEDCM, which contains no milk pulp-derived stem cells (SHED removed).
Another method of removing SHED, such as passing through a separation membrane that does not allow SHED to pass through, can be used to obtain a SHED-free conditioned medium (milk pulp-derived stem cells removed).

There is no restriction on the number of SHED passages used to produce SHEDCMs, but it is preferred to be between 5 and 15 in terms of the ability to improve or prevent target tissues and the range of target tissue types.

2) Results
The results of the CMAP measurements are shown in Figure 3. In control group 2 (saline-treated ALS mice), the potential decreased continuously to less than 5 mv on day 110.
On the other hand, the CMAP of the control group 1 (healthy mice) and the experimental

group ALS mice treated with SHEDCM remained above 10 mv until day 110, with no significant difference between the two. And there was a significant difference (1 per cent level, by T-test) in the conditioned medium between the control group2 and the experimental group group group.

The normal range of CMAP in mice is 10 mv-20 mv.

CMAP remained in the normal range up to day 70 in all three experimental groups, but The CMAP of the control group 2 (mSOD1) decreased and were below the normal range by day 90, and by day 110 they were below 5 mv. The control group 2 (mSOD1) declined to below the normal range on day 90 and 5 mv by day 110. On the other hand, control group 1 (WT) and the experimental group (SHEDCM-treated group) maintained the normal range until day 110.(Figure 3 Figure 4)

Fig 3

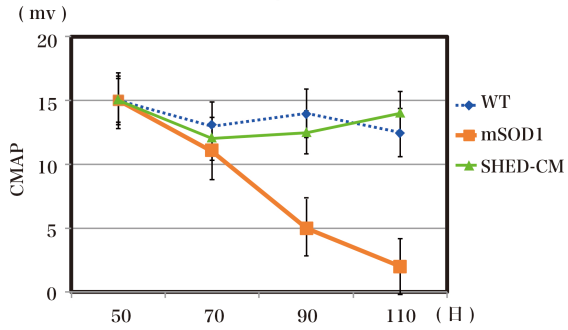
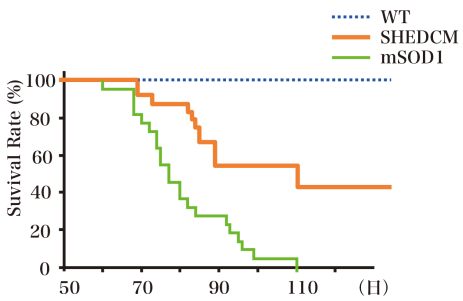


Fig 4



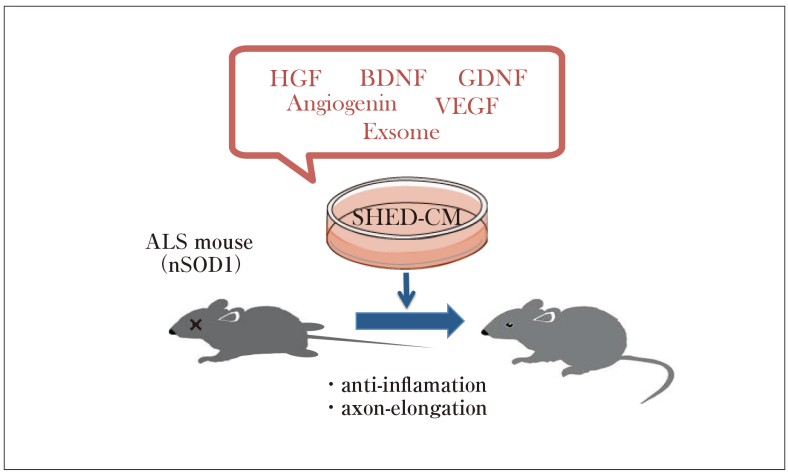
The survival rate of mice in each group is then shown in Fig. 4: at P70, all three groups were almost 100% alive. The survival rate at P110 was 100% in the WT, 0% in the control group 2 and 50% in the experimental group. The significance test method was based on the T-test.

3. Discussion.

Shimojima et al. (12) also studied the therapeutic effect of EAE in a mouse model by administering (SHEDCM). The results demonstrated that ED-Siglec-9 in the SHEDCM was a key factor in alleviating spinal cord inflammation. Meanwhile, Bonafede (15) et al. tested the effects of adipose stem cell exosomes in vitro using neural stem cells isolated from ALS model mice. They stated that exosomes protected neural stem cells from oxidative damage; both Ed-Siglec-9 and exosomes were present in the SHEDCM, and their co-operation with

other bioactive substances may have led to the maintenance of the CMAP potential and improved survival (Fig. 5).

Figure 5.



CLINICAL STUDIES OF SHEDCM FOR ALS

The therapeutic effect of SHEDCM on amyotrophic lateral sclerosis was then tested in human.

1.Severe and Elderly Patient.

Patient：

The patient (68 years old, male) was diagnosed with ALS in June 2020 and transferred to a specialised ALS hospital for observation, but the progression of symptoms did not stop. The patient was then transferred to a specialised ALS hospital for observation, but the progression of symptoms also did not stop.

The patient was referred to us in January 2021 because he wanted to be treated with SHEDCM.

The patient showed characteristic symptoms of ALS. In particular, there was a decrease in respiratory function (% lung capacity, 66.5%).

(June 2020) to 46.1% (August 2020)) was particularly severe, and the patient also had significant limb spasticity, confirming the rapid deterioration of his condition.

Treatment and Progress：

The patient was treated with intranasal SHEDCM (5 m1/week) from January 2021 to August 2021. During this period, respiratory function deteriorated (ventricular air, Spo2 < 90%) and limb spasticity developed.

The patient developed spasticity of the extremities. In September of the same year, he was started on SHEDCM infusion (240 m1/week).

One week after the start of intravenous SHEDCM treatment, the limbs and fingers (especially the thumb and little finger) showed spasticity relief and automatic spasticity in the limbs and fingers. showed a rapid increase in automatic joint range of motion and continued to improve in automatic movement and respiratory function.

Automatic movement and respiratory function have continued to improve since then.

This was observed before the start of treatment (January 2021) and after three months of intravenous administration of SHEDCM (December 2021).

The patient's range of motor movement was measured before the start of treatment (January 2021) and after three months of intravenous administration of SHEDCM (December 2021). Figure 6 shows how the range of motor movement was measured

Table 1 shows the results. Table 1 also shows the range of motor movement of this patient before treatment and after 3 months of SHEDCM administration.

The range of motion is defined as the range that can be moved by another person's hand, whereas the automatic range of motion is defined as the range that can be moved by the patient's own will.

Automatic range of motion means the range that can be moved at the patient's will (voluntary movement). The range of motion is measured as the angle of rotation of the limb or fingers about the joint axis. For measurement, a joint angle meter (angle meter) is used.

In January 2022, the patient's respiratory function was improved in the room, with Sp02>97%. As of March 2022, the patient is still receiving SHEDCM intravenously and his range of motion has further improved.

The patient's range of motion has further improved.

The benefits of SHEDCM are clear. Progressive atrophy of the respiratory muscles and spasticity of the limbs are caused by inflammation and degeneration of motor neurons.

The progressive atrophy of respiratory muscles and spasticity of the limbs are characteristic of ALS, caused by inflammation and degeneration of motor neurons. After onset, progression can be slowed. Although the progression of the disease can be slowed after onset, there have been no successful cases of halt or recovery in the past. The fact that the treatment of severe ALS such as this one has improved rapidly deteriorating respiratory function and continued to improve limb motor range of movement indicates that

SHEDCM has a high anti-inflammatory and neuroregenerative effect.

Figure 6.

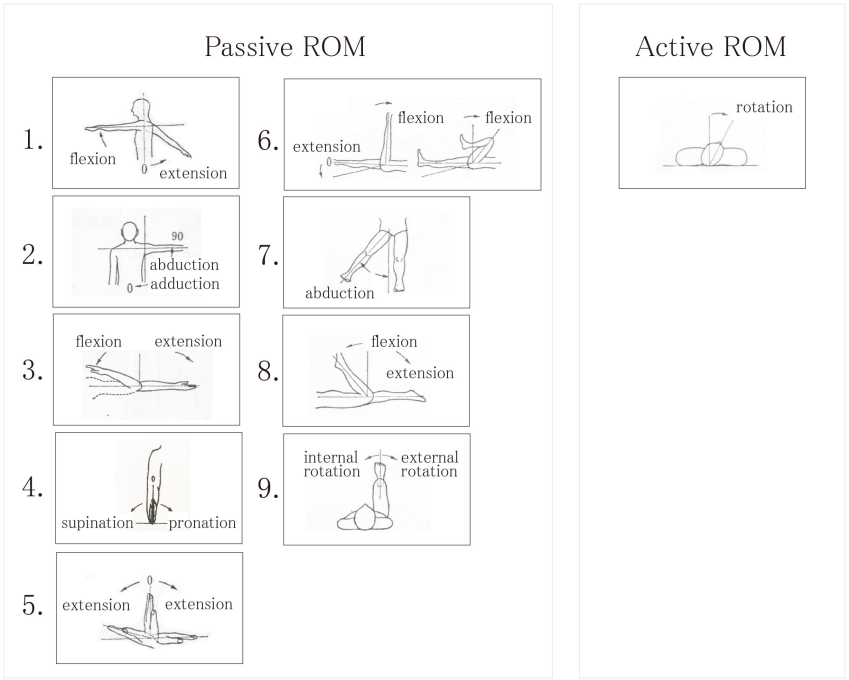


Table 1.

Passive ROM

	Site		Before	After
1.	Shoulder forward flexion	(R)	30	50
		(L)	40	60
2.	Shoulder abduction	(R)	25	35
		(L)	25	30
3.	Elbow flextion	(R)	50	90
		(L)	40	70
4.	forearm supination	(R)	-35	-5
		(L)	-45	-5
5.	Wrist extension	(R)	0	10
		(L)	-45	10
6.	Hip flextion	(R)	40	50
		(L)	35	60
7.	hip abduction	(R)	10	25
		(L)	15	25
8.	hip external rotation	(R)	10	20
		(L)	10	20
9.	Knee flextion	(R)	70	80
		(L)	70	85

Active ROM

Site		Before	After
Cervical rotation	(R)	0	30

2. Moderate Cases.

Patients：

Subjects: 5; Age: 42-81 years; Gender: 4 males, 1 female;.

Severity: 2-4; ALSRS-R score: 17-43.

Treatment and progress：

Number of culture supernatant doses (duration): 8-12 (2-3 months).

Changes in ALS symptoms were assessed based on ALSFRS-R score.

Results: 1 patient improved, 2 patients remained unchanged and 2 patients worsened. There were no discontinuations of intravenous infusion due to adverse events (Table 2).

Table 2.

case	age	sex	siverity
1	42	M	3
2	48	M	3
3	65	M	2
4	81	M	4
5	46	F	3

	ALSRS-R		times / duration	
case	before	after		ALS slope
1	22	15	12 / 3	-2.3points/M
2	36	36	9 / 3	0
3	43	40	12 / 2.5	-1.2points/M
4	32	32	12 / 2.5	0
5	17	24	8 / 2	+3.5points/M

The results of this clinical study 1 and 2 confirm that intravenous administration of SHEDCM is safe. In addition, despite the short observation period (approximately 3 months), there was marked improvement of symptoms in one patient and cessation of disease progression in two patients. Most importantly, in all cases, patients were aware of an improvement in their quality of life even after the end of treatment. The results strongly suggest that culture supernatant treatment with SHEDCM for ALS is effective.

DISCUSSION

1. Pharmacotherapy, stem cell therapy and stem-cell-free therapy

As far as we have been able to ascertain, previous drug therapies for ALS have only slowed the speed of disease progression and have not resulted in the cessation or improvement of disease progression.

In stem cell therapy, the results of most clinical trials have been as unsatisfactory as those of drug therapies. In addition, the risk of thrombosis and cancer cannot be taken into account when increasing the dose of stem cells without limit.

On the other hand, stem cell-free therapy using stem cell-free conditioned medium, allows for higher doses without the risk of cancer or thrombosis. In fact, in our clinical studies, no adverse events were observed even with a single dose of more than 240 ml of SHEDCM. The efficacy of the treatment for ALS was also unprecedented, with improvement of symptoms and arrest of the progression of the disease. Further improvements are expected in the future.

2. The future of stem-cell-free therapy

In stem cell-free therapy, strategies using the conditioned medium as conditioned medium is or extracting exosomes and specific cytokines from the conditioned medium and administering them as purified drugs have been investigated.(8) Shimojima et al.(12) demonstrated that ED-Siglec-9 alone in the conditioned medium had anti-inflammatory and neuroprotective effects comparable to those of the conditioned medium stock solution. Bonafedea(23) also reported that exosomes alone have a strong antioxidant effect. Nevertheless, such a strategy of mass administration of key factors alone is reasonable, but may increase the risk of adverse effects.

A similar case from the past is that of osteogenic proteins (16). This substance is known as BMP(Bone-Morphogenic-Protein), which was discovered in bone matrix by Urist in 1965 and subsequently successfully analysed genetically for 20 subtypes as a superfamily. Of these BMP-2 was found to have the highest activity, and was mass-produced based on genetic engineering technology and put on the market in 2002 under the trade name Amplify. Clinicians welcomed the drug as an alternative to bone grafts for bone regeneration, but 'Amplify' contained 'milligrams' of BMP-2. However in the blood the amount of BMP-2 is in 'nanograms'. It was found that a million times the physiological dose of BMP-2 was required to achieve the expected clinical results. Soon after, statistics were published showing a 4-5

times average cancer risk in patients receiving high doses of BMP-2 (17), and BMP-2 disappeared from clinical practice.

The realisation of cell-free therapy is still subject to the selection of the strategy. such as crude or key factor, administration methods, effective concentrations and side effects, and careful and intensive clinical research is required before it can be put into practice.

3. Cost-effectis of stem-cell-free therapies

It is said that one of the reasons stem cell-based therapies did not become widely available was the issue of cost-effectiveness. This means that the expected results are not achieved in proportion to the cost of the treatment. This is also true for stem-cell-free therapy. The production of conditioned medium is also expensive, although not to the same extent as stem cells. In addition, a large amount of conditioned medium is required to isolate exosomes, etc., and extraction (ultracentrifugation) requires additional time and money. However, in a serious disease such as ALS, for which there is no other treatment, this problem could be eliminated. The development of treatments should be promoted even at the expense of the cost of treatment.

Clinical trials for stem cell-free therapies are currently being planned all over the world. The clinical trials are required to show a treatment effect that can be clearly seen by the layperson, remembering that ALS patients do not want to 'slow down the progression' but to 'halt the progression and improve it'.

New technologies have begun to be developed to reduce costs: Deng (18) et al. showed that ultrasound stimulation increased the release of exosomes from human stellate cells by approximately fivefold. Similarly, Ueda et al. found that ultrasound stimulation of deciduous dental pulp stem cells increased the amount of cytokines such as MCP-1, Siglec-9 and HGF in the conditioned medium (personal communication).

Cost-effectiveness is an unavoidable issue in the development of new therapies. However, as far as ALS is concerned, the development of treatments must be successful even if this issue has to be ignored. There is no room to argue with this.

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