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ORIGINAL ARTICLE

Two Japanese familial cases of Caffey disease with and without the common *COL1A1* mutation and normal bone density, and review of the literature

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Abstract Caffey disease, also known as infantile cortical hyperostosis, is a rare bone disease characterized by acute inflammation with swelling of soft tissues and hyperostosis of the outer cortical surface in early infancy. The common heterozygous mutation of the *COL1A1* gene, p.Arg1014Cys, has been reported in patients with Caffey disease. However, its pathogenesis remains to be elucidated, and the reason for the incomplete penetrance and transient course of the disease is still unclear. In the present study, we performed mutation analysis of the *COL1A1* and *COL1A2* genes and measured bone mineral density in two Japanese familial cases of Caffey disease. The index case and two clinically healthy members of one family carry the common heterozygous mutation; in contrast, no mutation in *COL1A1* or *COL1A2* was identified in the affected members of the second family. In addition, we found normal bone mineral density in adult patients of both families who have had an episode of cortical hyperostosis regardless of the presence or absence of the common p.Arg1014Cys mutation. *Conclusion*: The results reveal that Caffey disease is genetically heterogeneous and that affected and unaffected adult patients with or without the common *COL1A1* mutation have normal bone mineral density.

Keywords Cortical bone · Hyperostosis · Type 1 collagen · Mutation · Bone mineral density · Bone formation

Abbreviations

MRI	Magnetic resonance imaging
NSAIDs	Non-steroidal anti-inflammatory drugs

Introduction

Caffey disease (OMIM 114000), also known as infantile cortical hyperostosis, is a rare bone disease characterized by acute inflammation with swelling of soft tissues and hyperostoses of the outer cortical surface in early infancy [7, 11, 13]. Radiographs of long bones, mandible, clavicles, ribs, and scapulae indicate massive periosteal bone formation and consequently increased cortical thickness. In a separate clinical situation, cortical hyperostosis is sometimes observed after long-term administration of prostaglandin E for ductus-dependent cyanotic congenital heart disease, suggesting inflammatory events in Caffey disease [8, 15, 22]. Magnetic resonance imaging (MRI) of bone can also detect characteristic diaphyseal thickening and inflammatory signals in adjacent muscle, connective tissue, and in the bone marrow of patients with Caffey disease [14, 17, 18]; hence, the disease seems not to be confined to bone. Caffey disease resolves spontaneously, but sometimes recurs in childhood. Non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids are sometimes used to improve inflammation and pain [4, 21].

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The common heterozygous mutation of the *COL1A1* gene, p.Arg1014Cys (counted from the initiator methionine or p.Arg836Cys with respect to the first glycine of the triple helical domain of the alpha 1 chain of type I collagen [α1(I)]), has been reported in patients with Caffey disease in Canada, Australia [10], Thailand [20], Korea [3], India [16], and Italy [2]. This heterozygous mutation is also found in patients with severe prenatal cortical hyperostosis [12]. On the other hand, parents who had no features of Caffey disease were reported to carry the mutation [3]. The reason for the incomplete penetrance of the disease has not been elucidated [19], and how the mutated collagen leads to hyperostotic bone lesion is still unknown [10, 11]. In addition, the mutation is not identified in some cases of Caffey disease. Thus, analysis of familial cases of Caffey disease may contribute to the understanding of the pathogenesis of the disease and bone biology.

It is well known that *COL1A1* mutations are responsible for osteogenesis imperfecta, a disorder characterized by bone fragility, ligamentous laxity, blue sclerae, dentinogenesis imperfecta, and low bone mineral density [5]. In contrast, the patients with the *COL1A1* p.Arg1014Cys mutation in Caffey disease have cortical bone thickening, but no bone fragility. However, fractures possibly due to bone fragility were reported in two members of a Thai family with Caffey disease [20]. These two patients harbor the common mutation, but the correlation between the p.Arg1014Cys mutation, bone mineral density, and fractures has not been evaluated in this family [20].

Here, we report two Japanese familial cases of Caffey disease, one of which has the common mutation whereas the other has no mutation in the *COL1A1* or *COL1A2* genes. In addition, we examined bone mineral density in these patients.

Patients and methods

Patients

Family A (COL1A1 mutation positive)

The proband (II-1), a 6-month-old female infant, was referred for evaluation of swelling and deformity in both legs and forearms noted since the age of 3 months. The antenatal, perinatal, and neonatal periods had been uneventful, and all developmental milestones had been attained normally. Her parents stated that they had no history of leg swelling during infancy or childhood. Radiographs of the bones revealed cortical bone thickening of both femora, tibiae, radii, ulnae, and swelling of the surrounding soft tissues (Table 1, Fig. 1). The diagnosis of Caffey disease was made on the basis of symptoms, signs, and radiographic findings; periodic examination was continued without medication. The swelling resolved spontaneously at the age of 1 year and 6 months. Serial radiographs revealed periosteal thickening and widening of the long bones over 3 years (Fig. 1). She developed recurrence at the age of 11 years, which resolved again spontaneously. She is now 12 years old with no medical problems except for mild deformity of her legs. She has had no features of osteogenesis imperfecta such as bone fracture, ligamentous and joint laxity, blue sclerae, deafness, and dentinogenesis imperfecta. The growth parameters were appropriate for her age; height 148 cm (−0.3 SD) and body weight 39.2 kg (−0.4 SD). Her brother (II-3) had a normal antenatal and perinatal history. Right lower leg swelling and deformity and left thigh swelling manifested at the age of 11 months. Radiographs demonstrated deformity and subperiosteal resorption of the right tibia and left femur, thus confirming cortical hyperostosis (Fig. 1). At the age of 2 years and 2 months, the right lower leg and left thigh remained swollen. In contrast, the mother (I-2) had no abnormal radiographic findings of the lower legs at the age of 36 years. According to her memory, she had no symptoms and signs of cortical hyperostosis during infancy or childhood. She could not recall any clinical history suggesting either joint laxity or skin hyperelasticity.

Family B (COL1A1 mutation negative)

The proband (III-1) had swelling of the left thigh and the right lower leg and irritability since the age of 1 month. Radiographs of the bones at the age of 2 months (Fig. 2) revealed cortical bone thickness of the right tibia and swelling of the surrounding soft tissues. MRI showed large lesions with increased T2-weighted signal intensity in the surrounding soft tissue and the bone marrow of the lower legs. She had difficulty walking due to the length difference of her legs and was referred to our hospital at the age of 2 years and 8 months. Physical examination revealed tender, diffuse, immobile swellings over the anterior aspects of both legs, which were hard in consistency. The neighboring knee and ankle joints appeared to be normal. There were no swellings over the jaw, clavicles, ribs, or elsewhere in the body (Table 1). She has had no evidence of joint laxity or skin hyperextensibility. Developmental milestones were not delayed. The patient is now 11 years old, her height and weight are within the normal range but the leg deformity persists, and her leg length discrepancy is 1.2 cm. The mother (II-2) had a history of bone swelling in childhood. Her brother (III-2) was born uneventfully at 39 weeks of gestation and had left lower leg swelling and deformity at the age of 3 months (Fig. 2). The maternal granduncle (I-1) had a history of swelling of legs, although no medical history or radiographs were available. The parents were not consanguineous and did not come from the same community.

Table 1 Radiological features in individuals evaluated for the *COL1A1* p.Arg1014Cys mutation

Family no.	Pedigree no.	p.Arg1014Cys	Sex	Onset	Hyperostosis								
					Femur	Tibia	Fibula	Humerus	Radius	Ulna	Clavicle	Mandible	Ribs
A[a]	II-1	+	F	3 months	R/L	R	R	−	R	R	−	−	−
	II-2	+	F	−	−	−	−	−	−	−	−	−	−
	II-3	+	M	2 years	L	R	−	−	−	−	−	−	−
	I-2	+	F	−	−	−	−	−	−	−	−	−	−
B[a]	III-1	−	F	1 month	+	+	+	−	−	−	−	−	−
	III-2	−	M	months	+	+	+	−	−	−	−	−	−
	III-3	−	F	−	−	−	−	−	−	−	−	−	−
	II-2	−	F	3 years	+	+	+	−	−	−	−	−	−
Australia [4, 9]	II-1	+	F	3 weeks	+	R/L	+	+	+	+	+	+	−
	II-2	+	F	2 weeks	+	+	+	+	R/L	R/L	+	+	−
Thai [19]	I-3	+	M	/	−	R/L	−	−	R/L	−	−	−	+
	II-2	+	M	0 month	−	R/L	/	−	R/L	/	−	−	−
	II-7	+	M	/	−	−	−	−	−	−	−	−	+
	II-11	+	F	S	−	R	/	−	−	−	−	−	−
	III-15	+	F	11 days	−	R/L	−	−	−	−	−	−	−
	III-3	−	F	/	−	−	−	−	+	/	−	−	−
Korea A [3]	II-1	+	M	2 months	R	R/L	−	−	R/L	R	−	+	−
Korea B [3]	II-1	+	M	9 months	−	R/L	−	−	−	−	−	+	−
	II-2	+	F	4 months	R	R/L	−	−	R/L	R/L	−	+	−
Korea C [3]	II-1	+	F	1 month	−	−	L	−	R/L	−	−	+	−
Korea D [3]	II-1	+	F	2 months	−	R/L	−	−	R	−	−	+	−
	I-1	−	M	−	−	−	−	−	−	−	−	−	−
	I-2	+	F	−	−	−	−	−	−	−	−	−	−
Korea E [3]	II-2	+	M	0 month	−	R/L	−	−	−	−	−	−	−
	I-1	+	M	−	−	−	−	−	−	−	−	−	−
	I-2	−	F	−	−	−	−	−	−	−	−	−	−
France [11]	Pt	+	M	0 day	+	+	+	+	−	−	−	+	+
India [15]	Pt	+	M	3 months	R/L	R/L	−	−	−	−	−	−	−
Italy [2]	VI-1	+	F	14 days	+	+	−	−	+	+	−	/	/
	VII-2	+	M	2 months	−	+	+	−	+	+	−	/	/
	V-2	+	M	15 days	−	+	+	−	−	−	−	/	/
	VI-4	+	F	2 months	−	+	+	+	+	+	−	/	/
	VII-3	+	M	10 days	−	+	+	−	+	+	−	/	/
	V-5	+	M	20 days	+	+	−	+	+	+	+	/	/
	IV-11	+	M	/	/	/	/	/	/	/	/	/	/
	VI-11	+	M	−	−	−	−	−	−	−	−	−	−
	V-3	+	M	/	/	/	/	/	/	/	/	/	/

F female, *M* male, / no data available, *R* right, *L* left, + presence of the common mutation or clinical signs, − absence of the common mutation or clinical signs, *S* soon after birth

[a] Families presented on this paper

Mutational analysis of *COL1A1* and *COL2A2*

The mutation analysis was approved by the ethics committees of Osaka University Graduate School of Medicine and Keio University School of Medicine, and informed consent was obtained from the proband and family members for the analysis of the *COL1A1* and *COL2A2* genes. Genomic DNA was extracted from peripheral blood by using the QuickGene-810 and QuickGene DNA whole blood kit (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions. We analyzed the previously reported mutation in *COL1A1* by digestion of the purified polymerase chain

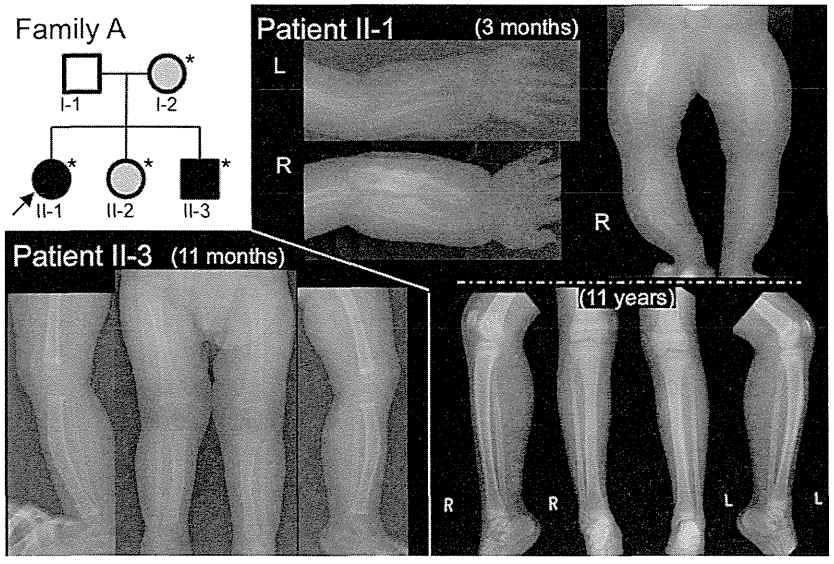

Fig. 1 *Left upper panel* shows the pedigree of family A (*COL1A1* mutation positive). *Black symbol*, affected individual; *white symbols*, no symptoms according to history; *gray symbols*, mutation-positive, but healthy family members; *asterisk*, mutation-positive individual. The *arrow* indicates proband of family A. *Right upper panel* (II-1 at the age of 3 months): cortical bone thickness of the right radius, right ulna, both femora, and the right tibia. *Right lower panel* (II-1 at the age of 11 years): mild deformity of the legs. *Left lower panel* (II-3 at the age of 11 months): mild deformity of both tibiae

reaction (PCR) product with HypCH4IV [10]. In family B, we analyzed all coding exons and flanking introns of *COL1A1* and *COL1A2* by PCR and direct sequencing.

Dual-energy X-ray absorptiometry analysis

The bone mineral density (BMD) *Z* score or *T* score of the lumbar spine for L_{2-4} was determined by dual-energy X-ray absorptiometry (DXA; Discovery A, Hologic).

Results

Mutation

The proband of family A had the common heterozygous p.Arg1014Cys mutation in *COL1A1*, as did her affected younger brother. In addition, her mother and younger sister also carry the same mutation, but they have had no history of soft tissue swelling and bone deformity. They also have no

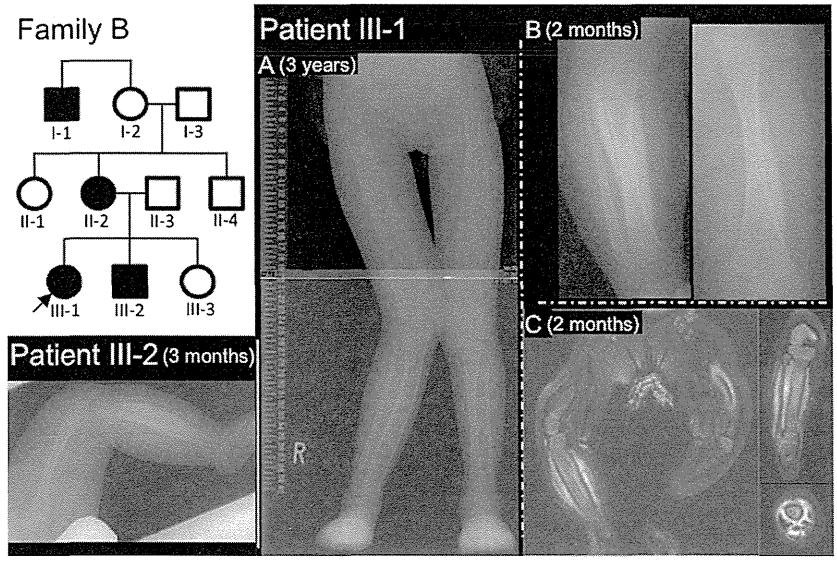

Fig. 2 *Left upper panel* shows the pedigree of family B (mutation negative). *Black symbol*, affected individual; *white symbols*, no symptoms according to history. The *arrow* indicates the proband of family B. *Right panel*: **a** (III-1 at the age of 3 years) bone deformity of both femora and the right tibia, **b** (III-1 at the age of 2 months) cortical bone thickness of the right tibia and swelling of the surrounding soft tissues, and **c** (III-1 at the age of 2 months) MRIs (fat-saturated T2-weighted images) revealing large lesions in the surrounding soft tissues and bone marrow with increased signal intensity on lower legs. *Left lower panel* (III-2 at the age of 3 months): cortical bone thickness of left lower leg

evidence of either joint laxity or soft skin. Her healthy father could not be examined.

The three patients in family B had no detectable mutation in either *COL1A1* or *COL2A2*. Exome sequencing was not feasible in this family.

BMD

The proband of family A showed a BMD of 0.852 g/cm^2 of L2–L4 (*Z* score +0.3) at the age of 12 years. Her mother, who has regular menstrual cycles, had a L2–L4 and total hip BMD of 1.096 g/cm^2 (*T*score +0.8) and 0.990 g/cm^2 (*T*score +1.2), respectively, at the age of 36 years.

The mother (II-2) of family B, who also has regular menstrual cycles, had L2–L4 BMD of 1.138 g/cm^2 (*T*score +1.1) at the age of 40 years.

Discussion

We found the common *COL1A1* mutation, p.Arg1014Cys, in the proband of family A (II-1) and the younger brother (II-3) who both presented with clinical and radiographic findings consistent with Caffey disease, whereas the same mutation was detected in her mother (I-2) who was asymptomatic and with normal radiological findings. Furthermore, her sister (II-2) who had no signs or symptoms suggesting this disease had also the same mutation. This family indicates rather low penetrance of Caffey disease as has been reported previously [3, 9, 10]. Gensure et al. reported that only 79 % of the family members with this mutation had signs or symptoms of Caffey disease [10]. Although the precise mechanism has yet to be elucidated, it is likely that an additional genetic or environmental condition is required for the manifestation of the disease. Likewise, the factor(s) contributing to the recurrence of the disease is unknown [1]. However, it is most likely that the p.Arg1014Cys substitution in *COL1A1* is linked to Caffey disease because several familial cases have been reported to have this mutation (Table 1). In the "osteogenesis imperfecta & Ehlers-Danlos syndrome variant databases" [6], this mutation was not found in patients with osteogenesis imperfecta or healthy subjects.

No mutations in *COL1A1* or *COL1A2* were identified in the patients of family B. However, bone lesions consistent with the diagnosis of familial Caffey disease were found in the femora, tibiae, and fibulae of the affected individuals in family B (Table 1). Thus, there can be considerable overlap between familial cases with and without the *COL1A1* mutation (Table 1). Since we could not identify a novel mutation in *COL1A1* or *COL1A2*, it appears likely that another heterozygous mutation in an as-of-yet unknown gene is responsible for the affected members of family B and possibly other cases of Caffey disease, too, either infantile or prenatal forms. This is strong evidence that similar phenotypic findings can be caused by different mutations. Exome analysis may be necessary to identify the responsible gene, although relatively low penetrance and few family members may prevent identification.

Increased T2-weighted images in patient II-1 of family B reflected increased water content consistent with edema surrounding the hyperostotic cortical bone and bone marrow [17, 18]. Although the pathogenesis of Caffey disease remains unclear, this edema suggested inflammatory events in the soft connective tissues surrounding the affected bone and bone marrow [11]. Inflammatory cytokines including prostaglandin E may stimulate periosteal reaction, although we did not examine inflammatory cytokines in our patients.

Bone mineral density of patients with Caffey disease has been reported in only one patient [20]; however, the relation between bone mineral density and the p.Arg1014Cys mutation has not been investigated. The patients in family A, the girls and their asymptomatic mother, had normal bone mineral density. In addition, the mother of family B who was symptomatic during childhood and does not carry the common mutation also had normal bone mineral density. These results suggest that Caffey disease, regardless of the p.Arg1014Cys mutation, is not associated with alterations in bone strength.

In conclusion, we obtained further evidence for genetic heterogeneity of Caffey disease and demonstrated normal bone mineral density in adult patients with this disease.

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Conflict of interest All the authors do not have anything to declare.

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Regenerative Medicine for Spinal Cord Injury Using Olfactory Mucosa Autografts

Koichi Iwatsuki and Toshiki Yoshimine

Abstract. The central nervous system has limited regenerative capacity, and functional restoration of the damaged system is difficult. Treatment is still limited to cerebrospinal protection after injury and reconstruction of neural networks by rehabilitation. In case of incomplete spinal cord injury, neural networks can be reconstructed because nerve tissues remain at the site of the injury. However, in case of complete spinal cord injury, all scaffolds for reconstruction of neural networks are lost. Thus, functional recovery through rehabilitation cannot be expected. At present, enhancement of residual function is the only treatment approach. Regarding spinal cord injury, expectations have been placed on regenerative medicine using stem cells including induced pluripotent stem cells, which have recently captured attention. However, regenerative medicine using stem cells is effective only during the acute-to-subacute phase, a period before scar tissues are formed in the injured spinal cord. Regenerative medicine using stem cells is completely ineffective in the chronic phase. In case of planning treatment for chronic-phase spinal cord injury, it is necessary to supply scaffolds where neuronal axons can grow to form neural networks, neurons, and neurotrophic factors for growth and protection of neuronal axons. In other words, all of the 3 elements, i.e.,scaffolds, neurons, and neurotrophic factors are necessary. In addition, transplantation including these 3 elements requires avoiding ethical problems and immunological rejection. These conditions are not satisfied by cell transplantation, but by tissue transplantation, particularly by autologous tissue transplantation. The olfactory mucosa contains olfactory nerves associated with olfaction and is an extracranial region with exceptionally active nerve regeneration. The mucosa, which is embryologically derived from the central nerve as primordium, contains stem cells, olfactory ensheathing cells that have axonal growth effects, and various neurotrophic factors. The mucosa is endoscopically resectable and spontaneously regenerates after resection. Because the olfactory mucosa allows active nerve regeneration under physiological conditions, it is considered useful as a scaffold for neuronal axon regeneration. We conducted an animal study and then a human clinical study on treatment of chronic spinal cord injury using olfactory mucosa autografts. At the end of 2011, this treatment was designated as an advanced medical treatment.

Keywords: spinal cord injury, olfactory mucosa, regeneration

1 Introduction

Olfactory mucosa autografts for complete paraplegic patients with chronic-phase spinal cord injury were first performed by Lima et al. at Egas-Moniz Hospital in Lisbon, Portugal, in 2001. [17], [18] Olfactory mucosa is the only extracranial region where nerve regeneration is observed under physiological conditions [7]; the mucosa contains olfactory ensheathing cells (OEC) and neural stem cells that contribute to the repair of spinal cord injury. [22] Especially, because an autologous graft can be endoscopically harvested from the nasal cavity of the patient, [38] neither immunological rejection nor ethical problems become an issue. We have been performing this procedure since 2007 in Japan, and are currently investigating its safety and efficacy.

2 History of experimental studies on spinal cord injury

In the late 1970s, transplantation of fetal nerve tissue was introduced as an experimental therapy for central nerve injury. In a model of brain injury, neurons arising from an embryonic graft and neurotransmitters secreted from the injured region in the host brain, which indicated enhanced

axonal growth and functional recovery, were confirmed. [1] Because fetal nerve tissue contains neurons, the mechanism of neuronal axonal restoration based on this transplantation procedure was attributed to formation of bidirectional synaptic connections between neurons contained in the transplanted tissue and the host spinal cord. [4] This discovery gave a new direction to basic studies on spinal cord injury towards clinical practice. However, this procedure could not be applied in humans because, as transplantation for one patient with spinal cord injury required 10 to 15 fetuses, the procedure was ethically unacceptable. Then, neural stem cells or embryonic stem (ES) cells contained in fetal tissue attracted attention.

Many therapies using stem cell transplantation to treat spinal cord injury are under investigation, and great expectations are placed especially on induced pluripotent stem (iPS) cells and ES cells. On the other hand, survival of transplanted stem cells is low in cell-based therapies including stem cells. It has been revealed that many of them die within 24 hours after transplantation. [14] In recent studies, development of scaffolds for survival of these cells is a major trend of research. [32]

3 Acute and chronic phases of spinal cord injury

The pathological conditions of spinal cord injury in the acute-to-subacute and chronic phases are completely different. Thus, treatment strategies in each phase also differ.

In the injured spinal cord, cells are destroyed, and both nerve fibers and blood vessels are sheared. This is the so-called primary injury, followed by the secondary cytotoxic injury, such as inflammation. [31] However, inflammatory responses after the primary injury that are considered the secondary injury are reported to be important for repair. [28] There are ongoing studies to clarify the cytokines or cells associated with exacerbation of pathological conditions or repair in the acute phase. [2]

The injury site loses neurons and, in the chronic phase after the secondary injury, is covered by glial scar tissue that is unlikely to allow axonal regeneration. [9] Thus, treatment in the chronic phase requires replenishment of neurons, facilitation of axonal growth, reduction of glial scar tissue that inhibits axonal regeneration and improvement of the spinal microenvironment that inhibits spontaneous axonal regeneration. [21], [33] In other words, it is necessary to create the conditions that favor the formation of scaffolds for axonal growth. [17]

4 Cell transplantation therapy in the acute-to-subacute phase

Regarding transplantation therapy using stem cells, such as ES and iPS cells, or using neural cells differentiated from these stem cells, functional recovery owing to remyelination of axons, etc. has been reported, and there are great expectations concerning the use of stem cells. [13], [24] Geron Corporation, an American entrepreneurial venture, developed a therapy for spinal cord injury using human ES cells, which was approved by the Food and Drug Administration of the United States in 2010. Although this therapy was performed in the first case in October 2010, Geron recently withdrew from its development. Regarding the use of human ES cells in clinical studies or treatment in Japan, neither safety issues nor ethical problems, similar to those concerning aborted fetuses, have been solved. Because human iPS cells derived from autologous cells are associated with fewer ethical problems, studies aiming at clinical application of iPS cells are being vigorously conducted. However, their mechanism to produce effects is considered to be basically the same as that of ES cells. The effects of iPS cells may be limited to the first 8 days after injury.

Bone marrow stromal cells contain abundant neurotrophic factors that have neuroprotective effects, [26] and clinical studies have been conducted in several countries for a long period. The clinical studies on transplantation of bone marrow stromal cells in the acute-to-subacute phase conducted in South Korea and Czechoslovakia demonstrated neurological functional recovery. [35], [36], [39] In Japan, bone marrow stromal cells were transplanted into 3 patients with acute-phase spinal cord injury at Kansai Medical University Hospital. No serious adverse event has been reported until now. Further development of the technique is expected.

In these clinical studies, bone marrow stromal cells were transplanted into patients in the acute-to-subacute phase of the lesion. Because this phase corresponds to a period when some spontaneous recovery may be possible even in cases with complete paraplegia, the efficacy of the cell transplantation is difficult to discuss. However, the future progress of several studies is expected.

5 Transplantation therapy in the chronic phase

In the chronic phase of spinal cord injury, death of neurons, rupture of neuronal axons, and glial scar occur. Thus, replenishment of neurons, axonal growth factors for neural network construction, and scaffolds for axonal growth, are necessary. Many investigators have confirmed that, if peripheral nerves or cultured Schwann cells are transplanted, central neuronal axons will grow in the transplant as a permissive scaffold. Cheng et al. resected spinal cord segments of adult rats and bridged the gaps with several intercostal nerve grafts with the aim to reduce an inhibitory environment.[5] Transplantation of OEC performed by Li et al. was also an attempt to reduce the inhibitory effect of the environment.[16] Thus, 3 factors consisting of cells that can replenish lost neurons, axonal growth factors, and scaffolds permissive for axonal growth are necessary in the chronic phase.

5.1 Neural stem cells

Although neural stem cells can serve for replenishment of neurons, simply transplanted stem cells cannot survive for a long period.[25] However, recent studies have revealed that, if the local microenvironment at the spinal cord injury is improved, nerves and axons can regenerate.[4],[8] Transplanted neural stem cells not only differentiate into neural cells but also secrete various and numerous factors that facilitate axonal growth.[10] Transplantation of neural stem cells is promising in terms of cell replenishment and axonal growth. However, it has been revealed that transplantation of neural stem cells alone cannot exert a sufficient regenerative effect without improvement of the local microenvironment at the spinal cord injury.[10]

5.2 Olfactory ensheathing cells (OEC)

The olfactory system that extends from the olfactory mucosa to the olfactory bulb of the central nervous system is an exceptional tissue where nerves and axons regenerate under physiological conditions throughout nearly the entire life.[7] This exceptional neuroregenerative effect is attributed mainly to neural stem cells and OEC contained in the olfactory mucosal epithelium.[29] Of these cells, OEC are cells that have axonal growth effects in the chronic phase of spinal cord injury.[20] Unlike oligodendrocytes in the central nervous system or Schwann cells in peripheral nerves, OEC can extend neuronal axons from the olfactory nerves at the periphery to the olfactory bulb at the center.[6],[29] Unlike Schwann cells, it has been revealed that adult OEC extend axons from retinal ganglion cells which are classified as central nerves,[15] and that OEC cocultured with

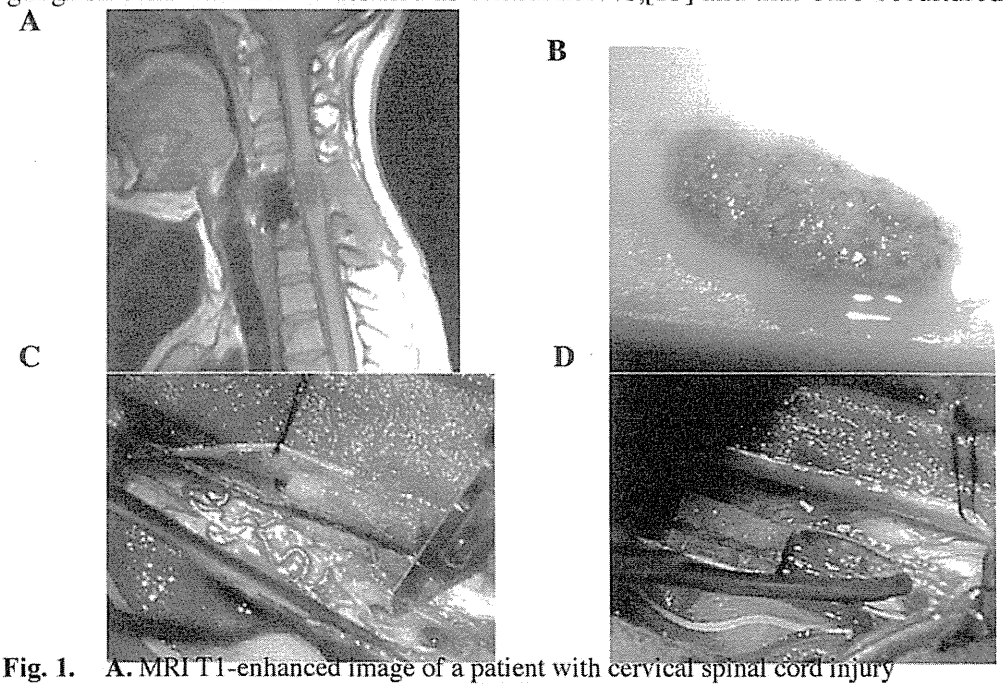


Fig. 1. A. MRI T1-enhanced image of a patient with cervical spinal cord injury

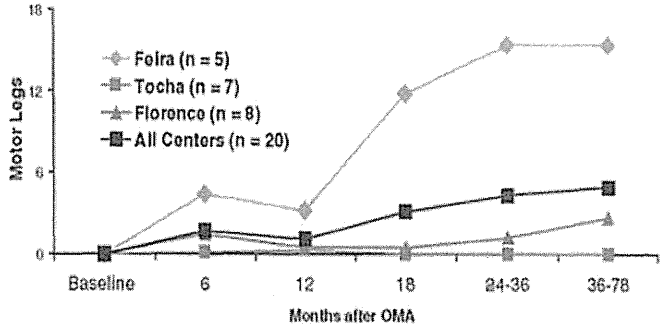
B. Autologous olfactory mucosa cut into small pieces
C. Removal of intramedullary scar tissue. Opening of posterior median sulcus
D. Transplantation of olfactory mucosa into the cavity after removal of scar tissue

hippocampal neurons better integrate with astrocytes as compared to OEC cocultured with Schwann cells.[37] OEC have a protective action on extended axons against axonal inhibitory factors in adult central nerves.[6] OEC transplanted into the injured spinal cord secrete abundant axonal growth factors and form a scaffold for axonal growth.[12],[16] In addition, it has been revealed that OEC promote myelination of axons to improve nerve conduction velocity[19],[34] and regenerate the transected descending axonal pathway to restore function.[16],[30] Furthermore, OEC infiltrate the glial scar tissue to some extent and secretevarious neurotrophic factors and adhesion molecules to facilitate axonal growth.[12] Mackay-Sim et al. at Griffith University, transplanted OEC into 6 patients with chronic-phase spinal cord injury, and reported that no serious adverse event occurred during 3 years after transplantation.[20] In this clinical study, neurological functional recovery was observed in 1 patient.

5.3 Olfactory mucosa

Because the spinal cord is a naturally inhibitory environment for axonal growth,[21],[33] formation of scaffolds is necessary for the survival of transplanted stem cells and axonal growth.[17],[32] The olfactory mucosa contains neural stem cells that can replenish neurons and OEC that exert a neuronal axonal growth action; thus, neurons actively regenerate in the olfactory mucosa. Based on these findings, the olfactory mucosa itself would be useful as a scaffold of regeneration of neuronal axons.[7] Basic studies using rats have also confirmed the usefulness of the olfactory mucosa.[3],[11] The olfactory mucosa may be an ideal graft to treat chronic-phase spinal cord injury.[17],[18]

Fig.2.



ASIA Motor Legs scores and WISCI and FIM after OMA with rehabilitation at individual centers. (A) ASIA motor legs scores at given times after OMA. After preoperative rehabilitation (mean = 8 months; range = 1-27 months), all 20 patients had a motor leg scores of 0. The greatest improvement after OMA was the primarily paraplegic patients receiving rehabilitation at SS (♦) with 5/5 patients improving, some improvement primarily in tetraplegics at GC (▲) with 4/8 patients improving, and no improvement (7/7 patients) in the primarily tetraplegics at RP (□). (Lima C. et al. Olfactory mucosal autografts and rehabilitation for chronic traumatic spinal cord injury. Neurorehabil Neural Repair 2010 Jan; 24(1):10-22)

6 Olfactory mucosa transplantation to treat spinal cord injury

According to autologous olfactory mucosa transplantation to treat spinal cord injury in the chronic phase, that is at least 6 months after spinal cord injury (Fig. 1A), the mucosa is endoscopically removed and cut into small pieces (Fig. 1B). After the intramedullary scar tissue at the injury site is removed (Fig. 1C), the pieces of olfactory mucosa are transplanted into the cavity left by the scar tissue (Fig. 1D). Although the scar tissue is just partially removed not to damage normal spinal cord tissue, sufficient opening of the posterior median sulcus is necessary along the cephalocaudal axis of the injury site for bridging the gap in the spinal cord.[17],[18] Lima et al. performed autologous olfactory mucosa transplantation in 20 patients with chronic-phase spinal cord injury that caused complete motor paralysis of both lower limbs; there were 17 men and 3 women whose ages ranged from 19 to 37 years. The patients underwent intensive rehabilitation. After a follow-up period of 12 to 45 months after the operation (mean: 27.7 months), American Spinal Injury Association (ASIA) classification scores improved from A to C in 6 patients, from B to C in 3 patients, and from A to B in 2 patients. Electromyography (EMG) of lower limb muscles revealed voluntary contractions in 15 patients. Furthermore, Lima et al. reported that improvement in bladder function test results was achieved in 5 patients (Fig. 2). All patients recovered olfaction, and recovery was achieved within 2 months after the operation in 95% of the patients. Regarding adverse events, subcutaneous accumulation of spinal fluid was observed in 3 patients, all of which resolved naturally or after a simple suture. In 1 patient, hypersensitive enteritis was observed 1 year after the operation and reported to have persisted for 5 years. This event is considered to be visceral neuropathic pain. Moreover, another patient concomitantly developed bacterial meningitis caused by methicillin-resistant Staphylococcus aureus, which was cured with vancomycin. However, the ASIA classification score worsened from B to A. Two months later, it improved to B again.[17],[18]

The team at Detroit Medical Center provided a similar rehabilitation program to 38 patients who underwent olfactory mucosa transplantation and 22 patients without transplantation. Although no statistically significant difference was observed, the team reported that motor function improved in 58% of the patients with transplantation, versus 27% of those without transplantation (The 26th Annual National Neurotrauma Society Symposium held in July 2008).

Since 2002, we have been conducting a clinical study to develop therapies for functional recovery of injured spinal cord by autologous olfactory mucosa transplantation. The inclusion criteria are: at least a 6-month period after the occurrence of spinal cord injury, age 40 years or younger, complete motor paralysis of the lower limbs of class A or B in the Frankel or ASIA classification, a 3-cm or shorter injury site on magnetic resonance imaging, and no infection in the nasal cavity. As of April 2010, 4 patients with thoracic spinal cord injury causing complete motor paralysis of both lower limbs underwent autologous olfactory mucosa transplantation: 2 patients on February 7 to 8, 2008, 1 on July 17, 2009, and 1 on March 19, 2010. In these 4 patients, there has been neither infection nor development of a malignant neoplasm associated with this procedure. Although hyposmia, headache, and pain at the site of the spinal cord injury occurred as adverse events in some patients, all events resolved without progressing to a serious condition. There has been no safety problem that would affect the continuation of the study. EMG revealed waveforms generated from the lower rectus abdominis muscle, paraspinal muscle, and tensor fasciae latae muscle in 1 of the 4 patients and EMG waveforms generated from the lower rectus abdominis muscle in another patient. Moreover, EMG waveforms were generated from the quadriceps femoris muscle in 1 of the 2 remaining patients. Lima et al. emphasize the importance of providing long-term rehabilitation in combination with olfactory mucosa transplantation. They discuss that recovery cannot be expected by olfactory mucosa transplantation or rehabilitation alone, and that rehabilitation for remodeling of skeletal muscles, blood vessels, and nerves is necessary. Although the ideal type of rehabilitation remains unknown, Lima et al. especially advocate the importance of walking rehabilitation with weight bearing that is called brain-initiated overground nonrobotic/nonweight supported training (BIONT).[17],[18]

7 Conclusion

Regarding spinal cord injury, there is evidence that combination therapy using transplantation with other factors is more effective than cell-based transplantation therapy alone.[23],[27] It may be difficult to achieve success using a single factor, such as specific cells. Olfactory mucosa is an ideal transplantation tissue at present because it contains the cells, axonal growth factors, and provides a scaffold for regeneration. Because reconstructed nervous tissue obtained by transplantation is not

natural, remodeling of skeletal muscles, blood vessels, and reconstructed neural networks is necessary. Thus, rehabilitation after transplantation is very important.

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Involuntary muscle spasm expressed as motor evoked potential after olfactory mucosa autograft in patients with chronic spinal cord injury and complete paraplegia

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ABSTRACT

Object: The efficacy of olfactory mucosa autograft (OMA) for chronic spinal cord injury has been reported. New activity in response to voluntary effort has been documented by electromyography (EMG), but the emergence of motor evoked potential (MEP) reflecting electrophysiological conductivity in the central nervous system, including the corticospinal pathway, after OMA, and the best indications for OMA, have not been clarified. Here, we report the emergence of MEPs after OMA and offer recommendations for appropriate indications based on the presence of involuntary muscle spasm (IMS). We used analysis of MEP to examine the efficacy of OMA for patients with complete paraplegia due to chronic spinal cord injury. To clarify the indications for OMA, we investigated the association of IMS and efficacy of OMA. Methods: Four patients, 3 men and 1 woman, were enrolled. The mean age of the cases was 30.3 ± 9.5 years (range, 19 to 40 years). All 4 cases were American Spinal Injury Association (ASISA) grade A. The mean duration from injury to OMA was 95.8 ± 68.2 months (range, 17 to 300 months). Samples of olfactory mucosa were removed, cut into smaller pieces, and grafted into the sites of spinal cord lesions after laminectomy. Postoperative subcutaneous fluid collection, postoperative meningitis, postoperative nosebleed, postoperative infection in the nasal cavity, impaired olfaction, neoplastic tissue overgrowth at the autograft site, new sensory disturbance, and involuntary muscle spasm were investigated as safety issues. Improvements in ASIA grade, variations in ASIA scores, EMG, SSEP, and improved urological function were evaluated as efficacy indicators. Results: There were no serious adverse events in this series. In 2 of the 4 cases, an improvement in motor function below the level of injury was recognized. In one, the motor score was 50 until 16 weeks after surgery, and it increased to 52 from 20 weeks after surgery. In the other, the motor score was 50 until 20 weeks after surgery, and it increased to 52 at 24 weeks after surgery with a further increase to 54 at 48 weeks after surgery. The emergence of MEP was recognized in the latter case at 96 weeks after surgery. The other 2 cases had no improvement in ASIA motor score. Both of these cases who showed improvements in the ASIA motor scores exhibited relative IMS compared with those who had no ASIA motor score recovery. Conclusions: We recognized the emergence of MEPs in a case with complete paraplegia due to chronic spinal cord injury after OMA. IMS might be a candidate of indication of OMA.

Keywords: Olfactory Mucosa Autograft; Spinal Cord Injury; Transplantation; Voluntary Movement; Motor Evoked Potential

1. INTRODUCTION

The olfactory mucosa is an excellent autologous source of adult neuronal precursor cells. The neurons and sustentacular cells of the olfactory mucosa constantly renew themselves throughout life by proliferation of basal global stem cells [1-3]. Furthermore, the mucosa contains olfactory ensheathing cells, which have previously received much attention for their potential application in

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the repair of spinal cord injuries (SCIs) [4-7]. Recent studies of spinal cord axon regeneration have reported good long-term results using various types of tissue scaffolds [8-10]. Olfactory tissue, which allows autologous transplantation, is easily accessible, and can be obtained by a simple biopsy performed through the external nares [11].

We have previously reported that grafts of the olfactory mucosa are effective in restoring functional recovery in rats following spinal cord transection, with histological evidence of neuronal regeneration [12-14]. Lima *et al.* performed a clinical trial of olfactory mucosal autograft (OMA) in humans with chronic traumatic SCI and reported restoration of voluntary electromyography (EMG) responses in 15 of 20 cases (75%) and mean American Spinal Injury Association (ASIA) motor score improvement of 4.95 ± 7.1 over a mean follow-up period of 27.7 months. The range of improvement was various, and some cases demonstrated no response to OMA. Therefore, evaluating the possible factors that could predict the efficacy of OMA will be useful. Inclusion criteria for the study by Lima *et al.* included age, extent of the lesion, and time from the injury, but the authors did not assess the neuronal condition of the severed caudal spinal cord [15]. Furthermore, they did not assess the emergence of motor evoked potential (MEP), which reflects electrophysiological conductivity in the central nervous system, including the corticospinal pathway [16,17], after OMA.

The emergence of involuntary muscle spasm (IMS) after SCI is an indirect measure of the recovery of motor neurons and general motor function [18]. It may indicate plasticity of the spinal cord and the potential for successful regenerative interventions in patients with chronic SCI.

Our pilot study was conducted to examine the emergence of MEP and IMS in OMA patients with chronic SCI and complete paraplegia.

2. MATERIAL & METHODS

2.1. Patient Selection and Inclusion Criteria

This phase I/II nonrandomized, non-controlled, prospective study was approved by the Ethical Committee of the Osaka University Medical School in Osaka, Japan. All procedures were performed after obtaining written informed consent, which included permission to culture and analyze a biopsy from the tissue to be grafted. Patients were fully aware of the experimental nature of the treatment, the uncertain outcomes, and possible side effects including pain, spasticity, autonomic dysreflexia, worsening of motor or sensory function, infection, and unforeseen adverse events.

Patients who had sustained SCI more than 6 months previously with chronic paraplegia (**Table 1**) were included. Our rationale for selecting chronic SCI patients (more than 6 months from injury) was to circumvent the spontaneous recovery bias [19]. The other inclusion criteria of this study were generally consistent with those of Lima *et al.* [15] and comprised ASIA Ggrade A or B; age ≥ 7 and ≤ 40 years; presence of a spinal cord lesion ≤ 3 cm; absence of significant nasal and paranasal sinus pathology; and absence of additional serious medical problems including respiratory disturbance, brain disease, or psychological disturbance.

Four patients were enrolled in the study, 3 males and 1 female. Demographic data, clinical findings, and imaging/radiological characteristics of the patients are presented in **Table 1**. The mean age of the patients was 30.3 ± 9.5 years (range, 19 to 40 years). Injuries were due to traffic accidents in 2 patients, fall in 1 patient, and hemorrhage of unknown origin in 1 patient. The mean maximum lesion size on the vertical axis as measured on both the T1- and T2-weighted MRI was 2.25 ± 0.57 cm (range, 1.55 to 2.94 cm). All 4 patients were ASISA grade A. The mean time from injury to OMA was 95. 8 ± 68.2 months (range, 17 to 300 months).

2.2. Transplantation Protocol and Surgical Procedure

Our procedure essentially followed that reported by Lima *et al.* [15,20]. Samples of olfactory mucosa were removed, cut into smaller pieces, and grafted into the spinal cord lesion site after laminectomy. Microbiological examinations of the nasal cavities were performed routinely before surgery and during the operation just prior to transplantation.

Table 1. Summary of demographic and clinical characteristics of 4 patients with olfactory mucosa autografts (OMA).

Case No. (years)	Age at OMA	Sex	Months Post-SCI	SCI Level	Length of Lesion	AIS Grade
1	40	Male	300	T4-5	2.2	A
2	19	Female	30	T7-9	2.3	A
3	26	Male	17	T12	1.55	A
4	36	Male	36	T7-8	2.94	A

Abbreviations: SCI, spinal cord injury; T, thoracic; AIS, ASIA Impairment Scale.

2.3. Pre- and Postoperative Rehabilitation

All patients underwent preoperative rehabilitation (15 h/week for 4 weeks) and postoperative rehabilitation (15 h/week for 48 weeks). The preoperative rehabilitation was carried out until immediately prior to the operation and baseline parameters were determined after the preoperative rehabilitation in order to confirm stabilized neurological status. Rehabilitation included standard physical therapy strategies encouraging motor function at and below the lesion to facilitate walking training as soon as possible.

2.4. Outcome Measures

Safety and efficacy measures are presented in **Table 2**. Any improvement in the ASIA grade scale or/and lower extremity motor scores was considered evidence of true gains since all patients had ASIA motor scores of 0 for both legs after the preoperative rehabilitation. The pre- and postoperative assessment protocol included an ASIA neurological exam, as described in the International Standards for Neurological and Functional Classification of Spinal Cord Injury Patients [21] as well as standard EMG, with recordings taken after patients were asked to move particular muscles, and somatosensory evoked potentials (SSEP), cortically recorded after tibial nerve stimulation; urodynamic studies; full spinal cord MRI scan; otolaryngological evaluation including a general ear, nose, and throat examination, nasal endoscopy, olfactory evaluation, and computed tomography scan of the nose and paranasal sinuses; and psychological assessment. Psychological testing was intended to detect conditions such as active psychosis, major depression, anxiety disorder, severe mood disorder, suicidal behavior, alcohol addiction, drug addiction, low cognitive resources, and unrealistic expectations about treatment results. Pain was assessed via interviews asking the patients to identify painful areas, describe the pain using standard descriptors, and identify temporal aspects of the pain.

Table 2. Outcome measures.

Safety Measures
Postoperative subcutaneous fluid collection
Postoperative meningitis
Postoperative nasal bleeding
Postoperative infection in the nasal cavity
Impaired olfaction
Neoplastic tissue overgrowth in the transplantation
New sensory disturbance
Involuntary muscle spasm (IMS)
Efficacy Measures
Ability to improve ASIA grade
Variation in ASIA scores
EMG
SSEP
Urological improvement

EMG: Electromyograph; SSEP: Somatosensory Evoked Potential.

We evaluated IMS. There is a variety of tests that attempt to quantify spasticity, but there is no uniformly accepted useful measure [22,23]. Our method was simply to note the emergence of IMS on EMG. IMS was evaluated in the bilateral biceps femoris, anterior tibial, flexor digitorum brevis, femoral quadriceps, gluteus maximus, and gastrocnemius muscles. We watched the emergence of IMS while patients rested in the supine position for 3 min (**Figure 1**).

MEP response to bifocal transcranial magnetic stimulation (TMS) was evaluated in the bilateral rectus femoris muscles. TMS was performed with a coil (7 cm diameter) using a MagPro ×100 (MagVenture A/S, Denmark). Navigation-guided TMS (Brainsight Frameless 1.5; Rogue Research Inc., Montreal, Canada) was used to target the optimal position of each stimulation point. The stimulation hot spot was determined starting about 4 cm rostral of Cz (vertex) [24]. Patients who were not able to produce force were asked to exert as much volitional innervation as possible. The duration of the monophasic transcranial single-pulse stimulus was 100 μs. The sample frequency was 2000 Hz, and a band-pass filter was set at 30 Hz to 1 kHz. TMS was delivered every 5 to 6 s. Three [25] to 5 representative MEPs at the desired stimulus intensity were applied if there was a well-defined response, and up to 10 stimuli were delivered if there was a visible but poorly defined muscle response in order to optimize 3 responses to be stored offline for further analysis [26]. The onset of the fastest response from 4 repeated MEP trials was determined to be the onset latency. The MEP amplitude was calculated from baseline to the negative peak for the largest response out of 4 trials. The intensity of the magnetic stimulus was expressed as a percentage of the maximal stimulator output.

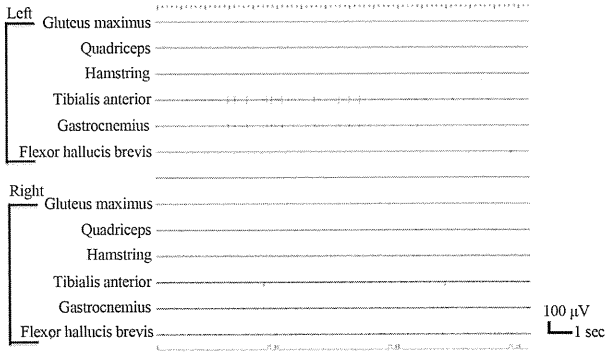

Figure 1. Involuntary muscle spasms were recorded in the left tibialis anterior.

The patients' neurological findings were evaluated preoperatively and 4, 12, 24, 36, and 48 weeks after OMA (MEP was evaluated additionally at 96 weeks).

3. RESULTS

3.1. Safety

No serious adverse event occurred in our series. There was no formation of subcutaneous collection of cerebrospinal fluid along the incision in any case. Two cases had postoperative nosebleed treated with tampon gauze and controlled within 7 days. All cases reported impaired sense of smell. Two regained smell within 12 weeks, and the others regained smell within 48 weeks without any further treatments. No case developed meningitis, nasal infection, or neoplasm after surgery. One case reported transient sensory disturbance consisting of pain around the level of injury (Th4 level) at evaluations both 4 and 12 weeks post-surgery. This disturbance resolved spontaneously without any treatment.

3.2. Efficacy

3.2.1. ASIA Scoring Assessments (Motor)

The data obtained for ASIA motor scores are summarized in **Figure 2**. No change in ASIA motor score was observed in cases 1 or 3, but cases 2 and 4 both demonstrated improved motor function below the level of injury. In case 2, the motor score remained at 50 until 16 weeks after surgery and then increased to 52 from 20 weeks until 48 weeks. In case 4, the motor score was 50 until 20 weeks after surgery and then it increased to 52 at 24 weeks after surgery and further increased to 54 at 48 weeks after surgery.

3.2.2. ASIA Scoring Assessments (Sensory: Pinprick and Light Touch Scores)

No remarkable changes were observed except in case 1, in whom the score decreased after surgery, from 17 to 2 in response to pinprick and from 15 to 2 in response to light touch.

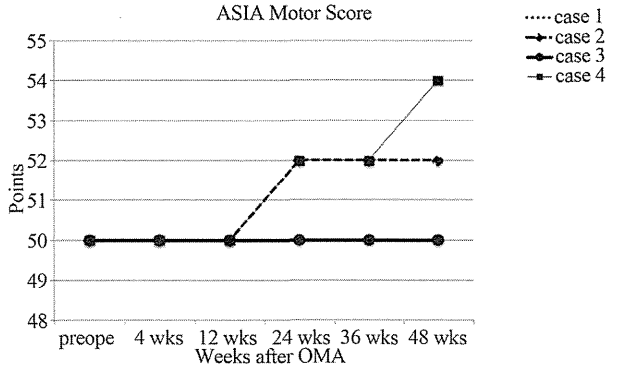

Figure 2. ASIA motor scores. No change in ASIA motor scores was observed in cases 1 and 3, but the score improved from 50 to 52 in case 2 and from 50 to 52 and ultimately 54 in case 4.

3.2.3. Electrophysiological Assessment

1) EMG assessment (voluntary movement)

New voluntary activity in response to voluntary effort was documented by EMG at 48 weeks after surgery in cases 2 and 4. In case 2, the activity was recognized in the bilateral tensor fascia lata muscles. In case 4, the activity was recognized in the bilateral hamstring, anterior tibial, femoral quadriceps, gluteus maximus, and gastrocnemius muscles (**Figure 3**). There was no activity in response to voluntary effort shown in cases 1 or 3.

2) SSEP assessment (somatosensory evoked potential)

There were no changes in SSEPs arising from tibial nerve stimulation recorded at the cortical level in any case.

3.3. Urodynamic Studies

No case experienced urge to urinate before or after OMA, and all cases remained unable to urinate by themselves.

3.4. MRI Findings

MRI 48 weeks after transplantation revealed fairly complete filling of cavities and heterogeneous intensities on T1- and T2-weighted images. Gd-enhanced MRI also showed that the grafts were enhanced heterogeneously. No evidence of neoplastic tissue overgrowth was observed in any case (**Figure 4**).

3.5. Involuntary Muscle Spasm

The evaluation for IMS in each case is documented in **Figure 5**. In case 3, IMS was not recognized preoperatively or throughout the follow-up period to 48 weeks after OMA (**Figure 5**). By contrast, in case 4, IMS was recognized preoperatively and throughout the follow-up period to 48 weeks after OMA (**Figure 5**). Each IMS emergence was calculated as 1 point, as summarized in **Figure 6**. At the beginning of the follow-up period, case 1 had more IMS points than case 2, but this difference reversed in the middle and latter half of the follow-up period. Thus, we concluded that that IMS could be recognized in cases 2 and 4 rather than the cases 1 and or 3.

3.6. Motor Evoked Potentials

MEPs were not observed before transplantation in any case, but they were recorded at 96 weeks after OMA in case 4 (**Figure 7**), although not in any other case.

4. DISCUSSION

The information about OMA derived from the studies of

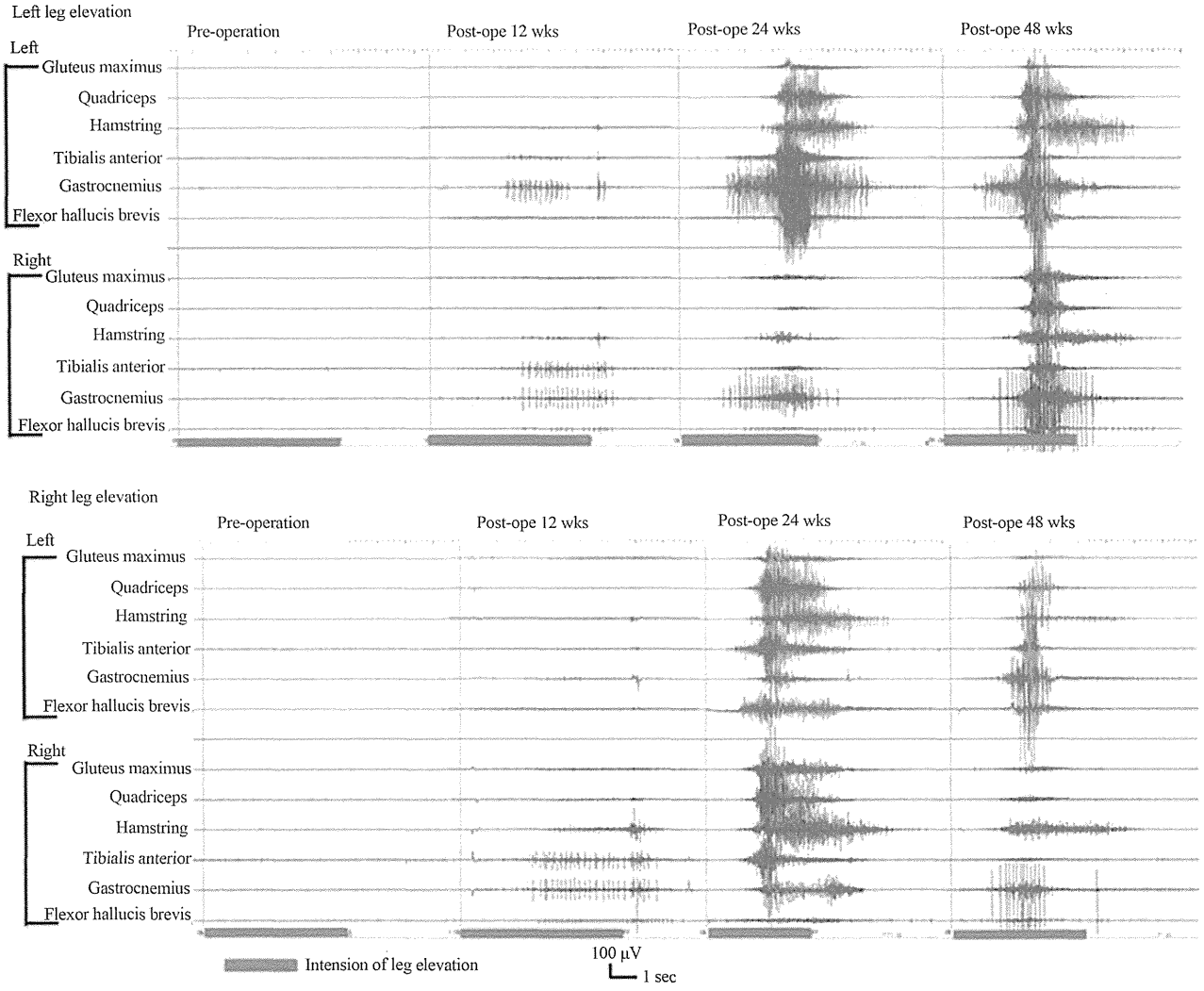

Figure 3. EMG in case 4. New EMG activity in response to voluntary effort was documented at 12, 24, 48 weeks after transplantation. The activity was recognized in the bilateral gluteus maximus, quadriceps, biceps femoris, anterior tibial, gastrocnemius, and abductor hallucis muscles.

Lima *et al.* is invaluable to basic and clinical researchers investigating regeneration in chronic SCI. Their pioneering work in this field revealed that OMA is fairly safe, feasible, and potentially beneficial [15,20]. OMA is advantageous in that it involves transplantation of whole tissue rich in factors that may facilitate neuronal regeneration. We have performed further basic studies of olfactory mucosa transplantation in rats that have supported its feasibility [12,13].

Spinal cord reconstruction using implantation of cells from various sources has gained attention in recent years [27,28]. Neuronal stem cells (NSCs) have the potential to differentiate into both neuronal and glial cells, and are therefore prime candidates for cell replacement therapy following CNS injury. NSCs constitutively secrete significant quantities of several neurotrophic factors that act to support host axonal regeneration after SCI [29]. Partial restoration of function after spinal cord contusion has been achieved by injecting neural/glial precursor NSCs differentiated *in vitro* from mouse embryonic stem cells into the lesion 9 days after injury [30]. However, implantation of NSCs alone did not produce any significant restorative effect because the majority of the NSCs grafted into the spinal cord differentiated into an astrocytic phenotype [29,31]. Although astrocytes can secrete neurotrophic factors and limit the extent of the inflammatory reaction, extensive astroglial scarring within the lesioned area blocks axon growth.

One of the major disadvantages associated with implantation or injection of cells alone is the limited proportion of viable cells surviving at the injury site after the procedure, as cells tend to migrate away from the injury site [32]. To achieve significant functional reconstruction of the spinal cord after SCI, it is necessary to

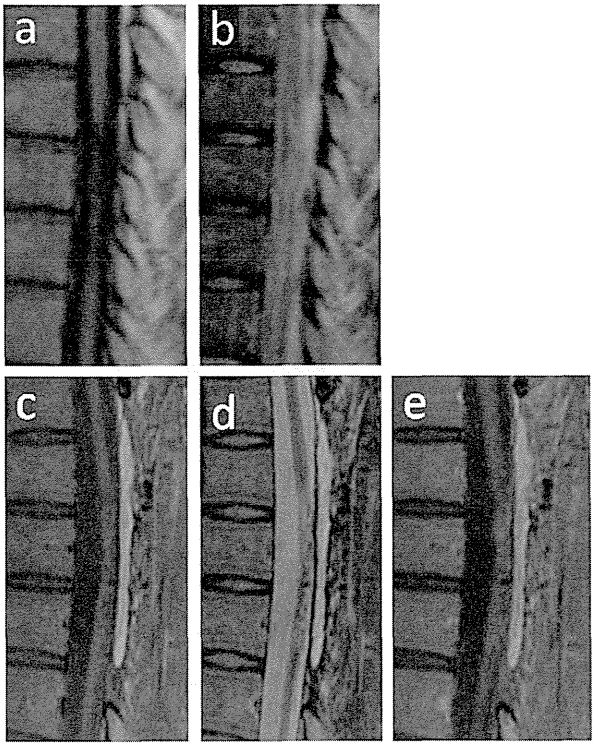

Figure 4. MRI findings in case 2. T1-weighted sagittal image before transplantation showed atrophic change of the thoracic spinal cord (a); T2-weighted sagittal image before transplantation showed an intramedullary high-intensity area (b); MRI scans at 48 weeks after transplantation showed fairly complete filling of cavities and heterogeneous intensity on T1- (c) and T2-weighted images (d); Gd-enhanced MRI showed that the grafts were enhanced heterogeneously (e). No evidence of neoplastic tissue overgrowth was observed.

either populate lesion sites with tissue-specific, regeneration-competent cells or activate endogenous neural progenitor cells to replace or rescue dying cells [33]. The olfactory mucosa seems to be an excellent autologous source of adult neuronal precursor cells. It provides an accessible site for sample biopsy [11] and it contains neurons and sustentacular cells that renew themselves throughout life [1-3] as well as olfactory ensheathing cells that have shown promise in the repair of SCIs [4-7]. These considerations make the nasal mucosa an attractive tissue for potential applications in axonal regeneration. However, while olfactory mucosa may be an ideal tissue for chronic SCI, whether all chronic SCI patients are candidates for OMA remains unclear. Lima *et al.* applied the following inclusion criteria: ASIA impairment grade A or B [34]; age 18 to 40 years; cervical spinal cord lesion below 3 cm or thoracic spinal cord lesion below 4 cm; absence of significant nasal and paranasal sinus pathology; and absence of additional serious medical problems, brain disease, or psychological disturbance [20]. Our present clinical trial generally followed these inclusion criteria and additionally investigated IMS. The emergence of IMS after SCI is an indirect measure of recovery of motor neurons and general motor function [18], and it may function as an indicator for the potential success of regeneration therapy in chronic SCI.

In considering the implications of IMS after SCI in our cases, improved motor function below the level of injury was observed in cases 2 and 4, both of whom exhibited relative IMS before OMA and during rehabilitation after OMA. However, no improvement in motor function was observed in cases 1 and or 3, and neither of them had exhibited relative IMS before OMA or during rehabilitation after OMA (**Figures 5** and **6**). In particular, in case 4, IMS was consistently observed during rehabilitation both before and after OMA (**Figure 6**), and new voluntary muscle activity was recognized in the bilateral hamstring, anterior tibial, femoral quadriceps, gluteus maximus, and gastrocnemius muscles (**Figures 2** and **3**). By contrast, in case 3, no IMS was observed during rehabilitation before or after OMA (**Figures 5** and **6**), and no new voluntary muscle activity was observed (**Figure 2**). The level of injury in this case was Th12. There are motor neurons to generate lower leg movement. These motor neurons were directly injured, and we might not have expected new voluntary muscle activity in such a case. Furthermore, the spinal cord at Th12 is lumbosacral spinal cord, where a central pattern generator (CPG) might exist [4]. The presence of CPG circuitry is thought to be responsible for generation of rhythmic activity within the lumbar cord isolated from brain influence. However, in the absence of descending brain control involved in the initiation of locomotion, CPG activity will be induced by activating afferents from muscles, tendons, and joints by means of peripheral afferent feedback [35]. The CPG in this case might have been injured, and CPG activity might not have been induced by gait training as a peripheral afferent feedback.

We were able to elicit MEPs in case 4. The MEP reflects conductivity in the central nervous system, including the corticospinal pathway [16,17]. MEP induced with transcranial magnetic stimulation allows objective assessment of the integrity of human motor circuitry comprising both the corticospinal tract and peripheral motor nerves [26,36]. The emergence of MEP in case 4 makes this the first report to indicate the recovery of electrophysiological conductivity after complete chronic SCI by any treatment.

In attempts to perform neuronal repair after SCI, strategies to improve the local environment to promote new neuron formation are essential. However, the condition of motor neurons remaining in the spinal cord should also be considered. Patients may not succeed with OMA without functional motor neurons, and the recov

Case 1

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt				■		
	Rt	■					
Anterior tibial muscle (L4,5)	Lt	■				■	
	Rt			■	■	■	
Flexor digitorum brevis muscle (L5,S1)	Lt		■		■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■	■		■		
	Rt	■			■		
Gluteus maximus muscle (L5-S2)	Lt		■				
	Rt						
Gastrocnemius muscle (S1,2)	Lt	■	■			■	
	Rt	■	■	■	■	■	

Case 2

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt				■		
	Rt	■	■		■	■	
Anterior tibial muscle (L4,5)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■
Flexor digitorum brevis muscle (L5,S1)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■				■	
	Rt				■	■	
Gluteus maximus muscle (L5-S2)	Lt						
	Rt		■		■		
Gastrocnemius muscle (S1,2)	Lt			■	■	■	■
	Rt	■	■	■	■	■	■

Case 3

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt						
	Rt						
Anterior tibial muscle (L4,5)	Lt						
	Rt						
Flexor digitorum brevis muscle (L5,S1)	Lt						
	Rt						
Femoral quadriceps muscle (L1-3)	Lt						
	Rt						
Gluteus maximus muscle (L5-S2)	Lt						
	Rt						
Gastrocnemius muscle (S1,2)	Lt						
	Rt						

Case 4

		Preope	4 wks	12 wks	24 wks	36 wks	48 wks
Biceps femoris muscle (L5-S2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Anterior tibial muscle (L4,5)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Flexor digitorum brevis muscle (L5,S1)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Femoral quadriceps muscle (L1-3)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Gluteus maximus muscle (L5-S2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■
Gastrocnemius muscle (S1,2)	Lt	■	■	■	■	■	■
	Rt	■	■	■	■	■	■

Figure 5. Evaluation for emergence of involuntary muscle spasm (IMS) in 4 cases. Black boxes represent the emergence of IMS. Blanks indicate no IMS. No emergence of IMS was recognized throughout the follow-up period in case 3. On the contrary, IMS was recognized throughout the follow-up period in case 4. In both cases 1 and 2, moderate IMS was recognized, although more so in case 2 than in case 1.

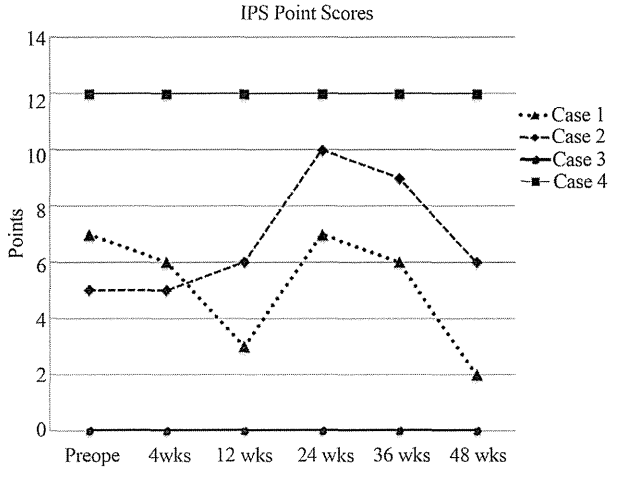

Figure 6. Each emergence of IMS was calculated as 1 point and is summarized in the figure. At the beginning of the follow-up period, case 1 had more points than case 2, but this difference reversed in the middle and latter parts of the follow-up period. Thus, we concluded that relative IMS could be recognized in cases 2 and 4 rather than in cases 1 and 3.

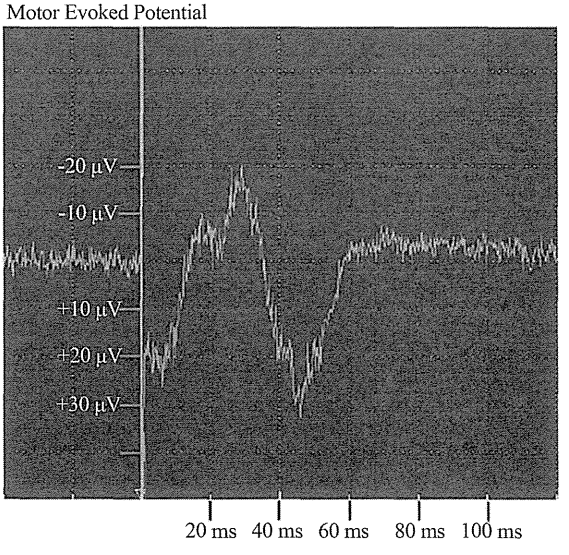

Figure 7. Motor evoked potential (MEP) in response to bifocal transcranial magnetic stimulation (TMS) was evaluated in bilateral rectus femoris muscles. Case 4 exhibited, MEPs in response to TMS.

ery of motor neurons indirectly indicated by IMS may be a predictor of the success of regeneration therapy in chronic SCI. But we have just only four cases in this study. We have to investigate this in our upcoming clinical trial.

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