significant difference in regression slopes in the correlation between MCV and age at examination (regression slope −0.073 for HNPP *v* −0.064 for controls). DL was very prolonged (179% of controls) and prolongation tended to worsen as age at examination increased (r = 0.47). The CMAP was reduced to various degrees in most patients and showed further reduction with advancing age (r = −0.67; fig 1). Worsening of both DL and CMAP with age was significantly more prominent than in controls, as evident from comparison of regression slopes ($p<0.0001$ and <0.01, respectively).

For the ulnar nerve, mild to moderate slowing of MCV and prolongation of DL were noted regardless of age at examination, while CMAP decreased with advancing age (r = −0.65). CMAP diminution with aging was significantly worse in patients than in controls (regression slope −0.109 for HNPP *v* −0.021 for controls; $p<0.0001$). For the tibial nerve, slowing of MCV and prolongation of DL also were mild to moderate in most patients of all ages. Reduction of CMAP was also present in all ages examined but, in contrast to other nerves, the relationship of reduction to aging was indistinguishable from that in controls (regression slope −0.062 for HNPP *v* −0.069 for controls). For the peroneal nerve, the age associated decrement in CMAP was significantly greater than in controls ($p<0.05$). Slowing of MCV and prolongation of DL were present in patients of all ages, but no significant worsening with aging was seen in comparison with controls.

As for sensory conduction studies, slowing of conduction velocity was present as in motor nerves. SCV of the median nerve tended to slow with increasing age at examination (r = −0.41). This age associated worsening was significantly greater than in controls ($p<0.05$), while SCV of the ulnar and sural nerves did not show a correlation with age. Reduction of SNAP was conspicuous in the median (24% of control amplitude), ulnar (28%), and sural (42%) nerves. Age associated reduction of SNAP was seen in the median (r = −0.50), ulnar (r = −0.45), and sural (r = −0.37) nerves, but the rate of change was not worse than in controls.

Duration of CMAP and SNAP was prolonged in all nerves examined compared to normal controls, suggesting the presence of temporal dispersion.[27] Compared to controls, significant age associated worsening was seen only in the SNAP of the median nerve ($p<0.0001$).

Histopathological features

Average total myelinated fibre density in patients' sural nerves was mildly, but not significantly, reduced compared to normal controls (7738 (SD 1253) *v* 8561 (SD 1289) fibers/mm^2; table 3). The density of large myelinated fibres was significantly reduced from that in controls (2458 (SD 730) *v* 3258 (SD 736) fibers/mm^2; $p<0.01$) but that of small myelinated fibres was not (5280 (SD 1025) *v* 5302 (SD 655) fibers/mm^2). Axonal sprouting was not conspicuous in any case. Although the density of large myelinated fibres decreased as age at examination increased (r = −0.70), the rate of reduction was indistinguishable from that in controls (regression slope −27.1 for HNPP *v* −26.0 for controls) because large myelinated fibres were reduced even at younger ages. Teased fibre preparations revealed frequent tomacular change (41.5% (SD 15.8%)). The frequency of segmental

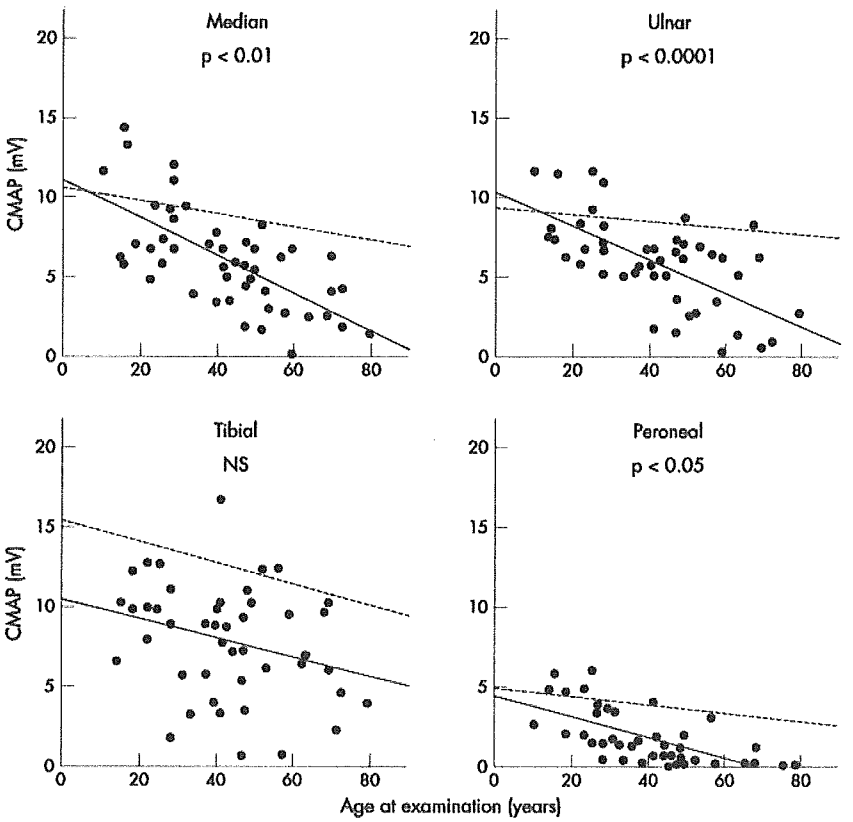

Figure 1 Correlation between CMAP and age at examination in HNPP patients and normal controls. Filled circles represent indices in HNPP patients, bold lines represent regression lines for HNPP patients, and broken lines represent regression lines for normal controls. Comparing regression slopes of normal controls and HNPP patients, CMAP of the median, ulnar, and peroneal nerves, but not the tibial nerve, in HNPP patients were significantly more reduced with increasing age at examination.

Table 2 Nerve conduction studies

	HNPP							Controls		
	Nerve conduction measures				Correlation to aging				Correlation to aging	
	n	Mean (SD)	% of controls	p Values for controls*	r †	Regression slope	p Values for controls‡	Mean (SD)	r †	Regression slope
Motor conduction										
Median nerve										
MCV (m/s)	47	46.0 (5.3)	80	<0.0001	−0.25	−0.073	NS	57.6 (3.8)	−0.27	−0.064
DL (ms)	47	6.1 (1.8)	179	<0.0001	0.47	0.046	<0.0001	3.4 (0.4)	0.19	0.005
CMAP (mV)	48	6.3 (3.2)	77	<0.0001	−0.67	−0.122	<0.01	8.2 (2.9)	−0.24	−0.042
Duration (ms)	32	5.4 (0.8)	115	<0.001	0.13	0.006	NS	4.7 (0.9)	−0.07	−0.004
Ulnar nerve										
MCV (m/s)	47	46.9 (8.3)	81	<0.0001	0.04	0.018	NS	58.0 (4.6)	−0.22	−0.062
DL (ms)	47	3.8 (0.8)	146	<0.0001	0.17	0.009	NS	2.6 (0.3)	0.06	0.001
CMAP (mV)	48	6.0 (3.0)	81	<0.001	−0.65	−0.109	<0.0001	7.4 (1.8)	−0.20	−0.021
Duration (ms)	28	5.9 (1.2)	116	<0.0001	−0.22	−0.016	NS	5.1 (0.7)	−0.01	−0.001
Tibial nerve										
MCV (m/s)	45	39.6 (4.5)	86	<0.0001	−0.02	−0.006	NS	46.0 (3.8)	−0.34	−0.079
DL (ms)	45	5.5 (1.3)	138	<0.0001	0.15	0.011	NS	4.0 (0.6)	0.11	0.004
CMAPs (mV)	45	7.9 (3.7)	67	<0.0001	−0.29	−0.062	NS	11.8 (3.5)	−0.33	−0.069
Duration (ms)	25	5.7 (1.3)	114	<0.01	−0.18	−0.012	NS	5.0 (0.7)	−0.17	−0.008
Peroneal nerve										
MCV (m/s)	38	35.7 (5.7)	76	<0.0001	−0.11	−0.042	NS	47.4 (4.5)	−0.38	−0.101
DL (ms)	38	7.7 (2.3)	167	<0.0001	−0.002	−0.00004	NS	4.6 (1.1)	0.04	0.002
CMAP (mV)	41	1.9 (1.8)	56	<0.0001	−0.65	−0.067	<0.05	3.4 (2.0)	−0.22	−0.027
Duration (ms)	16	6.4 (0.9)	131	<0.0001	−0.09	−0.006	NS	4.9 (0.9)	−0.17	−0.009
Sensory conduction										
Median nerve										
SCV (m/s)	42	38.6 (10.1)	69	<0.0001	−0.41	−0.235	<0.05	56.3 (5.3)	−0.26	−0.085
SNAP (μV)	48	6.8 (6.2)	24	<0.0001	−0.50	−0.178	NS	28.0 (11.5)	−0.45	−0.327
Duration (ms)	26	0.9 (0.4)	150	<0.0001	0.56	0.011	<0.0001	0.6 (0.1)	−0.11	−0.001
Ulnar nerve										
SCV (m/s)	41	36.8 (8.4)	68	<0.0001	−0.13	−0.069	NS	54.5 (5.5)	−0.28	−0.093
SNAP (μV)	48	6.6 (6.4)	28	<0.0001	−0.45	−0.170	NS	23.8 (10.3)	−0.37	−0.240
Duration (ms)	26	0.9 (0.2)	150	<0.0001	0.08	0.001	NS	0.6 (0.1)	−0.05	−0.00004
Sural nerve										
SCV (m/s)	43	36.4 (6.9)	74	<0.0001	−0.13	−0.052	NS	49.2 (4.8)	−0.12	−0.035
SNAP (μV)	48	7.1 (5.9)	42	<0.0001	−0.37	−0.124	NS	16.8 (7.8)	−0.38	−0.177
Duration (ms)	21	0.9 (0.3)	129	<0.05	0.23	0.004	NS	0.7 (0.1)	0.21	0.002

*Mann-Whitney U test; †Pearson's correlation coefficient; ‡regression slopes of HNPP and controls were compared.
Control values were obtained in 171 normal volunteers for the median nerve, 170 for the ulnar nerve, 161 for the tibial nerve, 171 for the peroneal nerve, and 163 for the sural nerve.
CMAP, compound muscle action potential; DL, distal latency; Duration, duration from the onset to the first crossing of the baseline in the CMAP and duration from the onset of the SNAP to the first negative peak; MCV, motor nerve conduction velocity; NS, not significant; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential.

de/re-myelination also was significantly high (25.6% (SD 13.9%), p<0.001). Axonal degeneration was slightly increased (3.6% (SD 3.8%)) and was seen even in young patients in contrast to controls.

DISCUSSION

This study demonstrated clinical, electrophysiological, and histopathological features of Japanese HNPP patients with the 17p11.2 deletion. Although recurrent transient nerve palsies are the characteristic feature of this disease, a minority of patients showed a symmetric polyneuropathy pattern, as previously reported.[16–18 36] Electrophysiological features of slowing of conduction velocities and varying degrees of abnormality among individual nerves, agreed well with previous reports of Western populations.[16–20] Slowing of MCV in our series seemed more marked than in previous reports.[16–18 20] The fact that we only examined probands of HNPP families and did not include affected siblings could

Table 3 Histopathological study of the sural nerve

	HNPP (n = 14)					Controls (n = 13)		
			Correlation to aging				Correlation to aging	
	Mean (SD)	p Values for controls*	r †	Regression slope	p Values for controls‡	Mean (SD)	r †	Regression slope
Myelinated fibre density (no./mm²)								
Total	7738 (1253)	NS	−0.45	−29.6	NS	8561 (1289)	−0.73	−39.9
Large	2458 (730)	<0.01	−0.70	−27.1	NS	3258 (736)	−0.83	−26.0
Small	5280 (1025)	NS	−0.05	−2.5	NS	5302 (655)	−0.50	−13.9
Teased fibre study (%)								
Tomacular change	41.5 (15.8)	–	−0.21	−0.18	–	–	–	–
Segmental de/re-myelination	25.6 (13.9)	<0.001	0.39	0.30	NS	6.9 (6.5)	0.82	0.22
Axonal degeneration	3.6 (3.8)	NS	−0.35	−0.07	<0.05	1.6 (1.8)	0.81	0.06

*Mann-Whitney U test; †Pearson's correlation coefficient; ‡regression slopes of HNPP and controls were compared.
NS, not significant.

account for the difference, or greater slowing might be characteristic of Japanese patients. In the peroneal nerve, it seems that the amplitude of CMAP is lower and the distribution of DL is wider than in Western populations even in normal controls.[26] Japanese people usually sit on the floor at home, rather than on chairs, and sometimes sit with their legs folded underneath them. This traditional Japanese sitting position may induce peroneal nerve injury.

A striking finding in our study was a reduction in CMAP with increasing age at examination. This feature was observed in the median, ulnar, and peroneal nerves but not in the tibial nerve. The median nerve passes through the carpal tunnel, predisposing it to entrapment injury, while the ulnar and peroneal nerves are vulnerable to repetitive compression injury at the cubital tunnel and fibular head, respectively, as suggested by the high frequency of episodic palsy of these nerves compared with the tibial nerve. Repetitive movement and nerve stretching at these sites also may contribute to injury. Thus, individual nerve-specific CMAP reduction with increasing age probably resulted from the cumulative effects of repetitive damage; conduction slowing caused by demyelination would be prominent at entrapment sites, as previously reported.[16–18 20] In the present study, demyelination also showed progression over time as demonstrated by age associated prolongation of DL and SCV in the median nerve for conduction through the entrapment site. However, in the ulnar and peroneal nerves, where electrophysiological indices were recorded distally from sites vulnerable to compression, no age associated worsening of MCV, SCV, or DL was observed, suggesting that myelin abnormality distal to the entrapment site does not worsen with advancing age. Thus, CMAP reduction in the median, ulnar, and peroneal nerves would reflect secondary axonal involvement complicating demyelination at the entrapment site. This age associated axonal involvement in a primarily demyelinating condition is similar to that observed in CMT1A with PMP22 duplication.[12 14 15] However, unlike CMT1A, axonal damage may not occur unless the nerves are subjected to compression. PMP22 duplication in Schwann cells results in disturbance of axonal cytoskeletal organisation, resulting in distal axonal degeneration and fibre loss.[13] However, the effect of PMP22 deletion on the axonal cytoskeleton is less severe.[15] PMP22 deletion in itself may not cause progressive axonal involvement associated with aging, though compression induced demyelination may elicit secondary axonal loss because of deficient Schwann cell signalling to the axonal cytoskeleton.[37]

SNAP of the median, ulnar, and sural nerves showed marked reduction even in nerves relatively free from compression and tended to decrease with increasing age at examination. Unlike findings for CMAP, however, rates of reduction with aging did not differ significantly from those in normal controls. Sensory axons may be less susceptible than motor nerves to changes caused by entrapment.

Reduction in CMAP and SNAP may be at least partly attributed to dispersion with phase cancellation as a result of demyelinating change, as suggested by significant prolongation of waveform duration.[27 38] Sural nerve biopsy specimens showed a reduction in large myelinated fibre density irrespective of age, which may indicate a developmental abnormality of axons or a loss of axons relatively early in life. This axonal loss also may contribute to reduction in amplitudes. At any rate, reduction in myelinated fibres of sensory nerves in HNPP patients did not appear to be associated with acquired damage at the entrapment sites. Thus, the electrophysiological features of HNPP are a mixture of abnormalities occurring from an early stage in life and acquired features caused by repetitive insults at entrapment sites. One therapeutic strategy in HNPP patients may be directed toward prevention of axonal damage associated with entrapment.

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Progression and prognosis in pure autonomic failure (PAF): comparison with multiple system atrophy

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PAPER

Progression and prognosis in pure autonomic failure (PAF): comparison with multiple system atrophy

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Objective: To clarify the progression of autonomic symptoms and functional deterioration in pure autonomic failure (PAF), particularly in comparison with multiple system atrophy (MSA).
Methods: The investigation involved eight patients with PAF (M/F = 7/1; mean age at onset, 57 years) and 22 with probable MSA matched for age at onset (M/F = 14/8; onset 56 years). Subjects were followed up for neurological symptoms, activities of daily living, and autonomic function for more than seven years. Autonomic functional tests were carried out.
Results: In PAF, fainting or sudomotor dysfunction occurred first, followed by constipation and syncope. Urinary dysfunction developed late, and respiratory dysfunction was not evident. This clinical course contrasted sharply with that in MSA, where early urinary dysfunction usually proceeded to sudomotor dysfunction or orthostatic hypotension ($p = 0.004$), followed by respiratory dysfunction ($p = 0.0004$). Results of pharmacological tests also distinguished PAF from MSA. Progression and prognosis in patients with PAF did not worsen, unlike the steady progressive autonomic dysfunction in MSA ($p < 0.0001$, $p < 0.0001$, $p = 0.0009$, and $p = 0.003$, for progression to modified Rankin scale grade III, IV, V, and death, respectively).
Conclusions: The time course and pattern of progression of autonomic failure differed significantly between PAF and MSA. Patients with PAF had slower functional deterioration and a better prognosis.

Pure autonomic failure (PAF) is a sporadic idiopathic neurodegenerative disorder characterised by gradually progressive severe autonomic disturbances without other neurological features. In the past, PAF was defined as severe orthostatic hypotension without other neurological deficits, and was referred to as idiopathic orthostatic hypotension. However, this has proved to be a heterogeneous condition, including diseases such as PAF, acute autonomic neuropathy, the early stages of Shy–Drager syndrome, and Parkinson's disease with autonomic failure.[1–6]

Bannister *et al*[7] classified primary autonomic failure into three categories: Parkinson's disease with autonomic failure, multiple system atrophy (MSA), and pure autonomic failure. In 1996, a consensus statement was established concerning PAF,[8] but it has remained uncertain whether the autonomic failure of PAF can readily be distinguished from those of MSA and Parkinson's disease with autonomic failure. In addition, although the clinical course of both MSA and Parkinson's disease with autonomic failure has been described to some extent, details of the natural history of PAF have not been fully assessed because of its rarity and very slow progression.[9–11] Previous reports have noted longer survival in patients with PAF than in those with MSA.[3 12–15] Orthostatic hypotension and anhidrosis/hypohidrosis are the main clinical symptoms in PAF, but their severity, prognosis, and progression have been only incompletely assessed. To clarify the clinical features, particularly the natural course of PAF, we observed eight patients who fulfilled the PAF consensus statement and maintained a follow up for at least five years. We show that their features are distinct from those of another form of primary autonomic failure, MSA.

METHODS

Patients

We examined eight patients with PAF (seven men, one woman; mean (SD) age at onset, 57 (14) years; mean age at first evaluation, 68 (12) years; mean duration from onset to most recent evaluation, 19 (10) years) who were referred to the Nagoya University Hospital or its affiliated hospitals in Aichi prefecture between 1988 and 1997. We evaluated these patients clinically from onset for between seven and 32 years. We reviewed the clinical records preceding our own follow up period, and also obtained information by interviewing the patients and family members.

According to the consensus statement,[8] PAF is characterised by orthostatic hypotension, various other autonomic signs without more widespread neurological involvement, and a low resting supine plasma noradrenaline concentration. The statement acknowledged that some patients would later prove to have other disorders such as MSA,[8] but did not state how long a period of follow up was required to confirm a diagnosis of PAF. Early MSA with predominant autonomic failure is particularly difficult to distinguish from PAF. We estimated that most MSA patients can be diagnosed by follow up for five years or more after onset,[8 16] and we therefore serially examined putative PAF patients for more than five years from onset to exclude those with MSA. We also excluded patients with acute autonomic neuropathy, Parkinson's disease with autonomic failure, and other diseases presenting with autonomic signs by neurological examination, imaging (magnetic resonance imaging and positron emission tomography), and neurophysiological tests.

We also investigated 22 probable MSA patients[17] matched according to age at onset (14 men, eight women; mean age at onset, 56 (8) years; mean age at first autonomic test, 61 (7) years; mean interval from onset, 8 (3) years) who had detailed clinical information particularly concerning autonomic features, and follow up intervals from over five years to 16 years after onset. All patients with MSA presented with autonomic failure as an initial symptom or with predominant autonomic failure at their first clinical visit, and fulfilled the criteria for a probable MSA diagnosis.[17]

Abbreviations: AVP, arginine-vasopressin; HUT, head up tilt test; MSA, multiple system atrophy; PAF, pure autonomic failure

Table 1 Clinical profiles of eight patients with pure autonomic failure at their first visit

Variable	Patient 1	2	3	4	5	6	7	8
Sex	M	M	M	M	M	M	M	F
Onset age (y)	35	68	72	78	50	52	51	50
Time until first evaluation (y)	17	1	10	5	27	7	5	13
Duration of observation (y)	32	7	12	12	32	14	15	29
Hypohidrosis	+	+	+	+	+	+	+	+
Faintness	+	+	+	+	+	+	+	+
Syncope	−	−	−	−	+	+	+	+
Constipation	+	−	−	−	+	−	+	−
Difficulty in urination	−	−	+	−	+	−	−	−
Incontinence/urinary urgency	−	−	−	−	−	+	+	+
Respiratory disturbance	−	−	−	−	−	−	−	−
Plasma noradrenaline (pg/ml) *	30	43	25	83	50	34	14	10
Orthostatic hypotension	+	+	+	+	+	+	+	+
Denervation supersensitivity	+	+	+	+	+	+	+	+
Modified Rankin scale	0	0	0	0	0	0	0	0

*Normal range 150 to 450 pg/ml.
F, female; M, male; y, years.

Procedures

We evaluated all eight patients with PAF and 22 with MSA with a passive multistage head up tilt test (HUT) and a noradrenaline infusion test. The HUT was performed as follows. Blood pressure and heart rate were measured continuously by tonometry (SA-250; Colin, Komaki, Japan). After blood pressure stabilised at the supine stage, changes in blood pressure and heart rate were recorded continuously through 20°, 40°, and 60° head up tilting for five minutes each. Orthostatic hypotension was defined as a fall in systolic blood pressure of more than 30 mm Hg during the 60° head up tilt.[18]

Blood samples were collected at the rested supine stage and after 60° head up tilting from all patients for evaluation of plasma noradrenaline and arginine-vasopressin (AVP). Differences in AVP between after 60° head up tilting and the supine position were calculated as ΔAVP. Additionally, a noradrenaline infusion test was carried out as follows. A very low (0.3 μg/min) or a low (3 μg/min) concentration of noradrenaline was infused intravenously while blood pressure was monitored for changes. If diastolic or systolic blood pressure rose by more than 10 mm Hg or 25 mm Hg, respectively, the patient was considered to have denervation supersensitivity involving the sympathetic nervous system.[19] Four patients were re-evaluated two, five, six, and 11 years later, respectively. We also carried out ^{123}I-metaiodebenzylguanidine (MIBG) scintigraphy and evaluated the heart/mediastinum (H/M) ratio from delayed images, as previously described.[20–22]

We followed up all eight patients and noted the time points when new autonomic symptoms appeared, including hypohidrosis, faintness and syncope, constipation, urinary dysfunction, impotence, and respiratory distress, and considered such clinical features in sequence to assess the natural clinical course. We evaluated hypohidrosis in terms of inspection of the skin and recording of patient symptoms. Dry skin or reduced perspiration was noted on some parts of the body, with compensatory hyperhidrosis elsewhere. Patients often noted their reduced perspiration in summer and felt severe fatigue, which sometimes limited their capacity for outdoor work. Faintness was defined as a floating sensation while in the upright position without loss of consciousness, or as symptomatic orthostatic hypotension during the head up tilt test. Syncope was defined as a blackout or loss of consciousness, including severe blurred vision. Constipation was defined by the passage of stools at intervals of three days or more, or complaints of straining. Urinary dysfunction was defined as urination twice at night or more than five times in the daytime, urinary urgency, incontinence, or difficulty in urination. Impotence was defined as difficulty in achieving normal sexual function. Respiratory disturbances were defined either as the presence of sleep apnoea, including heavy snoring, or as difficulty in respiration. Onset of an autonomic symptom was defined as the time when the patient first noted the symptom.

Statistics

The Mann–Whitney U test for non-parametric statistics was used as appropriate. Kaplan–Meier analyses were employed to estimate the natural course of autonomic features and disease progression, assessed by the modified Rankin scale in both PAF and MSA patients. Log-rank test statistics were used to determine whether the Kaplan–Meier curves differed between PAF and MSA. Calculations were done using the statistical software package Stat View (Abacus Concepts, Berkeley, California, USA). Statistical significance was defined as a probability (p) value of <0.05.

RESULTS

Clinical profiles of PAF on the first visit to the hospitals

Clinical profiles of the eight patients with PAF at their first examination at our hospital are presented in table 1. They had many complaints suggesting autonomic disturbances, but the specific features varied. The earliest age at onset was 35 years, and the latest was 78 years. The interval from onset to presentation at our hospital varied from one to 27 years. Each patient showed various autonomic disturbances at that time, but faintness and hypohidrosis had been experienced by all patients. Other autonomic symptoms were as follows: urinary dysfunction in five, syncope in four, constipation in three, and impotence in two. All patients had very low plasma noradrenaline concentrations, orthostatic hypotension, and denervation supersensitivity according to the noradrenaline infusion test.

Clinical manifestations of MSA

The initial symptoms in all 22 patients with MSA were those of autonomic failure. Median time from onset to the presence of concomitant autonomic and motor manifestations (evolution from onset to probable MSA) was 2.0 years (range 1 to 10). At the first clinical visit, seven of the 22 patients presented with severe autonomic failure but failed to fulfil consensus diagnostic criteria of MSA.

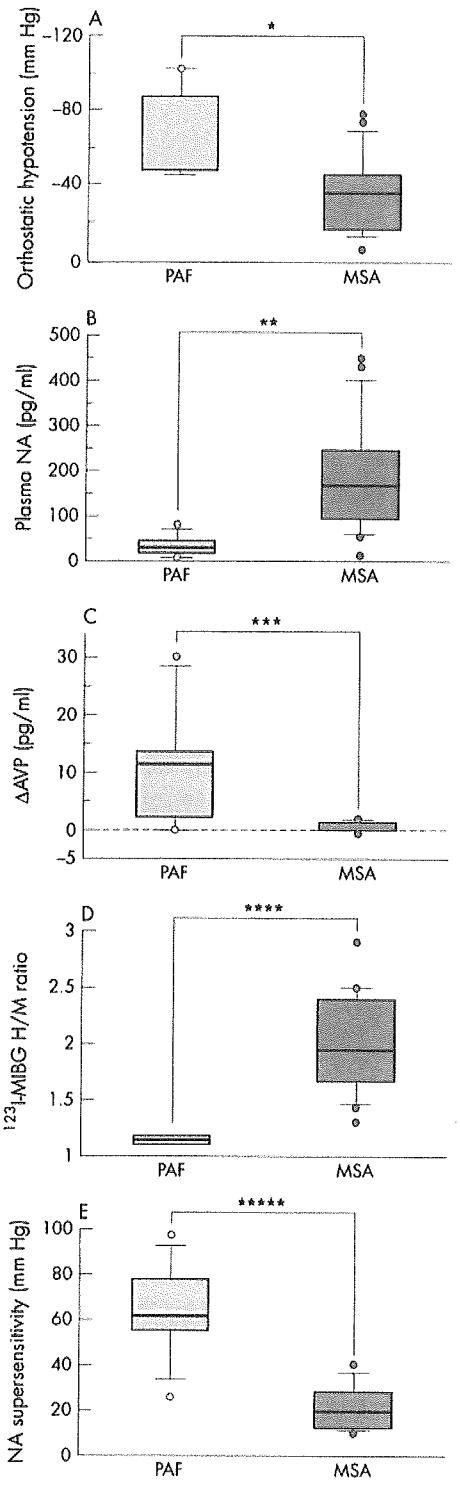

Figure 1 Box and whisker plot of the autonomic nervous testing comparing pure autonomic failure (PAF) with multiple system atrophy (MSA). (A) Systolic blood pressure fall during orthostatic hypotension. (B) Plasma noradrenaline (NA) concentration. (C) Differences in arginine-vasopressin (AVP) concentration between 60° head up tilt and supine posture calculated as ΔAVP. (D) Heart/mediastinum (H/M) ratio from ^{123}I-metaiodobenzylguanidine (MIBG) delayed imaging. (E) Systolic blood pressure increase during noradrenaline infusion test. *p = 0.004, **p = 0.0003, ***p = 0.003, ****p = 0.002, *****p = 0.0004, Mann–Whitney U test.

Autonomic nervous system testing in PAF and MSA

We found significant differences between PAF and MSA patients with respect to the following:

- *orthostatic hypotension* evaluated by the head up tilt test (mean (SD): PAF, 68.9 (22.5) mm Hg; MSA, 36.3 (20.4) mm Hg; p = 0.004 (fig 1A);
- *noradrenaline concentration*: PAF, 36.1 (23.2) pg/ml; MSA, 189.9 (121.9) pg/ml; p = 0.0003 (fig 1B);
- *ΔAVP*: PAF, (10.7) pg/ml; MSA, 0.34 (0.62) pg/ml; p = 0.003 (fig 1C);
- *H/M ratio*: PAF, 1.15 (0.05); MSA, 2.04 (0.44); p = 0.002 (fig 1D);
- *noradrenaline infusion test*: PAF, 70.1 (23.2) mm Hg; MSA, 23.7 (11.0) mm Hg; p = 0.0004 (fig 1E).

Clinical course of autonomic failure

Kaplan–Meier curves depicting the natural clinical course of PAF and MSA are shown in fig 2. Hypohidrosis, faintness and syncope, constipation, urinary dysfunction, and respiratory disturbance were assessed sequentially.

Hypohidrosis

Six patients noted hypohidrosis or anhidrosis as an initial symptom, and seven became aware of hypohidrosis within five years of onset. Hypohidrosis was one of the earliest and most important symptoms of patients with PAF. In contrast, patients with MSA noted hypohidrosis at a significantly later stage of disease (p = 0.027).

Faintness and syncope

These symptoms represented orthostatic hypotension. Usually faintness preceded syncope. Faintness was often noted as an initial autonomic symptom in PAF. Four of eight patients first noted hypohidrosis in the same year as they first experienced faintness. In our series, five patients complained of faintness as an initial symptom, and seven noted faintness within five years of onset. Syncope appeared at (mean (SD)) 6 (7) years after the onset of faintness, and half the patients had experienced syncope within five years. However, two patients first noted syncope more than 19 years after experiencing faintness. In patients with MSA, faintness was observed later in the course of illness, with risk of progression to syncope differing significantly between the two groups (p = 0.002).

Constipation

Constipation was among the early symptoms of PAF. In our series, three patients noted constipation as an initial symptom, and five noted constipation within five years of onset; all patients complained of constipation within 13 years. Constipation was the second earliest symptom in our PAF patients, while patients with MSA also complained of constipation at a relatively early stage of disease. No significant differences were seen between the two groups in time from onset of first symptom to development of constipation (p = 0.46).

Urinary dysfunction

In the early stages few PAF patients noted urinary dysfunction, while at a later stage most patients had this complaint. In our series, urinary dysfunction appeared at (mean (SD)) 9 (9) years after the onset of hypohidrosis, faintness, and constipation. Only three patients noted urinary urgency, urinary frequency, or incontinence in the first five years. Among types of urinary dysfunction, difficulty in urination was rare in PAF patients. We evaluated the results of urodynamic studies in five of the eight PAF patients, at four,

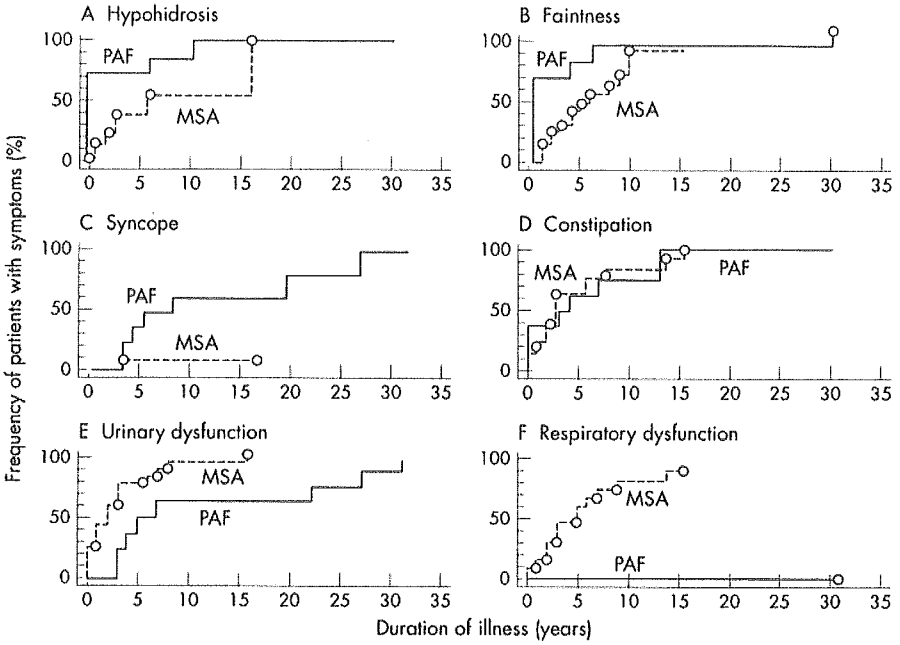

Figure 2 Progression of autonomic symptoms including hypohidrosis (A), faintness (B), syncope (C), constipation (D), urinary dysfunction (E), and respiratory disturbance (F) in patients with pure autonomic failure (PAF) and multiple system atrophy (MSA). Hypohidrosis was an earlier symptom in PAF than in MSA (panel A, $p=0.027$). Faintness and syncope were earlier symptoms in PAF than in MSA (panel B, $p=0.04$; panel C, $p=0.002$). Development of constipation was similar between the two diseases (panel D). Urinary dysfunction was a later symptom in PAF than in MSA (panel E, $p=0.004$). Respiratory disturbance did not occur in our PAF patients, but MSA patients had these problems at an early stage (panel F, $p=0.0004$).

six, 10, 13, and 17 years after the onset of PAF, respectively. Two of the five patients were essentially asymptomatic and had normal study results. Three patients were symptomatic, one of whom had an overactive bladder and the other an underactive bladder; the third had normal results. In our series, all eight patients had urinary dysfunction by 30 years after onset. Thus urinary dysfunction typically emerged in late stage PAF. In contrast, MSA patients developed urinary dysfunction at a very early stage of their disease ($p = 0.004$), often as an initial autonomic symptom in about a quarter of the patients. Within five years, more than 75% of MSA patients had urinary dysfunction, especially difficulty in urination. Thus urinary symptoms occurred early and were particularly prominent in MSA.

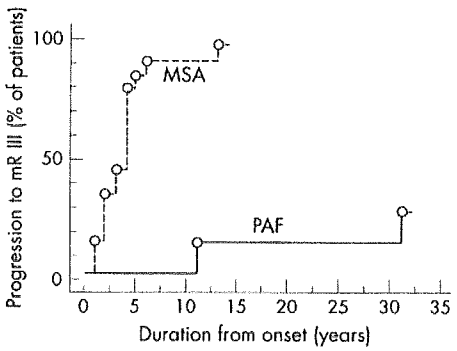

Figure 3 Differences in time remaining independent in activities of daily living (ADL) assessed by the modified Rankin scale between patients with pure autonomic failure (PAF) and multiple system atrophy (MSA). Round symbols represent censored data. Significant differences were seen between PAF and MSA for three ADL milestones and for survival, by Kaplan–Meier analysis and log-rank tests. mR III, modified Rankin scale, grade III (moderate impairment requiring minimal support such as a cane, stair rails, and so on): difference between PAF and MSA significant at $p<0.0001$.

Respiratory disturbances

Respiratory disturbances such as sleep apnoea were uncommon in patients with PAF. Indeed, in our series, no patient had respiratory difficulties in 30 years of follow up. In contrast, respiratory disturbance was one of the most important features in patients with MSA ($p = 0.0004$). About half the MSA patients had this complaint within five years, and subsequently the prevalence of respiratory disturbances increased. More than 80% of the MSA patients had respiratory disturbances by 10 years.

Progression of orthostatic hypotension and noradrenaline supersensitivity

Orthostatic hypotension

Orthostatic hypotension (fig 1A) was a major clinical feature in PAF, being marked even in the early stages of the disease. Blood pressure fall varied from 34 to 108 mm Hg at presentation to our hospital, and the extent of orthostatic hypotension progressed markedly in most patients over the next two to 11 years. In seven patients blood pressure fell by more than 50 mm Hg, and most patients experienced syncope.

Noradrenaline supersensitivity

The noradrenaline infusion test estimates denervation supersensitivity at peripheral noradrenaline receptors, suggesting disease involvement of the peripheral sympathetic nervous system. At an early stage, PAF patients all showed excessive rises in blood pressure of 30 mm Hg or more with infusion of a low concentration of noradrenaline (3 or 0.3 μg/min), indicating the presence of denervation supersensitivity (fig 1E). After two to 11 years, however, the extent of blood pressure rise in response to noradrenaline infusion was smaller than at an early stage, suggesting emergence of some compensatory mechanism or secondarily induced insensitivity of noradrenaline receptors.

Activities of daily living and prognosis

PAF patients did not show diminishing capacity for activities of daily living (ADL) up to a late stage (fig 3). In our series three patients died, but they maintained nearly normal ADL throughout their lives. One patient who died at 90 years, 12 years after disease onset, could walk alone without assistive devices until he was 89 years old (modified Rankin scale, 0 to1); rapid deterioration in the last year of life resulted from a subdural haematoma. Another patient who died at 82 years, 32 years after onset, could perform all his daily activities unassisted until he was 81. He was essentially bedridden for the last year of life because of myelodysplastic syndrome. The third patient, who died aged 84 years 12 years after disease onset, remaining healthy and active (modified Rankin scale 0 to 1) until he died suddenly of a severe stroke.

Although both MSA patients and PAF patients have severe autonomic disturbances, functional and survival prognoses[16] were significantly worse in MSA than in PAF. In our series, median time from onset to modified Rankin scale grade III in MSA was four years ($p<0.0001$ *v* PAF); grade IV, seven years ($p = 0.0009$); grade V, nine years ($p<0.0001$); and death, 11 years ($p = 0.003$). In contrast to MSA, PAF carried a relatively good prognosis for function and survival.

DISCUSSION

PAF is a chronic progressive neurodegenerative disease characterised by severe autonomic failure without other neurological deficits. Uniquely, PAF patients can maintain a long healthy life, in contrast to patients with other types of primary autonomic failure. Pathological reports of PAF have described Lewy bodies in the intermediolateral grey columns of the thoracolumbar spinal cord, suggesting that PAF is a form of Lewy body disease.[29-30]

Our study is the first assessment of long term progression of autonomic symptoms and ADL status in PAF, particularly in comparison with MSA. Although a consensus has been reached over the diagnostic criteria for PAF,[8] long term follow up observation of the clinical features is important to identify the differences between PAF and autonomic failure in other neurodegenerative diseases, particularly MSA and Parkinson's disease with autonomic failure.[7 8 12] We investigated clinical features of eight patients with PAF over follow up periods ranging from seven to 32 years.

It is generally accepted that patients with PAF have autonomic failure resulting in peripheral but not central involvement. The results of supine noradrenaline levels, ΔAVP, ^{123}I-MIBG, and the noradrenaline infusion test clearly confirm this. In contrast, patients with MSA have patterns suggesting a predominantly central involvement, although some patients with probable MSA also have low noradrenaline concentrations, increased ΔAVP, a reduced H/M ratio, and raised blood pressure during the noradrenaline infusion test. These neuropharmacological tests would be useful for differentiating PAF from MSA early in the course of the illness. Further studies are needed to clarify their sensitivity, specificity, and positive predictive value.

In our study, orthostatic hypotension and related faintness and syncope were the most important clinical features of PAF, and developed at a very early stage. Furthermore, orthostatic hypotension worsened gradually as the disease progressed in spite of medical treatment for hypotension. In contrast, MSA patients were less likely to have syncope than PAF patients. Progression of MSA is relatively rapid,[16] so MSA patients are often wheelchair bound or nearly bedridden before showing severe hypotension with syncope.[16] About half the patients with MSA noted faintness by four years after onset, at a time when most of them were wheelchair bound and spent a considerable amount of their waking time lying down. This may limit the exposure of MSA patients to syncope.

Another important autonomic abnormality observed in PAF was sudomotor impairment. Hypohidrosis or anhidrosis was a major complaint in patients with PAF. Emergence of orthostatic hypotension, sometimes with loss of consciousness, and sudomotor dysfunction at a very early stage were striking characteristic features in PAF, in contrast to MSA where these symptoms were absent in the early phase of the disease.

A striking clinical characteristic of PAF was the absence of respiratory dysfunction such as sleep apnoea until a very late phase of disease. This feature again contrasted with MSA, where respiratory dysfunction was a major problem, threatening life in the later phase of disease.

Constipation and urinary dysfunction are among the characteristic symptoms of primary autonomic failure syndrome including PAF, MSA, and Parkinson's disease with autonomic failure.[10 11] Urinary problems have been documented in the past to some extent,[9 11 12] representing a characteristic feature of PAF, especially in the late phase. Sakakibara *et al*[12] reported that all six of their patients with PAF who complained of urinary disturbances showed abnormalities on urodynamic studies. In our series, five of eight patients underwent urodynamic evaluation, and two with urinary symptoms showed a hyperactive or underactive bladder. However, the severity of the urodynamic abnormalities and associated symptoms was mild, in agreement with the previous report.[12] In contrast, patients with MSA have severe urinary dysfunction, especially difficulty in urination[13] and nocturnal urinary frequency, with residual urine, detrusor hyperreflexia, low compliance, and detrusor sphincter dyssynergy on urodynamic studies. Intermittent self catheterisation is often required even early in the course of the illness.

On the basis of these observations, we can assume that orthostatic hypotension and sudomotor dysfunction precede urinary dysfunction and particularly respiratory dysfunction in the development of autonomic disturbances in PAF, while in MSA urinary dysfunction precedes orthostatic hypotension and sudomotor dysfunction, and respiratory dysfunction is a serious problem even at an early stage. Modes of progression of autonomic symptoms seem to be an important way of distinguishing between PAF and MSA.

The evolution of the change in blood pressure during the noradrenaline infusion test in PAF is difficult to explain. While the clinical features became worse over the course of several years in PAF patients, in contrast the degree of blood pressure elevation during the test became smaller with time. The same method was used for the test on each occasion, and no previous reports provide an explanation for this phenomenon. Age related changes such as atherosclerosis or changes in drug treatment might have contributed, but further study is necessary.

Patients with PAF had a better prognosis than those with MSA. Even the three patients with PAF who died during follow up lived independently until one or two years before they died all died of concurrent diseases. Various factors contributed to this advantage in ADL and long term prognosis. First, patients with PAF did not have severe urinary disturbances, which would lessen the risk of recurrent urinary infections, and they also did not have life threatening respiratory failure. Second, while management of orthostatic hypotension remains challenging late in the course of illness, administration of plasma volume expansion fluids, fludrocortisone, and sympathomimetic agents can be effective in ameliorating symptoms and preventing faintness and syncope with resulting head injuries or bone fractures which could compromise ADL and survival. Third, patients

with PAF in this study showed no motor or cognitive impairment. No parkinsonism or dementia, which would have affected daily activities or required additional treatment, was evident during the course of their illness. Further studies are needed to evaluate the significance of the pathological background for temporal features of autonomic, motor, and cognitive involvements.

The precise epidemiology of PAF has not been assessed, either in Japan or in Western countries. To our knowledge, relatively few cases of PAF have been studied or described, and necropsy reports are far less common than for MSA. In our Japanese series, more than 200 patients with MSA were referred to hospital during the course of the study, but only eight patients with PAF were diagnosed during the same period. Although physician referral patterns may have on influence, PAF appears to be uncommon in Japan compared with MSA. Further studies should be undertaken to clarify the incidence and prevalence of PAF worldwide.

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Interferon alfa treatment for Sjögren's syndrome associated neuropathy

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SHORT REPORT

Interferon alfa treatment for Sjögren's syndrome associated neuropathy

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Treatment response to interferon alfa (IFNα) is described in three consecutive cases of two forms of Sjögren's syndrome associated neuropathy (SSN)—two with sensory ataxic ganglionopathy and one with sensorimotor neuropathy with demyelinating features. All responded well to IFNα in terms of neuropathic symptoms, sicca symptoms, antibody titres, and findings in salivary gland biopsy specimens. IFNα thus showed promise in treating both SSN and the underlying Sjögren's syndrome.

Although peripheral neuropathy is the most common extraglandular manifestation of Sjögren's syndrome, treatment of this complication is not well established.[1] Interferon alfa (IFNα) administration has been reported to alleviate Sjögren's syndrome associated sicca symptoms, as evidenced by increased salivary flow, and also to reduce histologically evident disease activity.[2] So far, the effects of IFNα on any extraglandular complications such as Sjögren's syndrome associated neuropathy (SSN) have not been reported. We describe the therapeutic effects of IFNα in three consecutive patients with two forms of SSN—two with sensory ataxic ganglionopathy and one with sensorimotor neuropathy.

CASE PRESENTATIONS

Patient 1

An otherwise healthy 46 year old man developed dysaesthesia which first involved the left foot and then progressed to affect the right foot and left hand in September 1997, finally progressing to his right foot and left hand. Over the next three years, the level of dysaesthesia gradually ascended to involve both thighs, and difficulty in walking led to hospital admission under our care. On admission, sensory examination revealed profoundly reduced vibratory and proprioceptive sensation affecting mainly the lower limbs, with slight loss of pain and temperature sensation. The heel to knee test showed marked dysmetria in both legs, particularly with the eyes closed. Deep tendon reflexes were absent in all limbs. Muscle strength was slightly decreased in the lower limbs. The patient could barely walk unassisted because of severe ataxia (sensory ataxia scale 5, table 1).[3] Romberg's test was strongly positive. No signs of autonomic dysfunction were evident. Routine haematological examination yielded normal results. Anti-SS-A/SS-B antibodies were positive at 42.0/18.0, respectively (by enzyme linked immunosorbent assay (ELISA); normal values are SS-A/SS-B <10.0/15.0). In cerebrospinal fluid (CSF), protein content was modestly raised (60 mg/dl), and the cell count was normal. Results of nerve conduction studies were normal in the upper limbs, but in the lower limbs the sensory nerve action potential (SNAP) in the sural nerve showed reduced amplitude (0.2 μV) with normal conduction velocity (CV, 48 m/s). Other nerve conduction study findings, as well as statokinesigraphy results[4], are summarised in table 1. Somatosensory evoked potentials (SEP) could not be elicited by lower limb stimulation. Cervical spinal cord magnetic resonance imaging (MRI) on T2* weighted images showed abnormal high intensity areas in the dorsal columns, reflecting the sensory ataxic ganglionopathy.[5] Findings on sural nerve biopsy were decreased numbers of large myelinated fibres, axonal degeneration without axonal sprouting, endoneurial oedema, and no evidence of vasculitis. The patient was treated with prednisolone (30 to 10 mg/day), cyclosporin (100 mg/day), and plasmapheresis, but without improvement. In August 2001, intravenous immunoglobulin treatment (IVIG) was given (0.4 g/kg for five days). After this treatment, dysaesthesias in the legs and left hand were reduced, and the patient was able to walk with less effort. However, he required repeated five day courses of IVIG every three to four weeks to halt the progression of the disease. Sicca symptoms developed and Schirmer's test gave a positive result (3 mm/5 mm, right/left). A labial salivary gland biopsy specimen showed marked lymphocytic infiltration and acinar cell destruction, graded as 3 by Daniels focus scores.[6]

In November 2003, IFNα treatment was initiated (3 MIU/day, three times weekly). Over the next two months, the patient showed dramatic improvement; dysaesthesias nearly disappeared and he was able to walk without effort. Nerve conduction studies revealed improvement of SNAP amplitude in the sural nerve (table 1). Statokinesigraphy also showed significant improvement ($p<0.01$, table 1). Sicca symptoms resolved and lacrimation increased (Schirmer's test; 18 mm/14 mm, right/left). Anti-SS-A/SS-B antibody titres fell to the normal range (9.1/9.2 respectively) and a follow up labial salivary gland biopsy showed fewer infiltrating lymphocytes graded as 2.[6] The clinical and therapeutic time course of patient 1 is summarised in fig 1A.

Patient 2

A 67 year old woman with Sjögren's syndrome developed sensory ataxic ganglionopathy over 14 years. She did not respond to prednisolone, cyclophosphamide, or plasmapheresis and required frequent (every two or three month) IVIG therapy to maintain her activities of daily living, as for patient 1. In December 2003, she was admitted to our department and treated with IFNα (3 MIU/day, three times weekly). After the initiation of IFNα, she had marked improvement in vibratory and proprioceptive sensation, leading to improvement of her activities of daily living. Nerve conduction studies showed improvement in SNAP amplitude in the sural nerve and statokinesigraphy demonstrated significantly

Abbreviations: CV, conduction velocity; IFNα, interferon alfa; IVIG, intravenous immunoglobulin; SEP, somatosensory evoked potentials; SNAP, sensory nerve action potential; SSA/SSB, Sjögren's syndrome associated antibody A and B; SSN, Sjögren's syndrome associated neuropathy

Table 1 Nerve conduction studies, statokinesigraphy, Rankin scale, and status of Sjögren's syndrome before and after interferon alfa treatment

Variable	Patient 1		Patient 2		Patient 3	
	Before Rx	After Rx	Before Rx	After Rx	Before Rx	After Rx
Motor conduction						
R median amplitude (mV)/CV (m/s)	4.8/56	4.7/58	10.6/55	12.9/53	3.1/35	3.8/42
R ulnar amplitude (mV)/CV (m/s)	7.8/47	8.0/48	7.2/44	7.0/47	10.5/38	11.6/40
R tibial amplitude (mV)/CV (m/s)	5.8/43	6.8/46	5.2/31	4.9/31	12.1/28*	11.6/35*
Sensory conduction						
R median amplitude (μV)/CV (m/s)	11.6/55	11.6/52	39.8/45	41.3/40	10.3/37	12.4/36
R ulnar amplitude (μV)/CV (m/s)	13.0/51	14.0/48	11.2/40	15.8/48	11.6/22	12.7/32
R sural amplitude (μV)/CV (m/s)	0.2/48	8.9/45	1.2/51	10.6/48	12.9/40	10.6/48
Statokinesigram (cm)†	217.7 (28.3)/ 346.3 (62.9)	72.5 (10.8)/ 195.8 (12.5)	125.5 (12.9)/ NP	7.79 (0.64)/ NP	NP	NP
Modified Rankin scale‡	2–3	1	2–3	1	2–3	0
Sensory ataxia scale§	5	3	5	2	2	1
Positive items for SS¶	I, II, III, IV, VI	II	I, II, IV, VI	II, IV	I, II, IV, VI	none
Anti-SS-A/SS-B antibody titres**	42.0/18.0	9.1/9.2	47.1/32.0	8.8/9.3	47.0/8.2	3.1/2.6
Schirmer's test (right/left, mm)	3/5	18/14	12/16	19/22	16/9	14/35
Daniels focus score (grade/focus)††	3/0	2/0	4/3	3/0	4/3	2/0

*Temporal dispersion was observed.
†Statokinesigram[4]: total movement length of the gravity centre with eyes opened/closed during 30 seconds, n=6 (mean (SD)).
‡Modified Rankin scale: 0, no symptoms at all; 1, no significant disability, able to carry out all usual duties and activities; 2, slight disability, unable to carry out delicate tasks but able to look after own affairs without assistance; 3, moderate disability, requiring some help, but able to walk without assistance; 4, moderately severe disability, unable to walk without assistance and unable to attend to own bodily needs without assistance; 5, severe disability, bedridden.
§Sensory ataxia scale[3]: 0, normal ability to stand on one foot with eyes closed; 1, stands/walks normally with eyes closed; 2, stands/walks with minor swaying with eyes closed but normally with eyes open; 3, stands/walks with some swaying with eyes open; 4, stands/walks on a large base with eyes open; 5, standing/walking impossible without support.
¶Items in the revised Euro-criteria for Sjögren's syndrome are: I, ocular symptoms; II, oral symptoms; III, ocular signs (positive Schirmer's test); IV, histopathological features; V, salivary gland involvement; VI, autoantibodies to SS-A or SS-B.
**Normative value for anti-SS-A/SS-B antibody titres in ELISA are <10/<15, respectively.
††Daniels focus score[6]: grade is based on infiltrate of lymphocytes, plasma cells, and macrophages per 4 mm^2 area of salivary tissue: grade 0, absent; grade 1, slight infiltrate; grade 2, moderate infiltrate, less than one focus; grade 3, one focus; grade 4, two or more foci. Focus score (extension of grade 4), 2–10: number of foci of >50 mononuclear cells per 4 mm^2 area of salivary gland.
NP, not performed; Rx, treatment with IFNα.

improved balance, as in patient 1. Her clinical and neurophysiological features are summarised in fig 1B and table 1.

Patient 3

A 45 year old woman with a history of hypothyroidism developed progressive weakness and dysaesthesias in both feet in December 2000. Within six weeks she became unable to walk, and was admitted to a hospital affiliated with Nagoya University. Neurological examination indicated mild weakness, mild loss of both positional and vibratory sensation, and slight loss of pain and temperature sensation involving all limbs, especially distally. Deep tendon reflexes were absent in all limbs. No autonomic symptoms were present. Routine haematological findings were normal. Serum anti-SS-A antibody was positive while anti-SS-B antibody was negative (anti-SS-A/SS-B 47.0/8.2, respectively). CSF protein content was raised (124 mg/dl), with a normal cell count. A labial salivary gland biopsy specimen showed marked lymphocytic infiltration graded as 4 (focus 3).[6] Nerve conduction studies showed a symmetrical sensorimotor polyneuropathy with reduced conduction velocities and the presence of temporal dispersion (table 1). The patient was treated with prednisolone (60 mg/day), cyclosporin (100 mg/day), and cyclophosphamide (100 mg/day) with no effect on the progression of disability. Plasmapheresis resulted in a slight improvement in activities of daily living lasting less than two weeks. After IVIG treatment (0.4 g/kg for five days), she had marked clinical improvement; dysaesthesias and weakness in all limbs gradually lessened, and she could walk without support. However, there were multiple relapses during the next two years. Beginning in April 2002, intervals between relapses shortened and sicca symptoms developed. A sural nerve biopsy specimen revealed subperineurial and endoneurial oedema with evidence of remyelination.

In June 2003, IFNα treatment (3 MIU/day, three times weekly) was started. Within 30 days, dysaesthesias and weakness nearly disappeared. After eight weeks, nerve conduction studies showed slight improvement (table 1). On follow up labial salivary gland biopsy specimen there were significantly fewer infiltrating lymphocytes graded as 2,[6] and sicca symptoms resolved. The serum anti-SS-A/SS-B antibody titres also fell to within the normal range (3.1/2.6, respectively). The clinical and therapeutic time course of patient 3 is summarised in fig 1C.

DISCUSSION

Sjögren's syndrome associated neuropathy includes a wide spectrum of manifestations such as sensory ataxic ganglionopathy, sensorimotor polyneuropathy, demyelinating polyradiculoneuropathy, multiple cranial neuropathy, and vasculitic neuropathy.[7]

Sensory ataxic ganglionopathy often develops in patients with Sjögren's syndrome and is characterised by severe impairment of kinaesthetic sensation with no obvious motor involvement.[8] This form of neuropathy is chronic and progressive, occasionally responding to treatment with IVIG.[9] In our patients with this type (patients 1 and 2), IVIG treatment partially lessened the neuropathic symptoms, but repeated courses were needed and they did not improve the overall status of the Sjögren's syndrome.

Previous reports have indicated that demyelinating polyradiculoneuropathy sometimes develops in patients with Sjögren's syndrome, and have shown that the concurrence of Sjögren's syndrome and demyelinating polyradiculoneuropathy is not coincidental but reflects a common underlying immunological derangement.[6] We diagnosed patient 3 as having SSN with mainly demyelinating features (demyelinating polyradiculoneuropathy), on the basis of the clinical

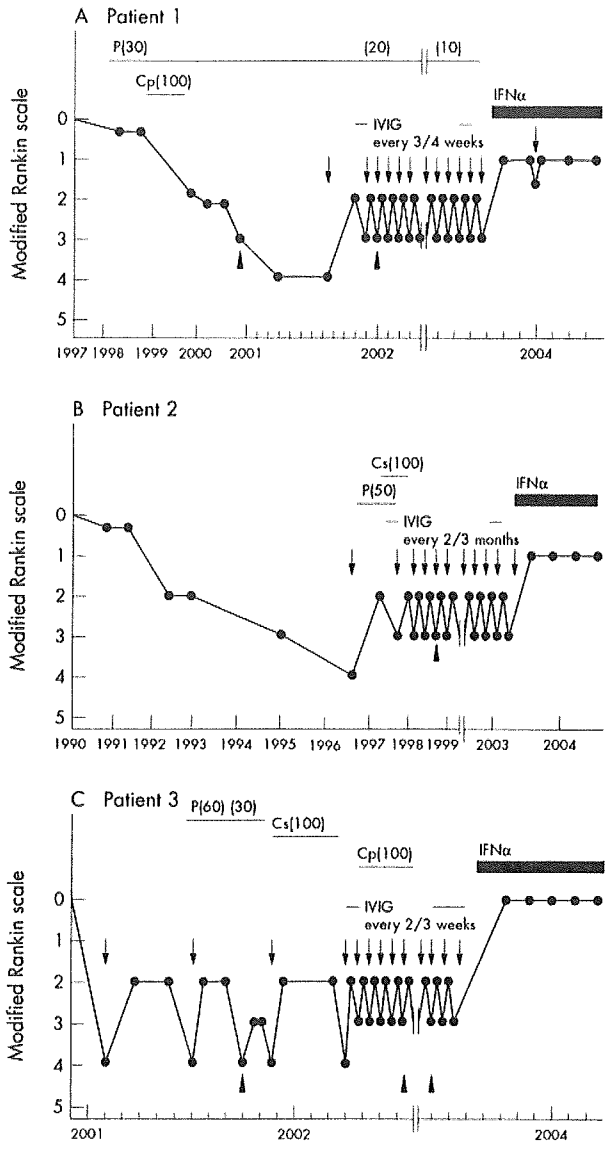

Figure 1 Clinical course of patient 1 (A), patient 2 (B), and patient 3 (C). All three patients needed repeated doses of intravenous immunoglobulin (IVIG) treatment to maintain their activities of daily living. After the initiation of interferon alfa (IFNα), their symptoms were markedly improved. Arrows = episodes of IVIG treatment, 0.4 g/kg for five days; arrowheads = plasmapheresis. Cp, cyclophosphamide; Cs, cyclosporin; P, prednisolone.

features and the findings on nerve conduction studies: reduced conduction velocities, presence of temporal dispersion, and histopathological evidence of remyelination.

Use of IFNα for Sjögren's syndrome first was described in 1993.[10] Since then, orally administered IFNα has been reported to be effective for the sicca symptoms of this condition, resulting in significant increases in salivary gland function and histological improvement in specimens from minor salivary glands.[2]

Before treatment with IFNα, all of our three patients had positive serum anti-SS-A/SS-B antibodies, characteristic salivary gland histopathological findings, and two of the following features: abnormal Schirmer's test result, oral symptoms, or ocular symptoms. Thus they fulfilled the diagnostic criteria of the American-European Consensus Group for Sjögren's syndrome.[11] After treatment, anti-SS-A/SS-B antibody titres fell dramatically to within the normal range in all patients, salivary gland lymphocytic infiltration decreased in patients with follow up specimens (1 and 3), and sicca symptoms resolved in all patients.

To our knowledge, this is the first report to show beneficial therapeutic effects of IFNα on SSN. The mechanism whereby IFNα induced marked improvement in SSN as well as in Sjögren's syndrome itself in our patients is uncertain, but could be related to its immunomodulating effects. As IFNα caused neurological improvement in patients with two different forms of SSN, these two forms appear likely to share a common immunopathogenic mechanism responsive to IFNα treatment, irrespective of the specific form of SSN. However, as our three patients all had chronic, progressive relapsing neuropathies that responded to treatment with IVIG, IFNα effects might conceivably reflect IVIG responsive neuropathic mechanisms, even though Sjögren's syndrome itself did not respond to IVIG.

Trials of IFNα in a variety of forms of SSN are needed to determine whether IFNα therapy represents a first line treatment.

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Dorfin prevents cell death by reducing mitochondrial localizing mutant superoxide dismutase 1 in a neuronal cell model of familial amyotrophic lateral sclerosis

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Abstract

Dorfin is a RING-finger type ubiquitin ligase for mutant superoxide dismutase 1 (SOD1) that enhances its degradation. Mutant SOD1s cause familial amyotrophic lateral sclerosis (FALS) through the gain of unelucidated toxic properties. We previously showed that the accumulation of mutant SOD1 in the mitochondria triggered the release of cytochrome *c*, followed by the activation of the caspase cascade and induction of neuronal cell death. In the present study, therefore, we investigated whether Dorfin can modulate the level of mutant SOD1 in the mitochondria and subsequent caspase activation. We showed that Dorfin significantly reduced the amount of mutant SOD1 in the mitochondria, the release of cytochrome *c* and the activation of the following caspase cascade, thereby preventing eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing the accumulation of mutant SOD1 in the mitochondria may be a new therapeutic strategy for mutant SOD1-associated FALS, and that Dorfin may play a significant role in this.

Keywords: amyotrophic lateral sclerosis, Dorfin, mitochondria, neuronal cell death, superoxide dismutase 1, ubiquitin ligase.

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective death of motor neurons. Approximately 10% of ALS cases are familial (FALS). Missense mutations in the gene coding superoxide dismutase 1 (SOD1) are responsible for approximately 20% of FALS cases (Rosen *et al.* 1993; Hirano 1996) through the gain of unelucidated toxic properties (Yim *et al.* 1996).

Many reports have documented that the mitochondria are involved in the pathogenic process in mutant SOD1-associated FALS. Mitochondrial degeneration, including swelling, dilatation and vacuolization, is an early characteristic pathological feature of FALS and FALS transgenic (Tg) mice models with SOD1 mutations (Dal Canto and Gurney 1994; Wong *et al.* 1995; Hirano 1996; Kong and Xu 1998; Jaarsma *et al.* 2000; Higgins *et al.* 2003). Recently, it was demonstrated that SOD1, considered to be a cytosolic enzyme, exists in the mitochondria (Sturtz *et al.* 2001; Okado-Matsumoto and Fridovich 2001; Higgins *et al.* 2002), and that the mitochondrial vacuoles in mutant SOD1 Tg mice were lined with mutant SOD1 (Jaarsma *et al.* 2001; Higgins *et al.* 2003). Many studies have suggested that the programmed cell death (PCD) pathway contributes to motor neuron death in FALS (Durham *et al.* 1997; Martin 1999; Li *et al.* 2000; Pasinelli *et al.* 2000; Guégan *et al.* 2001; Kriz *et al.* 2002; Raoul *et al.* 2002; Zhu *et al.* 2002). Moreover, we previously reported that accumulation of mutant SOD1 in the mitochondria triggered the release of mitochondrial cytochrome *c*, which subsequently activated the caspase cascade and induced neuronal cell death (Takeuchi *et al.* 2002a). Taken together, these results suggest that the accumulation of mutant SOD1 in the mitochondria is critical in the pathogenesis of mutant SOD1-associated FALS.

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Abbreviations used: ALS, amyotrophic lateral sclerosis; COX, cytochrome *c* oxidase; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; EGFP, enhanced green fluorescent protein; FALS, familial amyotrophic lateral sclerosis; MTS, 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCD, programmed cell death; PI, propidium iodide; SOD1, superoxide dismutase 1; Tg, transgenic.

Dorfin is the product of a gene that we cloned from the anterior horn tissue of the human spinal cord (Niwa *et al.* 2001); it contains a RING-finger/IBR motif (Niwa *et al.* 2001) at its N-terminus. It was reported that a distinct subclass of RING-finger/in-between RING-fingers (IBR) motif-containing proteins represents a new ubiquitin ligase (E3) family that interacts specifically with distinct ubiquitin-conjugating enzymes (Moynihan *et al.* 1999; Ardley *et al.* 2001). Dorfin is a juxtanuclearly located E3 that ubiquitylates various SOD1 mutants derived from patients with FALS, and enhances the degradation of mutant SOD1 (Niwa *et al.* 2002). Whether Dorfin can modulate the protein level of mutant SOD1 in the mitochondria, and the subsequent activation of the mitochondrial caspase cascade, is an important and interesting question.

Here we show that Dorfin significantly reduced the amount of mutant SOD1 in mitochondria, the release of cytochrome *c* from mitochondria into the cytosol and the subsequent activation of the caspase cascade, thereby preventing the eventual neuronal cell death in a neuronal cell model of FALS. These results suggest that reducing mutant SOD1 in the mitochondria may be a useful strategy for the treatment of mutant SOD1-associated FALS, and that Dorfin might play a significant role in this.

Materials and methods

Plasmids

Non-organelle-oriented plasmids expressing the enhanced green fluorescent protein (EGFP)-tagged human SOD1 (wild type, mutant G93A, and G85R) were described previously (Takeuchi *et al.* 2002a,b). These vectors express SOD1-EGFP fusion proteins ubiquitously in each organelle (Takeuchi *et al.* 2002a). They were designated Cyto-WT, Cyto-G93A and Cyto-G85R respectively. Mitochondria-oriented plasmids expressing EGFP-tagged human SOD1 (wil dtype, mutant G93A and G85R) with mitochondrial localizing signals were generated as described previously (Takeuchi *et al.* 2002a). These vectors express SOD1-EGFP fusion proteins mainly in the mitochondria (Takeuchi *et al.* 2002a). They were designated Mito-WT, Mito-G93A and Mito-G85R respectively. The plasmid pcDNA3.1/HisMax-Dorfin, which expresses Xpress-tagged Dorfin, was also described previously (Niwa *et al.* 2001). As a control, we used pCMV-β vector expressing LacZ (Clontech, Palo Alto, CA, USA). All constructs used here were confirmed by DNA sequence analysis.

Cell culture

Mouse neuroblastoma cell line Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen Corp.) as described previously (Takeuchi *et al.* 2002b). They were cultured on Laboratory-Tec II four-well chamber slides (Nalge Nunc International, Rochester, NY, USA) coated with poly-L-lysine (Sigma, St Louis, MO, USA). Transient expression of SOD1 plasmids (0.1 μg of DNA/well) and pcDNA3.1/His Max-Dorfin or pCMV-β (0.3 μg of DNA/well) in Neuro2a cells (2×10^4 cells/well) was accomplished with LipofectAMINE PLUS reagent (Invitrogen Corp.). After incubation for 3 h with transfection reagents, transfected cells were cultured in differentiation medium (DMEM supplemented with 1% fetal calf serum and 20 μM retinoic acid). To detect Xpress-Dorfin fusion protein, 0.5 μM proteasome inhibitor MG132 (Sigma) was added 16 h before collection, as described previously (Niwa *et al.* 2001).

Cell fractionation

At each time point (0, 24 and 48 h) after transfection, cells were collected and gently homogenized with a Dounce homogenizer in cold buffer [250 mM sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics, Basel, Switzerland)]. Cell fractionation was performed as described previously (Takeuchi *et al.* 2002a). To verify the fractionation, each fraction was subjected to western blotting for cytochrome *c* oxidase (COX) as a mitochondrial marker using anti-COX subunit IV mouse monoclonal antibody (1 : 1000; Molecular Probes, Eugene, OR, USA), and β-actin as a cytosolic marker using anti-β-actin mouse monoclonal antibody (1 : 5000; Sigma).

Western blot analysis

The protein concentration was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and western blotting was done as described previously (Takeuchi *et al.* 2002b). To evaluate the level of mitochondrially localized SOD1-EGFP fusion proteins, 20 μg protein from the mitochondrial fraction was loaded. For analyzing the release of cytochrome *c* from the mitochondria into the cytosol, 20 μg protein from the mitochondrial fraction or the cytosolic fraction was loaded.

To assess the levels of SOD1-EGFP fusion proteins, Xpress-Dorfin fusion proteins and the activation of caspase-9 and caspase-3, cells were collected at each time point (0, 24 and 48 h) after transfection, and lysed in TNES buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.1% sodium dodecyl sulfate and protease inhibitor cocktail) as described previously (Takeuchi *et al.* 2002a). For the analysis, 20 μg protein from the total lysate was loaded.

The primary antibodies used were as follows: anti-SOD1 rabbit polyclonal antibody (1 : 10 000; StressGen Biotechnologies, Victoria, BC, Canada), anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.), anti-caspase-3 rabbit polyclonal antibody and anti-caspase-9 rabbit polyclonal antibody (1 : 1000; Cell Signaling, Beverly, MA, USA) and anti-cytochrome *c* mouse monoclonal antibody (1 : 1000; Pharmingen, San Diego, CA, USA). After overnight incubation with primary antibodies at 4°C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (1 : 5000; Amersham Biosciences, Piscataway, NJ, USA). Blots were then visualized with ECL Plus western blotting detection reagents (Amersham Biosciences). The signal intensity was quantified by densitometry using NIH Image 1.63 software.

Immunocytochemistry

At each time point (0, 24 and 48 h) after transfection, cells were fixed with 4% paraformaldehyde for 30 min on ice and then

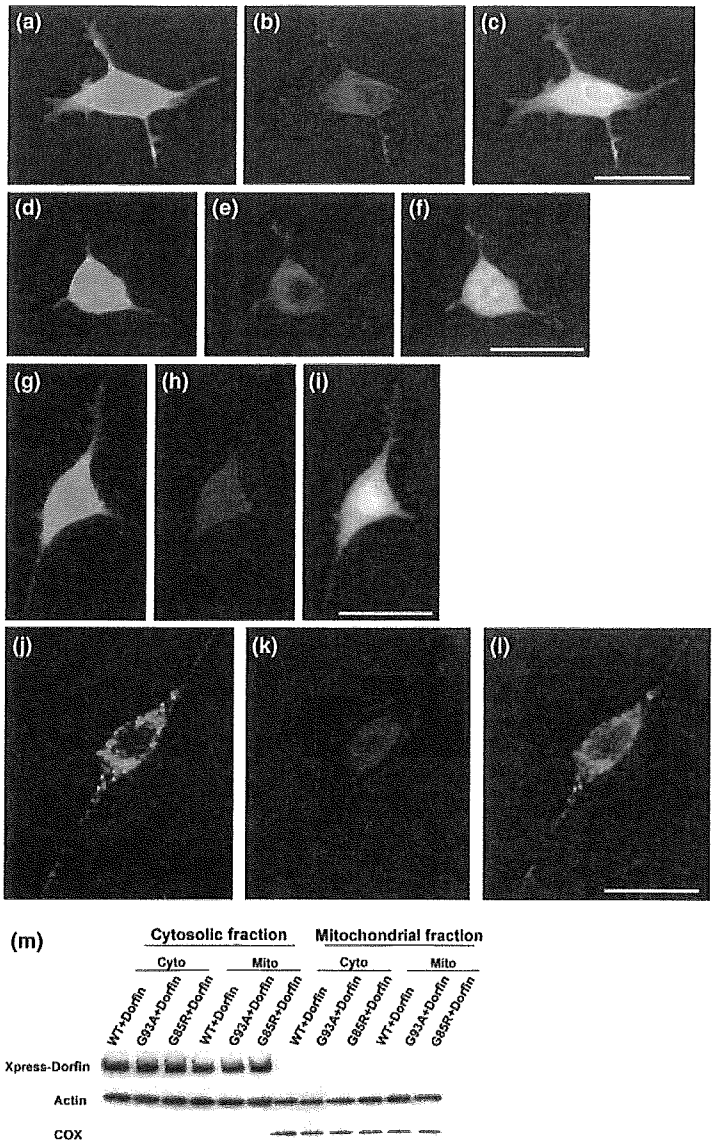

Fig. 1 Subcellular localization of SOD1-EGFP and Xpress-Dorfin in Neuro2a cells. (a–l) Confocal laser scanning microscopic images at 48 h after transfection. (m) Fractionation analysis of Xpress-Dorfin fusion protein. (a–c) Cyto-WT + Xpress-Dorfin, (d–f) Cyto-G93A + Xpress-Dorfin, (g–l) Cyto-G85R + Xpress-Dorfin; (j–l) Mito-G93A + Xpress-Dorfin. SOD1-EGFP fusion proteins (green; a, d and g) and Xpress-Dorfin fusion proteins (red; b, e and h) were observed ubiquitously in the cells with Cyto-SOD1 containing no organelle-oriented signals. SOD1-EGFP fusion proteins and Xpress-Dorfin fusion proteins were co-localized (yellow; c, f and i). In contrast, in the cells with Mito-SOD1, SOD1-EGFP fusion proteins were observed in the mitochondria (green; j) and Xpress-Dorfin fusion proteins (red; k) were observed mainly in the cytoplasm. They were not co-localized in the cells with Mito-SOD1 (l). Cells were counterstained with TO-PRO-3 (blue). Scale bars, 10 μm. Western blots also revealed that Xpress-Dorfin fusion proteins were absent in the mitochondrial fraction (m).

permeabilized with 0.05% Triton X-100 at room temperature for 10 min. They were stained with the anti-Xpress mouse monoclonal antibody (1 : 5000; Invitrogen Corp.) at 4°C overnight. They were subsequently stained with Alexa-568-conjugated secondary antibody (1 : 5000; Molecular Probes) at room temperature for 90 min. Then they were counterstained with 2 μg/mL TO-PRO-3 (Molecular Probes) at room temperature for 10 min, and mounted in Gelvatol. A confocal laser scanning microscope (MRC1024; Bio-Rad Laboratories) was used for the morphological analysis.

Quantitative assessment of mitochondrial impairment and cell death

To assess cell viability through mitochondrial impairment, we used the 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay with CellTiter 96 Aqueous one solution assay (Promega, Madison, WI, USA), as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, MTS assays were carried out in six independent trials. Absorbance at 490 nm was measured in a multiple plate reader as described previously (Ishigaki *et al.* 2002).

Cell death was assessed by the dye exclusion method with propidium iodide (PI; Molecular Probes) as described previously (Takeuchi *et al.* 2002a). At each time point (0, 24 and 48 h) after transfection, cells were incubated with 2 μg/mL PI in DMEM for 15 min at room temperature and mounted in Gelvatol. More than 200 transfected cells in duplicate slides were assessed blindly in three independent trials under a conventional fluorescent microscope. The ratio of dead cells was calculated as a percentage of PI-positive cells among EGFP-positive cells.

Statistical analysis

All results were analyzed by two-way ANOVA with Tukey–Kramer post-hoc test, using Statview software version 5 (SAS Institute Inc., Cary, NC, USA).

Results

Dorfin reduces the levels of total, cytosolic and mitochondrial mutant SOD1

Confocal laser scanning microscopic images revealed that expression of both non-organelle-oriented Cyto-SOD1 plasmid and pcDNA3.1/HisMax-Dorfin was diffusely present in the cells. SOD1-EGFP fusion proteins were co-localized with Xpress-Dorfin fusion proteins (Figs 1a–i), consistent with our previous study (Niwa *et al.* 2002; Takeuchi *et al.* 2002a). In contrast, the expression of mitochondria-oriented Mito-SOD1 plasmid was observed in the mitochondria, as in our previous report (Takeuchi *et al.* 2002a), and was not co-localized with Xpress-Dorfin fusion proteins (Figs 1j–l). Western blots also revealed that Xpress-Dorfin fusion proteins were absent from the mitochondrial fraction (Fig. 1m). At 48 h after transfection, co-expression of Dorfin had reduced the total cell lysate level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 2). In contrast, the amount of SOD1-EGFP fusion proteins expressed by Mito-SOD1 did not show any reduction even with co-expression of Dorfin (Fig. 2). In the cytosolic fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 40%, whereas it did not affect those expressed by Cyto-WT (Fig. 3). As we described previously (Takeuchi *et al.* 2002a), cells with Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction (Fig. 3). In the mitochondrial fraction, co-expression of Dorfin also reduced the level of SOD1-EGFP fusion proteins expressed by Cyto-G93A or Cyto-G85R by approximately 50%, whereas it did not affect those expressed by Cyto-WT (Fig. 4). This reduction in mitochondrial SOD1-EGFP was observed from 24 h after transfection, earlier than that of total or cytosolic SOD1-EGFP. In contrast, in the cells with Mito-SOD1, Dorfin did not reduce the amount of mitochondrial SOD1-EGFP fusion proteins (Fig. 4). The above results suggest that the mitochondrial accumulation of mutant SOD1 without organelle-oriented signals might be a result of mutant SOD1 in the cytosol, and we suggest that Dorfin, a cytosolic E3, reduced the accumulation of mutant SOD1 in the mitochondria by enhancing the degradation of mutant SOD1 in the cytosol, not in the mitochondria.

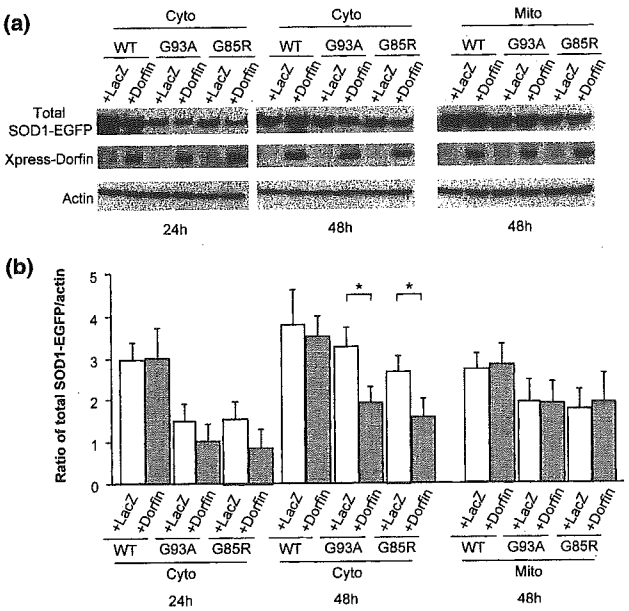

Fig. 2 Level of total SOD1-EGFP fusion protein. (a) Levels of total SOD1-EGFP fusion protein and Xpress-Dorfin fusion protein. (b) Densitometric analysis of total SOD1-EGFP fusion protein expressed as a ratio to actin. Dorfin significantly reduced the level of total SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

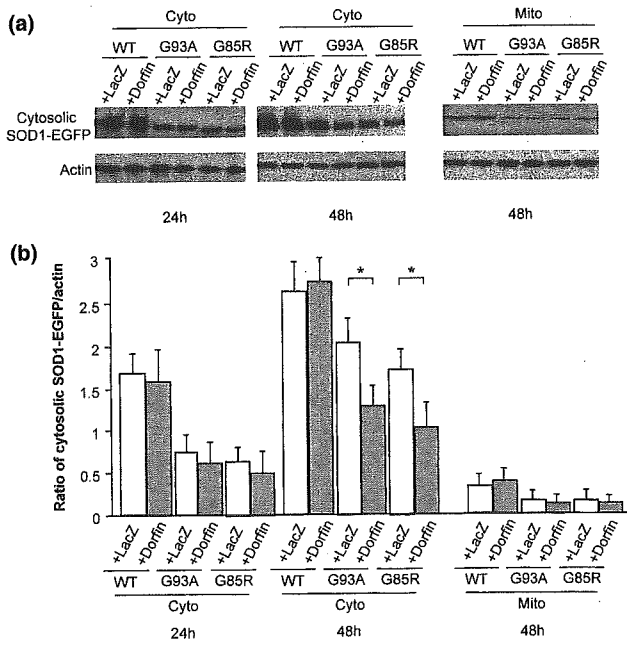

Fig. 3 Level of cytosolic SOD1-EGFP fusion protein. (a) Levels of cytosolic SOD1-EGFP fusion protein. (b) Densitometric analysis of cytosolic SOD1-EGFP fusion protein expressed as a ratio to actin. In the cytosolic fraction, Dorfin significantly reduced the levels of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R. Mito-SOD1 showed very small amounts of SOD1-EGFP fusion proteins in the cytosolic fraction. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

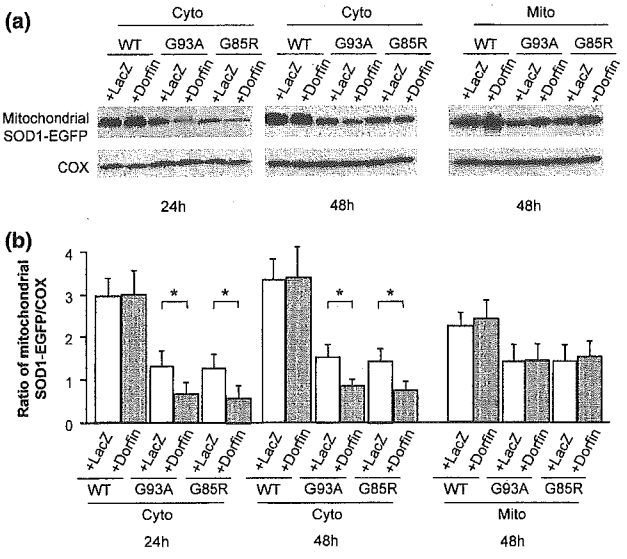

Fig. 4 Level of mitochondrial SOD1-EGFP fusion protein. (a) Levels of mitochondrial SOD1-EGFP fusion protein. (b) Densitometric analysis of mitochondrial SOD1-EGFP fusion protein expressed as a ratio to COX. In the mitochondrial fraction, Dorfin significantly reduced the level of SOD1-EGFP fusion protein expressed by Cyto-G93A or Cyto-G85R, whereas it did not reduce that expressed by Mito-SOD1. Values are mean ± SD ($n = 4$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

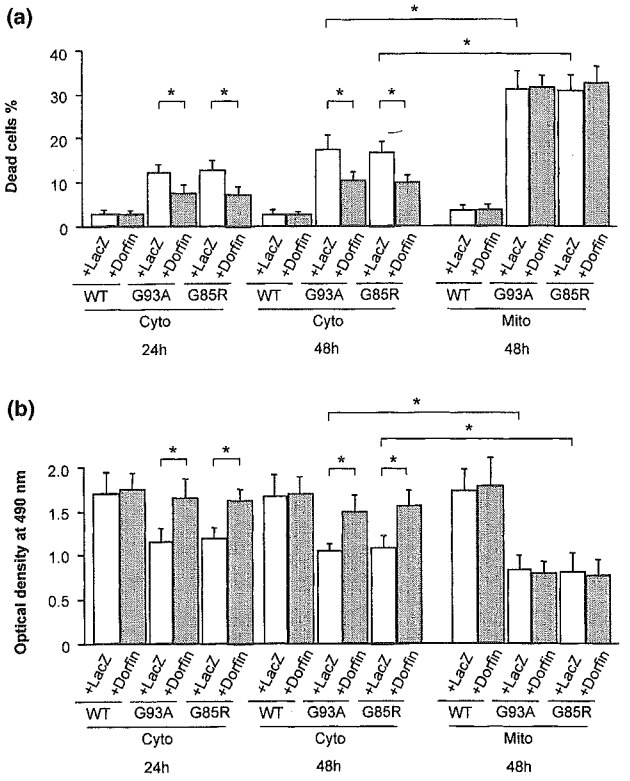

Fig. 5 (a) Frequency of dead cells and (b) mitochondrial impairment analyzed by MTS assay. The cells with Mito-G93A and Mito-G85R exhibited a significantly higher level of cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R. Dorfin significantly decreased cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R, whereas it did not affect those induced by Mito-SOD1. Values are mean ± SD ($n = 6$). *$p < 0.05$ (two-way ANOVA with Tukey–Kramer post-hoc test).

Dorfin protects neuronal cells from mutant SOD1-mediated neurotoxicity by reducing mitochondrial mutant SOD1

As we demonstrated previously (Takeuchi *et al.* 2002a), the cells with Cyto-G93A and Cyto-G85R underwent cell death (Fig. 5a) and mitochondrial impairment (Fig. 5b), whereas those with Cyto-WT did not. The cells with Mito-G93A and Mito-G85R exhibited significantly more cell death and mitochondrial impairment than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 5). Co-expression of Dorfin significantly ameliorated cell death and mitochondrial impairment induced by Cyto-G93A and Cyto-G85R (Fig. 5), as in our previous report (Niwa *et al.* 2002). In contrast, Dorfin did not affect cell death and mitochondrial impairment induced by Mito-SOD1 (Fig. 5), whose protein level Dorfin did not reduce. These findings suggest that Dorfin ameliorates mutant SOD1-mediated neurotoxicity by reducing the accumulation of mutant SOD1 in the mitochondria.

Dorfin reduces mitochondrial cytochrome *c* release and sequential activation of caspase-9 and caspase-3

We next assessed whether Dorfin reduced the mitochondrial death signal associated with the mutant SOD1-mediated cytotoxicity. Western blots revealed that Cyto-G93A and Cyto-G85R induced a gradual increase in the cytochrome *c* released from the mitochondria into the cytosol, whereas Cyto-WT did not (Fig. 6). The cells with Mito-G93A and Mito-G85R also exhibited a higher level of cytochrome *c* release than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Fig. 6). Co-expression of Dorfin significantly reduced the release of cytochrome *c* from the mitochondria into the cytosol induced by Cyto-G93A and Cyto-G85R (Fig. 6). In the cells with Mito-G93A and Mito-G85R, however, Dorfin did not reduce the cytochrome *c* release from the mitochondria into the cytosol (Fig. 6).

Next, we examined whether Dorfin affected the downstream signal cascade of the activation of caspase-9 and caspase-3 following the release of mitochondrial cytochrome *c*. As we demonstrated previously (Takeuchi *et al.* 2002a), western blots revealed that Cyto-G93A and Cyto-G85R induced gradual activation of caspase-9 and caspase-3, whereas Cyto-WT did not (Figs 7 and 8). The cells with Mito-G93A and Mito-G85R exhibited a higher level of activation of caspase-9 and caspase-3 than those with Cyto-G93A and Cyto-G85R, whereas those with Mito-WT did not (Figs 7 and 8). Co-expression of Dorfin significantly reduced