

Table 2 (Continued)

(C) Thymectomy (Tx)						
P-10	F	47	0	20.0		
			47	26.0	IIb	
			Tx →			
			95	47.2	IIIb	Pred
			270	19.8	IIb	Pred
P-11	F	52	0	22.6	IIb	
			58	23.2		
			Tx →			
			170	21.1		Pred
			255	25.0	IIb	
P-12	F	48	0	16.5	IIb	
			95	17.6		
			Tx →			
			210	15.7		Pred
			274	17.0	IIb	

PR, Pharmacological Remission; Pred, Prednisolone; Cyclo, Cyclosporine.

the last clinic visit after or during treatment. As shown in Table 2a, six patients (P1–P6) who underwent early steroid therapy showed impressive clinical improvement associated with a sharp decrease in anti-MuSK Ab titer; from 39.3 to 21.0 nM (P-1), 113.0 to 16.0 nM (P-2), 80.0 to 4.2 nM (P-3), 36.8 to 10.0 nM (P-4), 240.0 to 3.0 nM (P-5), and 33.0 to 0.2 nM (P-6). MG severities showed clinical improvement from class IIIb to pharmacological remission (PR) (P-1), class IVb to IIb (P-2), class IVb to PR (P-3), class IIIb to IIb (P-4), class V to IIb (P-5), and class IIb to PR (P-6).

Muscle-specific tyrosine kinase Ab titers of three patients were measured in serial samples taken before and after simple plasma exchange (Table 2b). The patients responded dramatically to that therapy, Ab titers decreasing from 74.4 to 9.0 nM (P-7), 113.9 to 28.0 nM (P-8), and from 30.0 to 20.5 nM (P-9), indicative of clinical improvement from class V to IIb (P-7), class IVb to IIb (P-8), and class IIIb to IIb (P-9). Moreover, conventional immunosuppression maintained the clinical improvement initially achieved by plasma exchange. In one patient (P-9), the effect had tapered off 45 days after plasma exchange, and Ab titer and disease severity returned to the level before treatment. Prednisolone and cyclosporin administered after MG relapse resulted in slower improvement.

Three patients who had histological abnormalities, including a small hyperplastic thymus, underwent thymectomies (Table 2c). After surgery one patient (P-10) immediately had worsening of dysphagia from class IIb to IIIb associated with a rapid increase in MuSK Ab titer from 26.0 to 47.2 nM. Thymectomy was not effective for the other two patients (P-11, P-12) who showed no change in disease severity and MuSK Ab titer.

We analyzed MuSK Ab titers in relation to quantitative clinical scores on the MGFA scale in serial studies of 12 individuals. MuSK Ab titers and disease severity were correlated ($P = 0.01$ by Kruskal–Wallis).

Epitopes in the extracellular domains of human MuSK

Eighteen sera with MuSK Abs were examined for ^{125}I -MuSK 1–2 and ^{125}I -MuSK 3–4 binding. All predominantly bound to ^{125}I -MuSK 1–2, range 68–97%. Only five of the 18 sera also showed slight binding (20–30%) to ^{125}I -MuSK 3–4 (Table 3).

IgG subclasses of MuSK Abs

In a solid phase RIA with sheep polyclonal antibodies to human IgG subclasses, in all the 18 sera tested MuSK Abs were exclusively IgG4 (Table 4).

Discussion

The MuSK Ab-positive rate found for generalized SNMG patients in Japan was 27% with female predominance (M:F = 5:18). This rate is lower than the 70% positivity originally reported [1] and the 40–50% recently reported [2–7]. It is consistent with the 27–33% reported for Japanese and Korean population [8–10] but significantly higher than the 3.8% Chinese positivity rate [11]. Age at onset ranged from 18 to 72 years old (median, 45 years); 61% of the patients presenting at >40 years of age, later than for Caucasians, and 57–71% of patients presenting at <40 years of age, but the differences was not significant [3,7,17,18].

Table 3 Ratio of MuSK Ig 1–2 and 3–4 Ab in MuSK 1–4 Ab titers

Case	MuSK Ab (nM)	Ig 1–2 domain (%)	Ig 3–4 domain (%)
1	8.4	97.1	2.9
2	19.3	68.1	31.9
3	32.5	81.2	18.8
4	50.0	82.7	17.3
5	33.4	97.3	2.7
6	31.8	95.7	4.3
7	19.9	91.0	9.0
8	11.4	95.2	4.8
9	40.7	87.9	12.1
10	32.0	91.0	9.0
11	33.6	95.0	5.0
12	74.7	95.4	4.6
13	72.0	76.2	23.8
14	46.2	80.8	19.2
15	74.7	96.9	3.1
16	82.2	74.2	25.8
17	110.7	89.5	10.5
18	114.6	71.2	28.8

Table 4 Ratio of IgG subclasses of MuSK Abs

Case	MuSK Ab (nM)	IgG 1 (%)	IgG 2 (%)	IgG 3 (%)	IgG 4 (%)
1	114.6	0.0	0.0	0.0	100.0
2	110.7	0.0	0.0	4.9	95.1
3	82.2	0.0	0.0	0.0	100.0
4	74.7	11.0	21.0	19.0	49.0
5	74.0	4.1	5.0	5.6	85.3
6	72.0	0.0	1.0	0.0	99.0
7	46.2	0.0	0.0	0.0	100.0
8	40.7	5.3	0.0	7.1	87.6
9	33.6	15.1	15.7	0.0	69.2
10	33.4	0.0	0.0	0.0	100.0
11	32.5	5.3	0.0	0.0	94.7
12	32.0	4.2	2.6	1.2	92.0
13	31.8	6.7	6.7	8.3	78.3
14	19.9	0.0	0.0	1.7	98.3
15	19.5	0.0	0.0	0.0	100.0
16	19.3	0.0	0.0	0.0	100.0
17	11.4	0.0	28.9	26.1	45.0
18	8.4	0.0	0.0	0.0	100.0

All the Ab-positive patients had similar patterns of muscle weakness, with prevalent involvement of the bulbar muscles in 100%, ocular symptoms (blepharoptosis and/or double vision) in 80%, and of the respiratory muscles in 35% with frequent myasthenic crises. Limb muscle involvement was comparatively less severe and inconsistent. Japanese MuSK Ab-positive patients therefore have clinical features similar in terms of the predominance of bulbar involvement to those reported for Caucasians.

We evaluated the correlation between MuSK Ab titers and disease severity. Table 2 shows patients who had a good response to early immunosuppressive therapy or simple plasma exchange. Their MuSK Ab titers sharply decreased in parallel with clinical improvement, whereas their Ab titers remained positive. We evaluated the effect of thymectomy in three individuals by measuring MuSK Ab titers in serum samples taken pre- and post-thymectomy. One patient's condition deteriorated after thymectomy and her Ab titer greatly increased. The two others showed neither progression nor Ab titer change during the observation period. Thymectomy therefore did not produce good results. Histological changes in the thymus of MuSK Ab-positive subjects are reported to be minimal and to include rare small germinal centers [19,20] in contrast to SPMG patients who had lymph node-type infiltrates. These findings, together with the lack of benefit of thymectomy, are evidence against a role for the thymus in antigen presentation and antibody production.

Serial studies showed a statistically close correlation between MuSK Ab titers and disease severity. MuSK Ab titers also recently were found to correlate with MG severity [21]. MuSK Ab titers were extremely high in all

the positive cases (Fig. 1). The close relationship between clinical status and MuSK Ab, found by monitoring Ab titers, suggests that MuSK Ab has a significant pathogenic role in MG patients. Circulating MuSK Abs, however, are reported not to cause a MuSK or AChR deficiency at the endplates [22]. Recent experimental models (rabbits [13] and mice [23]), developed by immunization with recombinant MuSK ectodomain protein, produced MG-like muscle weakness with reduced AChR clustering at neuromuscular junctions. These findings clarified the pathogenic MG mechanisms produced by MuSK Ab.

The paramount MuSK Ab IgG subclass in our eighteen patients was IgG4. Limb and intercostal muscle biopsies found neither reduction in AChR numbers nor complement deposition [9,24]. The absence of complement deposits at a patient's end plates is explained by the fact that MuSK Ab is mainly IgG class 4 which does not fix complement [5,25]. The MuSK extracellular domain consists of four MuSK immunoglobulin-like (Ig) domains. Binding analysis of MuSK Abs to ¹²⁵I-MuSK Ig 1-2 or ¹²⁵I-MuSK Ig 3-4 showed that the eighteen sera tested predominantly bound to the ¹²⁵I-MuSK Ig 1-2 domain. The epitope was the N-terminal of the extracellular domain of human MuSK as described previously [5]. Furthermore, MuSK Abs have been shown to inhibit agrin-induced clustering of AChRs [26]. In fact, MuSK Ig 1-2 domains are more responsible for agrin responsiveness of MuSK, in contrast to Ig 3-4 domains which are more responsible for rapsyn association. We postulate that this is relevant to our findings of predominant binding analysis to MuSK Ig 1-2. The characteristics of the MuSK IgG subclass and Ab binding epitope in Japanese patients therefore are similar to those of Caucasians.

Muscle-specific tyrosine kinase Ab-positive patients often suffer facial and tongue muscle atrophy [3,27]. Benveniste *et al.* [28] reported that MuSK Ab plasma may affect the expression of atrophy-related protein and that a facial muscle, the masseter, is the most susceptible. Amongst our MuSK Ab-positive patients, four patients had detectable tongue atrophy from a relatively early phase of illness; weakness was moderate in 2 patients and mild in two patients. More *in vitro* and *in vivo* studies are needed to clarify the pathologic mechanisms that cause the muscle weakness produced by MuSK Ab. MuSK Ab detection provides a valuable biological means of support for the clinical diagnosis of MG and a way to monitor its clinical course.

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REVIEW ARTICLE

Significance of SMP30 in gerontology

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The expression of senescence marker protein-30 (SMP30) was discovered by proteomic analysis. The fact that this molecule's structure is so highly conserved among numerous species strongly suggests that the age-dependent decrease of SMP30 may contribute to senescence. Research that targeted the *SMP30* gene showed deterioration in several organs accompanied by a shortened life span. We then identified SMP30 as gluconolactonase (GNL). The lactonase reaction with L-gulonono- γ -lactone is the penultimate step in vitamin C (L-ascorbic acid) biosynthesis. Our discovery has opened the door to a new aspect of aging studies.

Keywords: aging model, apoptosis, calorie restriction, gluconolactonase, SMP30, vitamin C.

Introduction

Senescence processes are time-dependent, deteriorative and functional changes in all living bodies. After middle age, we humans daily experience loss functions of several organs. However, the degree of deterioration varies in each individual. This variation suggests that senescence is pleiotropic, attributable to numerous candidate molecules. Therefore, to evaluate the extent of senescence, a convenient marker is an absolute requirement. Some modified substances such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) or a hormone like DHEA-S have been reported as useful for this purpose.^{1,2} However, these markers are not directly encoded by the genome. Furthermore, these substances are disadvantageous for modifying a gene to establish a model of aging. Recent advances in gene technology have now provided several useful mouse strains for aging research. In Japan, some well-known aging-prone murine strains (i.e. senescence-accelerated mouse, klotho mice) are presently available.^{3,4} Although experiments with those strains have revealed useful data about senescence, they do not cover the whole spectrum in aging research. In this review, we introduce the

molecules now considered critical for advancing this area of research and a widely applicable strain of mice.

Discovery of senescence marker protein-30

In 1991, to search for the molecular abnormality at work in the aging process, we surveyed age-associated changes in soluble proteins from rat livers by using proteomic analysis and two-dimensional gel electrophoresis (2D-PAGE). Thereby, we detected and isolated a novel rat liver protein, which as first calculated had a molecular weight of 30 kDa according to the commercial molecular weight markers then available. Because the amounts of this protein decreased androgen-independently with aging, we named it senescence marker protein-30 (SMP30).⁵ That designation of SMP30 was accurate until, in more sensitive resolutions, the mass of a SMP molecule proved to be 34 kDa (Fig. 1). However, the earlier name remained in use.

The next step was to prepare an antiserum to SMP30, which was applied to localize SMP30 and identified this protein most prominently in the liver and kidneys among the various organs tested (Fig. 2).⁶ Subsequently, we isolated and characterized two cDNA clones encoding rat SMP30.⁷ The open reading frame consisting of 897 bp encoded 299 amino acids. The estimated molecular weight and pI of the deduced polypeptide were 33 387 and 5.1, respectively. Genomic Southern hybridization analysis demonstrated that SMP30 was

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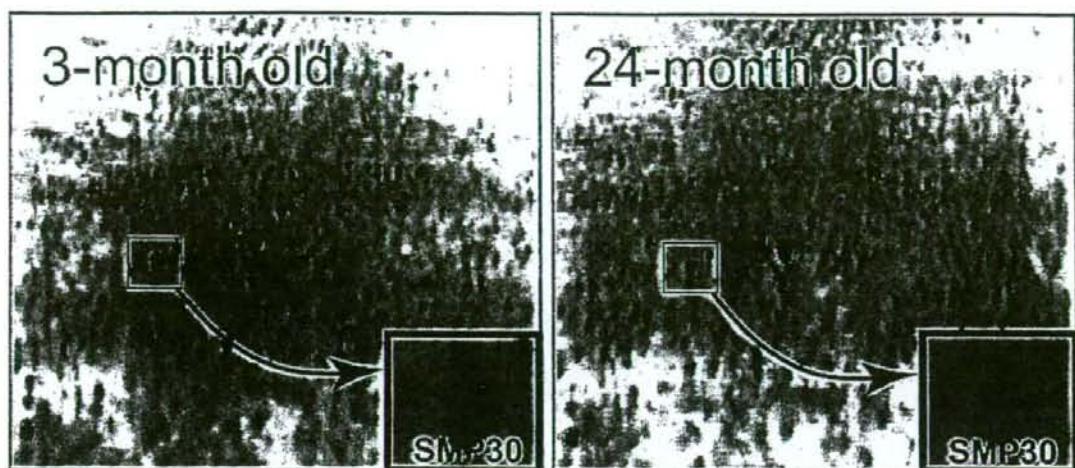


Figure 1 Proteomic profile of senescence marker protein-30 (SMP30) in soluble proteins from 3- and 24-month-old rats. SMP30 is identified as a molecule mass of 34 kDa. Amounts of this protein decrease with aging in an androgen-independent manner.

widely conserved among higher animals. At that time, a computer-assisted homology analysis of nucleic acid and protein databases revealed no marked homology with other known proteins. Therefore, SMP30 seemed to be a novel protein. Additionally, we cloned human SMP30 and documented its 88.6% homology with rat SMP30.⁸ The results of regional mapping using a panel of 11 rodent-human somatic hybrids indicated that the gene is located in the p11.3-q11.2 segment of the X chromosome.⁸ Analysis of the murine genomic clone revealed that the *SMP30* gene was organized into seven exons and six introns, spanning approximately 17.5 kb.⁹ Again, the accumulated genomic information showed that the *SMP30* gene is highly conserved among numerous animal species, and this finding was expanded to include non-vertebrates.^{10,11} These results indicate the critical biological functions of SMP30.

Functions of SMP30

At the time we discovered SMP30, no functional domain was recognized in the entire amino acid sequence. Subsequently, another research group reported a calcium-binding protein identified with SMP30. However, our purified rat SMP30 and the *Sarcophaga* homolog (anterior fat protein, AFP) failed to show calcium-binding activity.^{11,12}

In our laboratory, the evaluation of SMP30 continued for the purpose of examining its possible function in calcium homeostasis.^{13,14} Our results established that HepG2 (HepG2/SMP30), a human hepatoma cell line, expressed large amounts of SMP30 after transfection

with human cDNA. An investigation followed of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the Na^+ -independent Ca^{2+} efflux from these cells after extracellular adenosine triphosphate (ATP) stimulation. Although stimulation with ATP caused a transient increase of $[Ca^{2+}]_i$ in both HepG2/SMP30 and mock-transfected HepG2 cells, the rate of $(Ca^{2+})_i$ decrease after that peak was enhanced twofold by transfection with human SMP30 cDNA. Correspondingly, Ca^{2+} efflux was significantly increased in transfected HepG2/SMP30 cells compared with mock transfectants. In addition, more SMP30 transfectants survived than mock transfectants when cell death was induced by Ca^{2+} ionophore treatment. These results suggested that SMP30 regulates $(Ca^{2+})_i$ by modulating the Ca^{2+} -pumping activity on plasma membranes. Therefore, downregulation of SMP30 during aging may contribute to the deterioration of cellular functions.

In 1999, a report on the function of SMP30 appeared in which Billecke *et al.* characterized a novel soluble protein from the mouse liver as having enzymic activity; that is, hydrolysis of diisopropyl fluorophosphorofluoridate (DFP).¹⁵ This molecule also hydrolyzes sarin, soman and tabun, the poisons famously used by Japanese terrorists. However, it lacks paraoxonase and arylesterase activities with respect to paraoxon and phenyl acetate, respectively. Subsequent amino acid sequencing of the purified DFPase showed it to be identical with SMP30.¹⁶ Thus, SMP30 was classified as an enzyme. However, the substrates used in those studies were artificial chemicals developed after World War I.

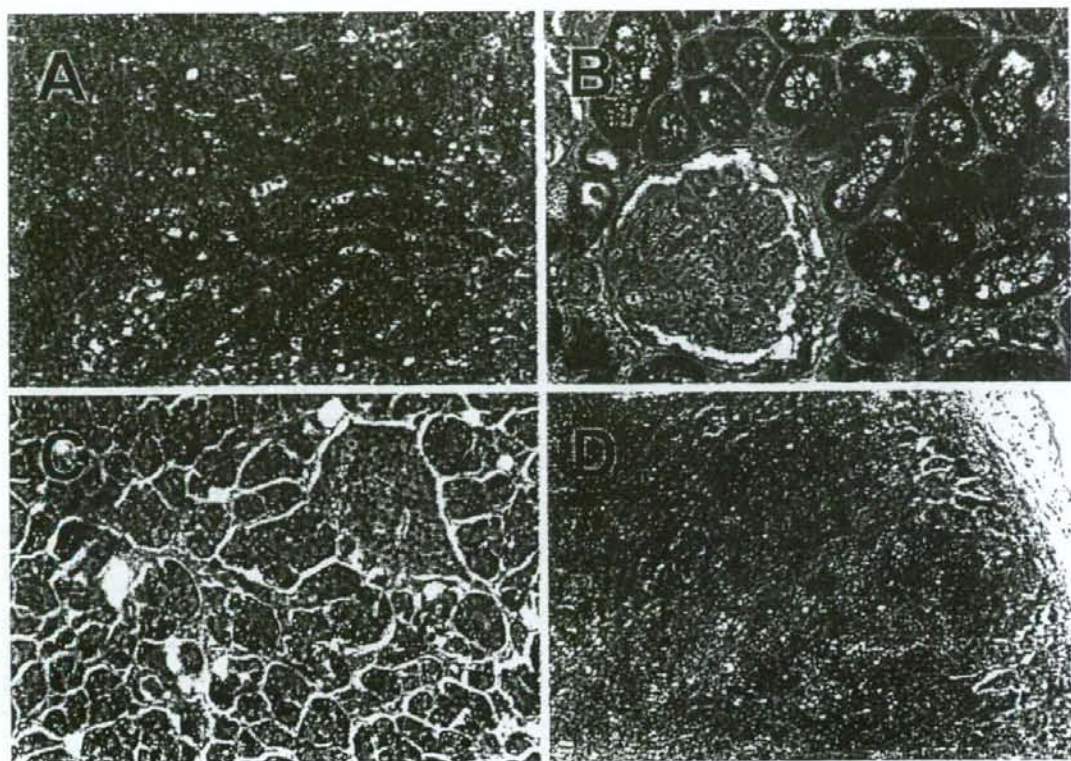


Figure 2 SMP30 expression in human organs. (a) Liver; SMP30 is expressed in parenchymal cells. (b) Kidney; SMP30 is abundant in proximal tubular cells. Localization of SMP30 at the brush border is prominent. (c) Pancreatic; acinar and ductal cells are positive for SMP30. No positive staining occurs in islets of Langerhans. (d) Adrenals; SMP30 is markedly expressed in fasciculata cells of the adrenal cortex.

We also characterized the nature of SMP30 as an organophosphatase.¹² Despite the sequence similarity between SMP30 and a serum paraoxonase (PON), the inability of SMP30 to hydrolyze PON-specific substrates such as paraoxon, dihydrocoumarin, γ -nonalactone and δ -dodecanolactone indicate that SMP30 is distinct from the PON family. The livers from normal mice contained readily detectable DFPase activity, whereas no such enzyme activity was found in livers from mice lacking SMP30. Moreover, the hepatocytes of mice lacking SMP30 were far more susceptible to DFP-induced cytotoxicity than those from the normal mice. This phenomenon accounts for the functional decrease of detoxification in elderly people.

The first report of a natural substrate for SMP30 came from Gomi *et al.*, who identified a SMP30 homolog designated as the luciferin-regeneration enzyme (LRE) in fireflies (*Photinus pyralis*).^{17,18} LRE converts oxyluciferin to luciferin via an intermediary substance. The deduced amino acid sequence based on cDNA analysis showed

at most a 39% identity with insect AFP and mammalian SMP30. However, only 1% of LRE is expressed in the lanterns of fireflies. Despite this possible link to LRE, no genuine function of SMP30 in the whole body has yet been clarified.

Recently, we found a homology between rat SMP30 and two kinds of bacterial gluconolactonase (GNL: EC 3.1.1.17) derived from *Nostoc punctiforme* and *Zymomonas mobilis*.¹⁹ Through subsequent biochemical study, we identified SMP30 as the lactone-hydrolyzing enzyme GNL of animal species.²⁰ SMP30 purified from the rat liver had lactonase activity toward the aldonolactones D- and L-glucono- δ -lactone, D- and L-gulono- γ -lactone, and D- and L-galactono- γ -lactone, with a requirement for Zn^{2+} or Mn^{2+} as a cofactor. Furthermore, in SMP30-knockout mice, no GNL activity was detectable in the liver. Thus, we concluded that SMP30 is a unique GNL in the liver. In the first report on the discovery of GNL in higher animal species, its molecules were not described in detail.²¹

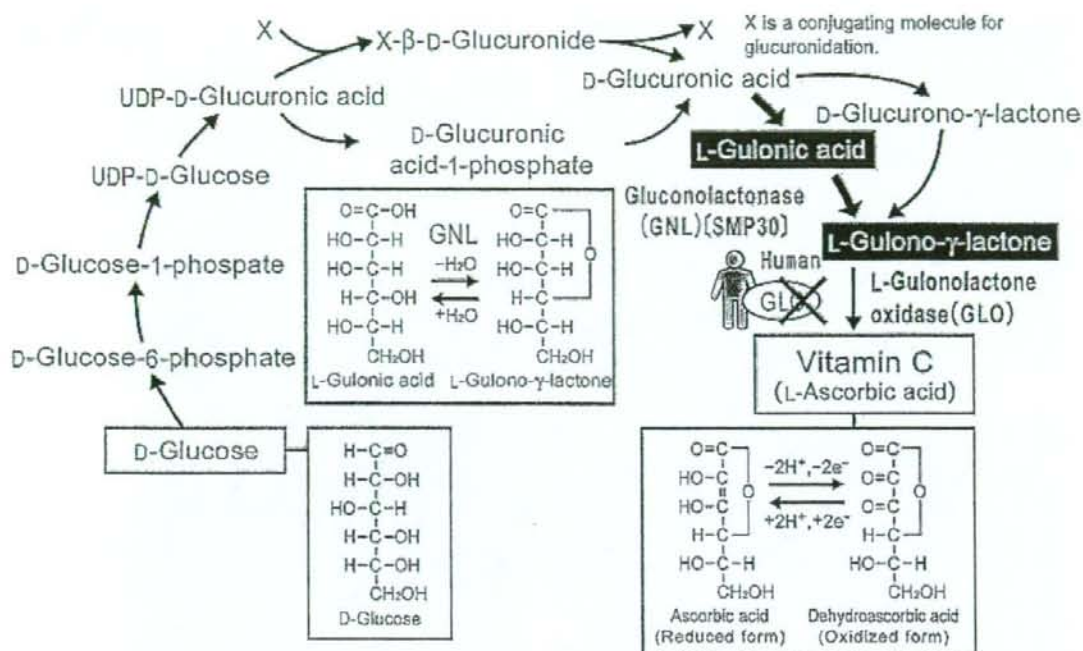


Figure 3 Vitamin C biosynthesis pathway. The pathway from D-glucose to L-gulonic acid is shared with that of early steps in the uronic acid cycle. X is a conjugating molecule for glucuronidation. SMP30 is a gluconolactonase (GNL), which catalyzes from L-gulonic acid to L-gulonolactone. In humans, L-gulonolactone oxidase (GLO) is absent because of mutation.

The lactonase reaction with L-gulonolactone is the penultimate step in vitamin C (L-ascorbic acid) biosynthesis (Fig. 3). SMP30-knockout mice fed a vitamin C-deficient diet did not thrive. They displayed symptoms of scurvy such as bone fracture and rachitic rosary and then died by 135 days after starting this vitamin C-deficient diet. The vitamin C levels in their livers and kidneys at the time of death were less than 1.6% of those in wild-type control mice. In addition, by using SMP30-knockout mice, we demonstrated that the alternative pathway of vitamin C synthesis involving D-gluconolactone operates *in vivo*, although its flux is fairly small.

Additional and unique functions of SMP30 have also been reported. In experiments with the aforementioned HepG2 (HepG2/SMP30) cells expressed large amounts of SMP30, we observed a slower rate and decreased amount of DNA synthesis than in control HepG2 cells (mock transfected with pcDNA3 vector only).

Ultrastructural studies by scanning electron microscopy revealed numerous microvilli covering the surfaces of HepG2/SMP30 cells, whereas few microvilli appeared on control HepG2 cells.²² Subsequently, transmission electron microscopy disclosed groups of HepG2/SMP30 cells with bile canaliculi and specialized adhe-

sion contacts, such as tight junctions and desmosomes, at interplasmic membranes. However, in controls, units of only two cells were seen, and these lacked specialized adhesion junctions. Moesin,²³ which plays a crucial role in the formation of microvilli structures, and ZO-1,²⁴ which concentrated in tight junctions and adherence junctions located at the apical end of epithelial cells, are known to be concentrated in microvilli and at tight junctions, respectively. The intensity of moesin and ZO-1 staining in the contact regions of each cell was markedly higher in HepG2/SMP30 than in control cells. Moreover, moesin stained more interior areas, which corresponded to the microvilli of bile canaliculi. Clearly, bile canaliculi with microvilli formed at the apical ends of HepG2/SMP30 cells. These results indicate that SMP30 has an important physiological function as a participant in cell-to-cell interactions and imply that the downregulation of SMP30 during the aging process contributes to the deterioration of cellular interactivity.

Deficiency of SMP30

Originally, SMP30 was discovered because of its decrease with aging. If this decrease is long lasting, the deficiency of SMP30 in animal models can be regarded

as an ultimate decrease approaching zero. To elucidate the effect of this SMP30 decrease with aging, we introduced a null mutation of the *SMP30* gene into the germ line of mice.²⁴

Despite the complete lack of SMP30, these mutant (SMP30-knockout) mice were indistinguishable from their wild-type littermates in terms of development and fertilization capability. We then investigated tissues' susceptibility for apoptosis induced by cytokines using primary cultured hepatocytes, because SMP30 could rescue cells from death caused by a calcium influx, using a calcium ionophore as previously described.^{13,14} In SMP30-knockout mice, hepatocytes were more susceptible to apoptosis induced by tumor necrosis factor- α (TNF- α) plus actinomycin D (ActD) than hepatocytes from wild-type mice. In addition, the TNF- α /ActD-induced caspase-8 activity in hepatocytes from SMP30-knockout mice was twofold greater than that in matched cells from wild-type mice. In contrast, no significant difference was observed in the TNF- α /ActD-induced NF κ B activation of hepatocytes from wild-type versus SMP30-knockout mice, indicating that SMP30 is not related to TNF- α /ActD-induced NF κ B activation itself.

Moreover, deletion of the *SMP30* gene enhanced the susceptibility to another apoptosis inducer. After we

treated SMP30-knockout mice with sub-lethal amounts of anti-Fas antibodies, liver injury was prominent in SMP30-knockout mice but not wild-type mice (Fig. 4).²⁵ Collectively, these results demonstrate that SMP30 acts to protect cells from apoptosis and other cell injuries.

Another molecular mechanism for the anti-apoptotic function of SMP30 was reported by our collaborators.²⁶ When cells were exposed to TNF- α plus ActD, cell viability was threefold higher in HepG2/SMP30 than control HepG2 cells. The presence of trifluoperazine, a calmodulin inhibitor, attenuated the anti-apoptotic effect of SMP30 in both cell types, but the effect was more prominent in HepG2/SMP30.

Western blot analyses revealed that Akt^{27,28} as a survival factor was activated in HepG2/SMP30 cells in the presence or absence of TNF- α plus ActD. However, the activation was not observed in control HepG2 cells. Further, trifluoperazine inhibited Akt activation in HepG2/SMP30 cells. We therefore propose that interplay between calmodulin and SMP30 regulates Akt activity and, thus, that SMP30 acts as a survival factor in hepatocytes.

Next, we evaluated the effect of a SMP30 deficiency on life span.²⁹ SMP30-knockout mice are viable and fertile but lower in bodyweight and shorter in lifespan

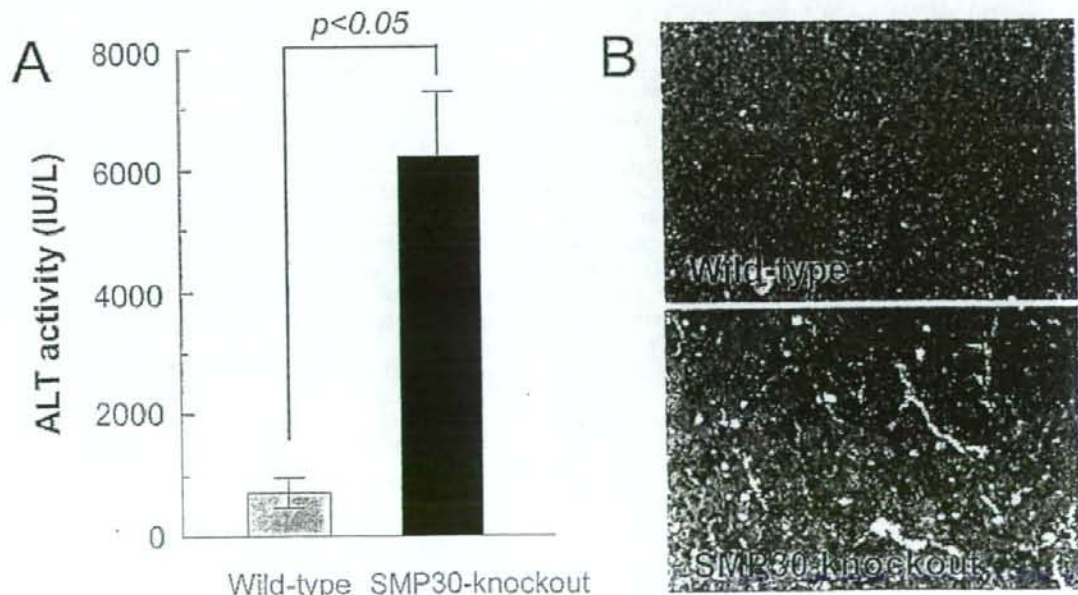


Figure 4 Anti-apoptotic activity of SMP30. Sub-lethal amounts of anti-Fas antibody were applied to SMP30-knockout and wild-type mice. (a) Serum alanine aminotransferase (ALT) levels of SMP30-knockout mice is higher than that of wild-type mice in peripheral blood after anti-Fas antibodies are applied. (b) Massive hemorrhage is visible in livers of SMP30-knockout mice but not wild-type mice.

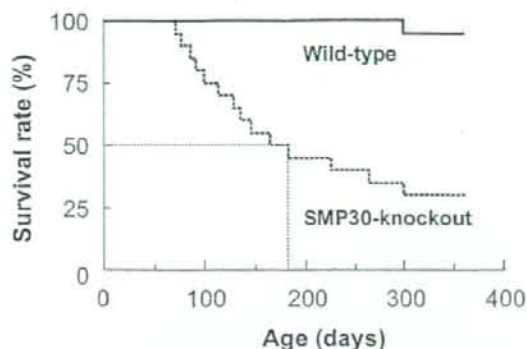


Figure 5 The lifespan is shortened in SMP30-knockout mice whose survival time was only 50%; that is, 180 days (6 months), in the 20 animals studied for comparison to the wild-type.

than their wild-type counterparts (Fig. 5). Histopathological examination showed no particular cause of the death in SMP30-knockout mice. The only noticeable event was marked emaciation in each individual. This finding suggests that the lack of SMP30 seems to be a model of "natural death." Because death often occurs in a background of malnutrition, the genuine cause deserves further study.

Via electron microscopy, hepatocytes from SMP30-knockout but not the wild-type mice at 12 months of age clearly contained many lipid droplets, abnormally enlarged mitochondria with indistinct cristae and enlarged lysosomes filled with electron-dense bodies. In liver specimens from SMP30-knockout mice, the marked number of lipid droplets visible around the central vein increased notably in size and amount as the animals aged. Biochemical analysis of neutral lipids, total hepatic triglycerides and cholesterol from SMP30-knockout mice showed approximately 3.6- and 3.3-fold higher levels, respectively, than those from age-matched wild-type mice. Moreover, values for total hepatic phospholipids from SMP30-knockout mice were approximately 3.7-fold higher than those for their wild-type counterparts.

By thin-layer chromatography analysis, phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine and sphingomyelin accumulations were detected in lipid extracts from the livers of SMP30-knockout mice. Conceivably, this abnormal lipid metabolism might be one cause of the shortened lifespan of these mice without SMP30.

Because SMP30 is expressed in almost all organs except those with hematopoietic function, we widened our search for the pathological features of aging beyond those in the liver and kidneys. As we previously reported, SMP30-knockout mice are novel models of

senile lungs with age-related airspace enlargement and enhanced susceptibility to harmful stimuli.³⁰ Aging and smoking are considered as major contributing factors for the development of pulmonary emphysema. For that reason, we evaluated whether SMP30-knockout mice are susceptible to oxidative stress associated with aging and smoking.³¹ In the lungs of SMP30-knockout mice, protein carbonyls tended to increase with aging and were significantly higher than in the age-matched wild-type mice. Exposure to cigarette smoke generated marked airspace enlargement with significant parenchymal destruction in the SMP30-knockout mice than in the wild-type mice. The protein carbonyls, malondialdehyde, total glutathione and apoptosis of lung cells were significantly increased after an 8-week exposure to cigarette smoke in the SMP30-knockout mice. Because our results suggest that SMP30 protects the lungs from oxidative stress associated with aging and smoking, the SMP30-knockout mouse could be a useful animal model for investigating age-related lung diseases such as emphysema.

In submandibular glands, the influence of a SMP30 deficiency is more intense than in other organs. For example, marked swelling of mitochondria and decreased numbers of secretory granules can be observed in 12-month old SMP30-knockout mice (Fig. 6).³²

The expression of SMP30 in the brain has been detected, although at a very low level. Nevertheless, the effect of this deficiency is prominent.³³ We showed that the generation of reactive oxygen species (ROS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activities were significantly elevated in the brains of SMP30-knockout mice. The increased oxidative status in these mice was further confirmed by their increases of oxidatively modified proteins such as dityrosine formation and carbonylation in the cerebral cortex. Moreover, the brains of SMP30-knockout mice manifested increased amounts of Mac-1,³⁴ which is regarded as the key mediator responsible for the migration of neutrophil, protein and myeloperoxidase activity, supporting the putative anti-oxidative action of SMP30. Interestingly, the activities of other major anti-oxidant enzymes (i.e. superoxide dismutase, catalase and glutathione peroxidase) in the brain were not affected by SMP30 depletion. Our results documented that, in the brain, SMP30 has a protective action against oxidative damage without influencing anti-oxidant enzyme status.

Two morphological features considered to be a hallmark of senescence are apparent in SMP30-knockout mice. At 12-months of age, SMP30-knockout mice had clearly visible deposits of lipofuscin and senescent-associated β -galactosidase in their renal tubular epithelia.³⁵ These features are compatible with high electron dense deposits in lysosomes. This observation supports

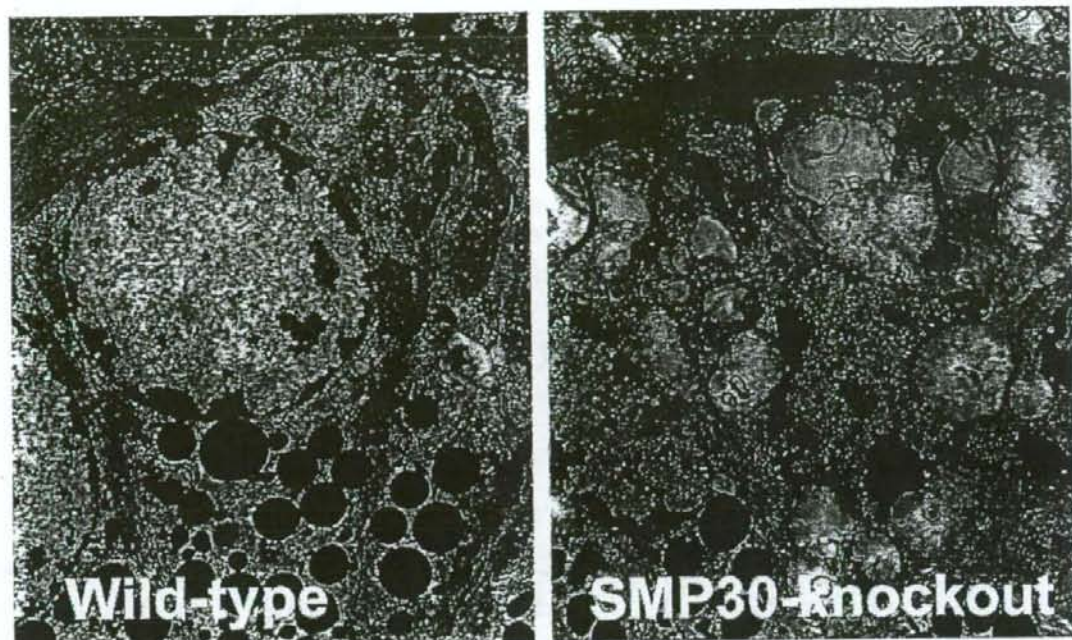


Figure 6 Degenerative subcellular components in SMP30-knockout mice. Granular duct cells of submandibular glands from a 37-week old SMP30-knockout mouse shows swollen mitochondria and a decrease of secretory granules.

the conclusion that the SMP30-knockout mouse is a useful model of ordinal senescence.

Most importantly, we have now identified an indisputable function of SMP30, that is, the production of vitamin C.²⁰ Because vitamin C is uniformly regarded as an anti-oxidant, we and others believe that a shortage of vitamin C induces senescence. This assumption has been proved by our observation that the lifespan of SMP30-knockout mice is significantly shorter than that of matched wild-type mice (Fig. 5).²⁹

Modulation of SMP30

The suppressive effect of SMP30 on senescence has been introduced in this review. The other side of that coin is also true; enhancement of SMP30 expression prevents the pleiotropic dysfunction of organs during the aging process. Based on our studies, one can conclude that, in this context, the most influential factor causing senescence is oxidative stress. The relationship between SMP30 expression and oxidative stress is critical for understanding the mechanisms of senescence.

We used calorie restriction to explore age-related changes in *SMP30* gene expression.³⁶ The restriction of calories is the most probable paradigm for manipulating the development of senescence in humans.³⁷ The thrust

of our investigation was based on the ability of calorie restriction to defend against age-related oxidative stress and the inflammatory process. The rats used for these experiments were divided into two groups: those fed ad libitum and those given a 40% calorie restricted diet. As expected, the animals' SMP30 expression declined with age, but in the calorie restricted group, this decline was clearly blunted (Fig. 7). Our data showed that the downregulation of SMP30 was accompanied by an increased generation of ROS, the oxygen-reactive entity. Therefore, the potent anti-aging and anti-oxidative action of a low-calorie diet effectively suppressed the age-related downregulation of SMP30 by ROS reduction.

Because age-related changes in SMP30 expression can be modulated by anti-oxidative action, the modulation of *SMP30* gene expression was explored by: (i) anti-oxidative calorie restriction of rats; (ii) pro-inflammatory lipopolysaccharide (LPS) administration to aged rats; (iii) oxidative stress promoter, *tert*-butylhydroperoxide (t-BHP) injection of mice; and (iv) t-BHP-treatment of Ac2F cells, a normal rat liver cell line.³⁸ We focused on the binding activity of an unidentified transcription factor at two sites located in the SMP30 promoter region.³⁹ Our results showed, first, that a calorie-restricted diet prevented the age-related decrease in SMP30 expression. Second, the binding of

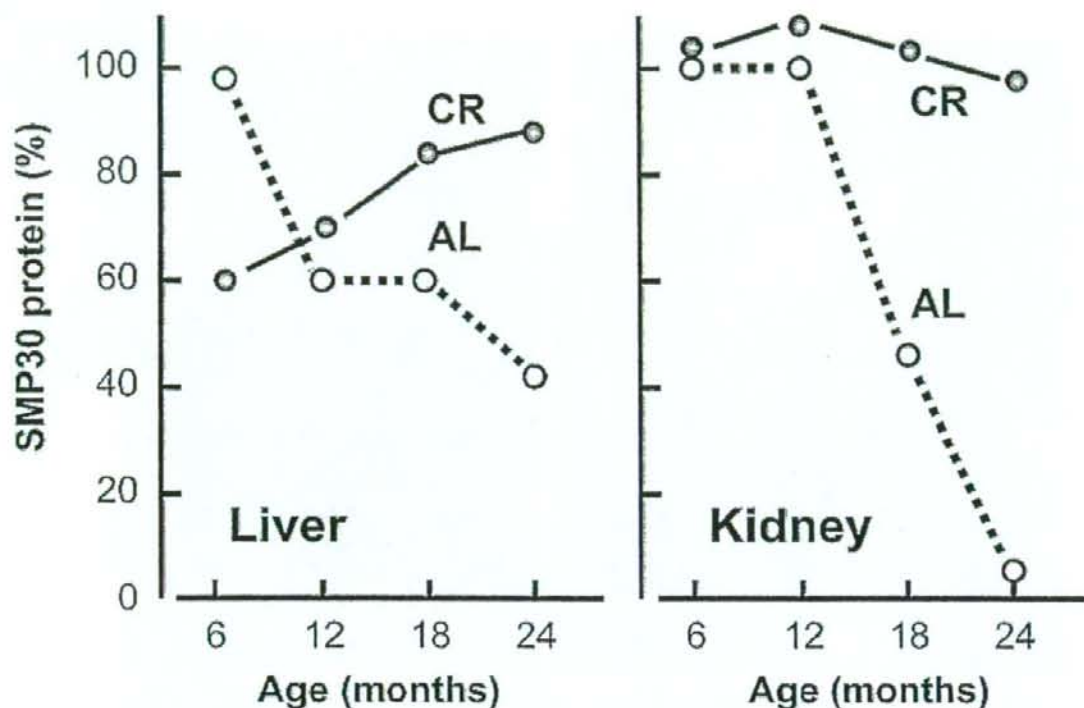


Figure 7 Enhanced SMP30 protein levels in the liver and kidneys from a calorie restricted (CR) diet. Rats were divided into an ad libitum fed group (AL) and a 40% CR group.

this transcription factor to the two sites in the SMP30 promoter region decreased after treatment with t-BHP or LPS. These findings were confirmed by using the anti-oxidant NAC and the ERK-specific inhibitor PD098059, both of which blunted the decrease in SMP30 gene expression. Third, the binding by t-BHP also diminished at both sites in the Ac2F cell system. These outcomes strongly indicate that the SMP30 transcriptional process is redox-sensitive and that its modulation occurs at DNA binding sites in the promoter region. The downregulation of SMP30 likely involves the ERK signal pathway.

Conclusions

Proteomics analysis has provided us with a large amount of information about aging in general and, in particular, about age-associated molecules including SMP30. This factor is one of the best prospects for elucidating the mechanism of senescence, as we have done in functional analyses of multiple organs. We propose that the SMP30-knockout murine strain, in which SMP30 is completely absent, is the most useful model available for understanding human aging. In fact,

the absence of SMP30 is the reason why this strain lacks an enzyme responsible for the synthesis of vitamin C as an anti-oxidant. Further research on the biological functions of SMP30 will assuredly produce useful tools for treating or offsetting the deleterious effects of aging in humans.

Acknowledgments

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神経筋接合部疾患における抗 MuSK 抗体と病態機序

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Key words: 重症筋無力症, 筋特異的カイネース, 神経筋接合部, 自己抗体

30年前に重症筋無力症が、神経筋シナプスの筋側部に集まる神経伝達分子レセプター（アセチルコリンレセプター；AChR）に対する自己抗体により発症することが、Lindstromらによって証明された¹⁾。2001年、Hochらはseropositive重症筋無力症の患者の2/3の血清中に抗MuSK (Muscle Specific Kinase)抗体を検出した²⁾。われわれは重症筋無力症患者の抗MuSK抗体の定量アッセイシステムを神戸薬科大学・太田教授と共同で開発して国内の患者をしらべたところ、seronegative重症筋無力症の約30%で抗MuSK抗体が高値で検出されることを報告した³⁾。その他の臨床研究報告から、抗MuSK抗体が重症筋無力症との因果関係はきわめて深いと予想されたにもかかわらず、抗MuSK抗体による動物発症モデルを作成することができず、抗MuSK抗体が重症筋無力症の真の発症原因となりうるのかどうか激しい議論がおきた⁴⁾。つまり、抗MuSK自己抗体と重症筋無力症発症の因果関係を明らかにするには、動物モデルで抗MuSK自己抗体によって症状を誘導できるかどうか重要な問題であった。われわれは、精製したMuSK蛋白をウサギに免疫することにより、抗MuSK自己抗体により重症筋無力症様の症状を発症することを世界ではじめて示した(Fig.1)⁵⁾。発症したウサギの神経筋シナプスでは、AChRの凝集が減少しており、筋電図も重症筋無力症と同じパターンを示すことから、抗MuSK自己抗体がMuSKの機能を阻止して神経筋シナプスの維持を障害することを明らかにした。いいかえればMuSKが、神経筋シナプスを維持するのに必須であることを明らかにした。

抗AChR抗体による重症筋無力症の発症メカニズムは、これまで多くの研究者により明らかにされてきた。それは大きく分けて3つの機序に分けることができる。(a)抗AChR抗体に結合して活性化された補体による組織破壊。(b)抗体結合によるAChRの発現減少とその結果による神経伝達の減少(antigenic modulation)。(c)抗体によるAChRの機能阻害⁶⁾。とくに(a)の補体によるシナプス後膜の破壊が、抗AChR抗体による重症筋無力症の発症機序として主要な原因と考えられているが、一方で抗MuSK抗体陽性の重症筋無力症患者血清に存在する抗MuSK抗体IgGのサブクラスは圧倒的にIgG4である(一部IgG2も検出することができるが大

変少ない)。しかしながらヒトIgG4には補体介在性作用はない。抗AChR抗体に関しては、これまでの研究から、補体介在性のIgG1やIgG3が主要なサブクラスであることがわかっている。このように抗MuSK抗体による病態機序には、補体介在性後シナプス膜破壊の関与はほとんどないと考えられることが、それまでの研究から示された。この点は抗MuSK抗体が重症筋無力症の発症原因となりうるかどうかの議論でも、抗MuSK抗体によって発症する動物モデルを示すことに成功していなかったことに加えて、謎とされていた一つである。

抗MuSK抗体による重症筋無力症の発症メカニズムを明らかにするには、MuSKの機能を知る必要がある。しかしながらMuSKの機能も未解明であり、リガンドは現在も明らかではない。agrinがMuSKのリガンドではないかと予想して、両方の分子が直接結合するかどうか多くの努力が払われたが結局誰も証明することができず、現在ではagrinはMuSKのリガンドではないというのがコンセンサスである。一方で、agrin蛋白を培養筋細胞に添加すると、その直後にMuSKのチロシンカイネースが活性化され、数時間後にはAChRの凝集が観察される。MuSKは他のレセプター型チロシンカイネースと同様に、細胞外ドメインに未知のリガンドが結合して2量体を形成することにより活性化されると予想される。MuSKのリガンドはagrinと未知の分子の複合体から成るのか、あるいはagrinと結合できる別の分子がMuSKのco-receptorとして存在する可能性が現在考えられている。ところでラミニンや植物レクチン(VVA:B4)によっても培養細胞AChRを凝集することができるが、これらのいずれの刺激もMuSKを活性化しない。したがってAChRを凝集するシグナル経路はagrin-MuSK活性化以外にも存在することは明らかである。生体内でも、そのシグナル経路が重要な役割を果たしていると考えられる。実際に胎児期に運動神経終末が進展して訪支配する前、すなわち神経終末から分泌されるagrinの非存在下であっても、MuSKの働きにより筋組織の中心領域でAChRの緩やかな集合が誘導されることが示されている。

そこで、上記の培養筋細胞のAChR凝集誘導の実験系を

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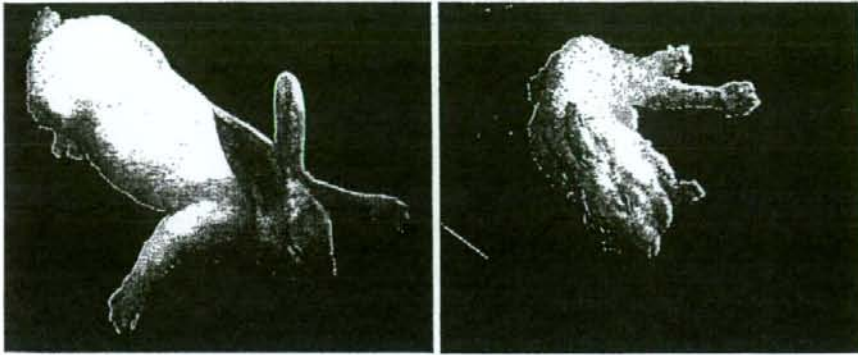


Fig. 1 Manifestation of muscle weakness after injection of purified MuSK proteins in experimental animals (a parietic rabbit).

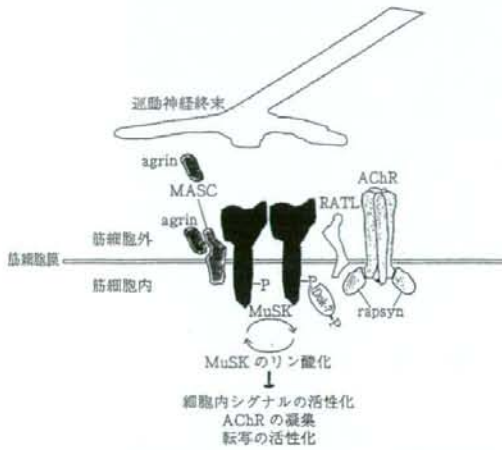


Fig. 2 Pathogenic mechanisms of the antibodies are composed of multiple events by the stall of MuSK functions as a multifunctional platform regulating synapse formation and maintenance. These reflect the complexity of clinical features ranging from a typical MG to the variants.

使って、われわれは抗 MuSK 抗体による重症筋無力症の発症メカニズムを解析した。大変興味深いことに、抗 MuSK 抗体で発症した筋無力症の動物モデルの自己抗体 (精製した IgG) は、agrin だけでなくラミニンやレクチンによる培養筋細胞の AChR 凝集のすべての誘導を抑制することを示した。これらの結果は発症動物の抗 MuSK 抗体は、補体の関与しない前述した (b) や (c) のメカニズムによって、MuSK 機能を抑制することを強く支持する。さらに発症したウサギの神経筋シナプスの AChR 凝集の顕著な減少はあるものの、補体による破壊像は観察されていない。これらの結果から、補体結合能の無い抗 MuSK 自己抗体 (IgG) であっても抗 MuSK 抗体は

性の重症筋無力症の発症の原因となることを、われわれの動物発症モデルは示していると考えられる。

MuSK が神経筋シナプスの形成に必要なことは、これまでノックアウトマウスを使って示されている。加えて、われわれの重症筋無力症の発症モデル動物の研究から、MuSK は成熟した神経筋シナプスの維持にも必須であることを示した (Fig. 2)。われわれは、抗 MuSK 抗体陽性重症筋無力症は自己抗体により MuSK のシナプスの維持機構が抑制されるために発症すると考えている。さらに、Oxford 大学の Beeson らが報告した Dok7 突然変異による先天性筋無力症と、MuSK 抗体陽性重症筋無力症の臨床症状およびわれわれの動物モデルの病理組織像に共通点があり大変興味深い¹⁰⁾。抗 MuSK 抗体による重症筋無力症と Dok7 突然変異による先天性筋無力症の発症メカニズムとの共通点は、それらの病態と MuSK の機能を理解する上で今後重要な鍵となると考えている。

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Abstract

Pathogenic mechanisms of myasthenia gravis induced by antibodies against muscle-specific kinase

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Antibodies to acetylcholine receptor (AChR) are major cause of the human autoimmune disease, myasthenia gravis (MG). Additionally, autoantibodies against Muscle-specific kinase (MuSK) were found in a proportion of patients with generalized MG. After the identification of MuSK antibodies in MG patients, laboratory test for measuring antibodies to MuSK is now required to confirm the diagnosis of MG and the clinical treatment as well as AChR antibodies. MuSK is critical to the clustering of AChR and plays multiple roles at neuromuscular junctions (NMJ). However, it has been dispute concerning the pathogenicity of MuSK antibodies in muscle weakness of MG, as the experimental autoimmune MG caused by MuSK antibodies was absent. Here we describe the recent progress to understand the pathogenic roles of MuSK antibodies in muscle weakness of experimental animals induced by MuSK protein.

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Key words: myasthenia gravis, muscle-specific kinase, neuromuscular junction, autoantibodies



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Myasthenia gravis experimentally induced with muscle-specific kinase

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Keywords:	myasthenia gravis (MG), experimental autoimmune MG (EAMG), muscle-specific kinase (MuSK), acetylcholine receptor (AChR), neuromuscular junction (NMJ), congenital myasthenic syndromes (CMS)



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Shigemoto *et al.*: EAMG with MuSK antibodies.

Myasthenia gravis experimentally induced with muscle-specific kinase

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KEY WORDS: myasthenia gravis (MG); experimental autoimmune MG (EAMG);
muscle-specific kinase (MuSK); acetylcholine receptor (AChR); neuromuscular

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