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Ⅴ．研究成果の刊行物・別冊
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ORIGINAL ARTICLE

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Twenty-four-week follow-up examination of a leukocytapheresis therapy in rheumatoid arthritis

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Abstract Several clinical trials have demonstrated that leukocytapheresis (LCAP) is a safe and effective therapy for patients with refractory rheumatoid arthritis (RA). However, most of those reports were limited to short-term clinical observation. We have treated 11 RA patients with LCAP and observed them for 24 weeks after the final administration. The 11 cases included 3 diabetes patients, 2 patients with interstitial pneumonia, 1 patient with diffuse panbronchiolitis, and 1 patient with old pulmonary tuberculosis. Alternative therapies for all of these patients were considered difficult. Once-a-week LCAP administration was added for 5 weeks to the previous therapeutic regime in all patients, and the treatment efficacy was prospectively qualified. At 4 weeks after the final LCAP therapy, 8 of the 11 patients (73%) had achieved an American College of Rheumatology (ACR) 20% response, and 3 of the 11 (27%) had achieved both ACR 50% and ACR 70% responses. Although the efficacy decreased after the observation periods, an ACR 20% response was maintained in 5 patients (45%) at 24 weeks. Although only a limited number of patients were examined in this study, the results suggested that LCAP therapy will be beneficial to RA patients, including patients who cannot be treated with tumor necrosis factor inhibitors or conventional disease-modifying antirheumatic drugs.

Key words DAS28-CRP · Leukocytapheresis (LCAP) · Rheumatoid arthritis (RA)

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Introduction

Cell populations as well as soluble factors have been targeted for the treatment of rheumatoid arthritis (RA). Plasmapheresis was first applied as a treatment for RA in 1963.[1] Furthermore, lymphocyte depletion has been used as an alternative to plasmapheresis in the treatment of RA.[2–4] In the past, lymphocytes were depleted by the total irradiation method,[5] thoracic duct drainage (TDD),[6] or the centrifuge method.[3] However, none of these therapies could become a standard regime because each had severe adverse effects and required both delicate surgical procedures and complex apparatuses. Recently, leukocytapheresis (LCAP) was developed as an alternative application to deplete circulating leukocytes in the treatment of RA.[2–4] Polyester fiber filters, used for LCAP, have been established as safely utilized materials to remove leukocytes from peripheral blood.[7] Leukocytapheresis is usually administered once a week for 5 weeks in patients with RA, and Hidaka et al.[8] as well as Ueki et al.[9] have reported the efficacy of LCAP therapy in patients with RA by short-term observation.

In Japan, LCAP therapy was approved as a therapeutic indication for refractory RA in April 2005.[10] However, there have been few reports on the mid- or long-term efficacy of LCAP after completion of the therapy. Here we present a 24-week follow-up examination of LCAP therapy in patients with refractory RA.

Patients and methods

We studied 11 patients (3 men and 8 women; mean age 56.6 ± 4.4 years, range 38–74 years) who met the American College of Rheumatology (ACR) criteria for RA[11] and who were treated at the First Department of Internal Medicine, Graduate School of Biochemical Sciences, Nagasaki University or the Department of Nephrology, Nijigaoka Hospital. Of the 11 patients, 8 were diagnosed with refractory RA and responded poorly to conventional disease-modifying

antirheumatic drugs (DMARDs). Two of the 11 patients were allergic to DMARDs. Three patients were complicated with diabetes mellitus, 2 with interstitial pneumonia, 1 with diffuse panbronchiolitis with *Pseudomonas aeruginosa* infection, and 1 with old pulmonary tuberculosis. Of the 11 RA patients, 10 were taking prednisolone (mean ± SEM: 8.6 ± 0.7 mg/day; range: 5–10 mg/day), 6 nonsteroidal anti-inflammatory drugs (NSAIDs), 7 methotrexate (mean ± SEM: 6.0 ± 0.6 mg; range: 4–8 mg/week), 3 sulfasalazine (1.0 g/day), 1 D-penicillamine (100 mg/day), 1 bucillamine (200 mg/day), 1 cyclosporin (100 mg/day), and 1 mizoribine (150 mg/day). At the beginning of the observation period, 6 patients were taking one DMARD each and 4 were taking two DMARDs each. The disease activity of RA before LCAP was not suppressed by the previous therapeutic regime, since only one patient had achieved an ACR 20% within the 3 months prior to the beginning of the study. Hand radiographs were obtained in all patients before the LCAP therapy. Each radiograph was given a Steinbrocker score, and the disability of each patient before LCAP therapy was measured according to those scores.[12] The characteristics of the patients are summarized in Table 1. Leukocytapheresis (Cellsorba column, CS-120; Asahi Medical, Tokyo, Japan) therapy, added to the previous therapeutic regime, was performed once a week for 5 weeks. The RA disease activity was assessed by ACR core set and disease activity score (DAS) 28 C-reactive protein (DAS28-CRP) values.

Table 1. Characteristics of rheumatoid arthritis (RA) patients

No.[a]	11
Complications[a]	
Type 2 diabetes mellitus	3
Interstitial pneumonia	2
Old pulmonary Tbc	1
Carrier of *Pseudomonas aeruginosa*	1
Age (years)[b]	56.6 ± 4.4
Sex (M:F)[b]	3:8
Disease duration (years)[b]	3.8 ± 1.0
Tender joint counts[b]	18.0 ± 2.9
Swollen joint counts[b]	8.6 ± 1.7
Patient's assessment of pain[b]	78.1 ± 4.1
Patient's global assessment of disease activity[b]	75.6 ± 2.9
Physician's global assessment of disease activity[b]	64.5 ± 6.1
Patient's assessment of physical function[b]	16.6 ± 1.7
C-reactive protein (mg/dl)[b]	4.4 ± 0.8
Stage[a]	
I	3
II	3
III	4
IV	1
Class[a]	
1	0
2	6
3	5
4	4
Combination drugs[a]	
NSAIDs	6
DMARDs (MTX use)	11(7)
Prednisolone	10

Tbc, tuberculosis; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying antirheumatic drugs; MTX, methotrexate
[a]Data represent the number of patients
[b]Values are mean ± SEM

Statistical analysis

Differences between the groups were examined for statistical significance using the Mann–Whitney *U*-test and the chi-square test. Changes within each group during LCAP therapy were analyzed using the Wilcoxon signed-rank test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

LCAP efficacy during therapy

Before LCAP therapy, the average DAS28-CRP score of the 11 cases was 5.98 ± 0.22 (mean ± SEM), a level considered as an active disease state. During the LCAP therapy, all of the patients' DAS28-CRP scores were decreased at the evaluation just before the 5th LCAP (Fig. 1) (5.98 ± 0.22 decreased to 4.62 ± 0.33, $P = 0.033$).

Evaluation at 4 weeks after LCAP therapy (Table 2)

We next evaluated the clinical response at 4 weeks after LCAP therapy. As previously described,[8,9] the efficacy was significant at 4 weeks, since 8 of the 11 patients (73%) achieved an ACR 20% response and 3 of the 11 patients (27%) achieved both ACR 50% and ACR 70% responses. However, CRP values tended not to be suppressed relative to the other variables. The DAS28-CRP score was also diminished at the same time point.

Evaluation up to 24 weeks after LCAP therapy (Fig. 1 and Table 3)

We evaluated the DAS28-CRP scores to monitor the disease activity up to 24 weeks after LCAP therapy. The thera-

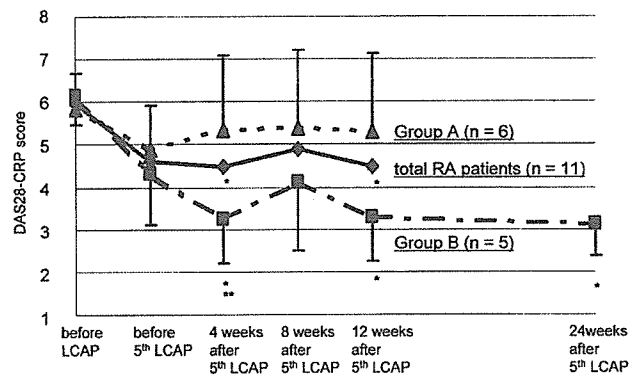

Fig. 1. Changes in disease activity score 28-C-reactive protein (*DAS28-CRP*) scores in rheumatoid arthritis (*RA*) patients. *Group A*: patients for whom the therapeutic regime was changed because of a flare-up of disease activity. *Group B*: patients for whom the therapeutic regime was unchanged or the dosages of drugs were reduced. $*P < 0.05$ vs DAS28-CRP score before leukocytapheresis (*LCAP*) in each group. $**P < 0.05$ vs DAS28-CRP score between Group A and Group B

Table 2. Efficacy of LCAP at 4 weeks in patients with RA

ACR core set	Before LCAP[a]	4 weeks after LCAP[a]	Significance level (P)[b]	≥20% improvement[c] at 4 weeks	≥50% improvement[c] at 4 weeks	≥70% improvement[c] at 4 weeks
Tender joint counts	18.0 ± 2.9	10.3 ± 2.9	0.041*	9 (82)	6 (55)	5 (45)
Swollen joint counts	8.6 ± 1.7	6.1 ± 2.7	0.21	9 (82)	7 (64)	4 (36)
Patient's assessment of pain	78.1 ± 4.1	36.1 ± 7.1	0.003*	10 (91)	5 (45)	4 (36)
Patient's global assessment of disease activity	75.6 ± 2.9	36.5 ± 7.9	0.004*	10 (91)	6 (55)	4 (36)
Physician's global assessment of disease activity	64.5 ± 6.1	36.3 ± 8.3	0.022*	8 (73)	4 (36)	3 (27)
Patient's assessment of physical function	16.6 ± 1.7	12.3 ± 2.0	0.006*	5 (45)	2 (18)	1 (9)
CRP (mg/dl)	4.36 ± 0.9	4.12 ± 1.5	0.48	7 (64)	4 (36)	1 (9)
Final number of improved patients by LCAP				8 (73)	3 (27)	3 (27)
DAS28-CRP	5.98 ± 0.22	4.47 ± 0.50	0.013*			

LCAP, leukocytapheresis; DAS28, disease activity score 28; CRP, C-reactive protein
[a] Values are mean ± SEM
[b] P value vs before LCAP; *significant
[c] Data represent the number of patients. Values in parentheses represent the percentage of the number of patients

Table 3. Efficacy of LCAP at 12 and 24 weeks in patients with RA

Measure	Before LCAP			12 weeks after LCAP			24 weeks after LCAP
	Total	Group A	Group B	Total	Group A	Group B	Group B
Tender joint counts (range 0–28)	12.8 ± 1.83	13.0 ± 3.16	12.6 ± 1.83	8.82 ± 3.02	11.2 ± 4.46	6.00 ± 4.09	2.60 ± 0.60*
Swollen joint counts (range 0–28)	7.36 ± 1.56	6.33 ± 2.08	8.60 ± 2.50	5.00 ± 2.30	8.00 ± 3.85	1.40 ± 0.98*	0.80 ± 0.49*
Patient's global assessment of disease activity	75.6 ± 2.90	72.8 ± 3.13	79.0 ± 5.12	38.3 ± 8.78*	54.0 ± 11.5	19.5 ± 7.99**	28.7 ± 13.4*
CRP	4.36 ± 0.85	4.23 ± 1.31	4.51 ± 1.17	3.68 ± 1.09	5.00 ± 1.75	2.09 ± 0.89*	1.76 ± 0.59*
DAS28-CRP	5.98 ± 0.22	5.84 ± 0.33	6.15 ± 0.30	4.47 ± 0.51*	5.29 ± 0.75	3.45 ± 0.37*	3.32 ± 0.21*

Values are mean ± SEM. Group A: patients whose therapeutic regimes were changed because of flare-ups of disease activity; Group B: patients whose therapeutic regimes remained unchanged or whose drug dosages were reduced
* $P < 0.05$ vs before LCAP
** $P < 0.05$ vs Group A and before LCAP

peutic regimes were not changed for 12 weeks after the final LCAP administration, since the RA disease activity was suppressed at this point compared with the activity level before LCAP therapy. However, the therapeutic regimes were modulated in 6 of 11 patients after 12 weeks because of flare-ups in disease activity. The DMARDs used were changed in all 6 patients. In addition, 1 patient received a synovectomy and the other 1 received a second regime of LCAP therapy. We defined those patients as Group A. Among the other 5 patients, the therapeutic regime was unchanged in 2 cases and the dosages of medications were reduced in 3 cases. We defined those 5 patients as Group B. In a comparison of the variables between the two groups, Group B had lower DAS28-CRP scores at both 4 and 12 weeks (Fig. 1). At 24 weeks after LCAP therapy, every variable of DAS28-CRP scores was significantly lower in Group B (Table 3).

We tried to determine whether any variables were present to differentiate the two groups at entry. However, there were no significant differences in disease duration, radiographic stage, functional class, methotrexate dosage, prednisolone dosage, tender joint counts, swollen joints counts, CRP, or DAS28-CRP score (Table 4).

Table 4. The characteristics of the two groups at entry

	Group A	Group B	P value
No.[a]	6	5	
Stage I–II/III–IV[a]	2/4	4/1	NS
Disease duration (years)[b]	4.8 ± 1.6	2.5 ± 0.6	NS
Tender joint counts[b]	19.3 ± 5.2	16.4 ± 2.8	NS
Swollen joint counts[b]	7.3 ± 2.1	10.2 ± 2.8	NS
MTX users[a] (%)	4 (67)	3 (60)	NS
Dosage of MTX (mg/week)[b]	5.5 ± 1.0	6.7 ± 0.7	NS
Prednisolone users[a] (%)	6 (100)	4 (80)	NS
Dosage of prednisolone (mg/day)[b]	9.2 ± 0.8	7.6 ± 1.0	NS
CRP (mg/dl)[b]	4.2 ± 1.3	4.5 ± 1.2	NS
DAS28-CRP[b]	5.84 ± 0.33	6.15 ± 0.30	NS

Group A: patients whose therapeutic regimes were changed because of flare-ups of disease activity; Group B: patients whose therapeutic regimes were unchanged or whose drug dosages were reduced
NS, not significant
[a] Data represent the number of patients
[b] Values are mean ± SEM

Evaluation of LCAP therapy by Steinbrocker stage classification (Fig. 2)

The 11 cases included 6 patients at stage I or II and 5 at stage III or IV. The change in DAS28-CRP score in

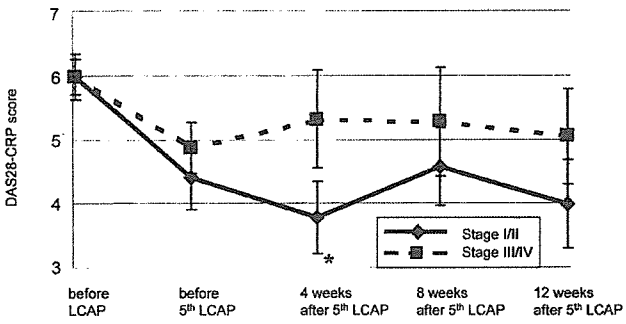

Fig. 2. Comparison of leukocytapheresis (*LCAP*) therapy efficacy by Steinbrocker stage classification. *$P < 0.05$ vs DAS28-CRP before LCAP

each group was comparably analyzed. DAS28-CRP scores at entry were not significantly different between the two groups. However, the score was clearly suppressed in the stage I/II group as compared with the stage III/IV group at 4 weeks. We also evaluated the scores at 8 and 12 weeks. Although a similar tendency was noted at the both time points, no statistical significance with the data from before LCAP therapy was found. We did not examine the efficacy at 24 weeks because some patients changed their therapies. However, in the stage I/II group, therapeutic regimes were unchanged at up to 24 weeks in 4 of 6 patients (66.7%), whereas in the stage III/IV group this was the case for only 1 of 5 patients (20%).

Adverse effects

Pseudomonas aeruginosa infection in the diffuse panbronchiolitis patient slightly worsened after the initial LCAP administration, but recovered shortly after antibiotics were administered, and thus the LCAP schedules could be completed. Except for this case, there were no abnormal clinical or laboratory findings during or after LCAP therapy.

Discussion

The efficacy of LCAP therapy after 4 weeks was similar to the findings of previous reports.[8,9] In addition, we examined the data obtained from 24 weeks of observation. Hidaka et al. reported that LCAP efficacy lasted up to 2 months, and recommended one LCAP session per month to maintain the improvement.[8] Although similar findings were reported by Ueki et al.,[9] there were no detailed descriptions in the previous reports regarding mid- to long-term observation.

Our study suggested that LCAP therapy remained effective at 24 weeks after completion, even in DMARD-refractory RA patients. Although statistical significance was not demonstrated, its efficacy appeared to be better in stage I/II cases than in stage III/IV cases. Our speculation may be supported by recent observations that early therapeutic intervention in RA improves its outcome.[13,14] Additionally, the outcome of RA patients is influenced by the presence of autoantibodies as well as HLA-DRB1 shared epitope alleles.[15] Since we did not find the indices to differentiate the future therapeutic response at entry among the present characteristics, further investigations, including genetic studies, will be important in identifying the contributory markers.

Our data included RA patients who could not be treated with tumor necrosis factor inhibitors or conventional DMARDs. Thus, we suggest that LCAP is an alternative therapeutic application in RA.

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Science
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The Muscle Protein Dok-7 Is Essential for Neuromuscular Synaptogenesis

Kumiko Okada, *et al.*

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changes at or near promoters, whereas Topoi inhibitors caused transcription complexes to stall in the midst of transcription units (*34*).

Collectively, our data reveal that a transient dsDNA break occurs at multiple regulated transcription units. This raises questions regarding the interplay between molecular machineries that are involved in the repair of dsDNA breaks and the activation of the gene transcription.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S5
References

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The Muscle Protein Dok-7 Is Essential for Neuromuscular Synaptogenesis

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The formation of the neuromuscular synapse requires muscle-specific receptor kinase (MuSK) to orchestrate postsynaptic differentiation, including the clustering of receptors for the neurotransmitter acetylcholine. Upon innervation, neural agrin activates MuSK to establish the postsynaptic apparatus, although agrin-independent formation of neuromuscular synapses can also occur experimentally in the absence of neurotransmission. Dok-7, a MuSK-interacting cytoplasmic protein, is essential for MuSK activation in cultured myotubes; in particular, the Dok-7 phosphotyrosine-binding domain and its target in MuSK are indispensable. Mice lacking Dok-7 formed neither acetylcholine receptor clusters nor neuromuscular synapses. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction with MuSK.

Skeletal muscle is controlled by motor neurons, which contact the muscle at the neuromuscular junction, a synapse that uses the neurotransmitter acetylcholine (*1*, *2*). To achieve sufficient sensitivity to the neurotransmitter, acetylcholine receptors (AChRs) on the muscle must be densely clustered on the postsynaptic side of the neuromuscular junction (*1*, *2*). Failure of AChR clustering is associated with disorders in neuromuscular transmission, including congenital myasthenic syndrome and myasthenia gravis (*3*, *4*). The presynaptic motor-nerve terminal secretes the glycoprotein agrin to activate postsynaptic MuSK (*5*). This agrin-dependent activation of MuSK is essential to establish the postsynaptic apparatus, including the clustering of AChRs, via the AChR-associated protein Rapsyn (*6*–*8*). Nevertheless, before innervation, MuSK-dependent AChR clusters can form at the endplate area of myotubes, suggesting a mechanism of postsynaptic specialization that is independent of agrin and innervation (*9*–*11*). Furthermore, neuromuscular synapses can form independently of agrin in mice that lack acetylcholine, which appears to antagonize postsynaptic differentiation (*12*, *13*). Thus, in addition to agrin, there may be another element that can achieve MuSK activation and trigger postsynaptic specializations at the neuromuscular junction. MuSK contains a phosphotyrosine-binding domain (PTB domain) target motif Asn-Pro-X-Tyr encompassing Tyr^{553} in the juxtamembrane region, which is essential for proper functioning in vivo (*14*). The binding partner for this motif has remained elusive.

By searching databases, including GenBank, the European Molecular Biology Laboratory, and the DNA Data Bank of Japan, for a previously unidentified member of the Dok-family of proteins, each of which has a PTB domain, we identified Dok-7 and cloned human cDNA encoding 504 amino acids. Like other members, Dok-7 has pleckstrin-homology (PH) and PTB domains in the N-terminal portion and Src homology 2 (SH2) domain target motifs in the C-terminal region (fig. S1) (*15*–*17*). Cloning of mouse (*Mus musculus*) and puffer fish (*Takifugu rubripes*) Dok-7 cDNA revealed a highly conserved structure (fig. S2). Like agrin and MuSK, no ortholog was found in invertebrates such as the fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*). Northern blot analysis of human tissues showed that Dok-7 mRNA is preferentially expressed in skeletal muscle and in the heart (fig. S3A), and immunoblot analysis identified a 55-kD Dok-7 protein in the thigh muscle, diaphragm, and heart but not in the liver or spleen (fig. S3B). Furthermore, immunostaining of mouse skeletal muscles, including the sternocleidomas-

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toid, extensor digitorum longus, and gastrocnemius, with antiserum to Dok-7 highlighted the accumulation of Dok-7 at neuromuscular junctions (Fig. 1, A to C), which are composed of the postsynaptic membrane with its densely clustered AChRs in close juxtaposition with the presynaptic nerve terminal. Therefore, we denervated a mouse gastrocnemius muscle by sciatic nerve resection to confirm the muscular, and thus postsynaptic, localization of Dok-7. One week after the operation, synaptophysin, a component of the presynaptic vesicle, was completely abolished in denervated muscles (fig. S4). However, the muscular localization of Dok-7 and AChRs remained intact, indicating a postsynaptic localization of Dok-7 at neuromuscular junctions (Fig. 1, D to F). Because postsynaptic differentiation and neuromuscular synapse formation are initiated at the endplate zone of skeletal muscle during embryogenesis (*9*–*11*), we performed a whole-mount in situ hybridization and found that Dok-7 transcripts are expressed in the central region encompassing the endplate area of the diaphragm muscles at day 14.5 of embryonic development (E14.5), when AChRs cluster in a nerve- and agrin-independent manner (fig. S5). Together, these results suggest that Dok-7 has the appropriate distribution to be involved in the neuromuscular junction.

Given the requirement for MuSK's PTB target motif and presumably its binding partner in postsynaptic specialization (*14*, *18*, *19*), we next examined the interaction of MuSK with Dok-7, which has a PTB domain, in 293T cells. These heterologous cells do not express either protein detectably, and forced expression of MuSK in these cells induced weak

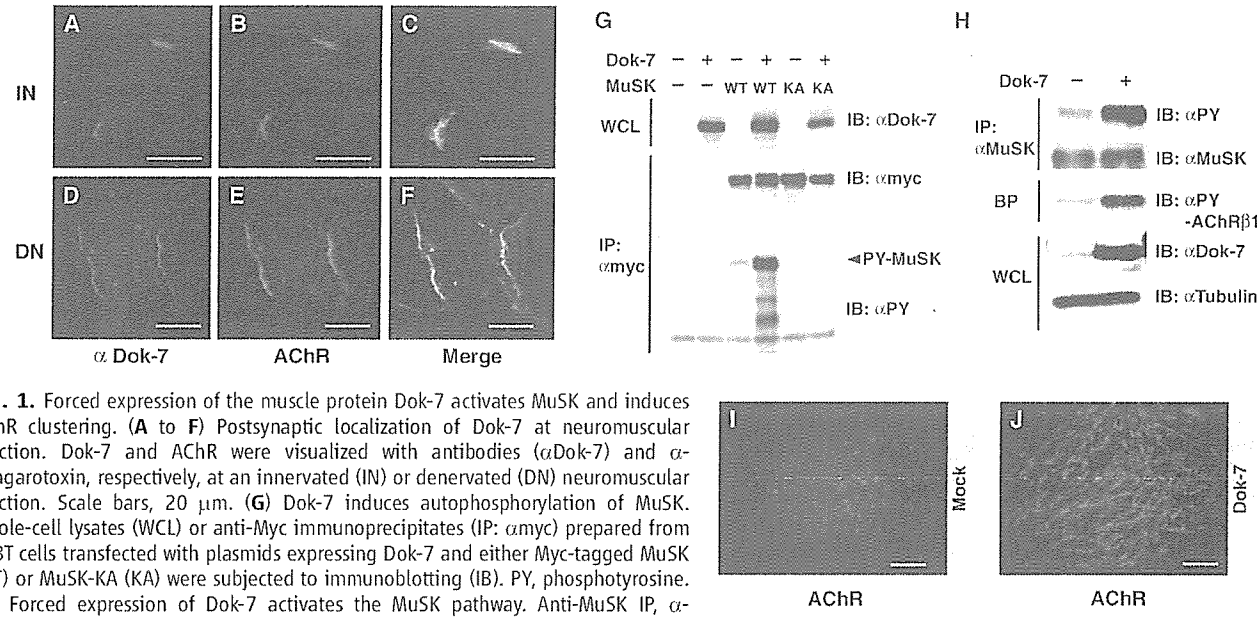

Fig. 1. Forced expression of the muscle protein Dok-7 activates MuSK and induces AChR clustering. (**A** to **F**) Postsynaptic localization of Dok-7 at neuromuscular junction. Dok-7 and AChR were visualized with antibodies (αDok-7) and α-bungarotoxin, respectively, at an innervated (IN) or denervated (DN) neuromuscular junction. Scale bars, 20 μm. (**G**) Dok-7 induces autophosphorylation of MuSK. Whole-cell lysates (WCL) or anti-Myc immunoprecipitates (IP: αmyc) prepared from 293T cells transfected with plasmids expressing Dok-7 and either Myc-tagged MuSK (WT) or MuSK-KA (KA) were subjected to immunoblotting (IB). PY, phosphotyrosine. (**H**) Forced expression of Dok-7 activates the MuSK pathway. Anti-MuSK IP, α-bungarotoxin precipitates (BP), or WCL from C2 myotubes transfected with plasmids for Dok-7 were subjected to IB. (**I** and **J**) Forced expression of Dok-7 induces aneural AChR clustering in C2 myotubes. Abundant clusters of AChRs formed in C2 myotubes transfected with Dok-7 expression plasmids (J), but only a few small clusters formed in the control (Mock) (I). Scale bars, 200 μm.

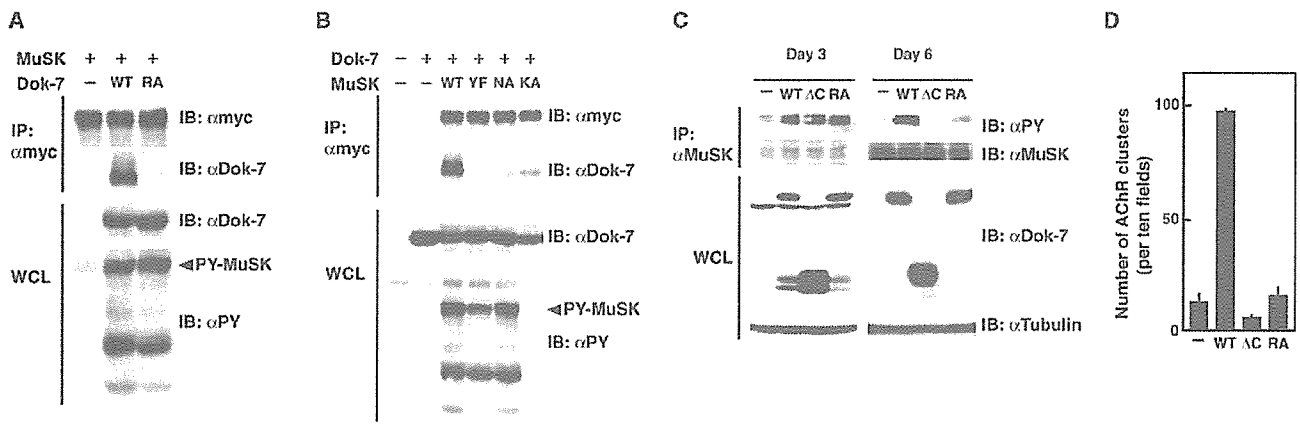

Fig. 2. Dok-7 interacts with MuSK by way of the PTB domain. (**A** and **B**) The PTB domain, its target, and kinase activity are essential for Dok-7 binding to MuSK. Anti-Myc IP or WCL from 293T cells transfected with plasmids for Dok-7 and Myc-tagged MuSK or their mutants, including MuSK-KA, were subjected to IB. (**C** and **D**) The PTB domain and C-terminal region are indispensable for the Dok-7–induced activation of MuSK and AChR clustering in fully differentiated C2 myotubes. Anti-MuSK IP or WCL from C2 cells transfected with expression plasmids for Dok-7 (WT), Dok-7–ΔC (ΔC), or Dok-7–RA (RA) were prepared at day 3 or 6 upon differentiation into myotubes and subjected to IB (C). The number of AChR clusters (mean ± SD) counted at day 7 is shown (D). Differentiation was achieved by day 6, whereas only a few myotubes had formed by day 3.