2. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, et al. A trial of etanercept, a recombinant human tumor necrosis factor: Fc fusion protein in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999;340:253.
3. Bathon JM, Martin RW, Fleishmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med 2000;343:1586–93.
4. Klareskog L, van der Heijde D, de Jager JP, Gough A, Kalden J, Malaise M, et al. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomized controlled trial. Lancet 2004;363:675–81.
5. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor α-neutralizing agents. N Engl J Med 2001;345:1098–104.
6. Gardam MA, Keystone EC, Menzies R, Manners S, Skamene E, Long R, et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 2003;3:148–55.
7. WHO report Global tuberculosis control-surveillance, planning, financing. http://www.who.int/tb/publications/global_report/en/
8. Report from the Ministry of Health, Labor and Welfare Japan (in Japanese). http://www.jata.or.jp/rit/rj/data_tp.html
9. Miyasaka N, Takeuchi T, Eguchi K: Proposed Japanese guidelines for the use of infliximab for rheumatoid arthritis. Mod Rheumatol 2005;15:4–8.
10. American College of Rheumatology-Hotline: 1. http://www.rheumatology.org/publications/hotline/0303tnfl.asp
11. Safety Update TNF-α blocking agents: 1. http://www.fda.gov/ohrms/dockets/ac/03/transcripts/3930T1.htm
12. Van Oosten BW, Barkhof F, Truyen L, Boringa JB, Bertelsmann FN, von Blomberg BM, et al. Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. Neurology, 1996;47:1531.
13. Genovese MC, Bathon JM, Fleischmann RM, Moreland LW, Martin RW, Whitmore JB, et al. Longterm safety, efficacy, and radiographic outcome with etanercept treatment in patients with early rheumatoid arthritis. J Rheumatol 2005;32:1232–42.
14. Miyasaka N. Research report of "Development of Guidelines for the Treatment of Rheumatoid Arthritis with New Biological Products and Research on Their Validation" supported by a Grant-in-Aid for Scientific Research (Prevention and Research of Immunologic and Allergic Diseases) from the Ministry of Health, Labour and Welfare, Japan in 2004; in press.
15. Study Group of the Ministry of Health, Labour and Welfare. Therapeutic manual of rheumatoid arthritis (revised). Diagnostic manual and evidence-based treatment guidelines. Japan Rheumatism Foundation; 2004.
16. Sakai F, Noma S, Kurihara Y, Yamada H, Azuma A, Kudoh S, et al. Leflunomide-related lung injury in patients with rheumatoid arthritis: imaging features. Mod Rheumatol 2005; 15:73–9.
17. Ogawa J, Harigai M, Nagasaka N, Nakamura T, Miyasaka N, et al. Prediction of and prophylaxis against pneumocystic pneumonia in patients with connective tissue diseases undergoing medium- or high-dose corticosteroid therapy. Mod Rheumatol 2005;15:91–6.
18. Keystone EC, Schiff MH, Kremer JM, Kafka S, Lovy M, De Vries T, et al. One-weekly administration of 50 mg etanercept in patients with active rheumatoid arthritis. Results of a multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 2004;50:353–63.

FEBS 28539 FEBS Letters 569 (2004) 235–239

Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro

Kiyoshi Migita[a,*], Seigo Abiru[a], Minoru Nakamura[a], Atsumasa Komori[a], Yuki Yoshida[a], Terufumi Yokoyama[a], Manabu Daikoku[a], Toshihito Ueki[a], Yasushi Takii[a], Koji Yano[a], Hiroshi Yastuhashi[a], Katsumi Eguchi[b], Hiromi Ishibashi[a]

[a]*Clinical Research Center, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 856-8562, Japan*
[b]*First Department of Internal Medicine, Nagasaki University School of Medicine, Japan*

Received 30 April 2004; revised 19 May 2004; accepted 27 May 2004

Available online 15 June 2004

Edited by Robert Barouki

Abstract To investigate the role of lipopolysaccharide (LPS) in hepatocyte activation, we examined the expression of Toll-like receptor 4 (TLR4), the putative receptor for LPS in human hepatocytes. TLR4 mRNA and protein expression was confirmed in human hepatocytes. Stimulation of human hepatocytes with LPS results in rapid degradation of IkappaB-α and mitogen activated protein kinase activation. Human hepatocytes stimulated by LPS produced serum amyloid A protein. Our data suggest that human hepatocytes utilize components of TLR4 signal transduction pathways in response to LPS and these direct LPS-mediated effects on hepatocytes may contribute to liver inflammation and injury.

Keywords: Hepatocyte; Lipopolysaccharide; Toll-like receptor; Serum amyloid A protein

1. Introduction

Bacterial lipopolysaccharide (LPS), an essential component of the outer membrane of gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that leads to septic shock and multiple organ failure [1]. LPS also causes liver injury [2]. The liver is an immunocompetent organ that plays a key role in the innate immune responses to pathogens [3]. The liver produces both inflammatory mediators and acute-phase reactants and functions to remove pathogens and microbial products from the blood [4]. Although hepatocytes have been reported to respond to LPS, the mechanisms by which LPS stimulates human hepatocytes remain uncertain. It has been suggested that Kupffer cells, as well as other macrophage populations, are responsive to LPS and produce TNF-α and IL-1 that activate hepatocytes [5]. Recently, Toll-like receptors (TLRs), several mammalian *Toll* homologues, have been identified and shown to play important roles in the recognition of various bacterial components [6]. In humans, the TLR family consists of 10 members, all of which are involved in the recognition of pathogen-associated molecular patterns [7]. Among these, Toll-like receptor 4 (TLR4) has been shown to be critical for LPS and endotoxin signaling [8]. TLR receptors have been identified on monocytes, macrophages, Kupffer cells and dendritic cells, and LPS binds to Kupffer cells via TLR4 [9]. LPS activation of Kupffer cells leads to upregulation of inflammatory cytokines, which are involved in liver damage [10]. Therefore, the general view is that the Kupffer cell is the major target of LPS in the liver.

Serum amyloid A protein (SAA) is a major acute-phase protein (APP) produced in the liver after various insults such as infection and inflammation [11]. SAA is an apolipoprotein that belongs to class 1 APP in which IL-1 and IL-6 are the main inflammatory mediators involved in its transcriptional induction [12]. Multiple *cis*-acting elements, including C/EBP and NF-κB, have been found to be important for SAA genes [13]. LPS injection in rabbit results in the activation of C/EBP and NF-κB, which may be responsible for LPS-induced SAA induction [14]. NF-κB and NF-IL6 are involved in the cytokine-induced SAA gene expression [15]. SAF-1 is a zinc finger transcription factor that is essential for cytokine-induced SAA induction [16]. It was demonstrated that MAPK signaling pathway regulated the DNA-binding activity and transactivation potential of SAF-1 and phosphorylation of SAF-1 in response to cytokines was markedly inhibited by MAPK inhibitors [17]. More recently, it was demonstrated that LPS-stimulated SAA protein induction was significantly reduced in TLR4-deificient mice, suggesting the critical role of TLR4 in SSA induction [18]. In the present study, to determine whether hepatocytes respond directly to LPS and produce SAA, we investigated the presence of TLR4, a ligand of LPS, in human hepatocytes.

2. Materials and methods

2.1. Cells

Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA). The cells were cultured in a basal medium composed of Ham's F-12 and Leibovitz L-15 (1:1) medium (Invitrogen, Carlsbad, CA), 0.2% (v/v) bovine serum albumin, 5 mM glucose (Wako Chemical Co Inc., Osaka, Japan), 10^{-8} M dexamethasone (Wako), and 10^{-8} M bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, Grand Island, NY). These hepatocytes prepara-

* Corresponding author. Fax: +81-957-54-0292.
E-mail address: migita@nmc.hosp.go.jp (K. Migita).

Abbreviations: ERK, extracellular signal-related kinase; IκB-α, IkappaB-α; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; SAA, serum amyloid A protein; TLR, Toll-like receptor

0014-5793/$22.00 © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2004.05.072

Science
AAAS

The Muscle Protein Dok-7 Is Essential for Neuromuscular Synaptogenesis

Kumiko Okada, *et al.*

Science **312**, 1802 (2006);
DOI: 10.1126/science.1127142

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.

References and Notes

1. C. K. Glass, M. G. Rosenfeld, *Genes Dev.* **14**, 121 (2000).
2. B. M. Spiegelman, R. Heinrich, *Cell* **119**, 157 (2004).
3. V. Perissi, M. G. Rosenfeld, *Nat. Rev. Mol. Cell Biol.* **6**, 542 (2005).
4. A. Tulin, D. Stewart, A. C. Spradling, *Genes Dev.* **16**, 2108 (2002).
5. R. Pavri *et al.*, *Mol. Cell* **18**, 83 (2005).
6. M. Y. Kim, S. Mauro, N. Gevry, J. T. Lis, W. L. Kraus, *Cell* **119**, 803 (2004).
7. B. G. Ju *et al.*, *Cell* **119**, 815 (2004).
8. M. Malanga, F. R. Althaus, *Biochem. Cell Biol.* **83**, 354 (2005).
9. S. Burma, D. J. Chen, *DNA Repair* **3**, 909 (2004).
10. J. M. Jeltsch *et al.*, *Nucleic Acids Res.* **15**, 1401 (1987).
11. J. C. Wang, *Nat. Rev. Mol. Cell Biol.* **3**, 430 (2002).
12. A. Dvir, S. R. Peterson, M. W. Knuth, H. Lu, W. S. Dynan, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11920 (1992).
13. T. Chibazakura *et al.*, *Eur. J. Biochem.* **247**, 1166 (1997).
14. C. A. Sartorius, G. S. Takimoto, J. K. Richer, L. Tung, K. B. Horwitz, *J. Mol. Endocrinol.* **24**, 165 (2000).
15. G. L. Mayeur *et al.*, *J. Biol. Chem.* **280**, 10827 (2005).
16. C. Leontiou, J. H. Lakey, R. Lightowlers, R. M. Turnbull, C. A. Austin, *Mol. Pharmacol.* **69**, 130 (2006).
17. R. Metivier *et al.*, *Cell* **115**, 751 (2003).
18. L. Ko, G. R. Cardona, W. W. Chin, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6212 (2000).
19. S. K. Lee *et al.*, *Mol. Endocrinol.* **14**, 915 (2000).
20. J. Torchia *et al.*, *Nature* **387**, 677 (1997).
21. M. Binaschi, R. Farinosi, M. E. Borgnetto, G. Capranico, *Cancer Res.* **60**, 3770 (2000).
22. M. N. Cervellera, A. Sala, *J. Biol. Chem.* **275**, 10692 (2000).
23. P. O. Hassa, M. Covic, S. Hasan, R. Imhof, M. O. Hottiger, *J. Biol. Chem.* **276**, 45588 (2001).
24. C. Le Page, J. Sanceau, J. C. Drapier, J. Wietzerbin, *Biochem. Biophys. Res. Commun.* **243**, 451 (1998).
25. A. J. Butler, C. P. Ordahl, *Mol. Cell. Biol.* **19**, 296 (1999).
26. M. Ku, S. Stewart, A. Hata, *Biochem. Biophys. Res. Commun.* **311**, 702 (2003).
27. F. R. Althaus *et al.*, *Mol. Cell. Biochem.* **138**, 53 (1994).
28. D. T. Brown, *Biochem. Cell Biol.* **81**, 221 (2003).
29. J. O. Thomas, *Biochem. Soc. Trans.* **29**, 395 (2001).
30. M. F. Ruh, J. C. Chrivia, L. K. Cox, T. S. Ruh, *Mol. Cell. Endocrinol.* **214**, 71 (2004).
31. D. Das, R. C. Peterson, W. M. Scovell, *Mol. Endocrinol.* **18**, 2616 (2004).
32. A. M. Nunez, M. Berry, J. L. Imler, P. Chambon, *EMBO J.* **8**, 823 (1989).
33. T. Barkhem, L. A. Haldosen, J. A. Gustafsson, S. Nilsson, *Mol. Pharmacol.* **61**, 1273 (2002).
34. I. Collins, A. Weber, D. Levens, *Mol. Cell. Biol.* **21**, 8437 (2001).

35. We thank C. Nelson and K. Ohgi for their assistance; S. Ogawa and J. Puc for reagents; and X. Zhu and T. Wang for discussions and advice. We also thank J. Hightower and M. Fisher for assistance in figure and manuscript preparation and M. Gonzalez (Santa Cruz Biotechnology) for advice on reagents. M.G.R. is an investigator with the Howard Hughes Medical Institute and B.J. is supported by the U.S. Army Medical Research and Materiel Command (grant DAMD17-01-1-0184). These studies are supported by NIH and National Cancer Institute grants to M.G.R. and C.K.G.

Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5781/1798/DC1
Materials and Methods
Figs. S1 to S5
References

9 March 2006; accepted 4 May 2006
10.1126/science.1127196

The Muscle Protein Dok-7 Is Essential for Neuromuscular Synaptogenesis

Kumiko Okada,[1*] Akane Inoue,[1*] Momoko Okada,[1] Yoji Murata,[1] Shigeru Kakuta,[3] Takafumi Jigami,[4] Sachiko Kubo,[3] Hirokazu Shiraishi,[5] Katsumi Eguchi,[5] Masakatsu Motomura,[5] Tetsu Akiyama,[4] Yoichiro Iwakura,[3] Osamu Higuchi,[1†] Yuji Yamanashi[1,2†]

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-

[1]Department of Cell Regulation, Medical Research Institute, [2]School of Biomedical Science, Tokyo Medical and Dental University, Tokyo 113–8510, Japan. [3]Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108–8639, Japan. [4]Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113–0032, Japan. [5]The First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852–8501, Japan.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: yamanashi.creg@mri.tmd.ac.jp (Y.Y.); higuchi.creg@mri.tmd.ac.jp (O.H.)

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

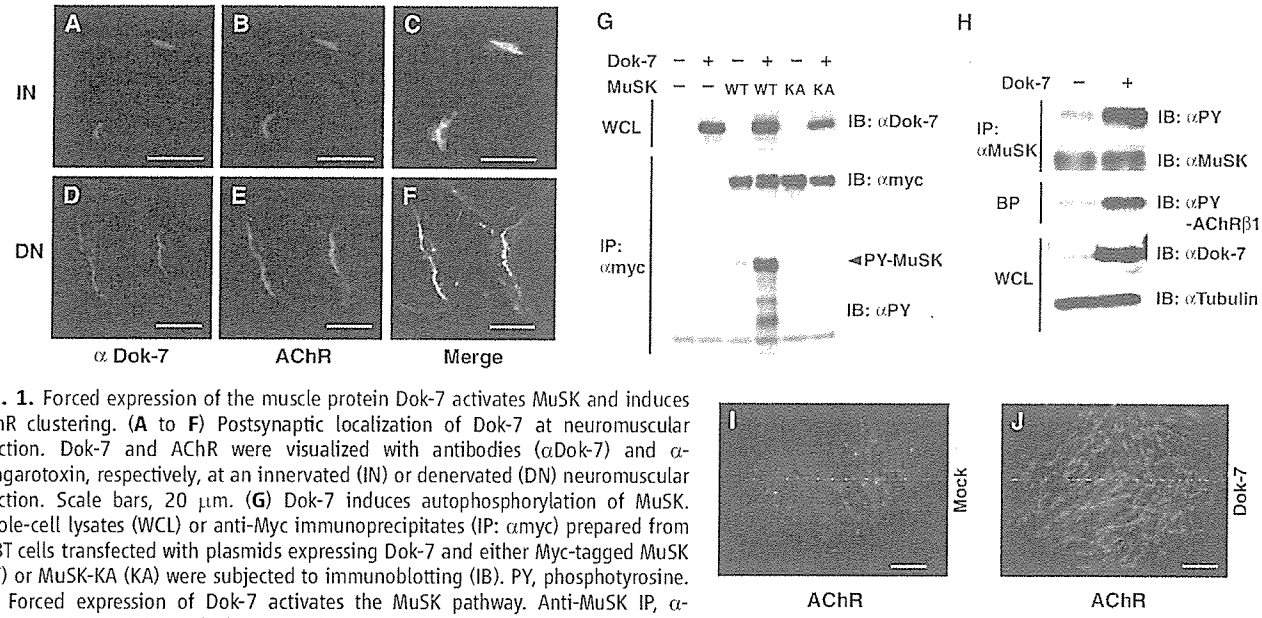

Fig. 1. Forced expression of the muscle protein Dok-7 activates MuSK and induces AChR clustering. (**A** to **F**) Postsynaptic localization of Dok-7 at neuromuscular junction. Dok-7 and AChR were visualized with antibodies (αDok-7) and α-bungarotoxin, respectively, at an innervated (IN) or denervated (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.

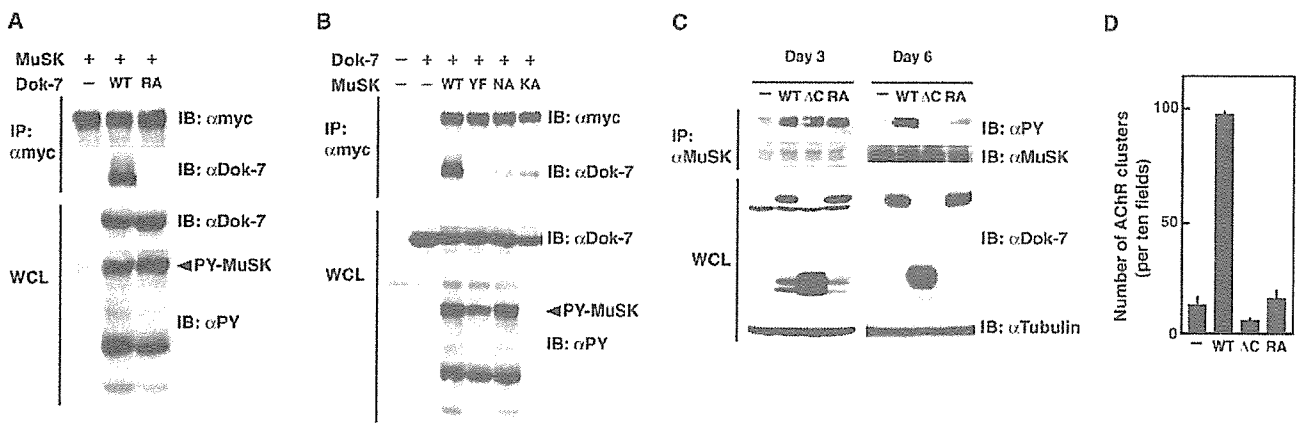

Fig. 2. Dok-7 interacts with MuSK by way of the PTB domain. (**A** and **B**) The PTB domain, its target, and kinase activity are essential for Dok-7 binding to MuSK. Anti-Myc IP or WCL from 293T cells transfected with plasmids for Dok-7 and Myc-tagged MuSK or their mutants, including MuSK-KA, were subjected 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.

autophosphorylation (*20*, *21*). Forced expression of Dok-7 induced an intense tyrosine phosphorylation of MuSK but not the kinase-inactive mutant with a Lys/Ala substitution (MuSK-KA), indicating that Dok-7 induced the autophosphorylation of MuSK (Fig. 1G). This activity was unique to Dok-7; no other mammalian Dok-family proteins induced phosphorylation of MuSK (fig. S6). It was also conserved; Dok-7 from puffer fish was able to activate even mammalian MuSK. Also, in C2 myotubes, the forced expression of Dok-7 induced tyrosine phosphorylation of MuSK and the β subunit (AChRβ1) of the AChR complex, which is known to be tyrosine-phosphorylated upon activation of MuSK (*22*) (Fig. 1H). Furthermore, this forced expression induced numerous clusters of AChRs, and the number of AChR clusters correlated with the amount of Dok-7 expression plasmid (Fig. 1, I and J; fig. S7A and supporting online material). The exogenous Dok-7–induced AChR clusters were elaborately branched, and their complicated architecture resembled the differentiated "pretzel-like" AChR clusters formed in vivo (fig. S7, B and C). In addition, forced expression in myotubes of Dok-7 that had been fused with enhanced green fluorescent protein (EGFP) induced Dok-7 and AChR coclustering (fig. S7, D to I), as observed at postsynaptic areas in vivo (Fig. 1, C and F).

Because the regulatory interaction of Dok-7 with MuSK as described above implies their physical interaction, we examined whether Dok-7 binds to MuSK by way of the PTB domain in 293T cells. MuSK was coimmunoprecipitated with Dok-7 but not with Dok-7 carrying three Arg/Ala substitutions (Dok-7–RA) in the PTB domain (Fig. 2A). Consistently, the mutant MuSK carrying either a Tyr/Phe substitution at Tyr^{553} (MuSK-YF) or an Asn/Ala substitution at Asn^{550} (MuSK-NA) in the PTB target motif was not coimmunoprecipitated with Dok-7 (fig. S9 and Fig. 2B). The failure of the MuSK-KA kinase-inactive mutant to be coimmunoprecipitated with Dok-7 confirms the requirement of tyrosine phosphorylation for the binding of MuSK with Dok-7 via the PTB domain. These results indicate that Dok-7 binds to MuSK through the PTB domain in a manner dependent on the tyrosine phosphorylation of its target motif in MuSK.

Nevertheless, mutations in the PTB domain (Dok-7–RA) or PTB target motif (MuSK-NA or -YF) did not block activation of MuSK, at least in heterologous cells (Fig. 2, A and B). In addition, the N- and C-terminal deletion mutants of Dok-7 (Dok-7-ΔN and -ΔC) revealed that the C-terminal moiety, but not the PH domain, of Dok-7 is dispensable for MuSK activation in heterologous cells (fig. S10). Also, the forced expression of Dok-7–RA or Dok-7–ΔC induced MuSK activation even in C2 cells at day 3 of differentiation into myotubes (Fig. 2C), when very few myotubes have formed. Unexpectedly, however, the PTB domain and C-terminal portion were indispensable for Dok-7–induced MuSK activation and AChR clustering in fully differentiated C2 myotubes at days 6 and 7 of differentiation (Fig. 2, C and D). In addition, the PH domain, responsible for membrane localization in general, was indispensable for the activation of MuSK in fully differentiated myotubes (fig. S11), as was seen in heterologous cells (fig. S10). Together these findings suggest that a negative regulatory mechanism preventing MuSK activation is established upon differentiation into myotubes, which is accompanied by increased expression of MuSK and Dok-7 (fig. S12). Trace phosphorylation of MuSK in myotubes might allow physical interaction with Dok-7, in turn facilitating dimerization and/or conformational changes in MuSK that are necessary for its sustained activation.

MuSK-deficient myotubes do not form agrin-dependent or -independent clusters of AChRs unless MuSK is reintroduced (*18*, *19*, *23*). To confirm whether Dok-7–mediated AChR clustering is dependent on MuSK, we introduced Dok-7 into MuSK-deficient myotubes. Unlike its effect in C2 myotubes, forced expression of Dok-7 induced no AChR clustering in the MuSK-deficient myotubes; however, additional expression of wild-type MuSK resulted in robust clustering of AChRs in these cells (Fig. 3A). Furthermore, the MuSK-KA and MuSK-YF mutant each failed to complement the MuSK deficiency, regardless of exogenous Dok-7. These findings demonstrate that Dok-7–induced AChR clustering in myotubes depends on Dok-7 interaction with MuSK and subsequent activation of MuSK catalytic activity. Thus, we examined the regulatory interaction of Dok-7 with a MuSK mutant [MuSK-Val/Met (MuSK-VM)] that carries a Val^{790} to Met substitution. This mutation is causally associated with the congenital myasthenic syndrome by way of an as yet unclear mechanism (*24*). As observed with MuSK-YF (Fig. 2B), forced expression of Dok-7 in 293T cells induced the autophosphorylation of MuSK-VM, but its coimmunoprecipitation with Dok-7 was barely detectable in these heterologous cells (fig. S13). Forced expression of Dok-7 with MuSK-VM induced only very weak AChR clustering in MuSK-deficient myotubes (Fig. 3A). Therefore, the congenital myasthenic syndrome–associated Val^{790} to Met mutation impaired interaction of MuSK with Dok-7, suggesting a possible cause of neuromuscular junction dysfunction in these patients.

To examine the effects of Dok-7 down-regulation in myotubes, we used a small interfering RNA (siRNA) designed specifically to block its expression. Inhibition of Dok-7 suppressed the tyrosine phosphorylation of

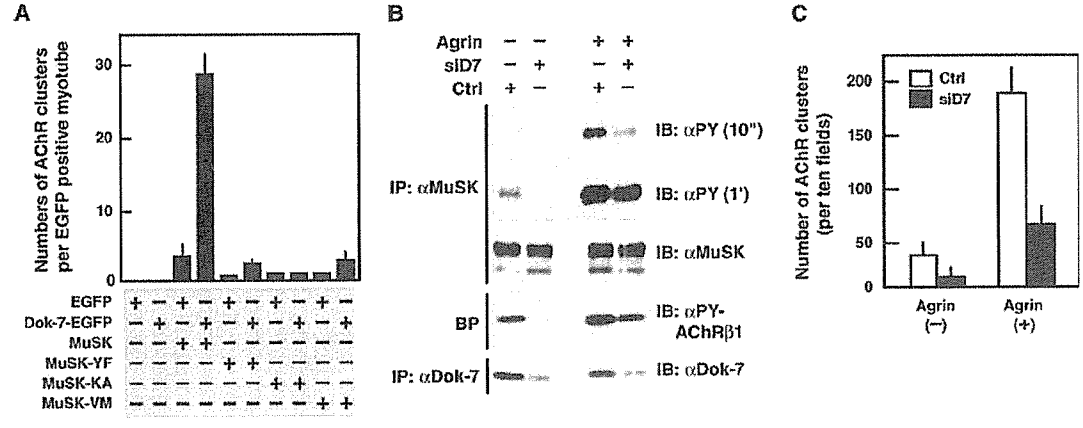

Fig. 3. Dok-7 is essential for activation of the MuSK-pathway to AChR clustering in myotubes. **(A)** MuSK is required for Dok-7–induced AChR clustering. MuSK-deficient myotubes were transfected with the indicated plasmids. The number of AChR clusters (mean ± SD) per EGFP-positive myotube is shown. MuSK-VM is a congenital myasthenic syndrome–associated mutant. **(B)** Activation of the MuSK pathway requires Dok-7. C2 myotubes transfected with Dok-7 siRNA (siD7) or the control (Ctrl) without (−) or with (+) agrin treatment for 15 min were studied as in Fig. 1H. Both short [10 s (10″)] and long [1 min (1′)] exposures are shown for the anti-PY IB of the anti-MuSK IP. **(C)** Dok-7 is essential for AChR clustering. C2 myotubes were transfected with Dok-7 siRNA (siD7) or the control (Ctrl) with or without agrin treatment for 12 hours. The number of AChR clusters (mean ± SD) is shown.

MuSK and AChRβ1 in C2 myotubes, demonstrating its essential role in the aneural, basal catalytic activity of MuSK (Fig. 3B). Indeed, MuSK-dependent spontaneous AChR clustering was suppressed by this siRNA-mediated inhibition (Fig. 3C). Moreover, the inhibition of Dok-7 impaired the agrin-dependent activation of MuSK, the phosphorylation of AChRβ1, and the subsequent formation of AChR clusters (Fig. 3, B and C). Thus, we conclude that Dok-7 is essential for aneural activation of MuSK and AChR clustering in myotubes and is also crucial for agrin-dependent activation of MuSK and AChR clustering. Nonetheless, our results do not exclude the possibility that Dok-7 might also play a role downstream of MuSK. Indeed, Dok-7 and MuSK were synchronously tyrosine phosphorylated upon treatment of myotubes with agrin (fig. S14).

We generated mice lacking Dok-7 to explore its role in vivo (fig. S15). Like mice lacking MuSK or agrin (*6*, *7*), all Dok-7–deficient (Dok-7$^{-/-}$) mice were immobile at birth and died shortly thereafter (26 homozygotes were observed among the first 137 pups), although their wild-type and heterozygous littermates appeared normal. Also, the alveoli of the mutant mice were not expanded at birth (fig. S15D), indicating a failure to breathe and suggesting a severe defect in neuromuscular transmission in the skeletal muscles. Consistently, there were no detectable AChR clusters in the endplate area of the diaphragm muscle in Dok-7$^{-/-}$ embryos at either E14.5 or E18.5 (Fig. 4, E and K). Because nascent AChR clusters are formed in a nerve- and agrin-independent manner at E13.5 to E16.5, whereas most neuromuscular junctions are formed in a nerve- and agrin-dependent manner at E18.5, our findings indicate a requirement for Dok-7 in both types of MuSK-dependent postsynaptic specialization, although we cannot exclude the possibility that nascent AChR clustering is a prerequisite for nerve- and agrin-dependent AChR clustering (*9*–*11*). Consistent with this finding, Dok-7 transcripts were expressed in the endplate area of the diaphragm muscle (fig. S5). In addition, axonal branches extending from the motor nerve trunk were aberrantly long in the endplate area of Dok-7$^{-/-}$ diaphragms at E18.5 and, unlike the controls, did not terminate near the nerve trunk (Fig. 4, G and J). Overall, these pre- and postsynaptic abnormalities are indistinguishable from those found in mice lacking MuSK (*7*), demonstrating an essential role in vivo for Dok-7 in neuromuscular synaptogenesis, a MuSK-dependent vital process.

MuSK-dependent postsynaptic specialization during neuromuscular synaptogenesis appears to be controlled by multiple regulatory mechanisms (*2*, *25*). We have shown that Dok-7 may be a muscle-intrinsic activator of MuSK by demonstrating its essential role in the aneural activation of MuSK and subsequent AChR clustering in cultured myotubes. This conclusion is further supported by our findings that mice lacking Dok-7 showed marked disruption of neuromuscular synaptogenesis that was indistinguishable from the disruption found in MuSK-deficient mice. Thus, neuromuscular synaptogenesis requires Dok-7 within the skeletal muscle. Dok-7 dysfunction may be involved in the pathogenesis of neuromuscular junction disorders.

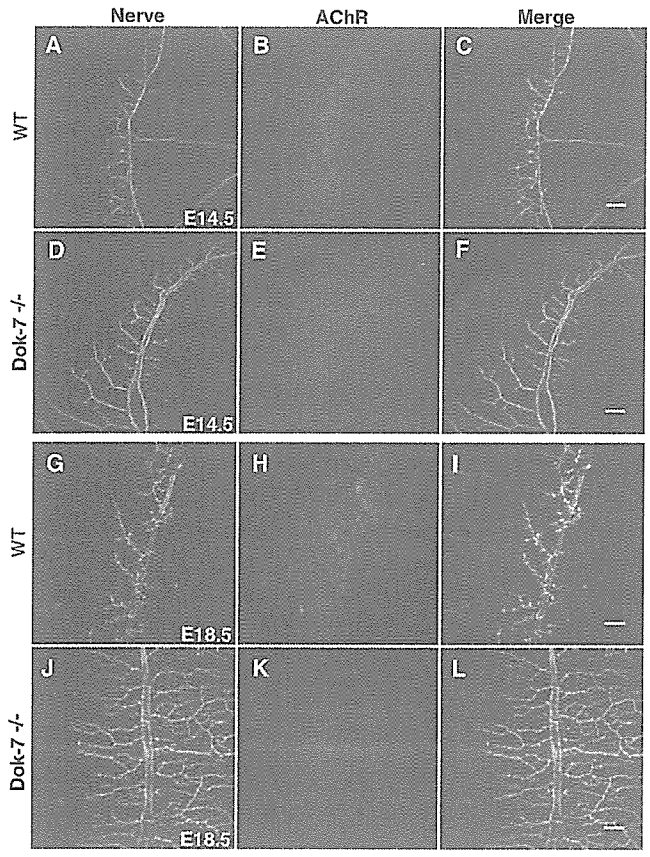

Fig. 4. Dok-7 is essential for neuromuscular synaptogenesis in vivo. Diaphragm muscles were prepared from the wild-type control (WT) or Dok-7$^{-/-}$ embryos at E14.5 (**A** to **F**) or E18.5 (**G** to **L**) and subjected to whole-mount anti-neurofilament and α-bungarotoxin staining, to visualize nerve and AChR, respectively. Scale bars, 100 μm.

References and Notes

1. S. J. Burden, *Genes Dev.* **12**, 133 (1998).
2. J. R. Sanes, J. W. Lichtman, *Nat. Rev. Neurosci.* **2**, 791 (2001).
3. A. G. Engel, K. Ohno, S. M. Sine, *Nat. Rev. Neurosci.* **4**, 339 (2003).
4. A. Vincent *et al.*, *Ann. N. Y. Acad. Sci.* **998**, 324 (2003).
5. D. J. Glass *et al.*, *Cell* **85**, 513 (1996).
6. M. Gautam *et al.*, *Cell* **85**, 525 (1996).
7. T. M. DeChiara *et al.*, *Cell* **85**, 501 (1996).
8. M. Gautam *et al.*, *Nature* **377**, 232 (1995).
9. W. Lin *et al.*, *Nature* **410**, 1057 (2001).
10. X. Yang *et al.*, *Neuron* **30**, 399 (2001).
11. X. Yang, W. Li, E. D. Prescott, S. J. Burden, J. C. Wang, *Science* **287**, 131 (2000).
12. T. Misgeld, T. T. Kummer, J. W. Lichtman, J. R. Sanes, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 11088 (2005).
13. W. Lin *et al.*, *Neuron* **46**, 569 (2005).
14. R. Herbst, E. Avetisova, S. J. Burden, *Development* **129**, 5449 (2002).
15. N. Carpino *et al.*, *Cell* **88**, 197 (1997).
16. Y. Yamanashi, D. Baltimore, *Cell* **88**, 205 (1997).
17. R. J. Crowder, H. Enomoto, M. Yang, E. M. Johnson Jr., J. Milbrandt, *J. Biol. Chem.* **279**, 42072 (2004).
18. H. Zhou, D. J. Glass, G. D. Yancopoulos, J. R. Sanes, *J. Cell Biol.* **146**, 1133 (1999).
19. R. Herbst, S. J. Burden, *EMBO J.* **19**, 67 (2000).
20. A. Watty *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4585 (2000).
21. S. K. H. Gillespie, S. Balasubramanian, E. T. Fung, R. L. Huganir, *Neuron* **16**, 953 (1996).
22. C. Fuhrer, J. E. Sugiyama, R. G. Taylor, Z. W. Hall, *EMBO J.* **16**, 4951 (1997).
23. J. E. Sugiyama, D. J. Glass, G. D. Yancopoulos, Z. W. Hall, *J. Cell Biol.* **139**, 181 (1997).
24. F. Chevessier *et al.*, *Hum. Mol. Genet.* **13**, 3229 (2004).
25. T. T. Kummer, T. Misgeld, J. R. Sanes, *Curr. Opin. Neurobiol.* **16**, 74 (2006).

26. We thank Regeneron Pharmaceuticals for MuSK-deficient cells; K. Manji, J. Hamuro, H. Moriya, and A. Ishii for technical assistance; and M. Shirakata, T. Yasuda, S. Sugano, T. Tezuka, R. F. Whittier, S. Tronick, and T. Yamamoto for discussions. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and by a grant from the Uehara Foundation.

Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5781/1802/DC1
Materials and Methods
SOM Text
Figs. S1 to S15
References

8 March 2006; accepted 19 May 2006
10.1126/science.1127142

INTERNAL MEDICINE

□ CASE REPORT □

Examination of IgM Rheumatoid Factor (IgM-RF) and Anti-cyclic Citrullinated Peptide Antibody (Anti-CCP Ab) in Japanese Patients with Palindromic Rheumatism

Mami Tamai[1], Atsushi Kawakami[1], Nozomi Iwanaga[1], Keita Fujikawa[1], Fumiko Tanaka[1], Toshiyuki Aramaki[1], Yasumori Izumi[1], Kouichiro Aratake[1], Kazuhiko Arima[1], Makoto Kamachi[1], Hideki Nakamura[1], Mingguo Huang[1], Hiroaki Ida[1], Tomoki Origuchi[2] and Katsumi Eguchi[1]

Abstract

We have studied the serology of 6 patients with palindromic rheumatism. None of the patients fulfilled the classification criteria for rheumatoid arthritis at the entry; however, 4 out of the 6 patients were seropositive for IgM rheumatoid factor (IgM-RF) at entry. Sequential serological study was performed in 4 patients; IgM-RF changed from seronegative to seropositive in one patient, and the titer increased in another patient. Anti-cyclic citrullinated peptide antibody (anti-CCP Ab) at the entry was detected in only one of the 6 patients; that patient later developed RA. Although follow-up is necessary, the present study may suggest the importance of serological examination, especially anti-CCP Ab, in patients with palindromic rheumatism.

Key words: palindromic rheumatism, IgM-RF, anti-CCP Ab

(DOI: 10.2169/internalmedicine.45.1407)

Introduction

Palindromic rheumatism is characterized by recurrent attacks of acute arthritis of short duration. In the long term, some of these patients develop a connective tissue disease, usually rheumatoid arthritis (RA) (1, 2). Previous studies in Europe and North America showed that the presence of IgM rheumatoid factor (IgM-RF) in palindromic rheumatism patients indicates a future risk for RA (1-4), and anti-cyclic citrullinated peptide antibody (anti-CCP Ab) was recently reported in palindromic rheumatism patients (5). Thus, we measured IgM-RF and anti-CCP Ab in the sera of Japanese palindromic rheumatism patients.

Case Presentation

We encountered 6 patients with palindromic rheumatism, as diagnosed by the criteria described by Gonzalez-Lopez et al (1). Informed consent was obtained from all of the patients.

Table 1 summarizes the profiles of the 6 palindromic rheumatism patients. All patients were prospectively followed-up, and the disease duration was estimated from the time of first attack until the last consultation. Seroposivities of IgM-RF and anti-CCP Ab shown in Table 1, were obtained from the data of the latest serological examinations. Joint involvement in Table 1 shows the total joints affected during the follow-up period. Serology was serially examined in 4 patients; IgM-RF turned to be positive in one patient and its titer increased in one case (Fig. 1). Among the 6 patients, one patient developed RA later. IgM-RF was seropositive in 4 out of 6 patients; however, only the one patient who developed RA was positive for anti-CCP Ab. Anti-CCP Ab in this case was consistently positive; however, IgM-RF was negative during the follow-up period

[1] First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki and [2] Nagasaki University of Health Sciences, Nagasaki University, Nagasaki

Received for publication May 9, 2005; Accepted for publication April 27, 2006

Correspondence to Katsumi Eguchi, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501

Table 1. **Clinical Findings and Serological Data from 5 Patients with Palindromic Rheumatism**

	Age (yr)	Sex (M/F)	Affected joints	Disease Duration (M)	IgM-RF (IU/ml)	Anti-CCP Ab (U/ml)
Case 1	58	M	ankle, MTP	19	98.6	0.6
Case 2	51	F	wrist, shoulder, ankle, knee	64	<9.8	<0.6
Case 3	40	F	wrist, MCP, PIP	24	90.0	0.6
Case 4	68	M	wrist, elbow, ankle, knee	88	37.0	3.0
Case 5	63	M	wrist, elbow, ankle	22	28.0	<0.6
Case 6	52	M	shoulder, ankle, wrist	7	<9.8	72.0

IgM-RF was measured by latex-enhanced immunonephelometric assay (Dade Behring, Marburg, Germany, cut-off value=14 IU/ml). Anti-CCP Ab was measured by enzyme-linked immunosorbent assay (DIASTAT Anti-CCP, Axis-Shield, Dundee, UK, cut-off value=4.5 U/ml). IgM-RF was positive in cases 1, 3, 4 and 5, whereas anti-CCP Ab was positive in case 6.

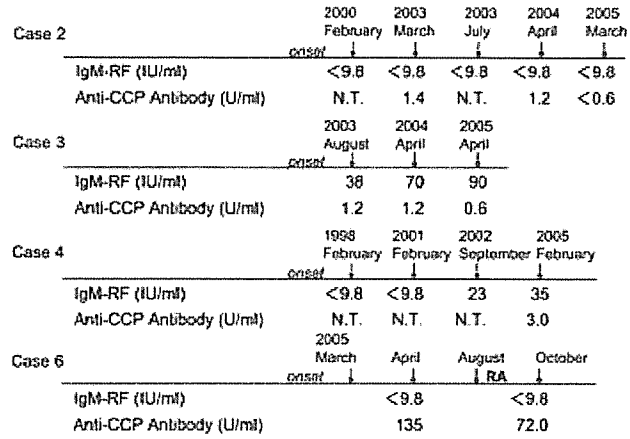

Figure 1. **Serial serological examinations of 4 patients with palindromic rheumatism. IgM-RF: IgM rheumatoid factor. Anti-CCP Ab: anti-cyclic citrullinated peptide antibody. N.T.: not tested. In case 2, the patient's condition developed into persistent arthritis in July 2003, whereas she did not fulfill the 1987 ACR classification criteria for RA. In addition, plain radiographs of the affected joints did not show any erosive change until now. In case 6, the patient was diagnosed as RA in August 2005; however, plain radiographs of the affected joints in October 2005 did not show any erosive change.**

(Fig. 1 and Table 1). Plain radiographs of the affected joints were examined serially (case 1 to case 6), but no erosive bone changes were not found in any of the patients (data not shown).

Discussion

Gonzalez-Lopez et al have reported that 28% of patients with palindromic rheumatism developed RA after a mean follow-up of 6 years (1). Previous investigations including that of Gonzalez-Lopez et al showed that seropositivity for IgM-RF could be one of the risk factors for subsequent development of RA (1-4). The present study includes 6 cases with a mean follow-up of 3.1 years. Among them, the one patient who developed RA later was seropositive for anti-CCP Ab and seronegative for IgM-RF. Thus, the presence of anti-CCP Ab might be a prognostic factor for future onset of RA. The involvement of proximal interphalangeal (PIP) joints, wrist joints and metacarpophalangeal (MCP) joints has been suggested to indicate future risk for RA in patients with palindromic rheumatism (1), and such involvement was observed in 5 out of 6 patients. Thus, further follow-up will be necessary to detect the onset of RA of our cases.

Van Gaalen et al recently revealed that the presence of anti-CCP Ab is the best predictor for the progression to RA in patients with undifferentiated arthritis (6). Anti-CCP Ab is reported to be found in 56.3% of patients with palindromic rheumatism in Spain (18 out of 32 patients); however, prediction of the future development of palindromic rheumatism to RA by anti-CCP Ab remains to be determined (5). Our prospective study of early-stage RA indicates that about 70% of the patients at baseline already show the seropositivity with anti-CCP Ab (7, 8); the difference in anti-CCP Ab seropositivity from that found by Salvador et al (5) in Spain may be due to arise from racial difference. Further study may answer question.

The present data are preliminary and limited; however, to our knowledge, this is the first prospective observation of Japanese patients with palindromic rheumatism regarding IgM-RF and anti-CCP Ab. Further clinical investigation, including a large number of patients will be necessary to clarify the significance of IgM-RF and anti-CCP Ab in the prediction of further onset of RA in Japanese palindromic rheumatism patients.

We thank Maiko Kubo, Nobuko Fukuda and Kouko Munechika for their technical assistance.

Abbreviations: ACR: American College of Rheumatology, anti-CCP Ab: anti-citrullinated peptide antibody, IgM-RF: IgM rheumatoid factor, MCP joint: metacarpophalangeal joint, MTP joint: metatarsophalangeal joint, PIP joint: proximal interphalangeal joint, RA: rheumatoid arthritis

References

1. Gonzalez-Lopez L, Gamez-Nava JI, Jhangri GS, Ramos-Remus C, Russell AS, Suarez-Almazor ME. Prognostic factors for the development of rheumatoid arthritis and other connective tissue diseases in patients with palindromic rheumatism. J Rheumatol **26**: 540-545, 1999.
2. Honnonen P, Mottonen T, Oka M. Palindromic rheumatism. A clinical survey of sixty patients. Scand J Rheumatol **16**: 413-420, 1987.

3. Williams MH, Sheldon PJ, Torrigiani G, Eisen V, Mattingly S. Palindromic rheumatism. Clinical and immunological studies. Ann Rheum Dis **30**: 375-380, 1971.
4. Youssef WA, Yan A, Russel AS. Palindromic rheumatism: A response to chloroquine. J Rheumatol **18**: 35-37, 1991.
5. Salvador G, Gomez A, Vinas O, et al. Prevalence and clinical significance of anti-cyclic citrullinated peptide and antikeratin antibodies in palindromic rheumatism. An abortive form of rheumatoid arthritis? Rheumatology **42**: 972-975, 2003.
6. van Gaalen FA, Linn-Rasker SP, van Venrooji WJ, et al. Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. Arthritis Rheum **50**: 709-715, 2004.
7. Tamai M, Kawakami A, Uetani M, et al. The presence of anti-cyclic citrullinated peptide antibody is associated with magnetic resonance imaging detection of bone marrow oedema in early-stage rheumatoid arthritis. Ann Rheum Dis **65**: 133-134, 2006.
8. Tamai M, Kawakami A, Uetani M, et al. Early prediction of rheumatoid arthritis by serologic variables and magnetic resonance imaging of the wrists and finger joints: results from prospective clinical examination. Ann Rheum Dis **65**: 134-135, 2006.

The presence of anti-cyclic citrullinated peptide antibody is associated with magnetic resonance imaging detection of bone marrow oedema in early stage rheumatoid arthritis

M Tamai, A Kawakami, M Uetani, S Takao, F Tanaka, H Nakamura, N Iwanaga, Y Izumi, K Arima, K Aratake, M Kamachi, M Huang, T Origuchi, H Ida, K Aoyagi and K Eguchi

Ann Rheum Dis 2006;65;133-134
doi:10.1136/ard.2005.04138

LETTERS

The presence of anti-cyclic citrullinated peptide antibody is associated with magnetic resonance imaging detection of bone marrow oedema in early stage rheumatoid arthritis

M Tamai, A Kawakami, M Uetani, S Takao, F Tanaka, H Nakamura, N Iwanaga, Y Izumi, K Arima, K Aratake, M Kamachi, M Huang, T Origuchi, H Ida, K Aoyagi, K Eguchi

Ann Rheum Dis 2006;**65**:133–134. doi: 10.1136/ard.2005.04138

Early prediction of erosive joint damage is very important in rheumatoid arthritis (RA) because significant articular damage in patients is evident radiologically within the first few years of the disease.[1] This study was designed to confirm whether anti-cyclic citrullinated peptide antibodies (anti-CCP Ab) define the subset of patients with early stage RA who have bone marrow oedema, observed by magnetic resonance imaging (MRI).

Patients were referred from the Early Arthritis Clinic, started in 2001 at the First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University. After prospective follow up, diagnosis of RA was made by the 1987 criteria for RA of the American College of Rheumatology.[2] Eighty patients who gave their informed consent to the protocol that was approved by the Institutional Review Board of Nagasaki University were enrolled in the study.

The disease duration of 80 patients with RA at the entry was <24 months (mean disease duration 4.8 months), and thus these patients had early stage RA. Serological variables at entry were as follows: mean (SD) C reactive protein 1.6 (2.5) mg/ml, matrix metalloproteinase 3 (MMP-3) positivity 46.3%, anti-CCP Ab positivity 67.5%, and IgM rheumatoid factor (IgM-RF) positivity 67.5%. The mean modified Genant-Sharp score of plain radiographs of both hands at entry was 0.41.

Magnetic resonance images of both wrists and finger joints were taken simultaneously using the 1.5 T system (Sigma, GE Medical Systems, Milwaukee, WI). Images were evaluated for the presence or absence of bone marrow oedema and synovitis in 15 joints of each finger and wrist—that is, the distal radioulnar joint, radiocarpal joint, mid-carpal joint, 1st carpometacarpal joint, 2nd–5th carpometacarpal joints (together), 1st–5th metacarpophalangeal joints separately, and the 1st–5th proximal interphalangeal joints separately (total 30 joints from both hands).

The severity of synovitis was assessed by the number of joints with synovitis and the rate of enhancement (E-rate), on a dynamic study by injection of gadolinium-diethylene-triamine pentaacetic acid. The E-rate means the vascularity,[3 4] by plotting the signal intensity against time in a selected region of interest (about 2–3 mm in diameter) of the site of maximum enhancement in the above-mentioned 15 joints. Determination of bone marrow oedema was also carried out[5–7] by two experienced radiologists (MU and ST), and decisions were reached by consensus.

We examined simply and automatically the wrists and finger joints, including proximal interphalangeal joints, by MRI, using the above-mentioned variables instead of the OMERACT 5 RA-MRI scoring system.[6 7] We divided the 80 patients with early stage RA according to the presence or absence of anti-CCP Ab (table 1).

The proportion of patients with bone marrow oedema was significantly higher in the anti-CCP Ab+ group than in the anti-CCP Ab- group. In contrast, there were no differences between the two groups for the other variables (for example, CRP, MMP-3 positivity, number of joints with synovitis, and mean E-rate of 30 joints).

Division of patients according to the presence or absence of IgM-RF also showed a higher proportion of patients with bone marrow oedema in those who were anti-CCP Ab positive than in those negative for the antibody, but the difference was not significant (table 2). However, because 81.5% of anti-CCP Ab+ patients also possessed IgM-RF (44/54 patients), anti-CCP Ab and IgM-RF are not independent factors for bone marrow oedema. Bone marrow oedema is a forerunner of bone erosion on plain radiography,[8] and thus our present data show the additional importance of the

Table 1 Comparison of anti-CCP Ab+ and anti-CCP Ab− patients

Variables	Anti-CCP Ab+ (n = 54)	Anti-CCP Ab− (n = 26)	p Value
CRP (mg/ml)	1.3 (2.0)	2.2 (3.3)	0.39*
MMP-3 (%)	50.0	38.5	0.33†
Number of joints with synovitis	12.2 (6.4)	10.3 (6.4)	0.30*
Mean E-rate of 30 joints	7.7 (3.0)	7.4 (2.9)	0.67*
Bone marrow oedema			
%	64.8	38.5	0.03†
No	2.8 (3.5)	1.1 (2.3)	0.01*

Data are mean (SD) unless stated otherwise. The proportion of patients with bone marrow oedema was significantly higher in the anti-CCP Ab+ group than in the anti-CCP Ab- group: *by Mann-Whitney U test; †by χ^2 test.

Table 2 Comparison of IgM-RF+ and IgM-RF− patients

Variables	IgM-RF+ (n = 54)	IgM-RF− (n = 26)	p Value
CRP (mg/ml)	1.5 (2.1)	2.0 (3.3)	0.96*
MMP-3 (%)	51.9	34.6	0.23**
Number of joints with synovitis	12.1 (5.6)	10.6 (7.8)	0.22*
Mean E-rate of 30 joints	7.6 (3.2)	7.6 (2.3)	0.58*
Bone marrow oedema			
%	63.0	42.3	0.08**
No	2.7 (3.5)	1.4 (2.7)	0.07*

Data are mean (SD) unless stated otherwise. Division of patients by IgM-RF seropositivity showed a higher proportion of patients with bone marrow oedema compared with those negative for the antibody, but the difference was not significant: *by Mann-Whitney U test; †by χ^2 test.

presence of anti-CCP Ab at baseline as an indication of future bone erosion in early stage RA.[9]

We thank Misses Maiko Kubo, Youko Uchiyama, Nobuko Fukuda, and Kouko Munechika for their technical assistance.
This study was supported by a grant from The Ministry of Health, Labour and Welfare, Japan.

Authors' affiliations
M Tamai, A Kawakami, F Tanaka, H Nakamura, N Iwanaga, Y Izumi, K Arima, K Aratake, M Kamachi, M Huang, H Ida, K Eguchi, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
M Uetani, S Takao, Department of Radiology and Radiation Research, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
K Aoyagi, Department of Public Health, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
T Origuchi, Nagasaki University School of Health Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8520, Japan

MT and AK contributed equally to this work.

Correspondence to: Professor K Eguchi, eguchi@net.nagasaki-u.ac.jp

Accepted 2 June 2005

REFERENCES

1 **Fuchs HA**, Kaye JJ, Callahan LF, Nance EP, Pincus T. Evidence of significant radiographic damage in rheumatoid arthritis within the first 2 years of disease. *J Rheumatol* 1989;**16**:585–91.
2 **Arnett EC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al.* The American Rheumatology Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
3 **Graffney K**, Cookson J, Blades S, Coumbe A, Blake D. Quantitative assessment of the rheumatoid synovial microvascular bed by gadolinium-DTPA enhanced magnetic resonance imaging. *Ann Rheum Dis* 1998;**57**:152–7.
4 **Reece RJ**, Kraan MC, Radjenovic A, Veale DJ, O'Connor PJ, Ridgway JP, *et al.* Comparative assessment of leflunomide and methotrexate for the treatment of rheumatoid arthritis, by dynamic enhanced magnetic resonance imaging. *Arthritis Rheum* 2002;**46**:366–72.
5 **McQueen FM.** Magnetic resonance imaging in early inflammatory arthritis: what is its role? *Rheumatology (Oxford)* 2000;**39**:700–6.
6 **Lassere M**, McQueen F, Ostergaard M, Conaghan P, Shnier R, Peterfy C, *et al.* OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 3: An international multicenter reliability study using the RA-MRI score, *J Rheumatol* 2003;**30**:1366–75.
7 **Conaghan P**, Lassere M, Ostergaard M, Peterfy C, McQueen F, O'Connor P, *et al.* OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 4: An international multicenter longitudinal study using the RA-MRI score, *J Rheumatol* 2003;**30**:1376–9.
8 **McQueen FM**, Benton N, Perry D, Crabbe J, Robinson E, Yeoman S, *et al.* Bone edema scored on magnetic resonance imaging scans of the dominant carpus at presentation predicts radiographic joint damage of the hands and feet six years later in patients with rheumatoid arthritis. *Arthritis Rheum* 2003;**48**:1814–27.
9 **van Gaalen FA**, Linn-Rasker SP, van Venrooij WJ, de Jong BA, Breedveld FC, Verweij CL, *et al.* Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis Rheum* 2004;**50**:709–15.

Early prediction of rheumatoid arthritis by serological variables and magnetic resonance imaging of the wrists and finger joints: results from prospective clinical examination

M Tamai*, A Kawakami*, M Uetani, S Takao, H Rashid, F Tanaka, K Fujikawa, T Aramaki, H Nakamura, N Iwanaga, Y Izumi, K Arima, K Aratake, M Kamachi, M Huang, T Origuchi, H Ida, K Aoyagi, K Eguchi

Ann Rheum Dis 2006;**65**:134–135. doi: 10.1136/ard.2005.043075

We aimed at characterising the serological variables and magnetic resonance imaging (MRI) early changes in the wrists and finger joints which would differentiate rheumatoid arthritis (RA) from rheumatic diseases other than RA (non-RA) at the earliest stage.

Patients were referred from the Early Arthritis Clinic, started in 2001 at the First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University. After prospective follow up, a diagnosis was made according to international classification criteria, and in particular, RA was defined by 1987 criteria of the American College of Rheumatology for RA.[1] Informed consent was obtained from all the patients, and the protocol was approved by the Institutional Review Board of Nagasaki University.

Eighty consecutive patients with RA and 33 non-RA patients were studied and a diagnosis evaluated 12 months after entry, by March 2005. The mean disease duration of the 80 patients with RA at entry was 4.8 months, and thus they were described as early stage RA.

MR images of both wrists and finger joints were acquired with a 1.5 T system (Sigma, GE Medical Systems, Milwaukee, WI, USA) with the use of an extremity coil. Coronal T_1 weighted spin echo (repetition time 450, echo time 13) and short time inversion recovery (repetition time 3000, echo time 12, T_1 160) images were acquired. The images were evaluated for the presence or absence of bone marrow oedema, bone erosion, and synovitis in 15 joints in each finger and wrist—namely, distal radioulnar joint, radiocarpal joint, mid-carpal joint, 1st carpometacarpal joint, 2nd–5th carpometacarpal joints (together), 1st–5th metacarpophalangeal joints separately, and 1st–5th proximal interphalangeal joints separately (total 30 joints from both hands). The extent of synovitis, bone marrow oedema, and bone erosion was determined, as previously described,[2 3] by two experienced radiologists (MU and ST), and decisions were reached by consensus.

Symmetric arthritis is a characteristic feature of RA.[1] The presence of symmetric synovitis on MRI was defined as bilateral involvement of wrist sites, metacarpophalangeal joints, or proximal interphalangeal joints without absolute symmetry. Because we focused on the presence or absence of early joint changes on MRI for the differentiation, we did not use the OMERACT 5 RA-MRI scoring system.[4 5] As expected, the positivity of matrix metalloproteinase 3 (MMP-3; measured by enzyme linked immunosorbent assay (ELISA; Daiichi Pure Chemicals, Fukuoka, Japan) (46.3% *v* 12.1%),

Table 1 Serological variables and MRI findings for the discrimination between early stage RA and non-RA

Variables	Odds ratio	Coefficient	SE	p Value	Weighted score
Anti-CCP antibody and/or IgM RF	7.42	2.00	0.57	*0.0005*	*1*
MMP-3	2.87	1.05	0.72	0.14	0
Symmetric synovitis	4.37	1.47	0.57	*0.009*	*1*
Bone marrow oedema and/or bone erosion	5.48	1.70	0.63	*0.007*	*1*

Logistic regression analysis identified the presence of anti-CCP antibody and/or IgM RF, symmetric synovitis on MRI, and bone marrow oedema and/or bone erosion on MRI as significant and independent measures for discrimination between early stage RA and non-RA. The weighted score was calculated based on the regression coefficient for each variable as described in the text.

Table 2 Evaluation of the prediction score (≥2) in early stage RA at the first visit

Total score	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
≥1	96.3	30.3			
≥2	82.5	84.8	93.0	66.7	83.2
3	50.0	96.9			

We calculated the sensitivity and specificity of our scoring system for the prediction of early stage RA according to the sum of weighted scores described in table 1.
Sensitivity and specificity are shown for patients classified as early stage RA according to the total score (sum of weighted score 1–3).
We evaluated the statistical character of prediction score (≥2) for the present 113 patients for the prediction of RA at entry.

anti-cyclic citrullinated peptide antibody (anti-CCP antibody; measured by ELISA; DIASTAT Anti-CCP, Axis-Shield, Dundee, UK) (67.5% *v* 12.1%), and IgM rheumatoid factor (IgM RF; measured by latex-enhanced immunonephelometric assay; Dade Behring, Marburg, Germany) (67.5% *v* 30.3%) as well as the frequency of symmetric synovitis (81.3% *v* 36.4%), bone marrow oedema (56.3% *v* 12.1%), and bone erosion (45.0% *v* 9.1%) were higher in early stage RA than in non-RA.

Logistic regression analysis using the statistical analysis system software demonstrated that the presence of anti-CCP antibody and/or IgM RF, symmetric synovitis and bone marrow oedema and/or bone erosion at entry could discriminate between patients with RA and non-RA patients (table 1).

At the first visit, a total score of two or more of the three objective measures (anti-CCP antibody and/or IgM-RF: 1, symmetric synovitis on MRI: 1, bone marrow oedema and/or bone erosion on MRI: 1) allowed the prediction of RA with 82.5% sensitivity and 84.8% specificity, respectively (table 2). (Statistical weights of the variables were calculated based on the regression coefficient for each variable, standardised by dividing by the coefficient for symmetric synovitis; values were rounded off to yield integers.)

Our present data may indicate that the prediction of autoantibodies as well as MRI detection of early joint changes contribute to the accurate diagnosis of early stage RA.

We thank Misses Maiko Kubo, Nobuko Fukuda, Kouko Munechika, and Junko Matsushita for their technical assistance.
This study was supported by a grant from the Ministry of Health, Labour and Welfare, Japan

Authors' affiliations

M Tamai, A Kawakami, F Tanaka, K Fujikawa, T Aramaki, H Nakamura, N Iwanaga, Y Izumi, K Arima, K Aratake, M Kamachi, M Huang, H Ida, K Eguchi, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
M Uetani, S Takao, H Rashid, Department of Radiology and Radiation Research, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
T Origuchi, Nagasaki University School of Health Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8520, Japan
K Aoyagi, Department of Public Health, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

*MT and AK contributed equally to this work.

Correspondence to: Professor K Eguchi, eguchi@net.nagasaki-u.ac.jp

Accepted 2 October 2005

REFERENCES

1 **Arnett EC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al.* The American Rheumatology Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
2 **McQueen FM.** Magnetic resonance imaging in early inflammatory arthritis: what is its role? *Rheumatology (Oxford)* 2000;**39**:700–6.
3 **Conaghan PG**, O'Connor P, McGoagle D, Astin P, Wakefield RJ, Gibbon WW, *et al.* Elucidation of the relationship between synovitis and bone damage: a randomized magnetic resonance imaging study of individual joints in patients with early rheumatoid arthritis. *Arthritis Rheum* 2003;**48**:64–71.
4 **Lassere M**, McQueen F, Ostergaard M, Conaghan P, Shnier R, Peterfy C, *et al.* OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 3: An international multicenter reliability study using the RA-MRI score, *J Rheumatol* 2003;**30**:1366–75.
5 **Conaghan P**, Lassere M, Ostergaard M, Peterfy C, McQueen F, O'Connor P, *et al* OMERACT rheumatoid arthritis magnetic resonance imaging studies. Exercise 4: An international multicenter longitudinal study using the RA-MRI score. *J Rheumatol* 2003;**30**:1376–9