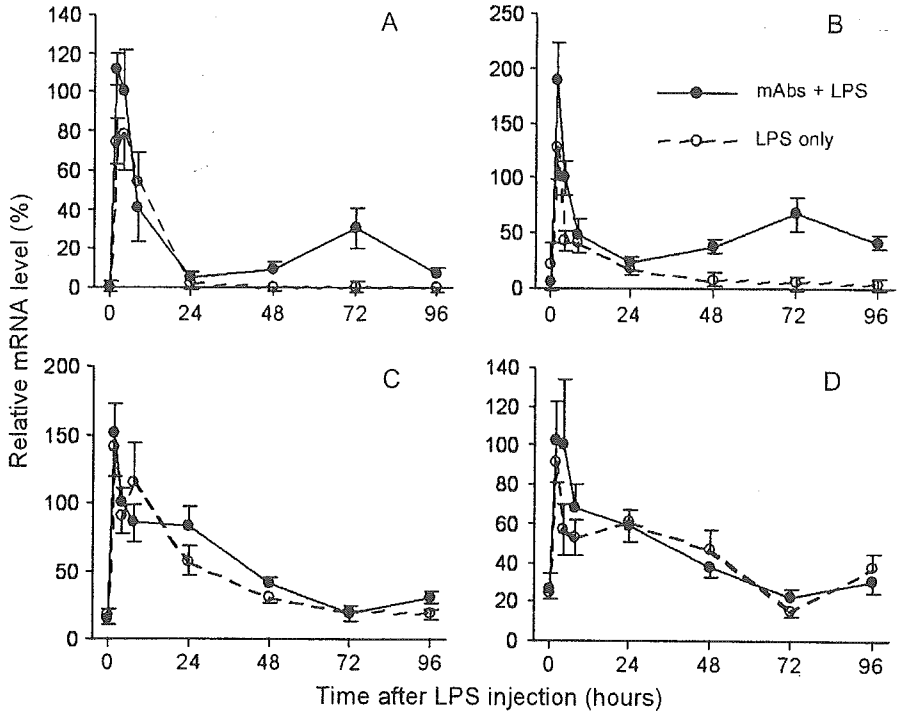


**Fig. 3.** Time courses of cytokine mRNA expression at the inflamed joints of mice with anti-type II CL antibody-induced arthritis. The arthritis was induced in 40 BALB/c mice, and 5 mice each were sacrificed 0, 2, 4, 8, 24, 48, 72 or 96 h after the LPS injection (solid lines). Other 40 mice were treated with LPS alone, and sacrificed similarly (broken lines). The mRNA levels of IL-6 (A), IL-1β (B), TNF-α (C) and IL-1α (D) at the joints were determined by quantitative RT–PCR. The quantities of the targeted mRNA were normalized by the quantities of control 18S rRNA. The normalized mRNA quantities were compared among the experimental groups. We fixed the mRNA quantities at the fourth hour in the mAbs + LPS group as 100%, and relative mRNA quantities were indicated. Values are means ± SEM.

considerable levels of IL-6 and that E-64-d significantly inhibited the production of IL-6 in a dose-dependent manner (Fig. 5A and B). The significantly effective concentration of E-64-d was 10–30 μg $ml^{-1}$, consistent with those of other reports (24, 28). The production of IL-6 in E11 cells was markedly enhanced by PMA plus IM, and E-64-d also decreased the production of IL-6 on stimulation with PMA plus IM (Fig. 5C and D). In contrast to E-64-d, E-64 and rCS showed only marginal effects (Fig. 5A–C). Although the MW of rCS (14 kDa) is much larger than that of E-64 (375.4 kDa) or E-64-d (342.4 kDa), even a high dose (4000 μg $ml^{-1}$) of rCS, equivalent to 100 μg $ml^{-1}$ of E-64-d in molar concentration, did not affect the production of IL-6 in E11 cells (supernatant IL-6 levels were 432 ± 78 and 403 ± 37 pg $ml^{-1}$ in the high-dose rCS-treated and the untreated E11 cells, respectively). Next, since IL-1α, IL-1β and TNF-α could not be detected in the supernatants of E11 cells, we examined intracellular levels of the cytokines by ELISA. Among the three cytokines, IL-1α (mature form) could be detected in E11 cells. E-64-d decreased the production of IL-1α in E11 cells significantly in a dose-dependent manner (Fig. 6B), whereas E-64 and rCS did not show significant effects (Fig. 6A). Suppression of the production of IL-1α by E-64-d was also observed on stimulation with PMA plus IM (Fig. 6C and D).

Since the inhibitory effects of E-64-d on cytokine production might have been due to its direct cytotoxicity, we examined the effects of E-64-d on the viability of E11 cells by a colorimetric WST-1 assay (29). As shown in Fig. 5(E and F), the three calpain inhibitors had neither cytotoxic nor proliferative effects on E11 cells. Finally, we examined the effects of the calpain inhibitors using crude synoviocytes, which were obtained from joint tissues of a disease-active RA patient. Consistent with the results for E11 cells, the crude RA synoviocytes spontaneously secreted IL-6, which was significantly decreased by E-64-d in a dose-dependent manner, while E-64 and rCS did not show significant effects (Fig. 7).

## Discussion

We and another group have found a new auto-antibody against calpastatin in RA patients (12, 13). Calpain, the target molecule of calpastatin, is a $Ca^{2+}$-dependent cysteine proteinase that cleaves a broad range of substrates (18), and is considered to participate in the pathogenesis of RA through the modulation of its substrates. First, calpain converts pro-IL-1α into its mature form (20). Second, it degrades inhibitor κB (IκB), leading to the activation of nuclear factor-κB (NF-κB) (30, 31). Third, it activates neutrophils and promotes granular exocytosis (21, 32).

To clarify the role of calpain in the pathophysiology of RA, we treated an animal model of RA with calpain-inhibitory compounds and examined their biological effects. We found that the administration of a high dose of E-64-d significantly ameliorated the clinical arthritis and the histopathological findings in murine mAb-induced arthritis (Figs 1 and 2). Cuzzocrea *et al.* have previously demonstrated that CL-induced

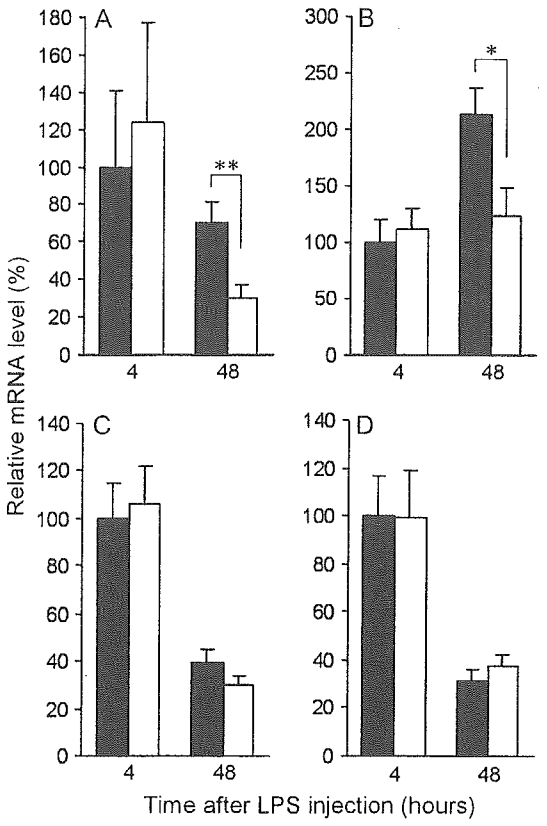


**Fig. 4.** Effects of E-64-d on cytokine mRNA expression at the inflamed joints of mice. The disease-affected mice were treated with a high dose (9 mg $kg^{-1}$ per day) of E-64-d (white bars) or control diluent (black bars), until they were sacrificed at the 4th or 48th h after the LPS injection. The mRNA levels of IL-6 (A), IL-1β (B), TNF-α (C) and IL-1α (D) at the joints were determined by quantitative RT-PCR. We fixed the mRNA quantities at the fourth hour in the control group as 100%, and relative mRNA quantities were indicated. Values are means ± SEM. $*P < 0.05$, $**P < 0.01$.

arthritis was successfully treated with calpain inhibitor I (*N*-acetyl-leucyl-leucyl-norleucinal) in rats and that the administration of calpain inhibitor I reduced the levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the inflamed joints (33). Since calpain can activate NF-κB through the degradation of IκB (30, 31), the therapeutic efficacy of calpain inhibitor I was assumed to be brought about by the inhibition of iNOS and COX-2, whose gene expression is induced by NF-κB (34). The contribution of calpain to the activation of NF-κB has been clearly demonstrated by the work of Chen *et al.*, in which the transduction of the *calpastatin* gene into a macrophage-like cell line inhibited the degradation of IκB and the activation of NF-κB in the cells (31).

IL-6 is recognized as an important factor in the pathophysiology of RA since it enhances the proliferation and differentiation of T and B cells, and IL-6 levels in synovial fluids correlate with the disease activity of RA patients (35). However, the relationship between calpain and cytokines such as IL-6 has not been well described. In the present study, we demonstrated that the expression of IL-6 mRNA at the inflamed joints of mice was decreased by the administration of E-64-d (Fig. 4A) and that the production of IL-6 in cultured synoviocytes was inhibited by E-64-d (Figs 5 and 7). Since the gene expression of IL-6 can be enhanced by NF-κB (36, 37), one of the reasons why E-64-d inhibited IL-6 might be its negative effect on NF-κB. However, Kagari *et al.* have reported that IL-6 was not as responsible for the progression of this experimental arthritis as TNF-α and IL-1β were (27). There may be other mechanisms by which E-64-d ameliorated the arthritis.

In the present study, we employed anti-type II CL antibody-induced arthritis, a kind of antibody-transfer model of autoimmune diseases, since the reproducibility of the disease is high (25) and cytokine levels change sharply in this model (27). In our analysis of the time course of cytokine mRNA expression at the affected joints (Fig. 3), the IL-6 and IL-1β mRNA levels showed elevations twice at around 4 and 48–72 h after the LPS injection (Fig. 3A and B, solid lines), whereas the TNF-α and IL-1α mRNA levels showed early peaks and declined gradually (Fig. 3C and D). When we examined the effects of LPS alone, the second peaks of the IL-6 and IL-1β mRNA levels were not observed (Fig. 3A and B, broken lines). These results suggest that the peaks at the fourth hour in all the cytokines, which were not affected by E-64-d (Fig. 4), are related to the direct effects of LPS, and the second elevations of IL-6 and IL-1β mRNA levels around the 48th–72nd h, which were significantly decreased by E-64-d (Fig. 4A and B), are associated with the local immunologic reactions induced by anti-type II CL mAbs. The results also imply that calpain may be responsible for the antibody-induced chronic local inflammatory reactions, but not for the LPS-induced acute systemic inflammation.

IL-1, which stimulates the degradation of cartilage matrices (28), is also an important mediator of RA. Interestingly, calpain is crucial for the activation of IL-1α since it processes pro-IL-1α into its mature form (20). This is confirmed by Fig. 6, which shows that E-64-d inhibited the production of mature IL-1α in E11 cells. Therefore, one of the mechanisms by which E-64-d ameliorated murine mAb-induced arthritis might be the suppression of conversion of IL-1α. Although we could not observe the negative effects of E-64-d on IL-1α mRNA expression *in vivo* (Fig. 4D), it is not inconsistent because the conversion of IL-1α occurs downstream of the mRNA transcription.

In the histopathological evaluation, the score for cell infiltration was significantly decreased by the administration of E-64-d (Fig. 2A). Calpain contributes to the migration of fibroblasts through the degradation of cytoskeletal proteins such as talin (38). Calpain is also associated with the spreading and polarization of neutrophils by regulating Rho-family GTPases (39). The chemotactic migration of cultured fibroblasts was inhibited by E-64-d (40), whereas the chemotactic movement of neutrophils was enhanced by calpain inhibitor I (39). Although the two results seem to be opposite, they at least make sure that calpain plays important roles in cellular migration. Taken together, these mechanisms can be applied to the ameliorative effects of E-64-d on murine mAb-induced arthritis.

Since an excessive amount of calpain is produced in synovial fluids of RA patients (1, 2) and calpain can degrade cartilage matrices including proteoglycans (41), the contribution of calpain to the pathogenesis of RA has been assumed to occur in the extracellular space. However, we found that

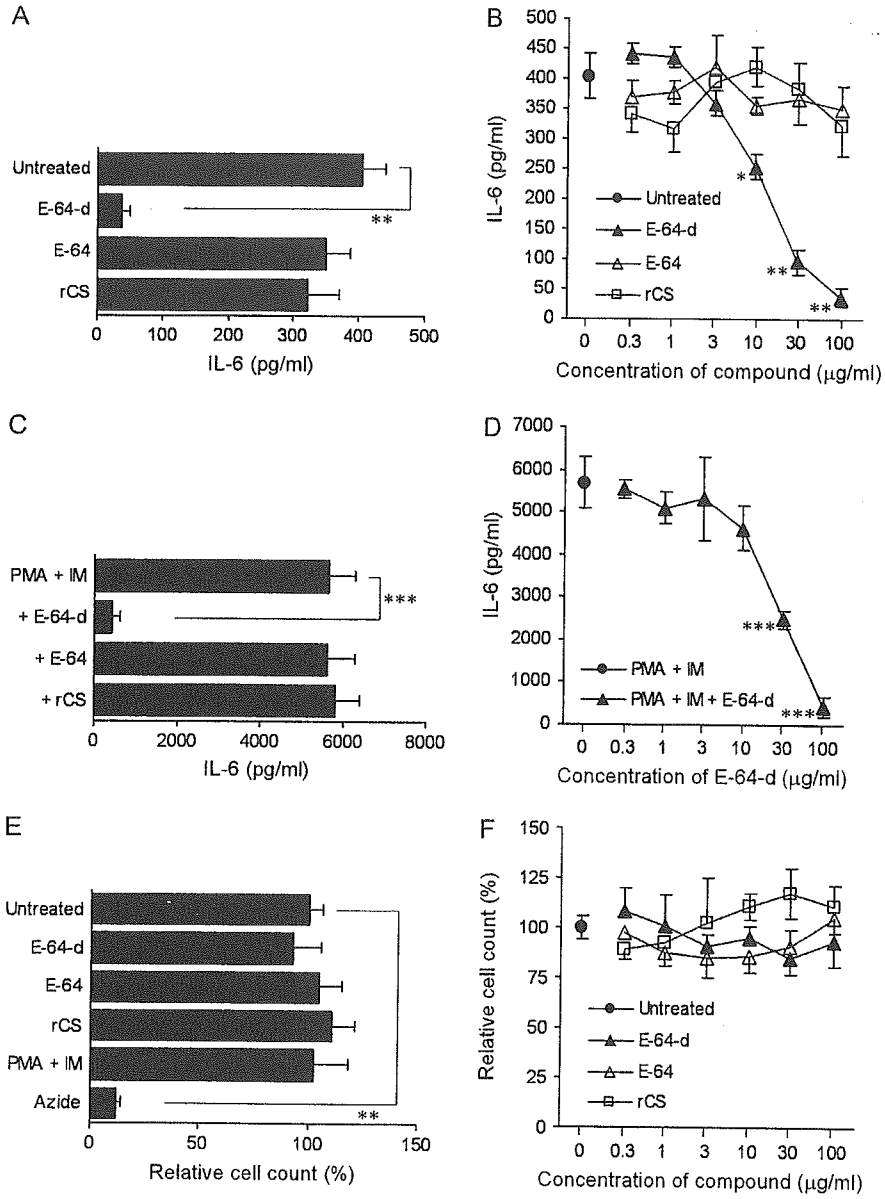


**Fig. 5.** Effects of calpain inhibitors on IL-6 production in E11 cells. After E11 cells were incubated with the indicated calpain inhibitors for 24 h in the presence (C and D) or absence (A and B) of 10 ng $ml^{-1}$ PMA plus 750 ng $ml^{-1}$ IM, the supernatant IL-6 was quantified by ELISA. (E and F) Effects of calpain inhibitors on viability of E11 cells. After E11 cells were cultured with the indicated calpain inhibitors for 24 h, a colorimetric cell viability assay was performed with WST-1 solution. Relative cell counts among the groups are indicated. In (A, C and E), concentrations of E-64-d, E-64 and rCS were 100 μg $ml^{-1}$. (B, D and F) Analysis of dose dependency. Values are means ± SEM. $*P < 0.05$, $**P < 0.01$ versus untreated, $***P < 0.01$ versus PMA + IM treated.

non-membrane-permeable E-64 and rCS did not have significant effects on cytokine production *in vivo* and *in vitro*, compared with lipophilic E-64-d (Figs 1A, 5–7). These results suggest that the intracellular calpain is more strongly related to the pathophysiology of RA than the extracellular calpain. We have previously described that anti-calpastatin antibodies of RA patients were able to block the physiological function of calpastatin *in vitro* (12). Theoretically, the blockade of calpastatin by the auto-antibodies results in the activation of calpain, leading to the above-mentioned induction of various inflammatory mediators. Although the mechanism by which the auto-antibodies can interfere with the intracellular calpain–calpastatin system remains unknown, Menard and el-Amine have pointed out the existence of extracellular calpastatin in RA patients, and speculated that the auto-antibodies can bind to the extracellular calpastatin and indirectly affect the inner balance of calpain and calpastatin (19).

E-64-d has been used in order to inhibit calpain *in vivo* and *in vitro*. Ray *et al.* applied E-64-d for the treatment of spinal cord injury in rats, demonstrating that 1 mg $kg^{-1}$ of intravenous E-64-d inhibited calpain and alleviated neuronal apoptosis at the injured spinal cord (23). Tram *et al.* found that

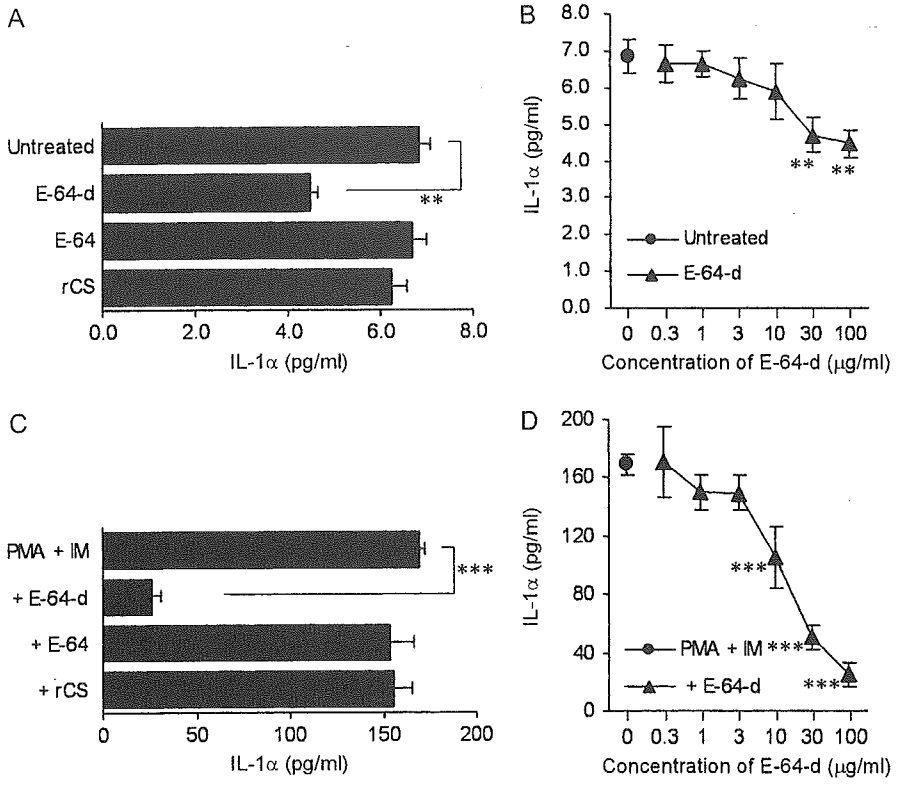


**Fig. 6.** Effects of calpain inhibitors on IL-1α production in E11 cells. After E11 cells were incubated with the indicated calpain inhibitors for 24 h in the presence (C and D) or absence (A and B) of 10 ng $ml^{-1}$ PMA plus 750 ng $ml^{-1}$ IM, the intracellular IL-1α was extracted and quantified by ELISA. In (A and C), concentrations of E-64-d, E-64 and rCS were 100 μg $ml^{-1}$. (B and D) Analysis of dose dependency. Values are means ± SEM. $^{**}P < 0.01$ versus untreated, $^{***}P < 0.01$ versus PMA + IM treated.

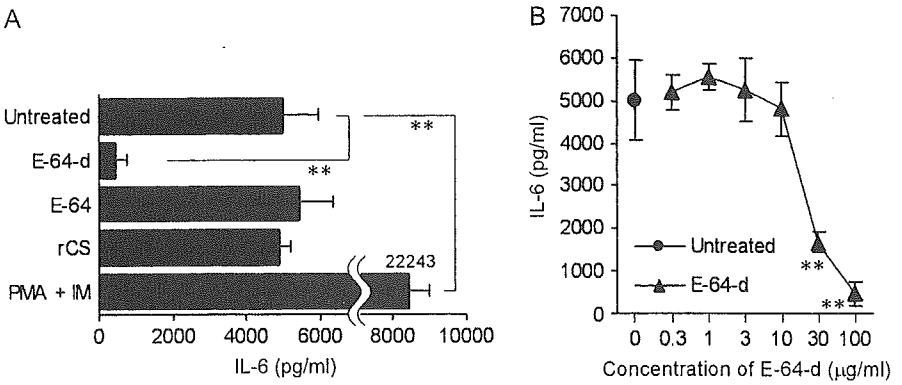


**Fig. 7.** Effects of calpain inhibitors on IL-6 production in crude RA synoviocytes. Synoviocytes obtained from a disease-active RA patient were incubated with the indicated calpain inhibitors for 24 h, and then the supernatant IL-6 was quantified by ELISA. In (A), concentrations of E-64-d, E-64 and rCS were 100 μg $ml^{-1}$. (B) Analysis of dose dependency. Values are means ± SEM. $^{**}P < 0.01$ versus untreated.

parathormone-induced contraction of cultured osteoblasts was inhibited by E-64-d but not by non-membrane-permeable E-64-c (24). Since E-64-d and calpain inhibitor I can inhibit not only calpain but also cathepsin (42, 43), it cannot be excluded that the effects of the two compounds on the animal models of arthritis might also be due to the inhibition of cathepsin. To target calpain more selectively, a trial is ongoing to treat cultured synoviocytes or an animal model of RA by transfection of the *calpastatin* gene.

In conclusion, we demonstrated the ameliorative effects of E-64-d, a calpain-inhibitory compound, on an animal model of RA, and presented new evidence for the negative potentiality of E-64-d on cytokine production. These results suggest that calpain contributes to the pathophysiology of arthritis and that

the regulation of the calpain–calpastatin system may be a new strategy to treat arthritic diseases such as RA.


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## Abbreviations

| | |
|---|---|
| CL | collagen |
| COX-2 | cyclooxygenase-2 |
| *Ct* values | cycle numbers to the threshold |
| FRP | follistatin-related protein |
| IκB | inhibitor κB |
| IM | ionomycin |
| iNOS | inducible nitric oxide synthase |
| i.p. | intra-peritoneally |
| MMP | matrix metalloproteinase |
| MW | molecular weight |
| NF-κB | nuclear factor-κB |
| PMA | phorbol 12-myristate 13-acetate |
| RA | rheumatoid arthritis |
| rCS | recombinant calpastatin |
| RT | reverse transcription |
| TNF-α | tumor necrosis factor-α |
| WST-1 | water-soluble tetrazolium salt-1 |

## References


1 Fukui, I., Tanaka, K. and Murachi, T. 1989. Extracellular appearance of calpain and calpastatin in the synovial fluid of the knee joint. *Biochem. Biophys. Res. Commun.* 162:559.

2 Yamamoto, S., Shimizu, K., Suzuki, K., Nakagawa, Y. and Yamamuro, T. 1992. Calcium-dependent cysteine proteinase (calpain) in human arthritic synovial joints. *Arthritis Rheum.* 35:1309.

3 Yamamoto, S., Shimizu, K., Niibayashi, H., Yasuda, T. and Yamamuro, T. 1994. Immunocytochemical demonstration of calpain in synovial cells in human arthritic synovial joints. *Biomed. Res.* 15:77.

4 Hashimoto, Y., Kakegawa, H., Narita, Y. *et al.* 2001. Significance of cathepsin B accumulation in synovial fluid of rheumatoid arthritis. *Biochem. Biophys. Res. Commun.* 283:334.

5 Yoshihara, Y., Nakamura, H., Obata, K. *et al.* 2000. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann. Rheum. Dis.* 59:455.

6 Maini, R. N., Breedveld, F. C., Kalden, J. R. *et al.* 2004. Sustained improvement over two years in physical function, structural damage, and signs and symptoms among patients with rheumatoid arthritis treated with infliximab and methotrexate. *Arthritis Rheum.* 50:1051.

7 Suzuki, A., Yamada, R., Chang, X. *et al.* 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.* 34:395.

8 Wipke, B. T., Wang, Z., Kim, J., McCarthy, T. J. and Allen, P. M. 2002. Dynamic visualization of a joint-specific autoimmune response through positron emission tomography. *Nat. Immunol.* 3:366.

9 Tanaka, M., Ozaki, S., Osakada, F., Mori, K., Okubo, M. and Nakao, K. 1998. Cloning of follistatin-related protein as a novel autoantigen in systemic rheumatic diseases. *Int. Immunol.* 10:1305.

10 Tanaka, M., Ozaki, S., Kawabata, D. *et al.* 2003. Potential preventive effects of follistatin-related protein/TSC-36 on joint destruction and antagonistic modulation of its autoantibodies in rheumatoid arthritis. *Int. Immunol.* 15:71.

11 Kawabata, D., Tanaka, M., Fujii, T. *et al.* 2004. Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis. *Arthritis Rheum.* 50:660.

12 Mimori, T., Suganuma, K., Tanami, Y. *et al.* 1995. Autoantibodies to calpastatin (an endogenous inhibitor for calcium-dependent neutral protease, calpain) in systemic rheumatic diseases. *Proc. Natl Acad. Sci. USA* 92:7267.

13 Despres, N., Talbot, G., Plouffe, B., Boire, G. and Menard, H. A. 1995. Detection and expression of a cDNA clone that encodes a polypeptide containing two inhibitory domains of human calpastatin and its recognition by rheumatoid arthritis sera. *J. Clin. Invest.* 95:1891.

14 Iwaki-Egawa, S., Matsuno, H., Yudoh, K. *et al.* 2004. High diagnostic value of anticalpastatin autoantibodies in rheumatoid arthritis detected by ELISA using human erythrocyte calpastatin as antigen. *J. Rheumatol.* 31:17.

15 Vittecoq, O., Salle, V., Jouen-Beades, F. *et al.* 2001. Autoantibodies to the 27 C-terminal amino acids of calpastatin are detected in a restricted set of connective tissue diseases and may be useful for diagnosis of rheumatoid arthritis in community cases of very early arthritis. *Rheumatology (Oxford)* 40:1126.

16 Lackner, K. J., Schlosser, U., Lang, B. and Schmitz, G. 1998. Autoantibodies against human calpastatin in rheumatoid arthritis: epitope mapping and analysis of patient sera. *Br. J. Rheumatol.* 37:1164.

17 Kanazawa, Y., Kaneshiro, Y., Sawa, M. *et al.* 2000. Domain reactivity of autoantibodies to calpastatin in patients with systemic rheumatic diseases. *Mod. Rheumatol.* 10:38.

18 Goll, D. E., Thompson, V. F., Li, H., Wei, W. and Cong, J. 2003. The calpain system. *Physiol. Rev.* 83:731.

19 Menard, H. A. and el-Amine, M. 1996. The calpain-calpastatin system in rheumatoid arthritis. *Immunol. Today* 17:545.

20 Watanabe, N. and Kobayashi, Y. 1994. Selective release of a processed form of interleukin 1 alpha. *Cytokine* 6:597.

21 Melloni, E., Pontremoli, S., Michetti, M. *et al.* 1985. Binding of protein kinase C to neutrophil membranes in the presence of Ca2+ and its activation by a Ca2+-requiring proteinase. *Proc. Natl Acad. Sci. USA* 82:6435.

22 Szomor, Z., Shimizu, K., Fujimori, Y., Yamamoto, S. and Yamamuro, T. 1995. Appearance of calpain correlates with arthritis and cartilage destruction in collagen induced arthritic knee joints of mice. *Ann. Rheum. Dis.* 54:477.

23 Ray, S. K., Matzelle, D. C., Wilford, G. G., Hogan, E. L. and Banik, N. L. 2000. E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. *Brain Res.* 867:80.

24 Tram, K. K., Murray, S. S., Lee, D. B. and Murray, E. J. 1993. PTH-induced osteoblast contraction is mediated by cysteine proteases. *Kidney Int.* 43:693.

25 Terato, K., Harper, D. S., Griffiths, M. M. *et al.* 1995. Collagen-induced arthritis in mice: synergistic effect of *E. coli* lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 22:137.

26 Abe, M., Tanaka, Y., Saito, K. *et al.* 1997. Regulation of interleukin (IL)-1beta gene transcription induced by IL-1beta in rheumatoid synovial fibroblast-like cells, E11, transformed with simian virus 40 large T antigen. *J. Rheumatol.* 24:420.

27 Kagari, T., Doi, H. and Shimozato, T. 2002. The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. *J. Immunol.* 169:1459.

28 Buttle, D. J., Saklatvala, J., Tamai, M. and Barrett, A. J. 1992. Inhibition of interleukin 1-stimulated cartilage proteoglycan degradation by a lipophilic inactivator of cysteine endopeptidases. *Biochem. J.* 281(Pt 1):175.

29 Umehara, H., Huang, J. Y., Kono, T. *et al.* 1998. Co-stimulation of T cells with CD2 augments TCR-CD3-mediated activation of protein tyrosine kinase p72syk, resulting in increased tyrosine

phosphorylation of adapter proteins, Shc and Cbl. *Int. Immunol.* 10:833.
30 Shumway, S. D., Maki, M. and Miyamoto, S. 1999. The PEST domain of IkappaBalpha is necessary and sufficient for *in vitro* degradation by mu-calpain. *J. Biol. Chem.* 274:30874.
31 Chen, F., Lu, Y., Kuhn, D. C. *et al.* 1997. Calpain contributes to silica-induced I kappa B-alpha degradation and nuclear factor-kappa B activation. *Arch. Biochem. Biophys.* 342:383.
32 Pontremoli, S., Melloni, E., Damiani, G. *et al.* 1988. Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. *J. Biol. Chem.* 263:1915.
33 Cuzzocrea, S., McDonald, M. C., Mazzon, E. *et al.* 2000. Calpain inhibitor I reduces the development of acute and chronic inflammation. *Am. J. Pathol.* 157:2065.
34 Barnes, P. J. 1997. Nuclear factor-kappa B. *Int. J. Biochem. Cell Biol.* 29:867.
35 Brozik, M., Rosztoczy, I., Meretey, K. *et al.* 1992. Interleukin 6 levels in synovial fluids of patients with different arthritides: correlation with local IgM rheumatoid factor and systemic acute phase protein production. *J. Rheumatol.* 19:63.
36 Libermann, T. A. and Baltimore, D. 1990. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol. Cell. Biol.* 10:2327.
37 Miyazawa, K., Mori, A., Yamamoto, K. and Okudaira, H. 1998. Constitutive transcription of the human interleukin-6 gene by rheumatoid synoviocytes: spontaneous activation of NF-kappaB and CBF1. *Am. J. Pathol.* 152:793.
38 Dourdin, N., Bhatt, A. K., Dutt, P. *et al.* 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J. Biol. Chem.* 276:48382.
39 Lokuta, M. A., Nuzzi, P. A. and Huttenlocher, A. 2003. Calpain regulates neutrophil chemotaxis. *Proc. Natl Acad. Sci. USA* 100:4006.
40 Fukai, F., Ohtani, T., Ueki, M. and Katayama, T. 1993. Involvement of calcium-dependent cysteine protease in fibronectin-induced chemotactic migration of NIH-L13 fibroblasts. *Biochem. Mol. Biol. Int.* 30:225.
41 Suzuki, K., Shimizu, K., Hamamoto, T., Nakagawa, Y., Murachi, T. and Yamamuro, T. 1992. Characterization of proteoglycan degradation by calpain. *Biochem. J.* 285(Pt 3):857.
42 Tamai, M., Matsumoto, K., Omura, S., Koyama, I., Ozawa, Y. and Hanada, K. 1986. *In vitro* and *in vivo* inhibition of cysteine proteinases by EST, a new analog of E-64. *J. Pharmacobio-dyn.* 9:672.
43 Sasaki, T., Kishi, M., Saito, M. *et al.* 1990. Inhibitory effect of di- and tripeptidyl aldehydes on calpains and cathepsins. *J. Enzyme Inhib.* 3:195.

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Journal of Gastroenterology



# A novel apoptosis-inducing monoclonal antibody (anti-LHK) against a cell surface antigen on colon cancer cells

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Editorial on page 1009

***Background.*** Apoptosis is a crucial element in the behavior of mammalian cells in many different situations. We here report the establishment of a novel monoclonal antibody (anti-LHK mAb) that has apoptosis-inducing activity against colon cancer Colo205 cells. ***Methods.*** The mechanism of anti-LHK mAb-induced cell death was assessed by microscopic morphology, Annexin V/Hoechst 33528 staining, and detection of DNA fragmentation. The molecular weight of LHK antigen was determined by Western blotting. Growth inhibition of Colo205 cells induced by anti-LHK mAb was determined by in vitro and in vivo studies. ***Results.*** Anti-LHK reacted with a 70-kDa antigen and completely blocked the proliferation of Colo205 cells bearing LHK in vitro in a manner characteristic of apoptosis. Strikingly, anti-LHK mAb suppressed tumor growth in a murine peritoneal dissemination model. ***Conclusions.*** LHK antigen, which is restricted to epithelial cells, may be a novel death receptor that plays a critical role in controlling the growth, invasion, and metastasis of human colon cancer cells.

**Key words:** anti-LHK mAb, apoptosis, Fas, colon cancer

## Introduction

Monoclonal antibodies (mAbs) are accepted as ideal adjuvant therapeutic agents for some diseases. In 1989, two groups independently isolated the same mouse-derived antibody, designated anti-Fas[1] and anti-APO-1 mAb,[2] respectively, which had a cytotoxic effect on various human cell lines.[1,2] Fas belongs to the tumor necrosis factor receptor (TNF-R) family.[3–6] The mechanism of cell death induced by Fas signaling is apoptosis. Furthermore, Fas is broadly expressed in a variety of normal organs and tissues, including hematopoietic cells and various normal and cancer epithelial cells.[7] It is well known that colon cancer cells express relatively lower levels of Fas and are more resistant to the Fas system than normal colonic epithelial cells.[6] In fact, Moller and colleagues[8] reported that Fas-negative subpopulations, which occur rarely in colon adenomas and normal cells, are common in colon cancers. In the immune response to neoplasms, cytotoxic T lymphocytes (CTLs) are activated by tumor cells and thereafter express Fasligand(L) on their own surfaces. FasL-expressing CTLs then bind to Fas-expressing target cells, and the binding induces the intrinsic apoptotic pathway in tumor cells. In contrast, it has been reported that colon cancer cells also express functional FasL on the surface and kill Fas-expressing Jurkat cells in a Fas-mediated manner as "Fas counterattack".[9] This converse phenomenon suggests that colon cancer cells can kill CTLs infiltrating around them through the Fas system and escape from immunological rejection.

Although it appears that an anti-Fas strategy is not feasible for the treatment of patients with colon cancers because of Fas resistance and its fatal side-effects, apoptotic cell death is still potentially of importance for clinical application. Here we report our establishment and analysis of a novel antibody (anti-LHK) that had a cytotoxic effect on Fas-resistant Colo205 colon cancer cells.

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*H.M. and T.K. contributed equally to this work.

## Methods

### *Reagents and antibodies*

Recombinant human (rh) interferon (IFN)-γ, with a specificity of $4 \times 10^6$ units/mg, was kindly provided by Shionogi Pharmaceutical (Osaka, Japan); rh TNF-α ($1.7 \times 10^7$units/ml) and the murine mAb anti-TNF-R IgG2a were purchased from GenZyme (Cambridge, MA, USA). Ethidium bromide, propidium iodide (PI), cycloheximide (CHX), Hoechst 33528, and dithiothreitol were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The murine mAb anti-Fas IgM (CH11) was purchased from MBL (Nagoya, Japan). Fluorescein iso thiocyanate (FITC)-conjugated goat polyclonal anti-mouse IgM or IgG was purchased from Tago (Burlingame, CA, USA). [$^{35}$S]-methionine was purchased from ICN Pharmaceuticals (Irvine, CA, USA). Dispase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Block ace was purchased from Dainihon Pharmaceuticals (Osaka, Japan). Streptavidin-enzyme conjugates (Vectastatin ABC kit) were purchased from Vector Laboratories (Burlingame, CA, USA).

### *Mice*

BALB/c and C.B.17 severe combined immunodeficiency (SCID) mice (6–8 weeks old, male) were purchased from Japan Clea (Tokyo, Japan). The mice were maintained in the Animal Care Facility of Keio University. The review board of the University has approved our experimental animal studies.

### *Cell lines and human tissues*

The human colon cancer cell lines (Colo205, WiDr, HT-29, and Caco2) and the human gastric cancer cell line (KATO-III) were purchased from ATCC (Rockville, MD, USA). The mouse myeloma cell line Sp2/0 was kindly given by Dr. S. Aiso (Keio University). The human hepatoma cell line derived from hepatitis B virus (HBV)-infected patients (Alexander) and the cervical epidermoid carcinoma cell line (HeLa) were kindly given by Dr. H. Saito (Keio University). The human T-cell leukemia cell line Jurkat was kindly given by Dr. Kayagaki (Juntendo University, Tokyo). The cell culture and all assays in this study were performed in RPMI 1640 or DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin/streptomycin, and 2 mercapto ethanol (ME) ($5 \times 10^{-5}$M). All normal human tissues and tumor tissues were obtained from biopsied or surgical specimens. The diagnostic criteria for the histological examinations were based on the Japanese classification of colorectal carcinoma (6th edition) of the Japanese Society for Cancer of the Colon and Rectum. Written informed consent was obtained from all patients prior to the study and all experiments were approved by the Committee on Human Subjects of Keio University Hospital.

### *Preparation of mAb*

Before immunization, $1 \times 10^7$ Colo205 cells were stimulated with 100 U/ml human rhIFN-γ for 48 h. Then, 6-week-old BALB/c mice were immunized intraperitoneally with IFN-γ-stimulated Colo205 cells ($4.0 \times 10^7$) four times, every 3 weeks. Four days after the last injection, spleen cells of immunized mice were fused with Sp2/0 cells by the standard hybridization technique. A hybridoma cell (termed B3D11-H12) producing an mAb with cytotoxicity to Colo205 cells was cloned twice by limiting dilution at a concentration of 0.3 cells per well. After the injection of B3D11-H12 cells into the abdominal cavity of BALB/c mice, ascites were purified. The mAb was termed anti-LHK mAb, and its isotype was determined by enzyme-linked immunosorbent assay (ELISA; Biomeda, Foster City, CA, USA).

### *Immunoprecipitation*

Prior to the immunoprecipitation assay, cell extracts were prepared from $5 \times 10^6$ Colo205 cells in methionine-free minimal essential medium, which were first labeled with [$^{35}$S]-methionine (500 μCi; ICN Pharmaceuticals) overnight. Cells were then harvested, washed with TBS (10 mM Tris-Cl, 150 mM NaCl, pH 7.5), resuspended in lysis buffer (50 mM Tris-buffered saline, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenyl methylsulfonyl fluoride [PMSF], 10 mM iodoacetamine, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin), and sonicated three times for 40 s each time with a Branson sonifer (Branson Ultrasonics, Danbury, CT). The supernatants were centrifuged at 15000 rpm for 10 min to remove soluble debris and used as sources of antigen. An immunoprecipitation assay was performed as described before.[10] Ten micrograms of anti-LHK mAb or isomatched anti-mouse IgM was incubated with 2 μg of protein A-Sepharose beads or protein A-Agarose beads (Sigma-Aldrich) pre-swollen in 500 μl of IPP buffer (500 mM NaCl, 0.1% Nonidet P-40 [Wako Chemicals, Tokyo, Japan], 10 mM Tris-Cl, pH 8.0) overnight at 4°C in a microfuge tube with end-to-end rotation. The antibody-coated beads were washed three times with 500 μl of IPP buffer, and then 400 μl of IPP buffer was added and the antibody-coated beads were incubated for 2 h at 4°C with 200 μl of [$^{35}$S]-labeled cell extract, which was equivalent to $5 \times 10^5$ Colo205 cells. After three washes, the beads were heated at 95°C

in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-Cl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue, pH 6.8) for 5 min to extract the bound proteins. The proteins were loaded onto 10% polyacrylamide-SDS gel and subjected to electrophoresis. The gel was then soaked for 30 min in 0.5 M sodium salicylate, dried, and exposed to X-ray film (XAR-5 film; Eastman Kodak, Rochester, NY, USA) with an intensifying screen at −70°C.

*Immunohistochemistry*

Serial sections (6 μm) from the fresh frozen tissues were air-dried for 2–3 h, fixed in acetone for 10 min at room temperature, and immunostained immediately. After the blocking of nonspecific binding sites with Block ace (Dainippon Pharmaceutical) for 10 min at 37°C, the sections were incubated with purified mouse anti-LHK IgM mAb (1 μg/ml) or isomatched mouse IgM (1 μg/ml, as a negative control) for 30 min at 37°C, then with biotinylated goat anti-mouse IgM for 30 min at 37°C, and finally with streptoavidin-enzyme conjugates (Vectastatin ABC kit). The localization of LHK antigen was visualized by incubation with diaminobenzidine solution, and faint counterstaining was performed with Mayer's hematoxylin.

*Flow cytometry*

An immunofluorescence technique[11] was used to estimate the quantity of the LHK antigen, Fas, or TNF-R expression by flow cytometry. Cultured cells were washed in phosphate-buffered saline (PBS) and suspended in 50 μl of staining buffer (PBS containing 2% FCS and 0.1% $NaN_3$), then 5 μg/ml of each primary antibody (anti-LHK, anti-Fas, anti-TNF-R) or isotype-matched control antibody (polyclonal mouse IgM or IgG) was added, and the samples were placed on ice for 20 min. FITC-conjugated goat anti-mouse IgM or goat anti-rat IgG was stained for 20 min. Samples were rewashed in staining buffer and suspended at $1 \times 10^6$ cells/ml in 50 μl of PBS. Fluorescence intensity on the surface was then analyzed using a FACScan (BD Sciences, San Jose, CA, USA). Changes in LHK antigen, Fas, and TNF-R expression after pretreatment with 100 U/ml IFN-γ for 48 h were also assessed. In addition, early apoptotic cells were detected by flow cytometric analysis, using FITC-labeled Annexin V, a protein with high affinity for phosphatidylserine exposed upon the cell membrane in the early apoptotic phase.[12]

*Morphological analysis*

Approximately $5 \times 10^4$ cells were seeded in 25-$cm^2$ culture flasks and incubated with isotype-matched control IgM (1 μg/ml), anti-LHK mAb (1 μg/ml), anti-Fas IgM (1 μg/ml), or TNF-α (50 ng/ml) in complete medium for 12 h, then washed twice with PBS. After exposure, the cells were trypsinized and harvested. Cell viability was counted by trypan blue staining. Morphological changes caused by these mAbs were observed by phase-contrast microscopy. In addition, apoptotic cells were assessed morphologically by staining with Hoechst 33258 (Sigma-Aldrich).

*Demonstration of DNA laddering*

DNA fragmentation was visualized by the method of Sellins and Cohen,[13] with some modification. Briefly, cells ($5 \times 10^6$) cultured for 3 h with anti-LHK mAb (1 μg/ml) or isotype-matched control IgM (1 μg/ml) were lysed in 1 ml of DNA extraction solution containing 20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM ethylenediamire tetraacetic acid [EDTA], and 0.5% SDS. The lysates were incubated with 100 μg/ml proteinase K at 37°C for 16 h. After incubation, the enzyme-digested cell lysates were carefully mixed with 1 ml of phenol/chloroform (1:1) and centrifuged at 15 000 *g* for 20 min. DNA in the aqueous phase was incubated with 5 μg/ml DNase-free RNase A at 37°C for 1 h and again extracted with phenol/chloroform. DNA was collected by precipitation with 1 ml isopropanol and 0.1 ml NaCl at −20°C overnight. After centrifugation, the resulting DNA was dissolved in 10 mM Trs-HCl and 1 mM EDTA, and its concentration was determined at 260 nm by spectrophotometry. DNA electrophoresis was carried out in 1.2% agarose gels containing 1 μg/ml ethidium bromide, and DNA banding was visualized by exposing the gels to UV light.

*Inhibition of Colo205 cell proliferation by anti-LHK IgM*

Colo205 cells and Fas-sensitive Jurkat cells ($5 \times 10^4$ cells/well) were incubated with the isotype-matched control IgM, anti-LHK mAb, anti-Fas IgM, or rhTNF-α at various concentrations in 100 μl of complete medium in a 96-well round culture plate for 24 h at 37°C in a 5% $CO_2$ incubator. Then cells were labeled with [$^3$H] thymidine by the addition of 50 μl of complete medium containing 0.5 μCi of [$^3$H]-thymidine for 4 h. The radioactivity incorporated was counted in a liquid scintillation counter to quantify the level of DNA synthesis.

*Preparation of isolated epithelial cells from the intestinal mucosa*

Human colonic epithelial cells were isolated from surgical specimens as previously described.[14] Briefly, resected samples of colonic mucosa were dissected from

the underlying musculature and washed in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS). Tissues were then treated with 1 mM dithiothreitol (Sigma-Aldrich) in CMF-HBSS for 15 min at 22°C. After three washes with CMF-HBSS, the tissue pieces were incubated twice in CMF-HBSS containing 1 mg/ml dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) for 30 min at 37°C. During the treatment, epithelial cells and intraepithelial lymphocytes were released from the tissues. The cell suspensions resulting from dispase treatment were washed twice, pelleted, and resuspended in 3 ml of 100% Percoll (Pharmacia Biotech, Piscataway, NJ, USA). Three-ml layers of 60%, 40%, and 30% Percoll were layered successively on top prior to centrifugation at 1500 rpm for 30 min at 4°C. Cells at the top 0/30% layer interface contained more than 95% pure epithelial cells. Cells were washed three times in RPMI, and viability was determined by trypan blue exclusion.

*Xenograft model of Colo205 cells*

A xenograft model of human colon cancer was made as previously reported.[15] Briefly, cultured Colo205 cells were harvested and the pellet of cells obtained by centrifugation was suspended in RPMI 1640 with 10% FCS to a concentration of $1 \times 10^7$ cells/ml. Aliquots of $5 \times 10^6$ cells were injected into the peritoneal cavities of 8-week-old BALB/c SCID mice. Twenty-four hours after the injection, 500 μg of anti-LHK IgM or control mouse IgM mAb in 0.5 ml of RPMI 1640 was injected intraperitoneally into Colo205-transplanted mice, and the injection was repeated weekly. The dosage was selected based on the preliminarily observed effects of several doses. Eight weeks after the first injection, mice were killed by cervical dislocation. The peritoneal tumors were dissected out and the total volumes were measured. Tumor volumes were calculated by using the formula ($A \times B^2/2$), where A and B are tumor caliper measurements expressed in millimeters, and $A > B$. The volume of tiny nodules in the mesentery was negligible, because they were less than 0.2 mm in diameter.

*Statistical analysis*

The values for results were expressed as means ± SD. Groups of data were compared by the Mann-Whitney U-test for correlations between the frequency of $LHK^+$ cells and histology of colon cancer, and tumor numbers and tumor volume examined in the xenograft model, and by the $\chi^2$ test for the frequencies of abdominal masses and peritoneal dissemination in the xenograft model. Differences were considered to be statistically significant when $P$ was less than 0.05.

**Results**

*Anti-LHK mAb induces apoptosis*

To establish novel mAbs, with cytotoxicity against colon cancer cells, BALB/c mice were immunized with IFN-γ-stimulated Colo205 colon cancer cells. Among more than 10000 hybridoma cells, one clone produced an mAb that had cytotoxicity against Colo205 cells. The isotype of the mAb was proven to be IgM, and the mAb was designated anti-LHK antibody. Anti-LHK mAb was purified to homogeneity, and the purified anti-LHK mAb killed Colo205 cells as well as crude culture supernatant or ascites of mice undergoing intraperitoneal injection of hybridomas. Anti-LHK mAb was added to Colo205 cells cultured in complete media at the concentration of 1 μg/ml, and cells were observed sequentially by phase-contrast microscopy. Budding, which is a characteristic of the apoptotic process,[16] was observed 3 h after the start of culture (Fig. 1A, upper right) and cell death was induced 12 h after the culture (Fig. 1A, lower right). In contrast, cell death was not induced in Colo205 cells cultured in the presence of control IgM (Fig. 1A, upper and lower left). Next, suppression of proliferation of Colo205 cells treated with anti-LHK mAb was assessed using [$^3$H]-thymidine. The proliferation of Colo205 cells was markedly suppressed by anti-LHK mAb in a dose-dependent manner (Fig. 1B).

To confirm the mechanism of cell death induced by anti-LHK mAb, three types of assays for apoptosis were performed. Hoechst 33528 staining of DNA revealed morphological features characteristic of apoptosis in Colo205 cells: fragmented and condensed nuclei were observed after 12 h of treatment with anti-LHK mAb (Fig. 2A). In the electrophoresis of DNA extracted from Colo205 cells treated with anti-LHK mAb, DNA fragmentation was visualized as DNA ladders (Fig. 2B). In the double-staining of Annexin V/propidium iodide (PI), Colo205 cells treated with anti-LHK mAb for 12 h were mainly Annexin $V^-/PI^+$ cells and Annexin $V^+/PI^+$ cells, as were many of those treated with CHX (Fig. 2C). These results suggest that apoptosis was critically involved in the mechanism of anti-LHK-induced cell death.

*The antigen for anti-LHK mAb*

Because Fas and TNF receptor (TNF-R) are known to be cell death receptors that induce apoptotic cell death, we next assessed whether the mechanism of anti-LHK mAb-induced apoptosis of Colo205 cells was identical to those of anti-Fas or TNFα. Phase-contrast microscopic time-course observation revealed that anti-LHK mAb and TNFα, but not anti-Fas mAb (CH11), had strong cytotoxicity for Colo205 cells (Fig. 3A, left). In

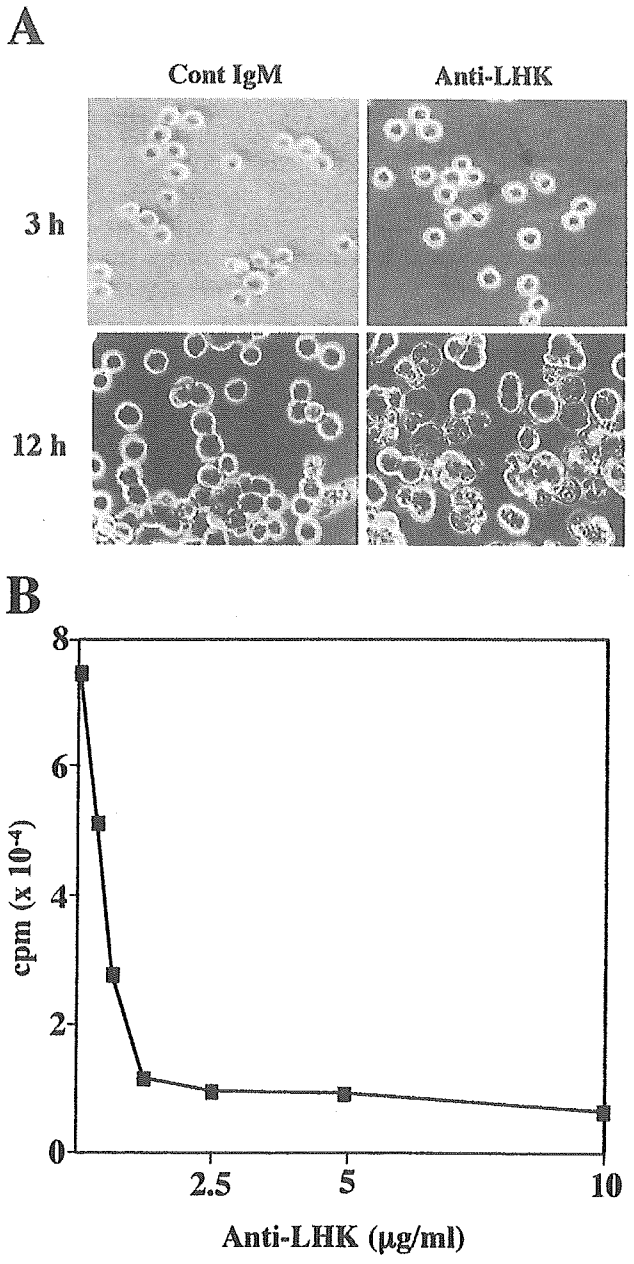


**Fig. 1.** **A** Morphological changes and proliferative responses of Colo205 cells induced by anti-LHK monoclonal antibody (mAb). Dying Colo205 cells observed under a phase-contrast microscope. Three h (*upper left*) and 12-h (*lower left*) with control IgM (1 μg/ml) and 3 h (*upper right*) and 12 h (*lower right*) with anti-LHK mAb (1 μg/ml). ×120. *Arrows* indicate budding phenomena. **B** Suppression of proliferative responses of Colo205 cells induced by anti-LHK mAb at various concentrations

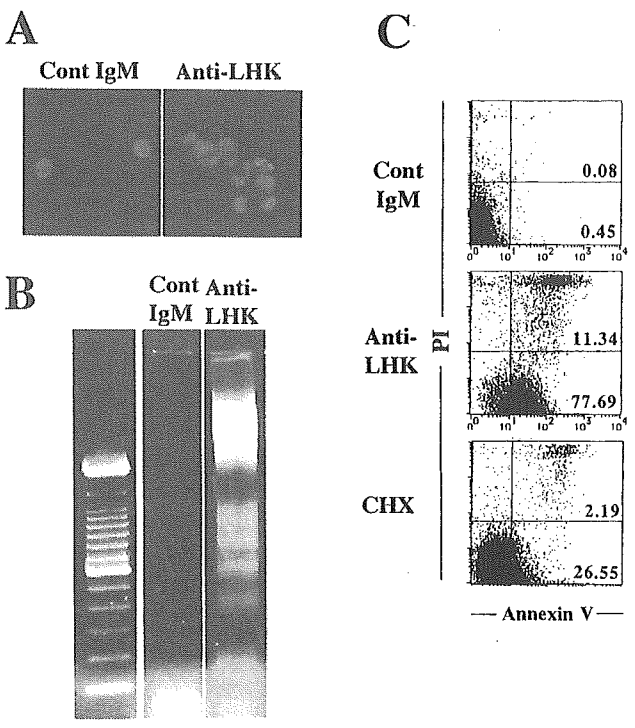


**Fig. 2A–C.** Anti-LHK-induced apoptosis. **A** Hoechst 33528 staining revealed morphological changes characteristic of apoptosis in Colo205 cells after treatment for 12 h with anti-LHK mAb (1 μg/ml; *left*). Not observed after treatment for 12 h with control IgM (1 μg/ml; *right*). ×120. **B** DNA ladders produced in Colo205 cells after culture with anti-LHK mAb (1 μg/ml; *right*) for 12 h. They were not observed after culture control (*cont*) IgM (1 μg/ml; *middle*). *Left*, size markers. **C** Double-staining of Colo205 cells with Annexin V and propidium iodide (PI). Colo205 cells incubated for 12 h with control (*cont*) IgM (1 μg/ml; *upper*), anti-LHK mAb (1 μg/ml; *middle*), or cycloheximide (CHX; 100 ng/ml; *lower*) were stained with Annexin V and PI

sharp contrast, anti-Fas mAb and TNFα, but not anti-LHK mAb, showed cytotoxicity toward to Jurkat cells, a human T-cell leukemia cell line (Fig. 3A, right). In the analysis of proliferation using [$^3$H]-thymidine, anti-LHK mAb, but not anti-Fas and TNFα, blocked the proliferation of Colo205 cells in a dose-dependent manner (Fig. 3B, upper). With Jurkat cells, the result corresponded with that of the microscopic observation (Fig. 3B, lower). Because Fas expression is reported to be upregulated by IFN-γ treatment,[17] we further investigated the modification of LHK antigen and Fas by IFN-γ treatment. As shown in Fig. 4, Fas expression on Colo205 cells was significantly upregulated by exogenously added IFN-γ, whereas LHK antigen was not affected at all. To further compare LHK, Fas, and TNF-R, we examined the expression profiles of LHK antigen, Fas, and TNF-R on various human cancer cell lines by flow cytometry. Although LHK antigen was markedly expressed on Colo205 cells, it was also expressed on other colon cancer cell lines, such as WiDr, HT29, and Caco2, albeit to a lesser extent (Fig. 5). Furthermore, LHK was moderately to highly positive on the gastric cancer cell line Kato-III, marginal on Jurkat cells, and negative on hepatocellular carcinoma Alexander cells

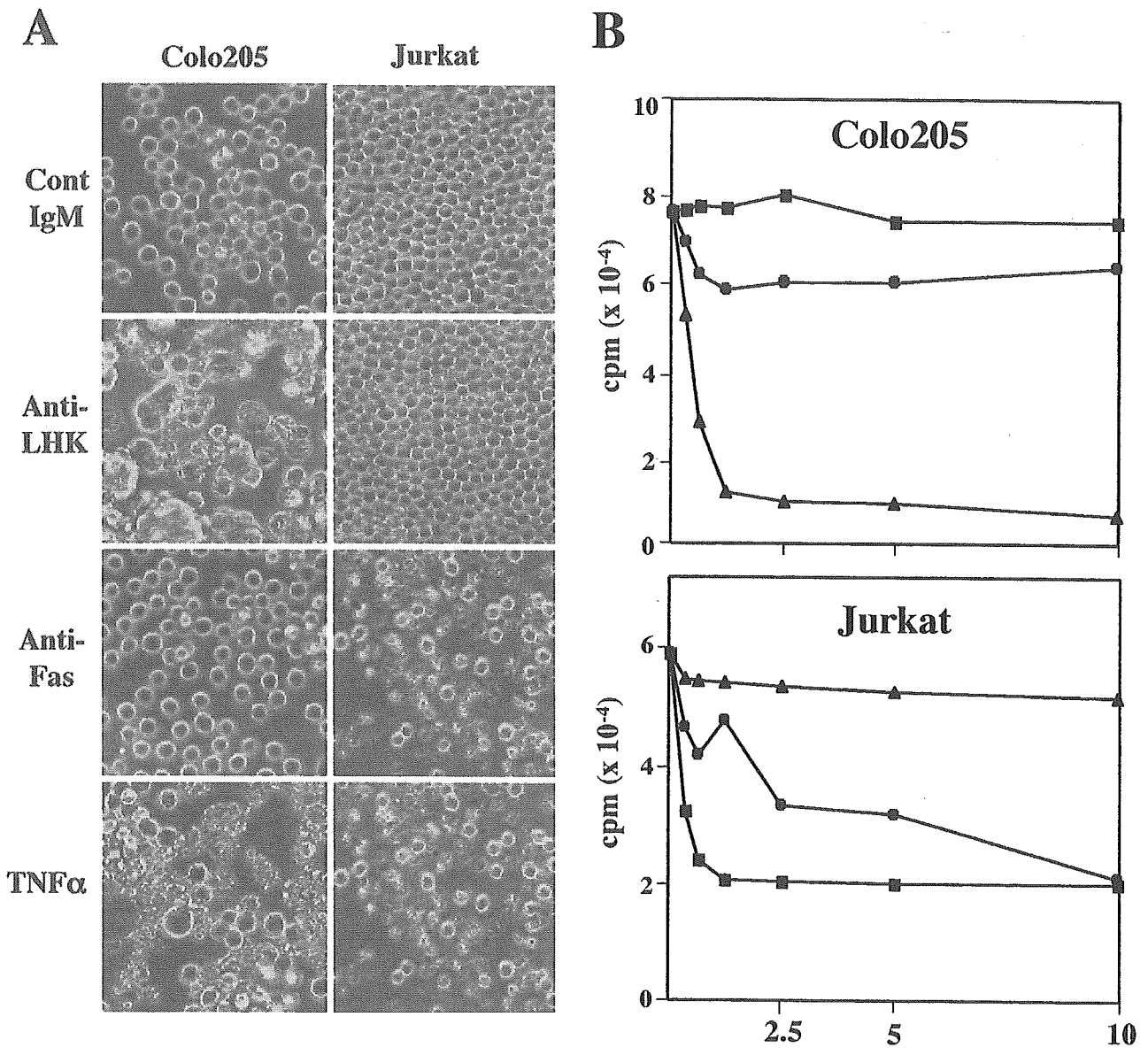


**Fig. 3A,B.** Distinct sensitivities of Colo205 and Jurkat cells to cell death induced by anti-LHK mAb, anti-Fas mAb, and tumor necrosis factor-α (*TNFα*). **A** Morphological changes of Colo205 cells and Jurkat cells induced by anti-LHK mAb (1 μg/ml), anti-Fas mAb (CH11; 1 μg/ml), and TNFα (50 ng/ml) after culture for 12 h. ×120 **B** Proliferative responses of Colo205 cells and Jurkat cells to control IgM, anti-LHK mAb (*triangles*; μg/ml), anti-Fas mAb (*large squares*; μg/ml), and TNFα (*small squares*; ng/ml). Cells were cultured in the presence of control IgM, anti-LHK mAb, anti-Fas mAb, or TNFα at various concentrations in triplicate for 48 h, and then labeled with [$^3$H]-thymidine for a further 4 h

and uterine cervical cancer HeLa cells. Based on these expression profiles, LHK was clearly distinguishable from Fas and TNF-R.

The molecular mass of LHK antigen was next assessed by immunoprecipitation assay. LHK antigen from Colo205 cells was specifically immunoprecipitated under reducing conditions and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using protein A-Sepharose beads (Fig. 6; lane 2, isomatched anti-mouse IgM; lane 3, anti-LHK mAb) and protein A-Agarose beads (lane 4, isomatched anti-mouse IgM; lane 5, anti-LHK mAb), where it appeared as a main band of 70 kDa (Fig. 6). Although several other bands were also observed, the band of 70 kDa was found only in lanes of anti-LHK, and not in lanes of control IgM by two different methods. Collectively, the results suggest that LHK antigen is a novel molecule that is distinct from Fas and TNF-R.

We next analyzed LHK antigen expression on human colon cancer cells and normal colonic epithelia by im-

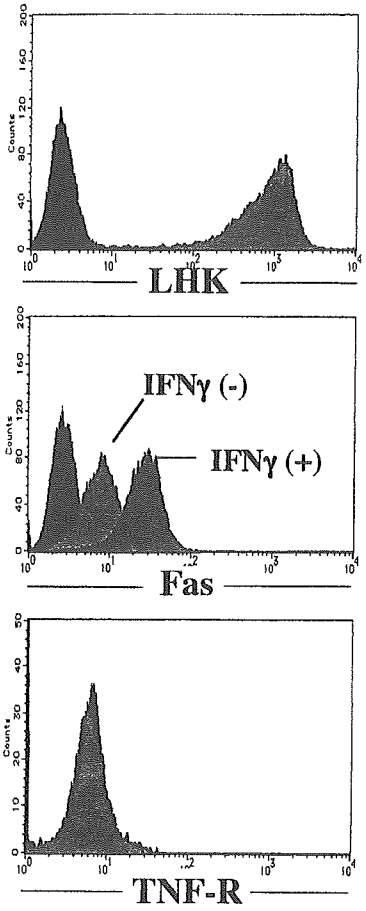


**Fig. 4.** Modulated expression of LHK, Fas, and TNF-receptor (*R*) by exogenously added interferon-γ (IFN-γ). The expression of LHK antigen (*upper*), Fas (*middle*), and TNF-R (*lower*) on Colo205 cells was determined after 48 h of culture with INF-γ (100 U/ml)

munohistochemical and flow cytometric analysis. Interestingly, immunohistochemistry revealed that LHK was expressed on epitheia in normal colonic epithelial cells (Fig. 7A, upper) and other epithelial cells such as tongue, esophagus, stomach, small intestine, pancreas, and skin, whereas it was never expressed in liver, spleen, tonsil, thymus, lung, heart, and kidney (data not shown). In assessing the expression of LHK antigen on human colon cancer tissues, we found that some colon cancer tissues expressed less or no LHK antigen (Fig. 7A, lower). This tendency was confirmed by flow cytometric analysis, using freshly isolated cancer and normal epithelial cells (Fig. 7B). To assess this in detail, we stained many colon cancer tissues and normal epithelial cells and found that the reactivity of anti-LHK mAb decreased significantly in parallel with the dedifferentiation of colon cancer (normal epithelial cells vs poorly differentiated adenocarcinoma; $P < 0.001$; Table 1), indicating a relationship between LHK antigen and the malignant potential of colon cancer.

**Table 1.** Immunohistochemical reactivity of anti-LHK mAb decreased in proportion with dedifferentiation of colon cancer

| Histology | % Positive LHK |
|---|---|
| Normal colonic epithelial cells | 100% (61/61[a]) |
| Tubular adenoma with severe dysplasia | 100% (10/10) |
| Well-differentiated adenocarcinoma | 74% (28/38) |
| Moderately differentiated adenocarcinoma | 49% (19/39) |
| Poorly differentiated adenocarcinoma | 40% (8/20) |

[a] Number of mice with positive staining/number of mice examined

### *Therapeutic effect of anti-LHK mAb in a xenograft model of human colon cancer*

Finally, we examined the therapeutic effect of anti-LHK mAb, using a xenograft model of Colo205 cells. BALB/c SCID mice that had received injection of $1 \times 10^6$ Colo205 cells intraperitoneally and had been treated with 1 mg of anti-LHK mAb weekly were kilted 8 weeks after the cell injection. We then examined the frequency of appearance of abdominal masses and tiny nodules in the mesentery (peritoneal dissemination) and compared the mean volumes of the tumors. An abdominal mass was observed in 7 of 17 (41%) mice treated with anti-LHK mAb, but in 16 of 18 (89%) mice treated with control IgM ($P < 0.01$). The risk ratio was 0.46 (95% confidence interval, 0.26–0.84; $P < 0.05$). Peritoneal dissemination (Fig. 8) was observed in 2 of 17 (12%) mice treated with anti-LHK mAb and in 8 of 18 (44%) mice treated with control IgM ($P < 0.01$). The risk ratio was 0.26 (95% confidence interval, 0.07–1.07; not significant). Mean tumor numbers were $1.0 \pm 1.41$ and $3.3 \pm 2.91$, respectively ($P < 0.01$). Mean total tumor volumes were $188.5 \pm 88.6$ and $514.8 \pm 152.5\,mm^2$, respectively ($P < 0.05$). These results indicate that anti-LHK mAb also exerts tumorcidal activity in vivo.

## Discussion

The goal of the present study was to establish a novel mAb capable of killing colon cancer cells that are resistant to Fas-induced apoptosis. We established an anti-LHK mAb produced by a hybridoma from mice immunized with Colo205 colon cancer cells. The mechanism of anti-LHK mAb-induced cell death was demonstrated to be apoptosis. The LHK antigen was a novel protein of 70 kDa that is distinct from Fas and TNF-R. Interestingly, the expression of LHK in colon cancer tissues decreased significantly in parallel with their grade of dedifferentiation. Furthermore, we demonstrated that the anti-LHK mAb suppressed tumor growth in a murine peritoneal dissemination model.

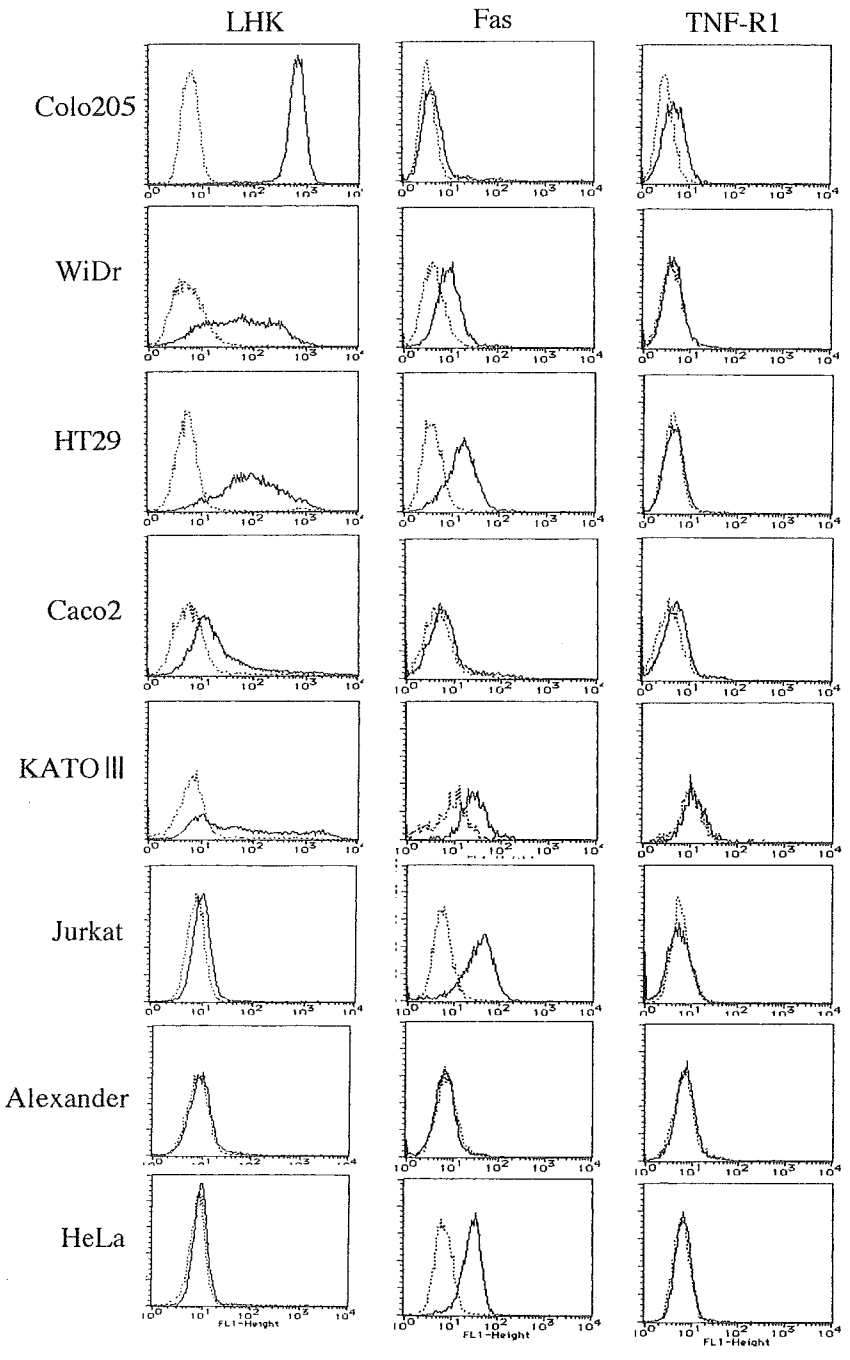


**Fig. 5.** Expression of LHK, Fas, and TNF-R on various human cancer cell lines. Aliquots of $1 \times 10^6$ cells were incubated on ice with control IgM and IgG, anti-LHK, anti-Fas, or anti-TNF-R mAb. Then the cells were washed, stained with fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgM or IgG, and analyzed by flow cytometry

Apoptotic cell death is morphologically characterized by membrane blebbing, shrinkage involving nuclear margination, and chromatin condensation. Moreover, the nuclear apoptotic events known as DNA ladder formation; that is, degeneration of chromatin induced by endogenous nuclease activation, have become a biochemical hallmark of apoptosis.[16] These characteristics were also observed in Colo205 cells treated with anti-LHK mAb, indicating that the mechanism of anti-LHK-induced death in the Colo205 cells was apoptosis. This was supported by two further assays for apoptosis; namely, Hoechst 33528 and Annexin V/PI staining.

Fas is functionally expressed on the basolateral surface of colonic epithelial cells regardless of their position along the crypt axis. In contrast to the normal epithelium, colon cancer cell lines are mostly resistant to Fas-induced apoptosis. In the normal situation, senescent epithelial cells in the gut mucosa are eliminated by apoptosis, but it is not yet clear how apoptosis is induced in these cells. Thus, death receptors including

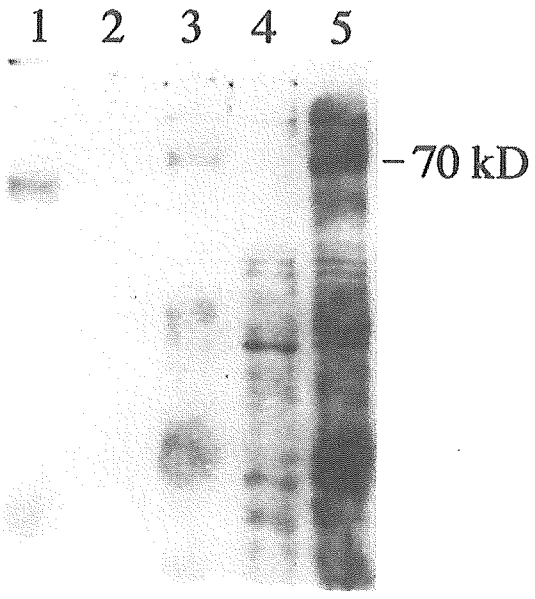


**Fig. 6.** Immunoprecipitation of LHK antigen on Colo205 cells. *Lane 1*, marker; *lane 2*, isotype-matched control IgM-conjugated protein A-Sepharose beads; *lane 3*, anti-LHK IgM-conjugated protein A-Sepharose beads; *lane 4*, isotype-matched control IgM-conjugated protein A-agarose beads; *lane 5*, anti-LHK mAb-conjugated protein A-Agarose beads

Fas may be involved in this elimination system. This may also be the case with the LHK antigen, because the expression of LHK in colon cancer and normal tissues decreased significantly in parallel with their grade of dedifferentiation. Indeed, these observations indicate that this mAb may not be ideal for cancer therapy, but, rather, that LHK antigen may be useful as a marker of malignancy for patients with colon cancer, because its down-regulation may enable colon cancer cells to escape from apoptosis. Of note, the expression of LHK antigen on colon cancer cells in patients with liver metastasis was marked, suggesting a useful predictor for their prognosis.

In the analysis of the LHK antigen, we first aimed to clarify whether LHK antigen was distinguishable from Fas and TNF-R. We concluded that LHK antigen is a novel death receptor, for the following reasons. First, the molecular mass of LHK is 70 kDa, whereas those of Fas, TNF-R1, and TNF-R2 are 48, 55, and 75 kDa, respectively. Second, the expression profiles from immunohistochemistry and flow cytometry revealed that Fas and TNF-R were broadly expressed on various cells, including hematopoietic cells and fibroblasts, whereas LHK was restricted to epithelial cells in the gastrointestinal tract and skin. Regarding the difference between LHK and Fas, it is noteworthy that Fas, but not LHK, was significantly upregulated on Colo205 cells by IFN-γ, and that Colo205 and Jurkat cells differed in their sensitivity to cell death induced by anti-LHK and anti-Fas (CH11) mAbs. Furthermore, because the TNF-R family is a still-growing group of homologous transmembrane proteins, it was important to assess whether or not LHK is identical to other recently-described molecules, such as TNF-related apoptosis-inducing ligand (TRAIL) receptors, DR3, DR4, and DR5. Like LHK, but unlike Fas ligand, TRAIL appears to preferentially induce apoptosis in tumor cells over normal cells.[18] Five distinct receptors for TRAIL have been identified (TRAIL-R1, R2, R3, R4, and osteoprotegrin [OPG]). Only TRAIL-R1 and R2, which bear a cytoplasmic death domain, can induce apoptosis. The latter three are thought to be nonsignaling decoy receptors and to be related to the regulation of apoptotic cell death via TRAIL systems.[19-22] Unlike LHK, TRAIL-R1 and R2 are expressed in most tissues, such as spleen, thymus, peripheral blood lymphocytes, activated T cells, and small intestine. The molecular masses of TRAIL-R1 and R2 were 46 and 52 kDa, respectively, indicating that LHK is also distinguishable from TRAIL receptors. Further study involving the direct cloning of the LHK molecule will be needed to resolve this issue.

Finally, we assessed the therapeutic effect of anti-LHK mAb in a murine peritoneal dissemination model. Although targeting specific death receptors in cancer therapy may have therapeutic effects on tumors, the clinical use of both TNF and FasL (or anti-FAS mAbs) has been prevented by their toxic side effects. The systemic administration of certain doses of TNF causes a severe inflammatory response syndrome that resembles septic shock, and injection of agonistic antibody to Fas in tumor-bearing mice can be lethal, apparently because of the induction of apoptosis in hepatocytes. Although TRAIL showed promising efficacy in a preclinical study and was suggested to be a safer agent than TNF and FasL,[23,24] a report indicated that human primary hepatocytes were also efficiently killed by TRAIL signaling.[25] In our murine model, anti-LHK mAb treatment had a potent tumoricidal effect. Importantly, no mice died or showed apparent adverse effects, such as severe inflammatory response syndrome, fulminant hepatic failure, or damage of normal colonic epithelial cells (data not shown). Of course, we cannot conclude that anti-LHK mAb is an effective and safe agent for the treatment of patients with colon cancer. Further investigation will be needed to clarify the mechanism of action of anti-LHK mAb before its clinical trials.

In conclusion, LHK antigen, which is uniquely restricted to epithelial cells in the gastrointestinal tract and skin, may be a novel death receptor that is critically involved in controlling the growth, invasion, and metastasis of human colon cancer cells. Therefore, this study might provide a basis for the practical application

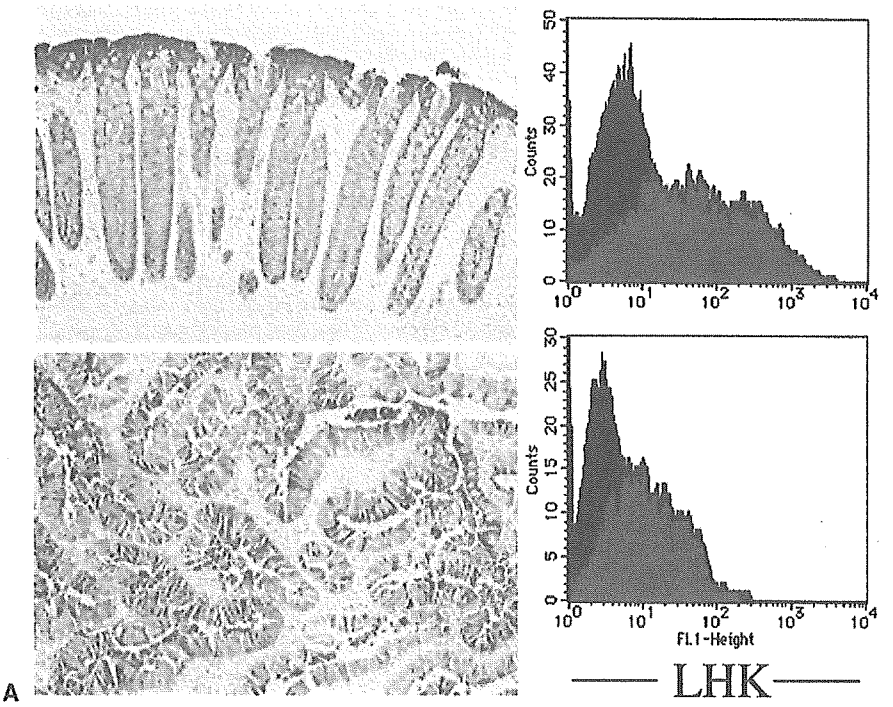


**Fig. 7A,B.** LHK expression in normal human colonic epithelial cells and colon cancer cells. **A** LHK antigen in normal human colonic epithelial cells (*upper*) and colon cancer cells (*lower*) was visualized by immunohistochemistry. ×100. **B** Flow cytometric analysis of freshly isolated human normal colonic epithelial cells (*upper*) and colon cancer cells (*lower*) is demonstrated

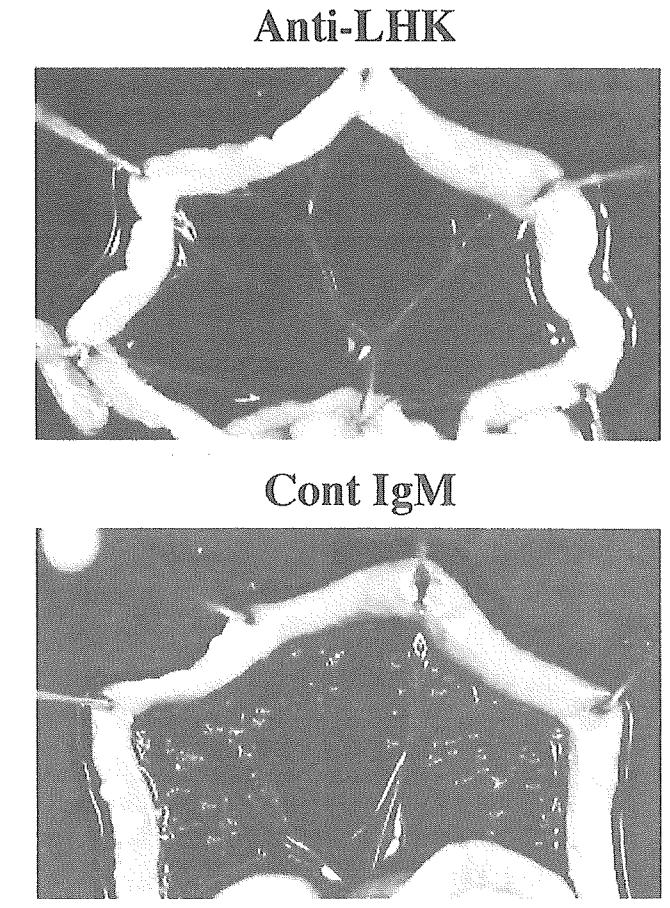


**Fig. 8.** Reduction of peritoneal dissemination in a murine xenograft model by anti-LHK mAb treatment. Colo205 cells ($5 \times 10^6$) were injected into the peritoneal cavities of 8-week-old BALB/c severe combined immunodeficiency (SCID)mice. Twenty-four hours after the injection, 500 μg of anti-LHK IgM or control (*cont*) mouse IgM mAb was injected intraperitoneally into Colo205-transplanted mice. The injection was repeated weekly for 8 weeks then mice were killed by cervical dislocation. Tiny nodules in the mesentery were observed in mice treated with control IgM, but not in mice treated with anti-LHK mAb

of therapy using anti-LHK mAb for the treatment of patients with colon cancer.

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## References

1. Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 1989;169:1747–56.
2. Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, et al. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 1989;245:301–5.
3. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, et al. The polypeptide encoded by the cDNA for human cell surface Fas can mediate apoptosis. Cell 1991;66:233–43.
4. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, et al. Purification and molecular cloning of the Apo-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J Biol Chem 1992;267:10709–15.
5. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland, NG, Jenkins NA, et al. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 1992;148:1274–9.
6. Nagata S, Goldstein P. The Fas death factor. Science 1995;267;1449–56.
7. Strater J, Moller P. Expression and function of death receptors and their natural ligands in the intestine. Ann NY Acad Sci 2000;915:162–70.
8. Moller P, Koretz K, Leithauser F, Bruderlein, S, Henne C, Quentmeier A, et al. Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer 1994;57:371–7.
9. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996;184:1072–82.
10. Okada N, Mimori T, Mukai R, Kashiwagi H, Hardin JA. Characterization of human autoantibodies that selectively precipitate the 7SL RNA component of the signal recognition particle. J Immunol 1987;138:3219–23.
11. Kanai T, Watanabe M, Okazawa A, Nakamaru K, Okamoto M, Naganuma M, et al. IL-18 is a potent proliferative factor for intestinal mucosal lymphocytes in Crohn's disease. Gastroenterology 2000;119:1514–23.
12. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 1995;184:39–51.
13. Sellins, KS, Cohen JJ. Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 1987;139:3199–206.
14. Okazawa A, Kanai T, Nakamaru T, Sato T, Inoue N, Ogata H, et al. Human intestinal epithelia-derived IL-18 is a potent proliferative factor for intraepithelial lymphocytes synergistically with IL-2, IL-7 and IL-15. Clin Exp Immunol 2004;136:269–76.
15. Okamoto K, Yamaguchi T, Otsuji E, Yamaoka N, Yata Y, Tsuruta H, et al. Targeted chemotherapy in mice with peritoneally disseminated gastric cancer using monoclonal antibody-drug conjugate. Cancer Lett 1998;122:231–6.
16. Ferguson DJ, Anderson TJ. Ultrastructural observations on cell death by apoptosis in the "resting" human breast. Virchows Arch A Pathol Anat Histopathol 1981;393:193–203.
17. Weller M, Frei K, Groscurth P, Krammer PH, Yonehara Y, Fontana A. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. Induction and modulation of sensitivity by cytokines. J Clin Invest 1994;94:954–64.
18. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996;271:12687–90.
19. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, et al. The receptor for cytotoxic ligand TRAIL. Science 1997;276:111–3.
20. Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J 1997;16:5386–97.
21. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, et al. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J Exp Med 1997;186:1165–70.
22. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG, et al. The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL mediated apoptosis, yet retains an incomplete death domain. Immunity 1997;7:813–20.
23. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–62.
24. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. Tumorcidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 1999;5:157–63.
25. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, et al. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med 2000;6:564–7.

# Clinical characteristics of Japanese patients with anti-PL-7 (anti-threonyl-tRNA synthetase) autoantibodies

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## Abstract

### Objective

*The clinical and laboratory features of seven Japanese patients with anti-aminoacyl-tRNA synthetase (ARS) autoantibodies against PL-7 (anti-threonyl-tRNA synthetase) were analyzed and compared with previously published findings.*

### Methods

*Serum samples from 1,135 Japanese patients with various autoimmune diseases were screened for anti-PL-7 antibodies using RNA and protein immunoprecipitation assays. The patients whose sera contained anti-PL-7 antibodies were assessed regarding clinical symptoms and clinical course.*

### Results

*Sera from seven patients were found to have anti-PL-7 antibodies. These autoantibodies were associated with polymyositis/dermatomyositis (PM/DM) and/or interstitial lung disease (ILD). The clinical diagnoses of these seven patients were PM - systemic sclerosis (SSc) overlap (5 patients), DM (1 patient) and idiopathic pulmonary fibrosis (IPF) (1 patient). All patients had ILD with a chronic course and six also had arthritis (85%) and five sclerodactyly (71%).*

### Conclusions

*These results indicate that anti-PL-7 autoantibodies are closely associated with PM-SSc overlap as well as ILD, arthritis and sclerodactyly in our series of Japanese patients.*

**Key words**

Polymyositis/dermatomyositis (PM/DM), interstitial lung disease (ILD), anti-aminoacyl-tRNA synthetases (ARS) antibodies.

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## Introduction

The aminoacyl-tRNA synthetases are a set of cellular enzymes, each of which catalyzes the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA. Autoantibodies to six anti-aminoacyl-tRNA synthetases (anti-ARS) have been identified in patients with inflammatory myopathies, as follows: anti-histidyl (anti-Jo-1), anti-threonyl (anti-PL-7), anti-alanyl (anti-PL-12), anti-glycyl (anti-EJ), anti-isoleucyl (anti-OJ), and anti-asparaginyl (anti-KS) tRNA synthetases (1-10). Among these anti-ARS antibodies, the most common, anti-Jo-1, are found in approximately 20-30% of polymyositis/dermatomyositis (PM/ DM) patients (8, 10-11).

Each of these anti-ARS antibodies has been reported to be associated with a similar syndrome. This syndrome is characterized by myositis with a high frequency of interstitial lung disease (ILD) (50-80%) and arthritis (50-90%), as well as an increase (compared with the overall myositis population) of Raynaud's phenomenon (60%), fever with exacerbations (80%), and the skin lesions of the fingers referred to as "mechanic's hands" (70%) (1, 7). Although the similarity of clinical features in patients with different anti-ARS antibodies is striking, further observation and analysis has shown that there are certain differences in clinical symptoms associated with each of the anti-ARS antibodies.

Hirakata *et al.* examined clinical features of anti-synthetase syndromes in detail and reported that anti-Jo-1 antibodies are common in patients with myositis, but anti-PL-12 and anti-KS antibodies are found in patients with ILD without any signs of myositis (10). The latter are more likely to have ILD and/or arthritis without clinical evidence of myositis (10,12-13).

Anti-PL-7 antibodies are the first non-Jo-1 anti-ARS, found in patients with PM/DM accompanied by ILD, the frequency of which is low (2). In previous studies, this antibody was found in only 3-4% of all patients with PM/DM (2,6, 8,14). Targoff *et al.* reported that patients with anti-PL-7 antibodies had a high incidence of arthritis and ILD (15). However, the presence of anti-PL-7 antibodies and their clinical significance has not been reported in Japanese patients so far.

In the present study, we describe the clinical and laboratory features of Japanese patients with antibodies against anti-PL-7 and review previously published reports from elsewhere.

## Patients, materials and methods

### *Patients and sera*

Serum samples were obtained from 1,135 Japanese patients suspected of having connective tissue diseases seen at the current or previous collaborating centers of the authors between 1990 and 2000. These included 120 with PM/DM, 400 with systemic lupus erythematosus (SLE), 192 with systemic sclerosis (SSc), 58 with rheumatoid arthritis (RA), 101 with mixed connective tissue disease (MCTD)/overlap syndrome, 114 with idiopathic pulmonary fibrosis (IPF), and finally, 150 patients who had arthritis or erythema but did not meet the criteria for other connective tissue diseases.

PM/DM was diagnosed based on the criteria of Bohan and Peter (16). The assessment of muscle weakness was performed using a manual muscle test (17). The diagnosis of SSc was based on the criteria for the classification of SSc defined by the American College of Rheumatology in 1980 (18). ILD was considered to be present if an interstitial change was observed on both chest radiography and computed tomography (CT) or a restrictive pattern found on pulmonary function testing in patients with IPF or PM/DM.

### *Detection of anti-PL-7 antibodies*

The immunoprecipitation (IPP) from HeLa cell extracts was performed as previously described (1, 6). For analysis of RNAs, 10 μl of patient sera was mixed with 2 mg of protein A-Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) in 500 μl of IPP buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 h at 4°C. The IgG-coated Sepharose was washed 4 times

in 500 μl of IPP buffer using 10-second spins in a microfuge and was resuspended in 400 μl of NET-2 buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.05% Nonidet P-40). This suspension was incubated with 100 μl of extracts, derived from $6x10^6$ cells, on the rotator for 2 h at 4°C. The antigen-bound Sepharose beads were then collected by centrifugation for 10 s in the microfuge, washed 4 times with NET-2 buffer, and resuspended in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0 M sodium acetate, 30 μl of 10% SDS, 2 μl of carrier yeast tRNA (10 mg/ml; Sigma, St. Louis, MO) and 300 μl of phenol/chloroform/isoamyl alcohol (50: 50: 1, containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and spinning for 1 min, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 μl of electrophoresis sample buffer composed of 10 M urea, 0.025% bromophenol blue, and 0.025% xylene cyanol-FF in TBE buffer (90 mM Tris-HCl, pH 8.6, 90 mM boric acid, and 1 mM EDTA). The RNA samples were denatured at 65°C for 5 min and then resolved in 7 M urea-10% polyacrylamide gels, which were then silver-stained (Bio-Rad Laboratories, Hercules, CA).

For the protein studies, antibody-coated Sepharose beads were mixed with 400 μl of [$^{35}$S] methionine-labeled HeLa extracts derived from $2x10^5$ cells, and rotated at 4°C for 2 h. After four washes with IPP buffer, the Sepharose beads were resuspended in SDS - sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.005% bromophenol blue). After heating (90° C for 5 min), the proteins were fractionated by 10% SDS-PAGE, enhanced with 0.5 M sodium salicylate, and dried. Radiolabeled protein components were analyzed by autoradiography.

With these assays, anti-ARS, anti-signal recognition particle, anti-Mi-2, anti-SSA, anti-SSB, anti-U1-RNP, anti-Scl-70, anti-PM-Scl and anti-Ku autoantibodies are detectable in comparison with corresponding standard sera (1). We also examined anticentromere antibody by ELISA (Medical & Biological Laboratories Co., Ltd. Nagoya, Japan).

*Clinical features*

Clinical information was retrospectively assessed in all PM/DM patients as well as non-PM/DM patients positive for anti-PL-7 antibodies. Clinical findings included clinical symptoms, serum creatine kinase (CK) level, electromyogram (EMG), muscle biopsy, chest radiograph and chest CT. The resolution of the myositis symptoms was defined as having both improvement of muscle weakness on a manual muscle test and the normalization of serum CK level. The two groups of PM/DM patients with or without anti-PL-7 antibodies were compared. Moreover, our cases were compared with those previously reported in the literature.

*Statistical analysis*

All comparisons between the two patient groups were performed using the $\chi^2$ test. Significance level was set at 5%.

## Results

*Identification of anti-PL-7 antibodies*

Of the 1,135 sera tested, samples from seven patients were found to immunoprecipitate a characteristic identical pattern of tRNAs. Representative examples are shown in Figure 1. This pattern of tRNAs was clearly distinguishable from those precipitated by the five other described anti-synthetases and identical in mobility and appearance to anti-PL-7 standard serum (Fig. 1a). The same sera also immunoprecipitated a protein band from [$^{35}$S] methionine-labeled HeLa cell extracts migrating at 80 kDa. This was clearly different from those immunoprecipitated by sera reactive with the other described anti-synthetases (Fig. 1b). Thus, it is concluded that they contained anti-PL-7 antibodies.

*Clinical features of patients with anti-PL-7 antibodies*

Clinical features of the 7 patients with anti-PL-7 antibodies are summarized in Table I. Five patients were clinically diagnosed as having PM-SSc overlap syndrome and the other two as DM and IPF. Six (86%) had muscle weakness and arthritis. Four (57%) had Raynaud's phenomenon. It was of note that 5 patients had scleroderma: the extent of skin thickness was diffuse scleroderma in 2 (29%), proximal scleroderma in one (14%) and sclerodactyly alone in 2 (29%). Although two (29%) had mechanic's hands, sclerodactyly of these patients was clearly distinguished from mechanic's hands. All 7 patients were diagnosed as having ILD from the results of chest radiography and chest CT or pulmonary function testing. One patient had anti-SS-A antibodies and another had anticentromere antibodies.

*Characteristics of myositis in patients with anti PL-7 antibodies*

The characteristics of 6 patients suffering from myositis are summarized in Table II. Only one patient manifested a DM rash and was accordingly diagnosed as having DM. The maximum level of CK (IU/l) was relatively low throughout the clinical course (maximum CK was 2,830 IU/l, seen in patient #1). EMG was performed in all 6 myositis patients and all showed a myogenic pattern: low-amplitude polyphasic units of short duration and resting fibrillation, complex repetitive discharges and positive sharp waves in needle EMG. The muscle biopsy revealed atrophic fibers, active necrosis with regeneration and infiltration of lymphocytes in all 3 patients tested. The administration of prednisolone (PSL) alone without other immunosuppressant in 5 resulted in an improvement of both muscle strength and serum CK value in all. One patient had no PSL medication due to concomitant tuberculosis infection. PSL was tapered gradually and 3 patients maintained inactive myositis by continuing on a low dose of PSL. Two patients (#1 and #3) died of cardiac failure and respiratory failure due to bacterial infection. The duration of the disease was 159 months and 44 months in these latter patients. All 7 PM/DM patients had ILD, classified as chronic course. The symptoms of ILD preceded muscle involvement in 5 patients.

Frequencies of several clinical mani-