YKL-39, a human cartilage-related protein, induces arthritis in mice

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Abstract

Objective

To determine whether YKL-39, a recently cloned secretory protein of articular chondrocytes, is arthritogenic in mice.

Methods

Recombinant YKL-39 (rYKL-39) was expressed and purified from E. coli. *To induce arthritis in mice, rYKL-39 (1, 10 or 50 g in Freund's incomplete adjuvant) was injected into the right footpad of mice from four different strains (BALB/c, DBA/1J, C57BL/6 and ICR). The mice received a second immunization with rYKL-39 by intradermal injection into the root of the tail 10 days after the first immunization. Severity of arthritis was assessed by scoring each paw on a scale from 0 to 4. Sixty days after the first immunization, the mice were sacrificed and the joints were examined by immunohistochemistry and radiography. The anti-YKL-39 and anti type II-collagen (CII) antibody titres were also assayed using ELISA.*

Results

Immunization with YKL-39 induced arthritis in all strains of mice tested, among which BALB/c was most susceptible. Histological examination showed synovial proliferation and irregularity of the cartilage surface in YKL-39-injected BALB/c mice. Moreover, radiographic analysis revealed pathological changes in these mice. The YKL-39-immunised mice produced not only anti-YKL-39 antibody but also antibody against type II collagen, suggesting a spreading of autoimmunity after YKL-39.

Conclusions

YKL-39, a cartilage-related protein, is found to induce arthritis accompanied by pathologic changes in bone and cartilage. A better understanding of the immune response against cartilage-related components including YKL-39 may help to elucidate the pathological processes of arthritic disorders.

Key words

YKL-39, arthritis, animal model, autoimmunity.

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Introduction

Recent reports have increasingly suggested that immune responses to major or minor articular joint-derived components might lead to arthropathy in animals and human beings. Type II collagen (CII), the major component of articular cartilage, is widely recognised to cause inflammatory arthritis when injected into susceptible animals (1). Collagen-induced arthritis (CIA) has clinical and pathological features similar to human rheumatoid arthritis (RA) and is therefore commonly considered to be an animal model of RA. In addition, other cartilage-derived proteins including aggrecan, link protein, human chondrocyte glycoprotein-39 (HC gp-39) (YKL-40 or chondrex), cartilage oligometric matrix protein (COMP), and cartilage intermediate layer protein (CILP) were reported to induce arthritis in animals (2-5). These observations support the notion that specific immune responses directed against articular component can arise, and that such responses might play a role in the pathogenesis of arthritis.

YKL-39, a protein analogous to HC gp-39, has been identified as a secretory protein in conditioned medium of human chondrocytes (6). YKL-39 contains the N-terminal sequence YKL, and has 52% amino acid and 56% nucleotide sequence homology to HC gp-39, which is a candidate autoantigen in RA. HC gp-39, which is synonymous with YKL-40 or Chondrex, belongs to the chitinase family and is a candidate autoantigen in RA (3), whereas YKL-39 lacks glutamate in its active site and in it no chitinase activity has been detected. YKL-39 mRNA is found mainly in chondrocytes and, to lesser extent, in synoviocytes, the lung, and the heart (6).

Recently, we have reported that a subset of RA patients had antibody against YKL-39 using a recombinant YKL-39 protein (rYKL-39) (7). We showed that the production of anti-YKL-39 antibody was independent of an anti-HC gp-39 response, suggesting that these two closely-related proteins are recognized differently in RA patients (7). This finding implies that autoimmunity to YKL-39 might be involved in the pathogenesis of arthropathies as well as HC gp-39. However, to date, the function of YKL-39 has not been clarified. Because HC gp-39 is arthritogenic in BALB/c mice (3), it seems likely that YKL-39 is also a potent inducer of arthritis in susceptible animals. This hypothesis was tested in the present study.

Materials and methods

Mice

Female BALB/c, DBA/1J, ICR, C57 BL/6 and ICR mice were purchased from Nippon SLC (Tokyo, Japan). They were maintained under routine laboratory conditions (23-25°C, relative humidity 60% with a 12-hour light-dark cycle) with standard rodent food and water *ad libitum*, and were acclimatised for at least 1 week in standard housing before studies were initiated. All mice were 9 weeks old at the time of initial immunization.

Preparation and isolation of recombinant YKL-39

The entire coding cDNA of the YKL-39 gene was obtained from cultured HCS-2/8 cells (a human chondrosarcoma cell line) (8) by a reverse transcriptase polymerase chain reaction (RT-PCR) technique as described previously (7). The amplified DNA was then subcloned into the EcoRI and SalI sites of the plasmid pMAL-His in order to generate YKL-39-MBP protein (7). For use in the ELISA, YKL-39 was also fused to glutathione S-transferase (GST) using plasmids of pMal-eHis and pGEX-4T-1 (9). These plasmids were expressed in the *Escherichia coli (E. coli)* strain pop-J 2104 (TOYOBO CO., LTD.). The recombinant proteins were purified from bacterial cell lysates following published procedures (7), and checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining. The concentration of the fusion protein was determined from its absorbance at 280 and 260 nm, which was corrected for background activity at 320 nm using appropriately diluted samples.

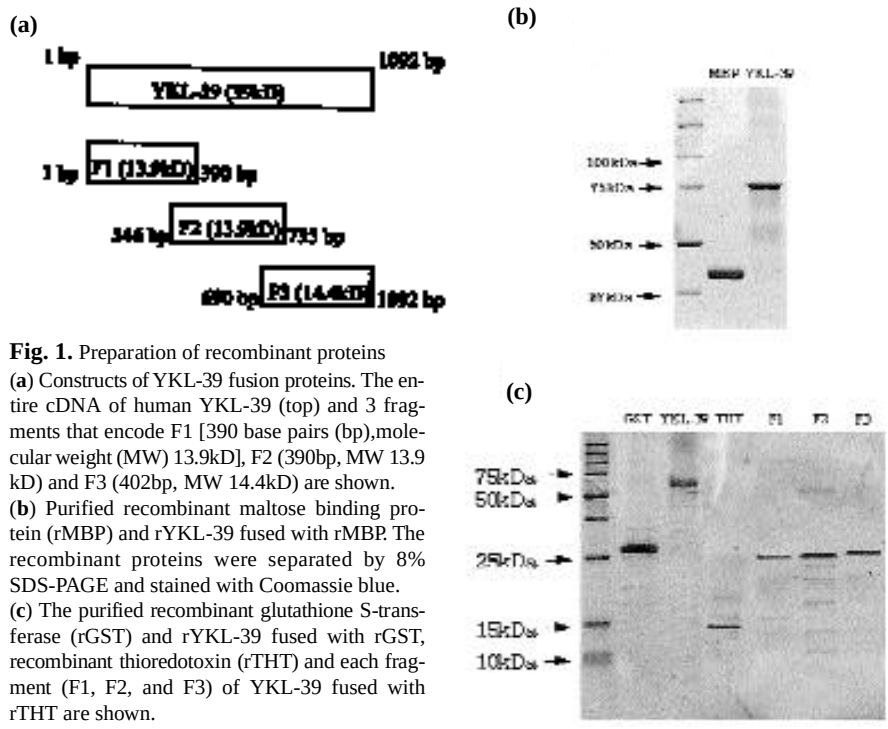


Fig. 1. Preparation of recombinant proteins
(**a**) Constructs of YKL-39 fusion proteins. The entire cDNA of human YKL-39 (top) and 3 fragments that encode F1 [390 base pairs (bp),molecular weight (MW) 13.9kD], F2 (390bp, MW 13.9 kD) and F3 (402bp, MW 14.4kD) are shown.
(**b**) Purified recombinant maltose binding protein (rMBP) and rYKL-39 fused with rMBP. The recombinant proteins were separated by 8% SDS-PAGE and stained with Coomassie blue.
(**c**) The purified recombinant glutathione S-transferase (rGST) and rYKL-39 fused with rGST, recombinant thioredotoxin (rTHT) and each fragment (F1, F2, and F3) of YKL-39 fused with rTHT are shown.

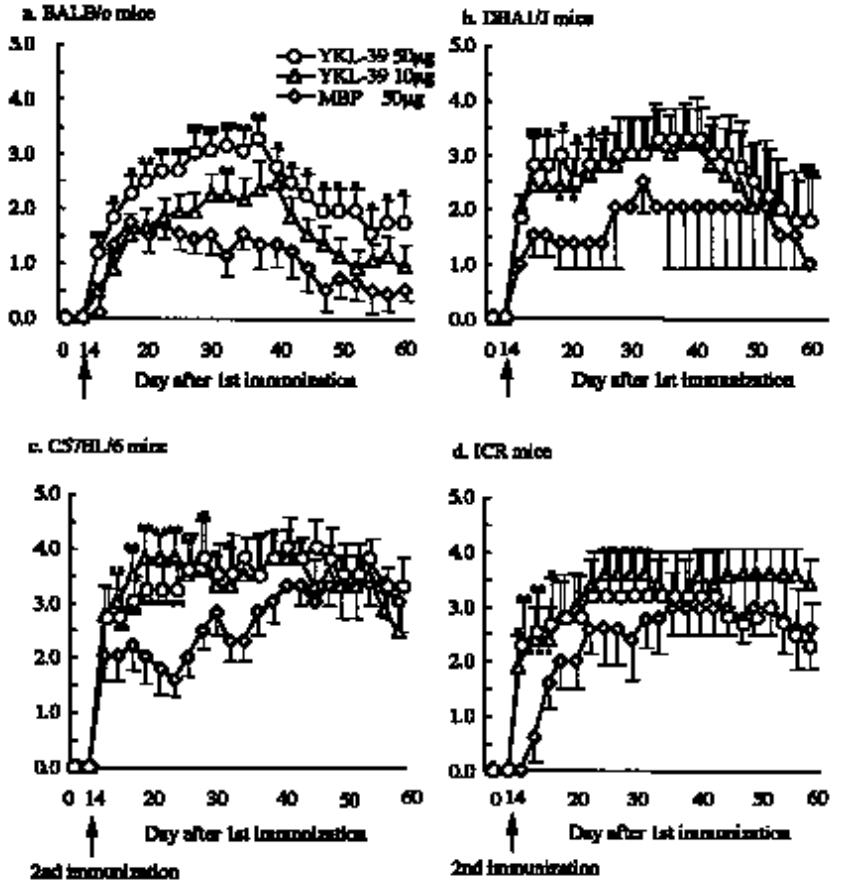
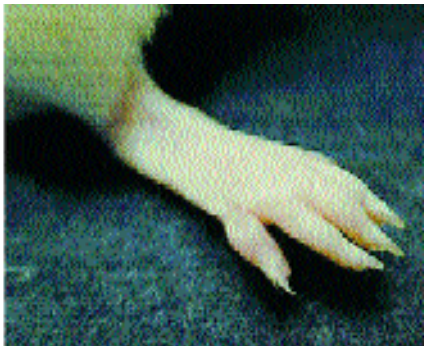


Fig. 2. Severity of YKL-39-induced arthritis. Arthritis was clinically evaluated using arthritic scores as described in Materials and Methods: (**a**) BALB/c, (**b**) DBA/1J, (**c**) C57BL/6, and (**d**) ICR. Ten mice were immunized with YKL-39 and 10 with MBP in BALB/c. Ten mice were immunized with YKL-39 and 5 were immunized with MBP in the other strains. Differences in the arthritis scores between YKL-39 immunized mice and controls (MBP) were analyzed by Student's t-test. Values are shown as the average ± S.E. *P < 0.05, **P < 0.01.

In addition to the entire protein, YKL-39 was further divided into three fragments designated F1 (390bp), F2 (390 bp) and F3 (402bp), respectively (as shown in Fig. 1a). The nucleotide sequences of the primers for PCR were as follows:
F1 sense: 5'-TTTGGATCCTACAAA-CTGGTTTGCTACTTTACC-3',
antisense: 5'-AAAGTCGACAGTGA-AATGAGTGTTTTCTTTCTG-3',
F2 sense: 5'-TTTGGATCCGTAA-GCTGGATCTACCCAGATCAG-3',
antisense: 5'-AAAGTCGACGTGCC-CATATGTGGGGATGCCCAT-3', and
F3 sense: 5'-TTTGGATCCATGCCA-TCAGAGAAGGTGGTCATG-3',
antisense: 5'-AAAGTCGACCAAGG-AGCCAAGGCTTCTCTTGAC-3'.
Nucleotide sequences of the PCR products were confirmed by sequencing. Each cDNA fragment was then subcloned into the expression vector pTHT-eHis, which carries six straight histidines $(His)_6$ at the C-terminus of multiple cloning sites for affinity purification (10). From these constructs, recombinant proteins containing an N-terminal fusion to a thioredotoxin (THT) and a C-terminal histidine tag were produced in *E. coli*.

Induction of arthritis

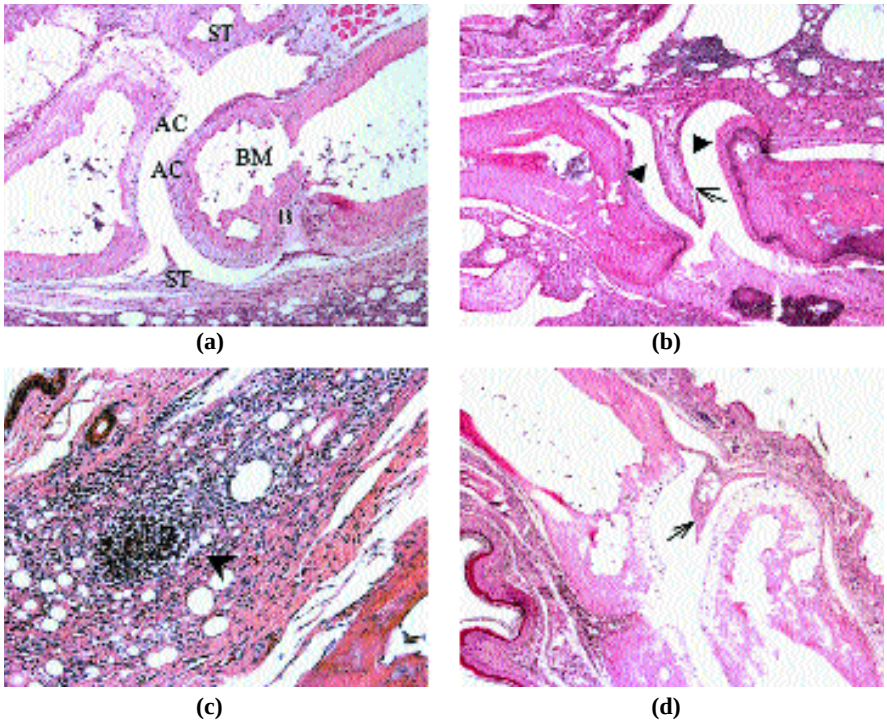
One, 10, or 50 g of rYKL-39 was mixed with Freund's incomplete adjuvant (FIA) at 1:1 and injected intracutaneously into the right footpad. Control mice of each strain received the same amount of the fusion partner, MBP, with FIA. Ten mice were immunized with rYKL-39 and MBP in BALB/c, and 10 mice were immunized with rYKL-39 and 5 were immunized with MBP in the rest of the strains. After 14 days, the mice were injected with the same amounts of the protein intracutaneously into the root of the tail. Then, the mice were observed daily for the incidence of arthritis by trained research assistants who were blinded to the treatments, and the severity of arthritis was assessed according to a published scoring method (3, 11) as follows: 0 = no change, 1 = mild but definite redness and swelling of the



(a) MBP 50 g

(b) YKL-39 50 g

Fig. 3. YKL-39-induced arthritis in BALB/c mice. (**a**) The right foot pad of a control mouse immunized with 50 g of rMBP and (**b**) the right foot pad of a YKL-39 (50 g)-immunized BALB/c mouse 30 days after the first immunization.



(a) **(b)**

(c) **(d)**

Fig. 4. Histopathologic findings in the paws of BALB/c mice.
Histological findings in the interphalangeal joint of a control mouse immunized with 50 g of MBP.
(**a**) The interphalangeal joint of a mouse immunized with 50 g of YKL-39 showed synovial proliferation (**arrow**) and an irregular articular surface (**arrowhead**)
(**b**) Accumulation of mononuclear cells was found in the synovial tissue of a YKIL-39 immunized mouse (**large arrowhead**)
(**c**) Findings in the contralateral (left) paw showed mild proliferation of synovial tissue (arrow)
(**d**) AC: Articular cartilage, B: Bone. BM; Bone marrow, ST: Synovial tissue. The magnification of panel c was 100 and of the other panel was 40.

ankle, 2 = moderate to severe redness and swelling of the ankle, 3 = redness and swelling of the entire foot including the digits, 4 = maximal inflammation of the entire foot including the digits. All the mice were observed for 60 days after the first immunization. Animal care was in accordance with institutional guidelines.

Enzyme linked immunosorbent assay

Microwell plates (Cook, Dynatech, Alexandria, VA) were coated overnight at 4°C with 2.0 g per well of rYKL-39 or rGST in 100 mM carbonate buffer (pH 9.4). Thereafter, the plates were washed 8 times with phosphate-buffered saline (PBS, at pH 7.4) containing 0.05% Tween-20 (PBST), and blocked with PBST containing 3% BSA for 1 hr at room temperature, and then washed again. To absorb the non-specific activity against bacterial protein and rGST, the murine serum samples were pre-incubated with bacterial lysate containing non-recombinant pGEX-His products, and then incubated in the coated wells in triplicate for 16 hr at 4°C. After washing, the plates were incubated for 1 hr with HRP-conjugated anti-mouse-IgG (Zymed Laboratories, Inc.), washed again and reacted with *o*-phenylenediamine as a substrate. After the reaction was terminated by the addition of 9N H_2SO_4, absorbance at 492 nm was measured using a Titertek Multiscan (Biorad). For each sample, the OD value for rGST was subtracted from the OD value for the fusion protein to obtain the corrected OD values (OD sample*). The reactivity of each serum to the fusion protein was expressed in units according to the formula: sample (binding units) = [OD sample */ (mean OD sample* + 3 SD of normal sera)] x 100. According to this formula, 100 binding units is the cut-off point.

Antibody to type II collagen (CII) was also titred. For this purpose, purified bovine CII (Biological Chemical Laboratory, Osaka, Japan) was used to precoat microwell plates, and ELISA was performed as described above.

Cellular proliferation to fragments of YKL-39

Five BALB/c mice from each immunization group were randomly selected for the cellular proliferation assay. Mice were sacrificed at the end of the observation period by cardiac evacuation, and peripheral blood mononuclear cells (PBMC) were separated from the collected blood samples using a standard gradient centrifugation method with Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). The mononuclear cells were maintained in RPMI 1640 medium supplemented with heat-inactivated 10% fetal calf serum.

The proliferative response of PBMC against fragments of YKL-39 (F1, F2 or F3) or rTHT was quantified using the [^{3}H]-labeled thymidine assay, which

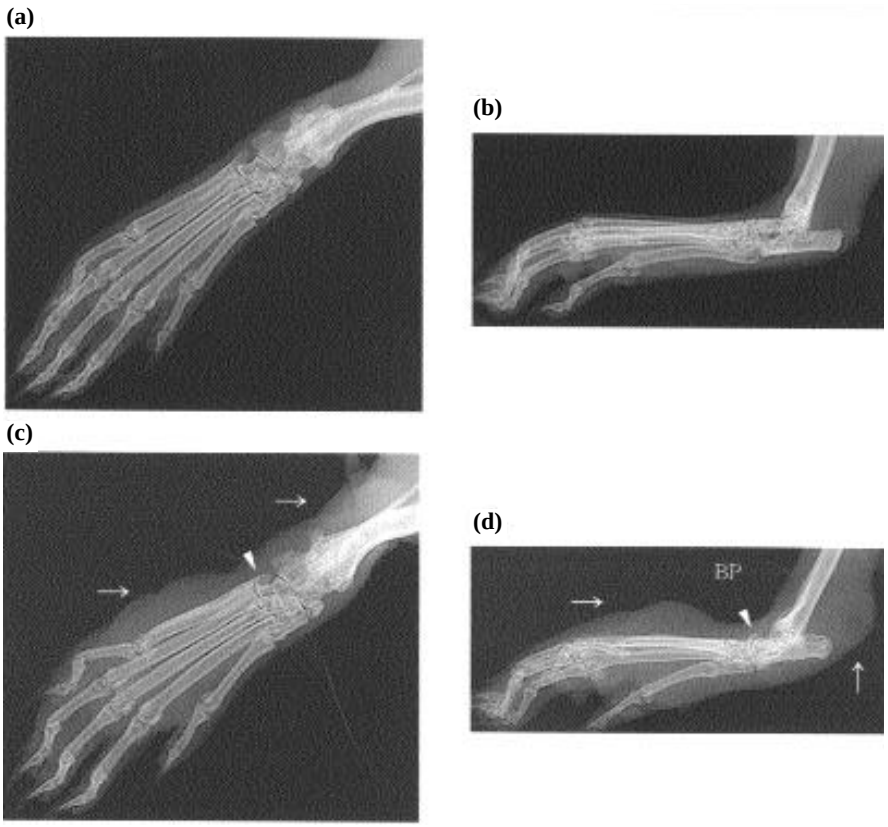


Fig. 5. Radiographs of the immunized BALB/c mice. Roentgenogram of the injected paws of mice immunized with 50 g of MBP (**a, b**) and rYKL-39 (**c, d**) are presented. Anteroposterior views (panel a and c) and lateral views (panel b and d) are shown. The swelling of soft tissue (**arrow**) and irregularity in the metatarsal articular cartilage (**arrow head**) are shown in panel c and d. In panel d, bony projections are clearly visible (BP).

has previously been described in detail (12). The assay was done in triplicate, and the response to each fragment was calculated by subtracting the thymidine incorporation for rTHT from each value for the respective fragment used.

Histopathologic analysis

At the end of the study, mice were sacrificed and their paws were removed, fixed with 10% formalin, and decalcified for 24 h. Subsequently, they were dehydrated in graded alcohol concentrations, and processed for embedding in paraffin wax. Each section through the joint was cut at 6 m on a microtome and stained with hematoxylin and eosin.

Radiographic analysis

The hind limbs of the sacrificed mice were removed and fixed in 10% formalin. Radiographs of the fixed limbs were taken in the anteroposterior and lateral plane orientations using X-rays (Hitachi, Japan) at 2 mA and 35 kV for 1 second. Film (Fuji fine-gain film) was processed by a Fuji automatic film developer (Fujifilm, Japan).

Statistical analysis

The values were analysed by Student's t-test to compare the differences between groups.

Results

Expression of recombinant YKL-39

In this study, we prepared YKL-39 as a recombinant fusion protein expressed in *E. coli* (Fig. 1a). For the first experiments, recombinant protein corresponding to the entire YKL-39 sequence was prepared as a fusion with either MBP or GST (Fig. 1b and c). Three fragments of YKL-39 (F1, F2 and F3) fused with THT were also prepared, which had the expected molecular weights shown in Figure 1c. These proteins were therefore considered to be of sufficient purity to use in the following examinations.

Arthritogenicity of YKL-39 in mice

Our previous study suggested an involvement of the immune response to YKL-39 in RA (7). Therefore we investigated whether immunization with YKL-39 could cause arthritis in mice. For this purpose, mice from four different strains (BALB/c, C57B/6, DBA/1J, and ICR) were immunised with rYKL-39 and the severity of arthritis was assessed. After the second immunization, 90.9% of ICR mice and 100% of mice from the other strains started to develop joint redness and swelling within 2 weeks. Interestingly, arthritis developed only in the injected paws and not in the remote joints in all the strains tested. Therefore the arthritis score represents the change in the injected paws. Although MBP induced swelling, YKL-39 immunization resulted in significantly more severe arthritis (Fig. 2). However, each strain showed a different clinical pattern of arthritis. For example, significant arthritis was observed up to day 28 in C57BL/6 mice (Fig. 2c), while it continued for longer in BALB/c mice (Fig. 2d). Thus, BALB/c mice were shown to be most susceptible to YKL-39 (Fig. 2a) among the four strains. In this strain, the arthritis persisted significantly for about 60 days after immunization.

Figure 3 shows the typical appearance of arthritis in a YKL-39-immunised BALB/c mouse, where marked redness and swelling around the joint is visible (Fig. 3b). A control mouse is shown in Figure 3a. Based on these results, we used susceptible BALB/c mice in the following assays.

Histopathologic and radiographic analysis

The paw joints of arthritic BALB/c mice were analysed histologically at 60 days after the initial immunization with rYKL-39 or MBP. Compared to the control mice (Fig. 4a), the joints of rYKL-39-immunised mice showed synovial proliferation and irregularity of

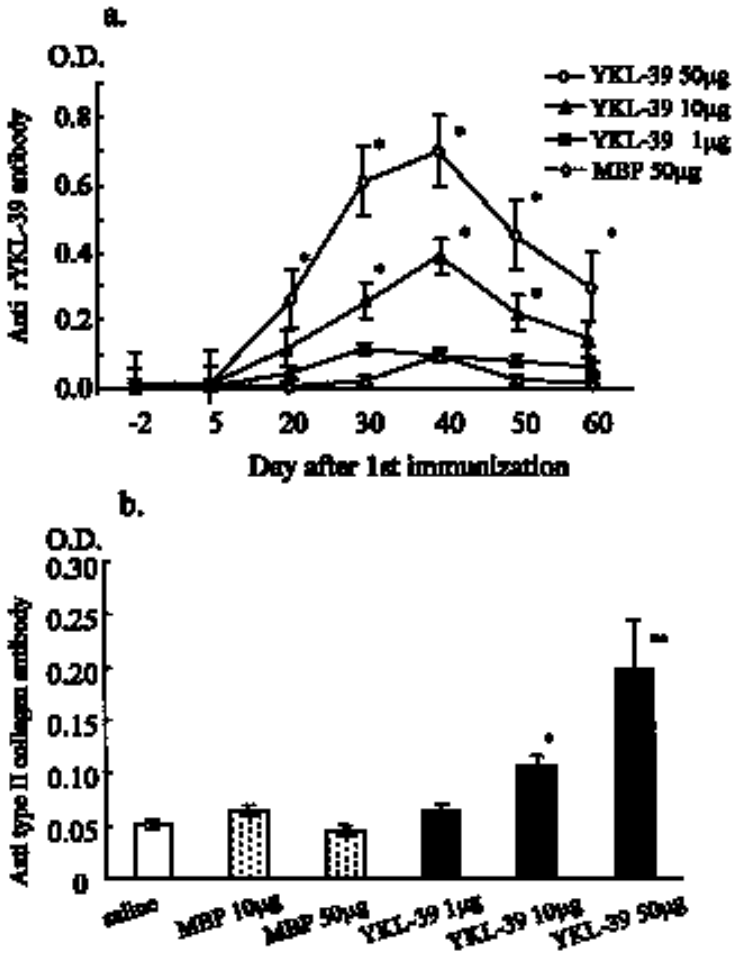


Fig. 6. Antibody production against rYKL-39 and type II collagen in the sera of YKL-39 immunised mice.
(**a**) A comparison of the humoral responses to rYKL39 in each group is shown. Titers of anti-YKL-39 antibody were very low in control mice (MBP) and in mice immunized with 1 g of YKL-39, whereas titers in mice immunized with 10 and 50 g of rYKL-39. Sera were diluted 1:2000. (**b**) Production of anti-type II collagen antibody at day 60 is shown. Only the sera of mice immunized with 10 and 50 μg of rYKL-39 had raised levels compared to the sera of control (MBP) mice. Sera were diluted 1:10. Values are given as the mean ± S.E. Statistical analysis was done by Student's t-test. *P < 0.05,**P < 0.01.

the articular surface (Fig. 4b). Follicular accumulation of mononuclear cells was observed in the synovial tissue of the ankle joints (Fig. 4c). Although swelling was not observed clinically, very mild synovial proliferation was found in the left hind paw (contralateral paw) of YKL-39-immunised mice (Fig. 4d and e).

To evaluate damage to the bone, we further performed radiographic analysis of the joints (Fig. 5). Compared to control mice (Fig. 5a, b), YKL-39 immunised mice showed marked swelling of the soft tissue, irregularity of the metatarsal bone and bony projections which were considered to be an arthritic reaction (Fig. 5c, d). These findings clearly indicated that immunization with YKL-39 induced not only inflammation but also bony destruction in BALB/c mice. No abnormal findings were found in the joints remote from the injected paws in the radiologic investigation.

Antibody production against YKL-39 and CII in immunised mice

Because arthritis was induced following immunization with rYKL-39, we investigated whether there was a specific immune response to rYKL-39 in these immunised BALB/c mice. The presence of IgG antibody specific for rYKL-39 was measured by ELISA in serially collected serum samples. As shown in Figure 6a, immunised mice were found to produce anti-YKL-39 antibody during the course of the disease. The antibody production response was dose-dependent; the titre was higher in the serum of mice receiving 50 g of YKL-39, whereas it was negligible in mice immunised with 1 g of the protein (Fig. 6a). In each case, the antibody titre peaked at day 40, shortly after the maximal arthritic score was noted (Fig. 2a).

Because YKL-39 is a human cartilage-derived protein, it is of interest to ask whether immunity to YKL-39 might trigger autoimmunity to other cartilage components such as CII. To address this issue, we measured the titres of anti-CII antibody in the sera of BALB/c mice 60 days after the first immunization. The responses were very low in mice immunized with MBP and low dose rYKL-39 (1 g), whereas significantly higher titres were shown in mice immunised with 10 and 50 g of rYKL-39 (Fig. 6b). This result suggests that the production of autoantibody to CII was induced after the onset of arthritis.

Proliferative response of PBMC to YKL-39 fragments

Finally, we investigated the presence of cellular immune responses to rYKL-39 in these arthritic mice. For this purpose, three fragments (F1, F2 and F3) of rYKL-39 were synthesized, and the responses of PBMC from un-immunized or immunised mice to these fragments were measured using [^{3}H]-thymidine incorporation. As can be seen in Figure 7, PBMC from mice immunised with rYKL-39 showed a higher proliferative response to the F1 fragment than other fragments (F2, F3).

Discussion

In this study, we have demonstrated that immunity to a recombinant protein of the recently cloned gene YKL-39 induced inflammatory arthritis with joint destruction in mice. Although the function of YKL-39 is still unknown, our results are the first to describe its arthritogenicity and possible involvement of

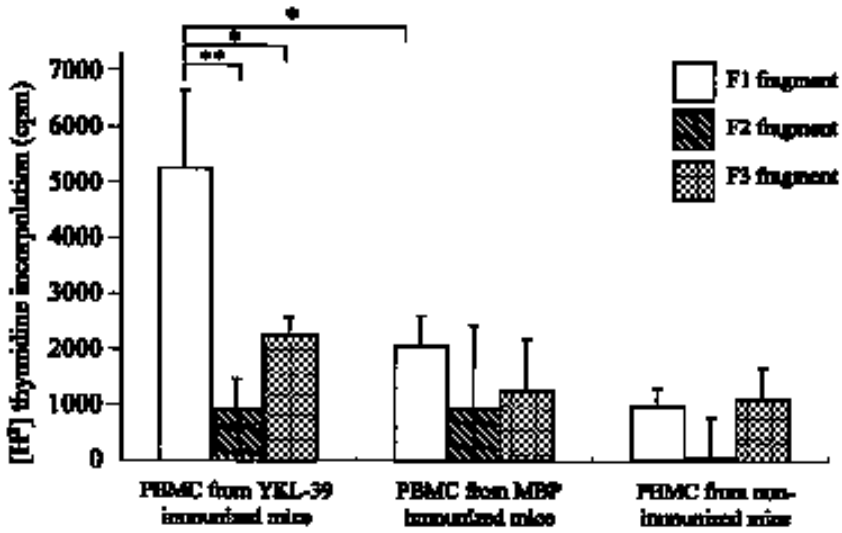


Fig. 7. Proliferative response of PBMC to YKL-39 fragments.
Proliferative responses of PBMC obtained from YKL-39-immunised mice (n = 5) , MBP immunized mice (n = 5) and non-immunized mice (n = 6) were measured by thymidine incorporation against YKL-39 fragments. The response to the F1 fragment was significantly higher in PBMC from YKL-39 immunized mice compared to PBMC from MBP immunized mice and non-immunized mice. Similarly, response of PBMC from YKL-39 immunized mice was higher to F1 fragment than F2 and F3 fragments. Values are the mean ± S.E. Differences between groups were analyzed by Student's t-test. *P < 0.05, **P < 0.01.

this cartilage-related protein in arthropathy.

YKL-39 was first identified as a protein secreted from cultured human chondrocytes (6) and it accounted for 4% of protein secreted into conditioned medium of chondrocytes *in vitro*. Two murine chitinase-like proteins, Brp39 and Ym1, were known (13). However, their amino acid homologies to YKL-39 are 49% and 45% respectively and their localization and function have not been thoroughly investigated. Therefore, it remains to be determined which self-component is recognised in the affected arthritic joints after immunization with recombinant human YKL-39.

The arthritis induced by immunization with YKL-39 represents a novel animal model of inflammatory arthropathy; however, it differs from established models such as CIA in several respects. First, arthritis of the model reported here was restricted to a single paw, whereas CIA causes severe polyarthritis. Secondly, the arthritis was quite mild; Histological examination revealed that YKL-39-induced arthritis was quite mild compared to CIA in terms of the degree of synovial proliferation, infiltration of mononuclear cells, and bone and cartilage destruction as shown by histological examination. One of the reasons is considered to be the amount of antigens; YKL-39 or its murine homologue, if any, seems to comprise only a minor part of the joint tissue, whereas CII is a major component of cartilage which is abundantly expressed in the joint. Antigenicity of epitopes in YKL-39 is also implicated in the intensity of the immuologic response. However, YKL-39 immunization triggered autoimmunity to CII (Fig. 6), suggesting that immunity to minor proteins might trigger an initial event for the induction of severe arthritis in which major components would be subsequently targeted in some conditions.

We examined arthritis induction in four different strains of mice, and found that BALB/c mice were the most susceptible to YKL-39-induced disease and for the longest time. For CIA, it has been reported that the H-2q (e.g. DBA1/J) and H-2r haplotypes determine susceptibility (14), whereas H-2d (BALB/c) is resistant (15) to arthritis. In addition, BALB/c mice did not produce antibody against CII after immunization with proteoglycan (16). Thus, it seems that the MHC restriction for the YKL-39-induced arthritis model is different from previously reported models. As murine T cells responded to recombinant human YKL-39, especially to the F1 fragment (Fig. 7), it is possible that an autoantigen homologous to the F1 region of human YKL-39 exists, and that activated T cells cross-react to murine self antigen. In addition, it could be hypothesised that the immune response to the murine YKL-39 homologue induced another autoimmunity against CII (Fig. 6). Taken together, it is suggested again that an immune response to a minor articular component would lead to the spread of autoimmunity to more major components in the joint.

To date, no mouse protein homologous to human YKL-39 has been found and no homology has been detected between the F1 region of human YKL-39 and previously reported arthritogenic antigens. Therefore, it is possible that the F1 fragment may contain an as yet unreported antigenic epitope which is responsible for arthritis induction.

In conclusion, we have demonstrated the arthritogenicity of cartilage-related protein derived from the recently cloned YKL-39 gene in mice. We have also documented cellular and humoral responses to recombinant YKL-39 in these immunised mice. Cartilage-derived secretory proteins, including YKL-39, may be targets for autoimmune responses in the joints and such immunity may further develop into destructive arthropathy. Identification of such cartilage-related proteins and characterisation of the autoimmune responses against them would help further our understanding of the pathogenesis and pathophysiology of human arthropathies such as RA.

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