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Suppression of T Cell Responses by Chondromodulin I, a Cartilage-Derived Angiogenesis Inhibitory Factor

Therapeutic Potential in Rheumatoid Arthritis

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Objective. **Chondromodulin I (ChM-I), a cartilage matrix protein, promotes the growth and proteoglycan synthesis of chondrocytes. However, it also inhibits angiogenesis. Since ChM-I is expressed not only in cartilage, but also in the thymus, we investigated the modulation of T cell function by ChM-I to assess its therapeutic potential in rheumatoid arthritis (RA).**

Methods. **The localization of ChM-I expression in mouse thymus tissue was examined by in situ hybridization. The proliferative response of peripheral blood T cells and synovial cells obtained from patients with RA was evaluated by ^{3}H-thymidine incorporation assay. The effects of ChM-I were examined using recombinant human ChM-I (rHuChM-I). Modulation of the antigen-specific immune response was evaluated by the recall response of splenic T cells and the delayed-type hypersensitivity response induced in the ear of mice primed with ovalbumin (OVA). Antigen-induced arthritis (AIA) was induced in mice by injecting methylated bovine serum albumin into the ankle joints 2 weeks after the priming.**

Results. **ChM-I was expressed in the cortex of the thymus. Recombinant human ChM-I suppressed the proliferative response of mouse splenic T cells and human peripheral blood T cells stimulated with anti-CD3/CD28 antibodies, in a dose-dependent manner. Production of interleukin-2 was decreased in rHuChM-I–treated mouse CD4 T cells. Ten micrograms of rHuChM-I injected intraperitoneally into OVA-primed mice suppressed the induction of the antigen-specific immune response. Finally, rHuChM-I suppressed the development of AIA, and also suppressed the proliferation of synovial cells prepared from the joints of patients with RA.**

Conclusion. **These results suggest that ChM-I suppresses T cell responses and synovial cell proliferation, implying that this cartilage matrix protein has a therapeutic potential in RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease in which massive synovial cell proliferation with leukocyte infiltration and abnormal capillary growth lead to the development of pannus and occasionally to disability due to the destruction of joints and bones. It has been suggested that T cells contribute to the pathogenesis of RA (1) on the basis of the massive infiltration of T cells into the synovial tissues (2), the oligoclonal expansion of T cells in the synovial fluid and synovial tissue (3–6), and the association between RA and particular HLA alleles (7,8). It has been proposed that these clonally expanded T cells play a role in disease pathogenesis by recognizing some

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arthritic antigens or by supporting synovial inflammation (3–6,9).

Since the formation of new blood vessels is one of the earliest histopathologic findings in RA and appears to be required for pannus development (10,11), it has been proposed that RA might be categorized as an "angiogenic disease." Since extension and flexion movements increase intraarticular pressure and collapse the capillaries, hypoxia and acidosis are induced in inflamed joints. The persistent growth of the synovial mass exceeds neovascularization, resulting in local ischemia (12). These metabolic demands and the decreased oxygen supply stimulate the production of angiogenic inducers, i.e., cytokines and growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, tumor necrosis factor α, interleukin-8 (IL-8), and vascular cell adhesion molecule 1 (13).

It has therefore been proposed that inhibition of angiogenesis might be a therapeutic strategy in the treatment of RA (10). In fact, it has been demonstrated that treatment with several angiogenesis inhibitors such as AGM-1470, which is a cyclic peptide antagonist of integrin $\alpha v\beta 3$ and anti-Flt1, and gene delivery using angiostatin or endostatin ameliorated arthritis in experimental animal models including collagen-induced arthritis (CIA), adjuvant arthritis, and antigen-induced arthritis (AIA) (14–19).

Increasing attention has been paid to chondroprotective and chondroregenerative treatment of arthritis, since it is known that cartilage does not spontaneously regenerate. During the progression of arthritis, cartilage has been shown to be damaged by the invasion of pannus from the synovium–cartilage junction, by degradation of the cartilage matrix by IL-1, metalloproteinases, and other factors, and by apoptosis of chondrocytes (20). Moreover, bony erosion sometimes progresses without any obvious arthritic inflammation. Numerous factors have been reported to promote chondrogenesis, and a therapy that combines these factors with antiinflammatory or immunosuppressive agents has been proposed recently (21).

We previously identified chondromodulin I (ChM-I) as an angiogenesis inhibitor (22). ChM-I is a 25-kd glycoprotein originally purified from bovine epiphyseal cartilage on the basis of its promotion of chondrocyte growth (23). Both ChM-I protein and ChM-I messenger RNA are richly expressed in cartilage. ChM-I has been shown to stimulate the growth, proteoglycan synthesis, and colony formation of cultured chondrocytes (24). However, it has also been shown to inhibit DNA synthesis, the proliferation of vascular endothelial cells, tube morphogenesis, and chorioallantoic membrane angiogenesis, thereby demonstrating its angiostatic ability (22,25,26). As confirmation of this ability, ChM-I has been shown to suppress chondrosarcoma growth via angiogenesis inhibition in vivo (27). Therefore, ChM-I is thought to participate in the angiogenic switching of cartilage by deterring vascular invasion (22,25).

During the biologic characterization of ChM-I, our Northern blotting analysis revealed ChM-I expression not only in cartilage, but also in the thymus, suggesting a correlation of ChM-I with T cell function (26). In the present study, we found that recombinant human ChM-I (rHuChM-I) suppressed the T cell proliferative response. In addition, rHuChM-I was able to inhibit the proliferation of synovial cells. Finally, rHuChM-I was able to reduce the severity of AIA. ChM-I therefore appears to act beneficially in the treatment of arthritis in 4 ways: protection of chondrocytes, inhibition of angiogenesis, prevention of synovial cell proliferation, and suppression of the immune system.

MATERIALS AND METHODS

Mice. BALB/c mice and DBA/1 mice were obtained from SLC (Shizuoka, Japan) and Charles River (Tokyo, Japan), respectively. DO11.10 transgenic mice whose T cells express a receptor specific for ovalbumin (OVA) peptide 323–339 (28) were kindly provided by Dr. T. Watanabe (Medical Institute of Bioregulation, Kyushu University, Japan). The mice were maintained in a temperature- and light-controlled environment with free access to food and water under specific pathogen-free conditions. Female age-matched BALB/c and DO11.10 mice and male DBA/1 mice were used in the respective experiments, and all mice were 7–10 weeks old at the start of each experiment.

Cell lines. RAW264.7 cells were kindly provided by Dr. Takayanagi (Department of Immunology, University of Tokyo, Japan). J558L and WEHI-231 cells were kindly provided by Dr. Tsubata (Medical Research Institute, Tokyo Medical and Dental University, Japan), and Jurkat cells were purchased from Riken Bioresource Center (Tsukuba, Ibaraki, Japan).

Preparation of rHuChM-I. Recombinant human ChM-I was prepared as described previously (26). Briefly, we subcloned the coding region for the human ChM-I precursor protein into a pcDNA3 expression vector, repetitively transfected the resulting vector into CHO cells, and then selected the drug-resistant clone. Our preliminary experiment indicated that the recovered rHuChM-I molecules were eluted in the aggregated forms with an apparent molecular size of >200 kd, which requires reduction with β-mercaptoethanol in the presence of 6*M* urea for dissociation. Therefore, the culture supernatant was first loaded on a butyl-cellulofine column, which was then eluted by 6*M* urea. The eluted materials were reduced by β-mercaptoethanol at a final concentration of 1 m*M*. Contaminant proteins were eliminated by successive chromatography on QAE-toyopearl, butyl-toyopearl, and

sulfate-cellulofine columns. The purified rHuChM-1 was confirmed to have the same biologic activity as the native bovine ChM-1 on chondrocytes and endothelial cells (26).

RNA in situ hybridization. To synthesize the digoxigenin-labeled riboprobes, a 0.5-kb polymerase chain reaction fragment of ChM-I complementary DNA (627–1,163 bp) was inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA). Linearized DNA was transcribed using T7 and SP6 polymerases. Thymus tissue was dissected from a 4-week-old male BALB/c mouse. Tissue was embedded in paraffin, sectioned at 7 μm thickness, and collected on silane-coated glass slides (Matsunami, Osaka, Japan). After deparaffinization with xylene, rehydration, and rinsing with 0.1*M* phosphate buffer, sections were treated with proteinase K (10 μg/ml) in Tris–EDTA at room temperature for 10 minutes, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and then treated with 0.2*M* HCl for 10 minutes. Acetylation of the sections was performed by incubation for 10 minutes with 0.1*M* triethanolamine–HCl, pH 8.0, and 0.25% acetic anhydrate for 10 minutes.

A hybridization mixture (50% formamide, 10 m*M* Tris–HCl, pH 7.5, 200 μg/ml transfer RNA, 1× Denhardt's solution, 10% dextran sulfate, 600 m*M* NaCl, 0.25% sodium dodecyl sulfate, 1 m*M* EDTA, pH 8.0) was preheated for 10 minutes at 85°C. Ten micrograms of the sense or antisense RNA probe was added to the hybridization mixture and denatured by heating at 85°C for 3 minutes, and then applied to the sections. Hybridization was performed overnight at 50°C. After hybridization, sections were washed with 50% formamide in 2× saline–sodium citrate at 55°C for 30 minutes and treated with a solution of 10 m*M* Tris–HCl, pH 7.5, 0.5*M* NaCl, and 1 m*M* EDTA (TNE) at 37°C. Nonspecific bindings of the probes were reduced by RNase A treatment (10 μg/ml in TNE) at 37°C for 30 minutes. Hybridization signals were visualized by using nitroblue tetrazolium salt and BCIP. The sections were counterstained with methyl green.

Lymphocyte proliferation assay. Naive T and B cells were purified with a magnetic cell sorting system (Miltenyi Biotech, Bergisch Gladbach, Germany), as previously described (29,30). Naive T cells were stimulated with 1 μg/ml of anti-CD3 antibody and 1 μg/ml of anti-CD28 antibody in the presence of various concentrations of rHuChM-I (from 0 to 1 μ*M*) in RPMI 1640 medium supplemented with 2 m*M* L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum for 24, 48, or 72 hours. Naive B cells were stimulated with 10 μg/ml of lipopolysaccharide (LPS) in the presence of various concentrations of rHuChM-I (from 0 to 1 μ*M*) for 24 hours. Naive OVA T cell receptor transgenic mouse DO11.10 T cells were cultured at 1×10^5 cells/well with irradiated antigen-presenting cells, various concentrations of OVA peptide (0.01, 0.1, and 1 μ*M*), and various concentrations of rHuChM-I (0, 1, 10, and 100 n*M*) for 24, 48, or 72 hours. This procedure was followed by a final 4 hours of culture in the presence of 1 μCi of ^{3}H-thymidine per well.

In some experiments, media without 2-mercaptoethanol contained either E-64 protease inhibitor (100 n*M*; Calbiochem, La Jolla, CA), iodoacetamide (50 n*M*; Sigma, St. Louis, MO), or *N*-ethylmaleimide (50 n*M*; Sigma) (31,32). The incorporated radioactivity was counted with a β-scintillation counter. The proliferative response was expressed as the mean ± SD counts per minute of test cultures.

Human peripheral blood T cell and synovial cell proliferation assays. Human peripheral blood T cells obtained from healthy volunteers were selected by lymphoprep (Axis Shield, Oslo, Norway) and stimulated with human anti-CD3 antibodies (0.001, 0.01, and 0.1 μg/ml) in the presence of rHuChM-I (0, 1, 10, and 100 n*M*) for 24 hours. Synovial cells were obtained from the joints of RA patients, who gave their informed consent, before undergoing total knee arthroplasty or total hip replacement. Synovial cells (1×10^4 cells per well), within 4 passages of culture (33), were seeded in culture plates with various concentrations of rHuChM-I (0, 10, 30, 100, and 300 n*M*) and cultured for 5 days. This procedure was followed by a final 16 hours of culture in the presence of 1 μCi of ^{3}H-thymidine per well. The cells were detached with 50 μl of 0.25% trypsin–0.2% EDTA, and harvested onto glass-fiber filters. The incorporation of ^{3}H-thymidine was measured by scintillation counting.

Naive T cells viability assay. Mouse naive T cells (1×10^6 cells per well) were cultured with various concentrations of rHuChM-I (0–1 μ*M*) for 24 hours. Viable cells were counted by trypan blue exclusion.

Evaluation of IL-2 production. The concentration of IL-2 was determined in the supernatant from mouse CD4+ T cells or CD8+ T cells. These T cells were activated with immobilized anti-CD3 (1 μg/ml) + anti-CD28 (1 μg/ml) for 24 hours, and the IL-2 concentration was determined by sandwich enzyme-linked immunosorbent assay (Genzyme, Cambridge, MA).

Assessment of delayed-type hypersensitivity (DTH). The evaluation of the DTH response was based on the degree of ear swelling. BALB/c mice were immunized with 100 μg of OVA in Freund's complete adjuvant (CFA) with or without a concomitant intraperitoneal injection of rHuChM-I. DTH was induced by an injection of 200 μg of OVA into the left ear pinnae of the mice 14 days after the priming. The right ear served as an untreated control. Both ear pinnae were measured immediately before the injection and 24 hours later with a dial-gauge caliper (Mitsutoyo, Kawasaki, Japan). The measurements were performed in triplicate.

Synovial cells viability assay. Synovial cells (1×10^5 cells per well) were cultured with various concentrations of rHuChM-I (0–1 μ*M*) for 5 days. Viable cells were counted by trypan blue exclusion. Each experiment was performed in triplicate.

MTT assay of synovial cells. MTT is a substrate that is cleaved by living cells. Since this process requires active mitochondria and even freshly dead cells do not cleave significant amounts of MTT, this colorimetric assay is able to determine the amount of live cells (34,35). Therefore, to evaluate the proliferation of synovial cells, we conducted an MTT assay according to the manufacturer's protocol (Chemicon, Temecula, CA). Briefly, synovial cells were seeded in a 96-well microtiter plate (1×10^4 cells/well) and were incubated in the growth medium in the presence or absence of rHuChM-I for 5 days. Four hours before the termination of culture, MTT (5 mg/ml) was added to each well. At the end of the incubation, 100 μl of isoproanol was added to each culture to dissolve the formazan complex. The optical density at 590 nm was measured using a 96-well multiscanner. Each experiment was performed in triplicate.

Induction of AIA. BALB/c mice were injected intradermally with 100 μg of methylated bovine serum albumin (mBSA) in CFA at the base of the tail on day 0. Mice received 10 μg of rHuChM-I in PBS on day 0 (for the single-injection protocol) or day 0 to day 3 (for the 3-consecutive-days delivery protocol), and control mice received PBS alone on day 0 or day 0 to day 3. Fourteen days later, 20 μg mBSA dissolved in 20 μl of PBS was injected intraarticularly into the left ankle joint. The right ankle joint was injected with 20 μl of PBS alone as a negative control. The joint thickness was measured with a dial gauge caliper, and the net increase in thickness was calculated (30,36).

Induction of CIA and treatment with rHuChM-I. Male DBA/1 mice were injected intradermally with 100 μg of bovine type II collagen (BII; Chondrex, Redmond, WA) in CFA (Difco, Detroit, MI) at the base of the tail on day 0. A booster was administered on day 21. The mice were injected intraperitoneally with 10 μg of rHuChM-I dissolved in PBS on day 0 (for the single-injection protocol) or from day 0 to day 3 (for the 3-consecutive-days delivery protocol). Control mice were injected with PBS alone on day 0 or from day 0 to day 3, and signs of arthritis appeared at around days 25–28, which is consistent with the findings in previous reports (37–40).

Assessment of CIA. Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or other parts of the paws. Arthritis was scored using the following scale: 0 = no change; 1 = redness or mild inflammation; 2 = swelling or inflammation; 3 = severe swelling or severe inflammation; 4 = ankylosis (41). The scoring was done by 2 independent observers.

Histologic examination. Ankles and knees were fixed in 10% phosphate-buffered formalin and decalcified. Tissues were then dehydrated in a gradient of alcohol, and then paraffin-embedded, sectioned, mounted on glass slides, and stained with hematoxylin and eosin (30,36). The histopathologic arthritis score of AIA was quantified according to the method of Brackertz et al (42), based on the degree of synovial hypertrophy, mononuclear cell infiltration, and pannus formation. Each section was studied by 3 blinded examiners in the AIA experiment.

The histopathologic arthritis score of CIA was assessed according to the method of Hietala et al (43), based on the degree of synovial hypertrophy, cartilage destruction, and pannus formation. Each section was studied by 2 blinded examiners in the CIA experiment. The average scores of each parameter from 4 joints (rear ankles and knees) in each mouse were calculated.

Statistical analysis. Statistical significance was determined by the Student's unpaired *t*-test. *P* values of less than 0.05 were considered to indicate a statistically significant difference. Results are reported as the mean ± SD.

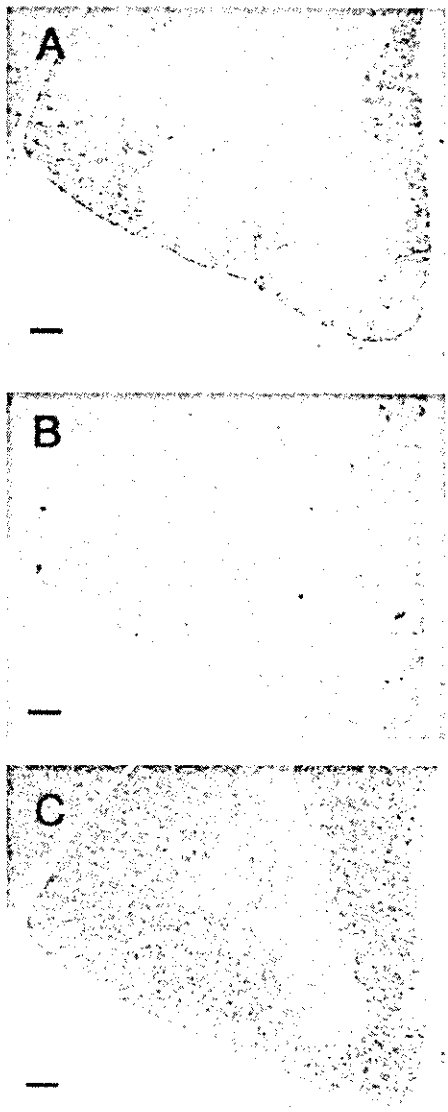

Figure 1. Expression of chondromodulin I (ChM-I) mRNA in the cortex of the thymus of 4-week-old mice. **A,** In this thymus section, which was hybridized with the antisense ChM-I cRNA probe, there are obvious hybridization signals in the cortex. **B,** In this semiserial section, which was hybridized with the sense probe as a control, no signal was detected. The sections were counterstained with methyl green. **C,** The cortex of mouse thymus tissue is purple stained with hematoxylin and eosin. Bar = 100 μm.

RESULTS

ChM-I expression in the cortex of the thymus. When we previously examined the tissue distribution of ChM-I in DDY mice by Northern blot analysis, we found that ChM-I is expressed not only in cartilage, but also in the thymus and the eye (26). In order to further verify the ChM-I expression in the thymus, we performed in situ hybridization using BALB/c mice in addition to DDY mice. We found that ChM-I is expressed in the cortex, but not in the medulla (Figure 1). The ChM-I–expressing cells seemed to be thymic stromal cells.

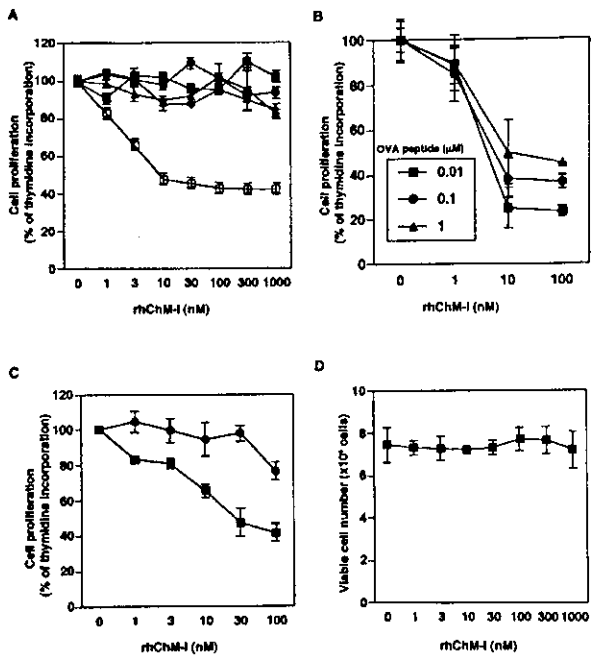

Figure 2. Suppression of the T cell proliferative response in vitro by recombinant human chondromodulin I (rhChM-I). A, Mouse splenic T cells (1×10^5/well) (□) were stimulated with immobilized anti-CD3 (1 μg/ml) + anti-CD28 (10 μg/ml) in the presence of varying concentrations (0.1–1,000 n*M*) of rhChM-I, and cultured for 24 hours. As a reference, the growth, in the presence of various concentrations of rhChM-I, of various cell sources derived from mouse blood cells is shown: 5×10^4 cells/well of the RAW264.7 mouse macrophage-derived cell line (■), J558L mouse myeloma cell line (▲), and WEHI-231 mouse lymphoma cell line (◆), as well as 10^5 cells/well of mouse splenic B cells stimulated with lipopolysaccharide (●). B, Splenic DO11.10 T cells were stimulated with ovalbumin (OVA) peptide and irradiated antigen-presenting cells in the presence of various concentrations of rhChM-I. C, Human peripheral blood T cells (■) were purified by lymphoprep and stimulated with human anti-CD3 antibodies (0.1 μg/ml) in the presence of rhChM-I (1, 3, 10, 30, and 100 n*M*) for 24 hours. As a reference, the growth, in the presence of rhChM-I, of the Jurkat human T lymphocyte cell line (●) is shown. Bars in A–C show the mean ± SD ^{3}H-thymidine incorporation as a proportion of that in the absence of rhChM-I. D, To demonstrate lack of toxicity of rhChM-I, mouse T cells were cultured in the absence or presence of various concentrations of rhChM-I. Bars show the mean ± SD number of live cells.

Suppression of the T cell proliferative response by rHuChM-I. The thymic expression of ChM-I suggested that ChM-I might be associated with the development or function of T cells. Therefore, we examined the possibility that rHuChM-I modifies the T cell immune response. As shown in Figure 2A, rHuChM-I suppressed the proliferative response of mouse T cells stimulated with anti-CD3 + anti-CD28 antibodies. Similarly, rHuChM-I suppressed the antigen-specific proliferation of OVA-stimulated T cells (Figure 2B). These inhibitions of T cell proliferative response occurred in a dose-dependent manner, and the maximum inhibition of 76.5% was obtained at an rHuChM-I concentration of 100 n*M*. This suppressive effect was not due to the toxicity of rHuChM-I, since incubation with variable amounts of rHuChM-I did not alter the number of live T cells (Figure 2D).

The proliferation of human peripheral blood T cells was also inhibited by rHuChM-I in a dose-dependent manner (Figure 2C). The dose–response curves revealed that the dose required for 50% inhibition (ID_{50}) of the T cell proliferative response was ~3 n*M* for mouse T cells and ~10 n*M* for human T cells (see Figures 2A and C). These ID_{50} values for mouse and human T cells are fairly consistent with our previous observation that the ID_{50} of endothelial cell proliferation was almost 8 n*M* (22). Since mouse and human ChM-I are 87% similar in their amino acid sequences, it is possible that human ChM-I can bind the receptor for mouse ChM-I with almost the same affinity.

Since ChM-I inhibits the spontaneous growth of endothelial cells, we examined the possibility that ChM-I is a general inhibitor of growth. As shown in Figure 2A, rHuChM-I did not inhibit the spontaneous growth of the RAW264.7 mouse macrophage-derived cell line, J558L mouse myeloma cell line, or WEHI-231 mouse lymphoma cell line, and it did not inhibit the proliferation of mouse splenic B cells stimulated with LPS. Moreover, rHuChM-I did not inhibit the spontaneous proliferation of the Jurkat human T lymphocyte line, although at higher doses, partial inhibition did occur (Figure 2C). Considering that the ID_{50} is ~3 n*M* for mouse T cells, ~10 n*M* for human T cells, and almost 8 n*M* for endothelial cells, the dose needed to inhibit Jurkat proliferation was extremely high, implying that the suppressive mechanism in Jurkat cells might be different. These results indicate that rHuChM-I is not a general growth inhibitor and that T cell proliferation is one of the selective targets of rHuChM-I.

Furthermore, IL-2 production in the supernatant was significantly decreased by rHuChM-I in CD4+ T cells, but not in CD8+ T cells (Figure 3). This result indicates that the inhibitory mechanism of T cell proliferation involves, at least in part, the suppression of IL-2 production in CD4 T cells, and again supports the idea that the biologic effect of rHuChM-I is specific to certain cell types.

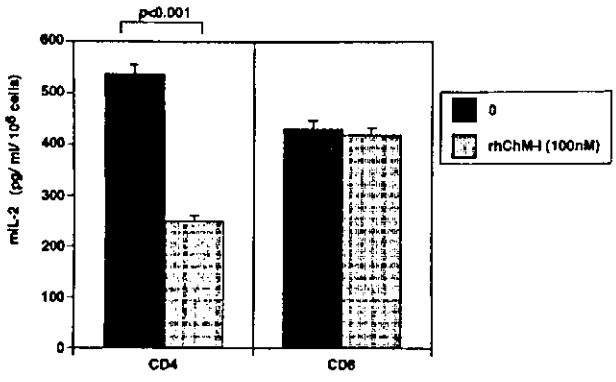

Figure 3. Reduction by rhChM-I of interleukin-2 (mIL-2) production from mouse CD4+ T cells. Levels of IL-2 in the supernatant of either CD4+ or CD8+ mouse splenic T cells stimulated with anti-CD3 + anti-CD28 antibodies in the presence of 100 n*M* of rhChM-I were evaluated by enzyme-linked immunosorbent assay. The IL-2 concentrations were normalized to the number of live T cells. Bars show the mean and SD IL-2 production from 10^6 T cells. See Figure 2 for other definitions.

Suppression of the antigen-specific immune response in vivo by rHuChM-I. To confirm that rHuChM-I is able to suppress an antigen-specific immune response in vivo, we immunized mice with a nominal antigen, OVA. Splenic T cells from mice primed with OVA exhibited a decreased recall response to OVA in vitro when they were injected with rHuChM-I at the time of their priming (Figure 4A). Ear swelling, which was induced by OVA injection into the ear of mice primed with OVA, was diminished in a dose-dependent manner (Figure 4B) in the mice treated with rHuChM-I in comparison with untreated control mice. Since the background level of ^{3}H-thymidine incorporation was not significantly altered between the rHuChM-I–injected mice and the control mice, rHuChM-I seems to suppress the immune response to the primed antigen preferentially. These results indicate that ChM-I suppressed the immune response to the antigen in vivo.

Duration of effect of rHuChM-I on the T cell proliferative response. The suppressive activity of rHuChM-I on T cells began to diminish by 48 hours, and it was completely abrogated by 72 hours in the in vitro culture experiments (Figure 5). When we repeatedly added rHuChM-I every 24 hours, the suppression lasted for at least 4 days. Recently, it was reported that plasma contains a reductase that can reduce disulfide bonds in proteins and reduce the average size of von Willebrand factor secreted by endothelial cells (31,32). Since ChM-I contains 4 intramolecular disulfide bonds, a feature that is assumed to be critical for its activity (22,25), we assumed that the short duration of rHuChM-I activity might be due to a reduction of disulfide bonds by some molecules contained in the culture. To verify this hypothesis, we examined the kinetics of the suppressive activity of rHuChM-I on T cells in the presence of reductase inhibitors. Although the reductase inhibitors were not toxic on T cells, rHuChM-I was able to retain its suppressive activity for 72 hours in the presence of reductase inhibitors (Figure 5). These results suggest that the short duration of the rHuChM-I suppressive activity in vitro might have been due to reductase in the culture.

Suppression of the proliferation of synovial cells by rHuChM-I. Since our studies of ChM-I have consistently revealed its potential to ameliorate arthritis, we decided to further examine the effect of ChM-I on synovial cell proliferation, which must be controlled to treat RA. As expected, the incorporation of ^{3}H-thymidine into synovial cells prepared from RA joints decreased in the presence of rHuChM-I. The maximal

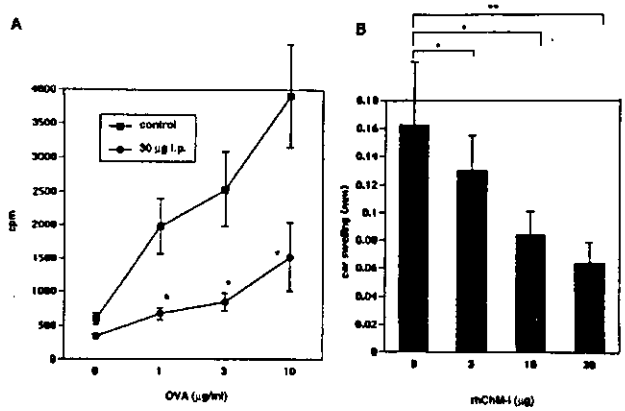

Figure 4. Suppression of the T cell response in vivo by rhChM-I. **A**, In splenocytes primed with OVA, rhChM-I reduced the recall response against OVA. BALB/c mice were immunized with OVA and intraperitoneally (i.p.) injected with rhChM-I at the time of OVA immunization. The secondary proliferative response of the splenocytes was examined 14 days later, by culturing for 72 hours with various concentrations of OVA (1, 3, and 10 μg/ml). **B**, The delayed-type hypersensitivity response, evaluated by ear swelling, was suppressed by rhChM-I. Fourteen days after the immunization, 200 μg of OVA was injected into the left ear pinnae of the mice. The right ear served as an untreated control. Both ear pinnae were measured immediately before and 24 hours after the injection. Bars show the mean ± SD of 5 mice per group. * = $P < 0.05$; ** = $P < 0.01$. See Figure 2 for other definitions.

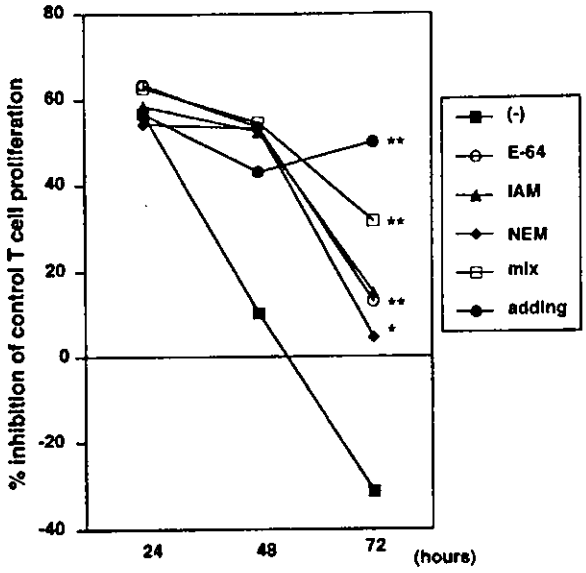

Figure 5. Preservation of the suppressive effect of recombinant human chondromodulin I (rHuChM-I) on the T cell response up to 72 hours by sequential addition of rHuChM-I or by the presence of reductase inhibitors. Splenic T cells (1×10^5/well) were plated in 96-well plates with 1 μg/ml of anti-CD3 + 10 μg/ml of anti-CD28. The rHuChM-I was added every 24 hours (adding). Either E-64, iodoacetamide (IAM), or *N*-ethylmaleimide (NEM) was added separately or mixed (E-64 + IAM + NEM) (mix) at the beginning of the culture. * = $P < 0.05$; ** = $P < 0.01$.

inhibition was 48% at 100 n*M* of rHuChM-I (Figure 6A). In order to discern whether the suppression of ^{3}H-thymidine incorporation was simply due to cytotoxicity of rHuChM-I, we conducted additional studies involving direct counting of the live cells and an MTT assay that is able to determine the amount of live cells. Both studies confirmed that rHuChM-I suppressed the proliferation of synovial cells (Figures 6B and C) and that the decreased ^{3}H-thymidine incorporation did not simply reflect the decreased cell number due to rHuChM-I cytotoxicity, because the number of cells after the culture increased compared with that at the start of culture, even at the 1 μ*M* concentration of rHuChM-I.

Suppression of the development of AIA by rHuChM-I. We next examined whether rHuChM-I is able to suppress the induction of experimental arthritis. We primed BALB/c mice with mBSA so that they would develop AIA after intraarticular injection of mBSA, and evaluated the severity of arthritis using the maximum hind-paw thickness. We injected rHuChM-I intraperitoneally at the time of the priming. The rHuChM-I significantly suppressed the development of AIA. In addition, when rHuChM-I was delivered to the mice for 3 consecutive days, the development of the arthritis was markedly suppressed (Figure 7A). Histologic examination of the ankle joints revealed that the number of inflammatory cells invaded into periarticular soft tissues and bone marrow in the tarsus was reduced in the rHuChM-I–treated mice in comparison with the control mice (Figures 7C and D). The evaluation of histopathologic severity revealed a significant amelioration by rHuChM-I treatment ($P < 0.001$) (Figure 7B). These

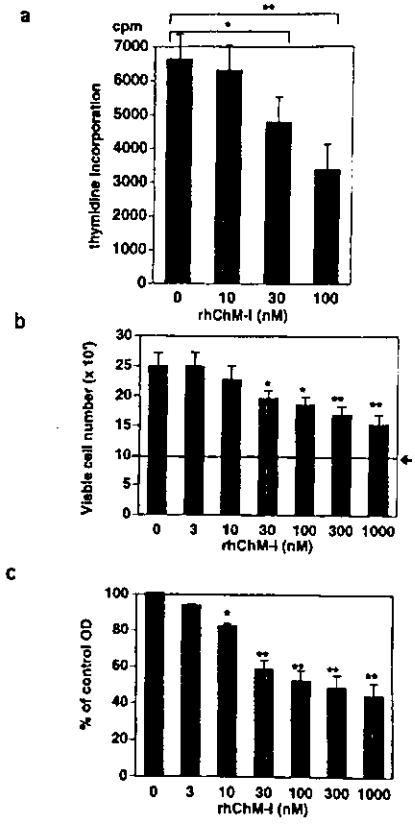

Figure 6. Reduction of rheumatoid arthritis (RA) synovial cell proliferation by recombinant human chondromodulin I (rhChM-I). a, Synovial cells (1×10^4) from RA patients were plated in 96-well plates and incubated with rhChM-I (10, 30, and 100 n*M*) for 5 days, and the proliferative response was measured by ^{3}H-thymidine incorporation. b, Synovial cells (1×10^5) from RA patients were plated in 24-well plates and incubated with rhChM-I (3, 10, 30, 100, 300, and 1,000 n*M*) for 5 days, and viable cells were counted by trypan blue exclusion. The arrow denoting the horizontal line indicates the initial number of cells at culture start (1×10^5 cells). c, Synovial cell proliferation was determined using MTT assay. Results are expressed as the percentage of the values detected in cells in the absence of rhChM-I. OD = optical density. Bars show the mean and SD. * = $P < 0.05$; ** = $P < 0.01$.

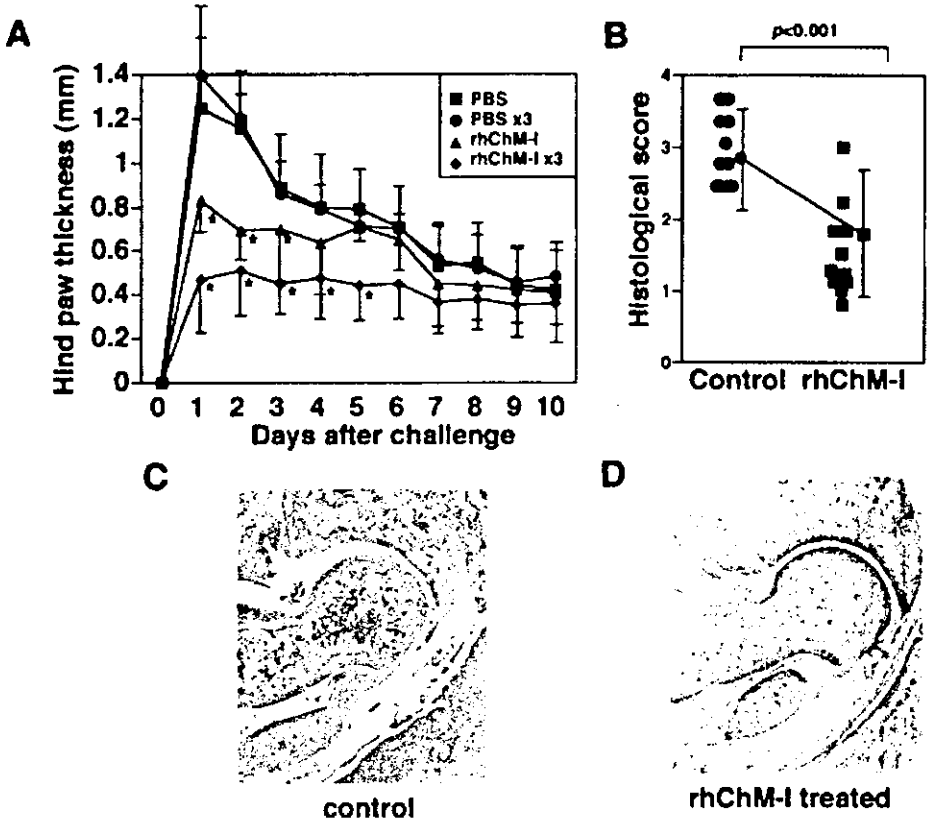

Figure 7. Suppression of the development of antigen-induced arthritis (AIA) by rhChM-I. **A**, For induction of AIA, BALB/c mice were immunized with 100 μg of methylated bovine serum albumin in Freund's complete adjuvant at the base of the tail. Ten micrograms of rhChM-I was intraperitoneally injected once on the same day or on 3 consecutive days (×3). The control mice received phosphate buffered saline (PBS) alone. The primed mice were challenged intraarticularly with the antigen on day 0. Bars show the mean ± SD increase in hind-paw thickness during the course of the disease (n = 10 per group). **B**, Histologic examination of the ankle joints. The histopathologic arthritis score for AIA was assessed by 3 blinded examiners as the extent of synovial hypertrophy, mononuclear cell infiltration, and pannus formation. **C** and **D**, Massive cell infiltration in the control AIA mice was ameliorated in the rhChM-I–treated AIA mice, respectively. See Figure 6 for other definitions.

results confirmed that rHuChM-I is able to modulate AIA.

Reduction of incidence of arthritis in CIA by rHuChM-I. In order to further evaluate the effect of ChM-I on arthritis, we also investigated its ability to suppress CIA. We injected 10 μg of rHuChM-I (or PBS for the control) intraperitoneally when we immunized the mice with BII. While all the control mice treated with PBS fully developed CIA, only 60% of the mice receiving a single injection of 10 μg rHuChM-I developed the disease (Figure 8A); however, this reduction was not statistically significant. The incidence of CIA significantly decreased to 50% in the mice receiving rHuChM-I injection for 3 consecutive days ($P < 0.05$). The mean arthritis score in the group of rHuChM-I–treated mice also decreased significantly (Figure 8B). Since the arthritis score in the mice that developed the disease in spite of rHuChM-I delivery eventually increased to the full value, similar to that in the control mice, this reduction might simply reflect a decrease in arthritis development.

The histopathologic examination revealed massive mononuclear cell infiltration and edema in the control mice (Figure 8C), whereas both of these features were suppressed in the rHuChM-I–treated mice (Figure 8D). The grading of histopathologic severity revealed

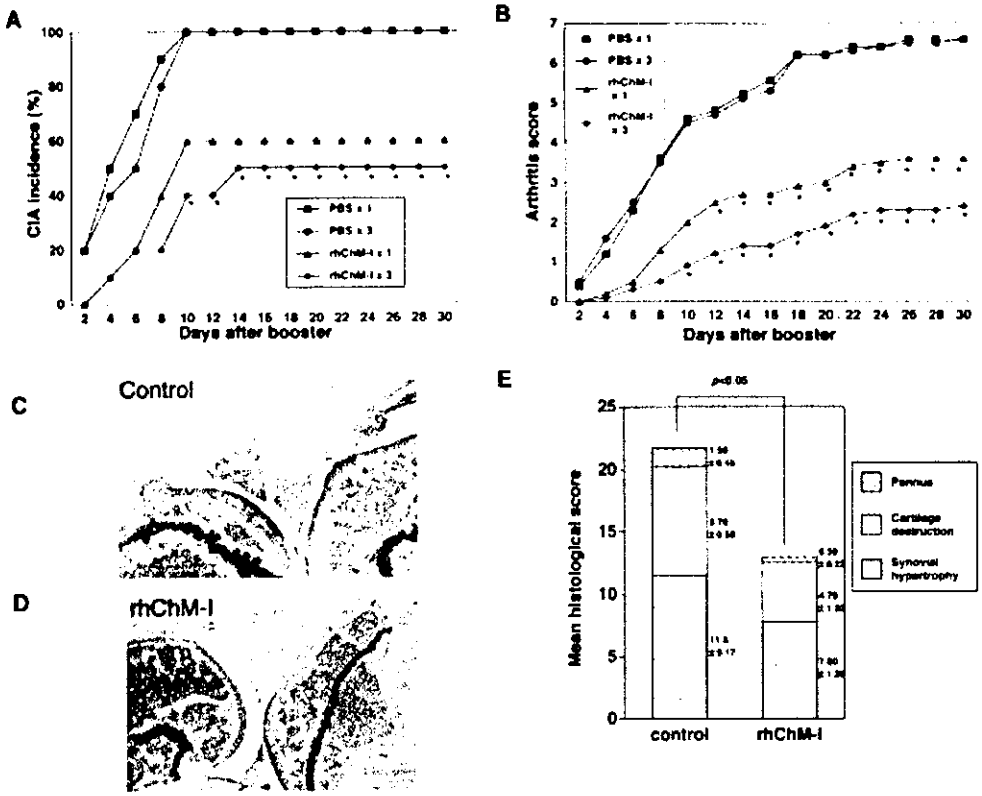

Figure 8. Decreased incidence of collagen-induced arthritis (CIA) by treatment with rhChM-I. A, For induction of CIA, mice were injected intradermally with 100 μg of bovine type II collagen in Freund's complete adjuvant at the base of the tail on day 0. A booster was administered on day 21. Mice received 10 μg of rhChM-I in phosphate buffered saline (PBS) on day 0 or from day 0 to day 3 (×3). Control mice received PBS instead of rhChM-I. Each group consists of 10 mice. B, For the arthritis score of CIA with or without rhChM-I treatment, 2 independent observers scored the ankle joints on a scale from 0 to 4. C and D, For histopathologic analysis, CIA control mice and CIA mice treated with rhChM-I were killed on day 50 and their knee joints were sectioned and stained by hematoxylin and eosin. C, The joints of control mice showed severe inflammation in the synovium and joint space with synovial hyperplasia. D, The joints of rhChM-I–treated mice showed mild inflammation in the synovium. E, To determine the histopathologic severity of arthritis, the histologic arthritis score of CIA was assessed by 2 blinded examiners as the extent of synovial hypertrophy, cartilage destruction, and pannus formation. The scores of each parameter from 4 joints of each mouse were summed. Values are the mean ± SD. * = $P < 0.05$. See Figure 6 for other definitions.

that rHuChM-I treatment significantly prevented the development of CIA ($P < 0.05$) (Figure 8E), but that once the mice developed arthritis in spite of rHuChM-I delivery, the pathology of the arthritic joints was almost the same as in the CIA control mice.

Taken together, these results show that rHuChM-I suppressed the proliferation of both T cells and synovial cells. In addition, rHuChM-I suppressed the development of AIA as well as CIA, although its effect on the latter was partial.

DISCUSSION

This study revealed 2 novel features of ChM-I, namely, that ChM-I suppressed both T cell activation and synovial cell proliferation. These findings combined with our previous findings (that ChM-I promotes chondrocyte growth and inhibits angiogenesis) would suggest a therapeutic potential for ChM-I in arthritis.

The therapeutic effect in CIA was partial, and we were unable to confirm that T cell suppression occurred in our CIA model. We did not observe a significant

decrease in the T cell proliferative response against BII, although the antibody titer against BII in the mice treated for 3 consecutive days was slightly decreased (data not shown). Therefore, we cannot conclude that ChM-I exerted its therapeutic effect on CIA via the suppression of the T cell response.

In contrast to the CIA experiments, rHuChM-I exhibited a distinct suppressive effect on the development of AIA. The prevention of T cell priming in vivo was also confirmed, as shown in Figures 4A and B, indicating that the therapeutic effect of AIA depends on T cell suppression. CIA requires a rather longer time course (almost 40 days) to develop in comparison with AIA. Therefore, we suspect that the short duration of the suppressive activity of rhChM-I on T cell proliferation, as demonstrated in Figure 5, might have been related to this discrepancy of the outcome between AIA and CIA, since the arthritis severity in AIA as well as the arthritis incidence in CIA decreased more significantly in the mice receiving rHuChM-I for 3 consecutive days than in the mice receiving a single injection.

It is possible that some factors expressed or secreted by activated T cells might have been involved in decreasing the rHuChM-I activity, since the suppression of T cells did not last as long as in our previous study using endothelial cells. In addition, this short duration of activity might have prevented us from observing chondrocyte protective and synovial cell growth retardation effects, which require a longer period to examine clearly. In order to dissect those effects, we would have to deliver rHuChM-I more frequently throughout the entire disease course, although at this time we cannot prepare a sufficient amount of rHuChM-I to conduct such a study.

In any case, the current form or protocol of ChM-I delivery might limit its practical use in arthritis in which activated T cells are involved. The development of methods to compensate for the short activity of ChM-I, e.g., the development of a form that is made less susceptible to reduction or joint expression by using adenoviral vector, would provide a new innovative therapy not only for RA, but also for other rheumatic diseases, including osteoarthritis and seronegative spondylarthropathy. In addition, identification of the mechanism of ChM-I activity would help in the development of a more refined therapy.

To date, no molecule derived from bone or joint tissues has been shown to modulate the immune response. Although the primary role of ChM-I must be related to its antiangiogenic activity in cartilage, one other physiologic role of ChM-I might be the control of T cell positive selection. It is interesting to note that the effective dosage of ChM-I is almost the same irrespective of its various biologic outcomes; that is, the dose required for a 50% effect in chondrocyte growth promotion is between 4–8 n*M*, while the suppressive ID_{50} values are almost 8 n*M* for endothelial cells (22) and ~3–10 n*M* for T cells. It seems that the opposite functions in the different cells share a single type of receptor. This interesting phenomenon should stimulate further studies to elucidate its mechanism.

During the inflammatory process or the drastic pressure change caused by joint movement, the molecules released from damaged joint tissues could be presented as antigens by synovial cells or dendritic cells. Once these molecules are recognized by the immune system, the resulting immune response might contribute to the exacerbation or initiation of arthritis. In fact, a number of joint-derived matrix molecules, including type II collagen, BjP, YKL-39, YKL-40, matrilin-1, proteoglycan aggrecan, and p205, have been demonstrated to be the target of autoreactive T cells and to be involved in the pathogenesis of not only RA, but also osteoarthritis and polychondroarthritis (44–50). In addition, since it is known that the autoreactive immune response becomes aggressive as the immunologic determinant spreads (51–53), it would be important to prevent the immune system from recognizing new antigens or additional epitopes. In this context, it is interesting to note that fetal bone is rich in ChM-I and the expression level decreases with age (54), whereas aged cartilage contains very little ChM-I and aging increases the susceptibility to arthritis. It would be interesting to examine whether ChM-I is able to prevent priming of these arthritic antigens under physiologic conditions.

The biologic features of ChM-I not only provide us with a therapeutic strategy, but also contribute new insights into the relationship between the cartilage matrix and the immune system. Future studies will be undertaken to clarify the mechanism or factors that promote the degradation or reduction of ChM-I or the loss of its activity, thereby contributing to the treatment of arthritis.

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T cells accumulating in the inflamed joints of a spontaneous murine model of rheumatoid arthritis become restricted to common clonotypes during disease progression

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Keywords: autoantigen, autoimmunity, clonal expansion, rheumatoid arthritis, TCR, transgenic mouse

Abstract

Although a number of studies have revealed that T cells expand clonally in the joints of patients suffering from rheumatoid arthritis (RA), the kinetics of T cell clonality in multiple joints of an individual throughout progression of the disease is not known. By employing a TCR β chain gene-specific RT-PCR and subsequent single-strand conformation polymorphism, which enables us to monitor T cell clonality, we analyzed transgenic mice (Tg) carrying the human T cell leukemia virus type I env-pX region. These mice spontaneously develop destructive progressive arthritis similar to RA as they age. In the early stage, the majority of accumulating T cell clones differed in each of four affected feet analyzed. However, in the advanced stage, many of the clones were common to all four feet. The total number of distinct clones gradually decreased as the disease progressed. When splenocytes from arthritic elder Tg were adoptively transferred into either nude mice or young Tg, the clones common to all four feet of the donor were detected again in four feet of the recipients. These findings suggest that, as arthritis progresses, the T cell clones accumulating in the arthritic joints are gradually restricted to certain common clonotypes, some of which are arthrotropic.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent inflammation of synovium, destruction of cartilage and bone, and systemic illness. Based on the association with particular HLA alleles (1–3), massive infiltration of T cells into the synovial tissues (4) and T cell oligoclonal expansion in the joints (5), T cells have been proposed to play an important role in RA pathogenesis: T cells would initiate or be involved in the inflammatory process by recognizing some antigens and producing cytokines (5,6), although this viewpoint has been controversial (7).

We and others have demonstrated that T cells expand clonally in the synovium as well as the synovial fluid of RA patients (5,8–14). Furthermore, some clones were found to be common in multiple joints of the same patient (14). These findings suggest that the oligoclonally accumulated common T cell clones are neither transiently nor randomly recruited into the inflammatory sites, but that they recognize some antigens which are associated with the pathogenesis of RA inflammation. Supporting this viewpoint, Mima *et al.* reported that T cell clones with an identical V_β CDR3 sequence frequently detected in the joints of some RA patients induced synovial hyperplasia in SCID mice when these clones were transferred (15).

In autoimmune disease murine models, it has been demonstrated that determinant spreading may be necessary for

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development of the full autoimmune syndrome (16–20). This epitope spreading occurs in experimental autoimmune encephalomyelitis, which is initiated by immunization of autoantigen peptides, as well as in NOD mice, a spontaneous autoimmune diabetes model. Both models are known to be mediated by T cells. This amplification of the autoimmune response by recruitment of T cells with additional specificities would lead to clonality diversification. Recently, it was reported that a shift towards a high-avidity pathogenic T cell population may be the key event in the progression of benign insulitis to overt diabetes in NOD mice (21). Alternatively, the clonality of the lesion might be restricted toward T cells which are able to accumulate preferentially and to surpass other clones, because of their advantage to expand clonally responding to the antigen released from the destroyed inflamed joint. Whether the clonality in the lesion continues to increase the diversity due to determinant spreading or decrease the variety is especially important in relation to T cell clonotype-targeted therapy. If the clonality becomes restricted, then regulation of a smaller number of clones should be sufficient to achieve antigen-specific immunotherapy. Thus, studies of the dynamics of clonality are of great significance.

So far, studies of T cell clonality have been performed using samples from patients with rather advanced disease. Although information on the dynamics of the clonality during the disease course from the early to the late stage could provide more profound insights into the pathogenic clones, kinetics studies of expanded clones in multiple joints at several time points throughout the disease course are not realistic, because of the difficulty of sampling during the long and heterogeneous clinical time course of RA. It is also difficult to appreciate and to compare the significance of expanded clones among patients, since RA patients are diverse and heterogeneous in terms of the disease severity, duration and modification by treatment.

Transgenic mice (Tg) carrying the env-pX region of the human T cell leukemia virus type I (HTLV-I) genome spontaneously develop chronic inflammatory arthritis as they age (22). The histopathologic characteristics of the joints of arthritic Tg closely resemble those found in patients with RA, showing proliferation of synovial lining cells, infiltration of inflammatory cells with lymph follicle-like structures, and formation of pannus-like granulation tissue with destruction of cartilage and subchondral bone (23). Genes for inflammatory cytokines, including IL-1α, IL-1β, IL-6, tumor necrosis factor-α, transforming growth factor-β1, IFN-γ and IL-2, as well as MHC genes are activated in joints. Moreover, these mice develop autoantibodies against IgG, type II collagen and heat-shock proteins (24). A T cell response to type II collagen (25) and loss of T cell tolerance in the periphery are also demonstrated (26). These pathological features are similar to those of RA. Therefore, the Tg are suitable for investigating T cell clonality during the disease course and its contribution to arthritis.

In the present study, using this spontaneous RA model HTLV-I env-pX Tg, we investigated the dynamics of the T cell clonotype in the affected four feet during the disease progression. We found clonally expanded T cells, some of which were common to all four inflamed feet. Surprisingly, as the disease progressed, the number of common clonotypes among the different feet increased, whereas the total number of clonotypes decreased. Adoptive transfer studies revealed that these common clonotypes of the donor feet migrated into the feet of the recipients, whose arthritis was exacerbated by the transfer. These results provide evidence that T cells in the inflamed joints are rather restricted to certain common clonotypes.

Methods

Mice

HTLV-I env-pX Tg were backcrossed to BALB/c mice for more than eight generations. The mice were maintained under specific pathogen-free conditions in the animal facility of the University of Tokyo, Graduate School of Medicine. All experiments using animals were performed according to the guidelines for animal experiments in our institution.

Clinical evaluation

The severity of arthritis was assessed using a clinical scoring system of 0–3: 0 = normal, 1 = redness and swelling, 2 = deformity, and 3 = ankylosis (27). Each foot was scored and the total score was determined; the maximum possible score per mouse was 12. Tg were divided into three groups by the arthritis stage: early stage (age: 5–10 weeks; score: 0–3), mid stage (age: 4–5 months; score: 6–8) and late stage (age: 7–8 months; score: 10–12).

Analysis of T cell clonality by RT-PCR/single-strand conformation polymorphism (SSCP)

Joint tissues were obtained from the front and back feet of Tg after removal of skin and muscle. Total RNA was prepared with Isogen (Nippon Gene, Tokyo, Japan) and then converted to cDNA with reverse transcriptase (Superscript II; Gibco/BRL, Gaithersburg, MD) and random hexamer oligonucleotide (Gibco/BRL) at 42°C for 2–3 h. PCR was performed with 100–200 ng of cDNA, 50 pmol of each of the 22 V_β primers and 50 pmol of a common C_β primer, dNTPs, and 1 U of Taq polymerase (Takara, Otsu, Japan) for 35 cycles (94°C for 1 min, 54°C for 2 min and 72°C for 3 min) in a Thermal Cycler 9600 (Perkin-Elmer, Norwalk, CT). The sequences of the V_β primers were obtained from published data (28). The sequence of C_β primer was 5′-GGCTCAAACAAGGAG-ACCTTG-3′. SSCP analysis was performed as follows. Amplified DNA was diluted (1:2–1:20) with a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), heat denatured at 90°C for 2 min and electrophoresed on non-denaturing 4% polyacrylamide gels containing 10% glycerol. After electrophoresis, the DNA was transferred onto GeneScreen (NEB, Beverly, MA) and then hybridized with a biotinylated internal common C_β oligonucleotide probe (5′-AGGATCTGAGAAATGTGA-3′). The bound C_β probe was detected using a Phototope-Star detection kit (NEB).

The number of T cell clonotypes was calculated by three researchers independently who did not know the sample sources.

Standardization of TCR mRNA

To adjust the amount of TCR mRNA in spleen to that of joint for SSCP analysis, we conducted real-time quantitative PCR on the iCycler iQ real-time detection system (Bio-Rad, Hercules, CA). PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the amplification of the C_β region, 5′-GGTCTCCTTGTTTGAGCCAT-3′ was used as the 5′ primer and 5′-ACTGATGTTCTGTGTGACAG-3′ as the 3′ primer. The housekeeping gene β-actin (5′-TCACCCACACTGTGCCC-ATCTAC-3′, 5′-ACGATTTCCCTCTCAGCTGTGG-3′) was chosen for internal normalization.

Sequence analysis

TCR V_β genes extracted from SSCP polyacrylamide gels were re-amplified using the same TCR V_β and C_β primer as primary PCR. The re-amplified TCR V_β gene was subcloned to a plasmid vector (pGEM-T Easy Vector; Promega, Madison, MI). Nucleotide sequences of the TCR V_β genes were determined by the dideoxy method using a 310 Genetic Analyzer (Perkin-Elmer/Applied Biosystems, Foster, CA).

Adoptive transfer experiment

Spleens were removed aseptically from arthritic Tg in the late stage and control mice. As a control, we used non-arthritic Tg aged 4–6 weeks which had not developed the arthritis. Single-cell suspensions were prepared by teasing apart the spleens in HBSS and pushing them through a metal sieve with a syringe barrel. After washing with HBSS, $2–5\times10^7$ splenic cells were injected into either young Tg (6–7 weeks) with the onset of overt arthritis (the total score was <1–2) or BALB/c *nu/nu* mice. After 3 weeks, these recipients were sacrificed and then subjected to RT-PCR/SSCP analysis to examine the T cell clonotypes accumulated in the joints.

Statistical analysis

Data was analyzed using statistical software (Statcel, Saitama, Japan) and expressed as the mean ± SEM. The rate of common T cell clonotypes in all four feet and the arthritis score were compared by Student's *t*-test, and the total number of T cell clonotypes by Mann–Whitney's *U*-test.

Results

Accumulation of T cell clonotypes in arthritic joints of HTLV-I env-pX Tg

Arthritis in the Tg was discerned as swelling and redness of the footpad, including the ankle. The abnormality began to occur at age 4–8 weeks and thereafter the grade of arthritis increased as the animal aged. The clinical score finally reached the maximum at ~6 months (Fig. 1). Thus, since the disease severity was closely related to the age, we divided Tg into three groups according to their age, i.e. early stage (age: 5–10 weeks; score: 1–3), mid stage (age: 4–5 months; score: 6–8) and late stage (age: 7–8 months; score: 10–12).

We analyzed the T cell clonotypes infiltrating the joints of all four feet from HTLV-I env-pX Tg at 5 months (the mid stage) using the TCR/RT-PCR/SSCP method (Fig. 2). While the spleen exhibited smear patterns indicating a diverse T cell population consisting of heterogeneous CDR3 regions of TCR, accumulation of several distinct bands was observed in the feet. These results indicate that T cells expand oligoclonally in the joints of all four feet, as has been shown for the joints of RA patients.

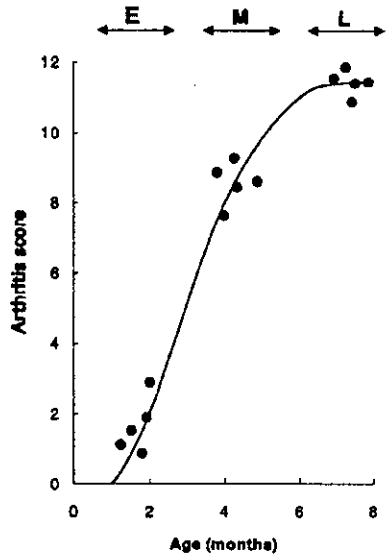

Fig. 1. Arthritic score increases with age of HTLV-I env-pX transgenic mice. The arthritic score starts to increase at age 4–8 weeks and reached the maximum score at ~6 months (n = 64). The arthritis was divided into three groups (early, mid and late stages) and five mice (solid circles) in each stage were used for T cell clonality analysis. E: early stage; M: mid stage; L: late stage.

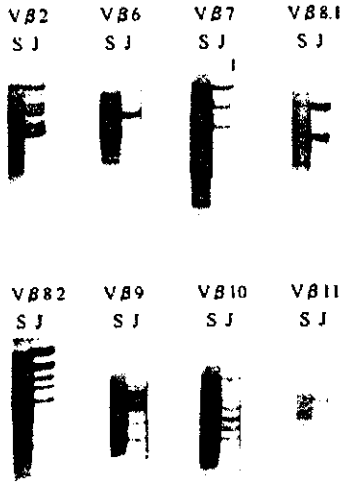

Fig. 2. Accumulation of T cell clonotypes in arthritic joints of HTLV-I env-pX transgenic mice analyzed by the RT-PCR/SSCP method. S: spleen. J: joint. TCR mRNA of spleen and joint were standardized by C_β products as described in Methods. The amount of TCR mRNA from all joints corresponded to ~1/16 of that from spleen. Thus, SSCP analysis was performed with PCR products using cDNA of spleen diluted by 1:16.

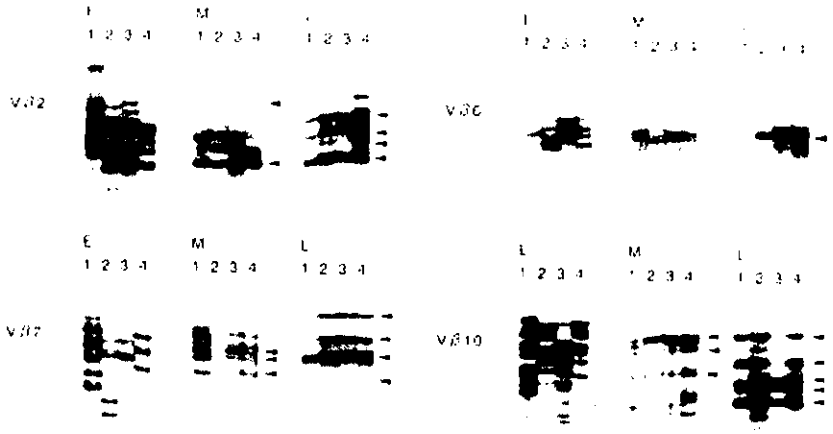

Fig. 3. Comparison of T cell clonotypes in the early, mid and late stages of arthritis. Lanes from 1 to 4 represent the right front foot, left front foot, right rear foot and left rear foot respectively. E: early stage; M: mid stage; L: late stage. Arrows indicate bands with identical migration in all four feet.

Table 1. Number of distinct T cell clonotypes accumulated in four feet of Tg in the early, mid and late stages[a]

Arthritis stage	V_β gene family																				Mean ± SEM	Percentage of common T cell clonotypes
	1	2	3	4	5.1	5.2	6	7	8.1	8.2	8.3	9	10	11	12	13	14	15	16	17	18	
E	–	0/8	–	0/7	–	–	1/6	1/10	0/7	1/7	2/6	0/7	3/12	0/5	–	1/7	1/7	2/8	1/4	1/7	2/6	1.0 ± 0.2/7.1 ± 0.5	14.1
M	–	3/7	–	1/8	–	–	1/4	1/10	3/6	2/5	3/4	2/8	4/11	1/5	–	2/7	1/10	0/7	2/6	0/9	1/4	1.7 ± 0.3/6.9 ± 0.6	24.6
L	–	4/5	–	1/6	–	–	3/3	4/5	3/4	4/5	4/4	5/9	8/10	2/4	–	2/6	1/7	3/7	2/4	3/10	1/5	3.1 ± 0.4/5.9 ± 0.5[b]	52.5

[a]T cell clonotypes were analyzed using three mice in each stage. Results are shown as common clonotypes/total clonotypes per foot (total number of bands in all four feet detected and the number of bands common to four feet were counted, and then divided by the number of feet to represent the value per foot). E: early stage; M: mid stage; L: late stage. –: bands were faint or not detected.
[b]$P < 0.05$.

The number of common T cell clonotypes increased, whereas the total number of T cell clonotypes decreased as the disease advanced

To investigate the change in T cell clonality during the progression of arthritis, we compared T cell clonotypes in the early, mid and late stages. The T cell clonotypes which accumulated in each joint of the four feet showed different mobility in the early stage, but they migrated to an identical position in the late stage (Fig. 3). Bands with the same mobility on SSCP gels had been demonstrated to possess the same nucleotide sequences (13,29,30) and we ourselves always confirm this rule by DNA sequencing. Therefore, bands with identical migration in SSCP represent identical clones.

We counted the total number of T cell clonotypes detected in each foot and the number of common clonotypes among the four feet. The results are summarized in Table 1. The proportion of T cell clonotypes common to all four feet was low in each V_β repertoire in the early stage (average 14.1%). However, it increased during progression of the stage. This increase during the transition from the early to late stage was observed in many V_β repertoires (V_β 2 and 6, $P < 0.0001$; V_β7, $P < 0.001$; V_β8.1 and 8.2, $P = 0.001$; V_β8.3, 10, 11 and 15, $P < 0.05$). Finally in the late stage, a high rate of >70% was found for V_β2, 6, 7, 8.1, 8.2, 8.3 and 10 (Fig. 4). Of interest, in contrast to the common clones, the total number of T cell clonotypes accumulated in joints decreased as the disease advanced ($P < 0.05$) (Table 1). In particular, V_β2, 6, 7 and 8.1 are striking, showing that the total number of T cell clonotypes in the late stage decreased by 40–50% compared with in the early stage. Taken together, the T cell clonotypes became identical while reducing their variety during progression of arthritis.

To examine amino acid sequences of the CDR3 region in common T cell clonotypes among four feet, DNA encoding the V_β genes were collected from SSCP gels and sequenced. As shown in Table 2, conserved amino acid motifs were found, e.g. QGW in the CDR3 region of V_β2 clones, RGTG in V_β9, and SXTGG, LTGG, QGA and YRG in V_β10. In addition, certain conserved amino acid motifs, such as RSG in V_β2 and V_β9, DWG in V_β2 and V_β10, and IQG and QGA in V_β10 and V_β11, were observed in the CDR3 region of different V_β clones. This sequence information implies the possibility that T cell clonotypes in multiple joints might recognize some common epitopes on antigens.

The common clones were able to migrate into the joints of the recipients

In order to investigate the pathological significance of the common clonotypes in four feet, we conducted an adoptive

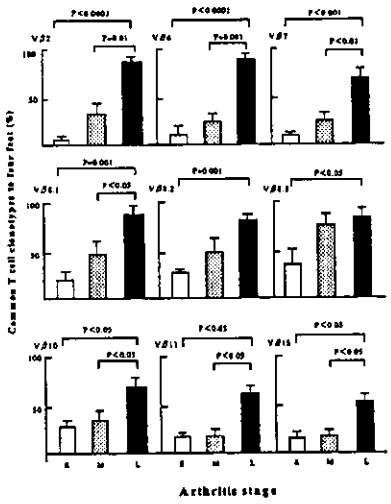

Fig. 4. The rate of common T cell clonotypes in all four feet increased as HTLV-I env-pX transgenic mice aged. The proportions of common T cell clonotypes in all four feet in the early, mid and late stages are demonstrated. Data given as the percentage of the number of bands common to all four feet against the total number of bands in all four feet. Values are expressed as the mean ± SEM (n = 5).

Table 2. The TCR VDJ region of common T cell clonotypes in four feet

V_β		N-D_β-N		J_β
V_β2	CSA	QGW	EQ	J_β2.6
V_β2	CSA	RSGRE	DTQ	J_β2.5
V_β2	CSA	RGI	SNERL	J_β1.4
V_β2	CS	IGQGW	GNTL	J_β1.3
V_β2	CS	EDWGG	AETL	J_β2.3
V_β9	CAS	RSGTI	SNERL	J_β1.4
V_β9	CAS	RGTGAN	TQ	J_β2.5
V_β9	CASS	RTGRG	EQ	J_β2.6
V_β9	CASS	RDR	SDY	J_β1.2
V_β9	CASS	RGTGQ	SYEQ	J_β2.6
V_β10	CASS	YQGAL	NQDTQ	J_β2.5
V_β10	CASS	VDWGD	QDTQ	J_β2.5
V_β10	CASS	SLTGG	YEQ	J_β2.6
V_β10	CASS	SGTGG	YEQ	J_β2.6
V_β10	CASS	IQGA	NSDY	J_β1.2
V_β10	CASS	YRGI	EQ	J_β2.6
V_β10	CASS	LTGGAD	TL	J_β2.4
V_β10	CASS	YRGP	NERL	J_β1.4
V_β11	CASS	LGQT	NSDY	J_β1.2
V_β11	CASS	SQGA	EV	J_β1.1
V_β11	CASS	IQG	TEV	J_β1.1

Underlined letters indicate conserved amino acid motifs.

transfer experiment. First, splenocytes derived from an arthritic Tg were transferred into nude mice. The joints of the feet of the recipients showed just mild swelling transiently. Nevertheless, clonotype analysis revealed that some common clones of the donors migrated into the feet of the recipients (Fig. 5).

Since non-T cell components are considered to be indispensable for inducing the persistent arthritis (31), we selected young Tg as recipients. In each experiment, splenic cells either from an arthritic Tg (experimental group) or a non-arthritic Tg (control group) were transferred into two young Tg with the onset of arthritis. Three weeks after the transfer, the arthritis score of the recipients from an arthritic Tg was significantly higher than that of recipients from non-arthritic Tg and that of non-treated Tg (Fig. 6). This result suggests that the progression of arthritis was accelerated by the transfer of splenocytes from arthritic mice and that the T cells from the donors were involved in the exacerbation of the arthritis. Three weeks after the transfer, these recipients were sacrificed and subjected to T cell clonality analysis. Some identical T cell clonotypes were detected in the donor and the two recipients, indicating that these identical T cell clonotypes accumulated in the joints of the recipients originated from the donors (Fig. 7). Since the smear pattern of the clonotype in the spleen of the recipients suggests that the degree of clonotype expansion is different between the joints and spleen, and since the common T cell clonotype between all four feet of the donor and recipient was not dominant in the spleen, we were able to rule out the possibility that the clonotype in joints simply reflects that of the spleen.

To verify that the clonotypes common to the donors and the recipients possess the identical TCR V_β chain sequences in transfer experiments, DNA was recovered from the bands with

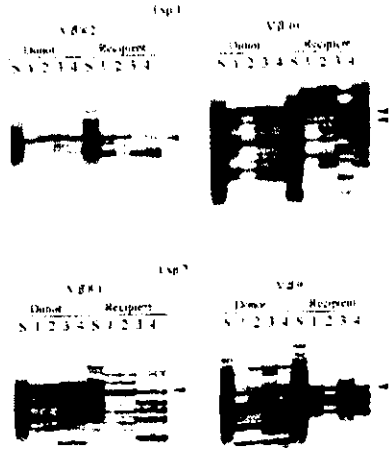

Fig. 5. The clonotype common to all four feet of a Tg donor migrated to the joints of nude mice. In each experiment, splenocytes from an arthritic Tg were transferred into a nude mouse recipient. Nude mice were sacrificed at 3 weeks after the cell transfer and subjected to T cell clonotype analysis. Representative V_β panels from two independent experiments are demonstrated. S: spleen. Lanes from 1 to 4 represent the right front foot, left front foot, right rear foot and left rear foot respectively. The common clones are indicated by the arrows. TCR mRNAs of spleen and joint were standardized by C_β products as described in Methods. The amount of TCR mRNA from all joints corresponded to ~1/10 of that from spleen.

same mobility on SSCP gels and sequences were determined. Table 3 shows the DNA sequences of SSCP bands from a donor and recipient, shown by arrows in Figs 5 and 7. As expected, the sequences were identical between the donor and recipient. These findings indicate that some of the common clonotypes in four feet of the donor infiltrated again

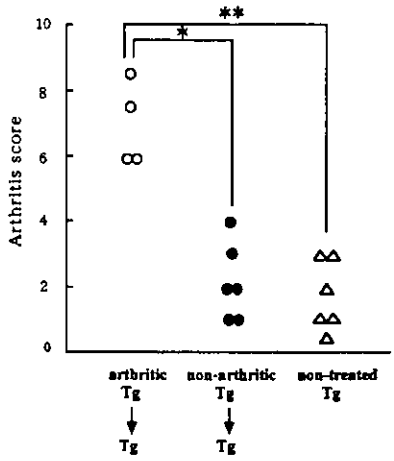

Fig. 6. Young Tg developed more severe arthritis after receiving splenocytes from an arthritic Tg. Splenocytes from an arthritic Tg or a non-arthritic Tg were transferred to young Tg on clinical onset, as described in Methods. The arthritis score at 3 weeks after cell transfer was judged. Note that the score of the recipients from an arthritic Tg (open circles) was significantly higher than that of recipients from a non-arthritic Tg (sold circles) and that of the non-treated Tg (open squares) ($^*P < 0.001$, $^{**}P < 0.0005$).

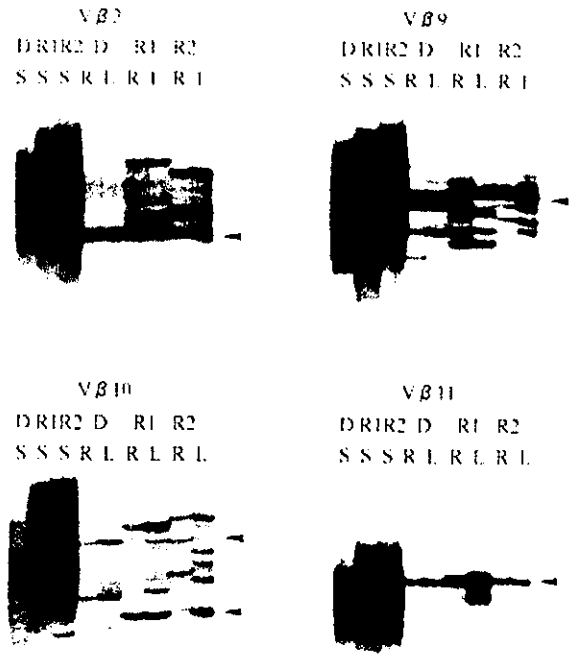

Fig. 7. The clonotype common to all four feet of an arthritic Tg donor migrated to the joints of young Tg recipients. Two young Tg were used as recipients in a transfer experiment. Recipients were sacrificed at 3 weeks after cell transfer and subjected to T cell clonality analysis. D: donor; R1: recipient 1; R2: recipient 2; S: spleen; R: right rear foot; L: left rear foot. Arrows indicate common T cell clonotypes among feet of the donor and two recipients. The amount of TCR mRNA from all joints corresponded to ~1/8 of that from spleen.

into the joints of the recipient. There were no clear conserved amino acids among migrated clonotypes in the amino acid sequences of the CDR3 region.

Discussion

We studied the T cell clonotypic change in arthritic joints of HTLV-I env-pX Tg during the development of the disease. In the early stage of the disease, T cell clonotypes diversified among the feet. On the contrary, in the late stage, most of the clonotypes were common to all four feet. The total number of accumulating clones decreased compared with in the early stage. Thus, the T cell clonotypes became identical and decreased in variety as the disease progressed. Some of these common clones remaining in the late stage joints might be arthrotropic, since the transfer studies revealed their ability to migrate into the joints. Due to the restricted availability of clinical samples, it might have been difficult to know the dynamic changes of clonality, which are diverse at the initial stage and finally are restricted to common clonotypes.

Analysis of T cell clonality in the joints of RA patients as well as in the affected organs of autoimmune disease animal models demonstrated the presence of common clonotypes among multiple joints or among different parts of an organ (13,28). Moreover, the amino acid sequences of TCR V_β junctional regions of clones which are identical in multiple joints of a patient were found to be the same as the sequences of the common clones of different patients (14). Considering that CDR3 interacts with the peptides presented by MHC molecules (32–34), these identical clones might recognize certain common antigens which are involved in the pathogenesis of RA. Our identification of common clonotypes in the four arthritic feet and some conserved CDR3 motifs among them is consistent with these previous findings.

In our present study of HTLV-I env-pX Tg, the accumulation of common clonotypes was distinct after the mid stage, whereas in the early stage it was not so pronounced. How can this change in clonality during disease progression be explained?

Several studies have implicated epitope spreading in the pathogenesis of autoimmune diseases (16–20). Disease progression is associated with a shifting of T cell autoreactivity from primary initiating self-antigenic determinants to defined cascades of secondary determinants that sustain the self-recognition involved in disease perpetuation. Intramolecular as well as intermolecular epitope spreading could presumably occur by antigen presentation of autoantigens derived from destroyed tissues (35–37). As recruitment of T cells specific for additional determinants continues, T cell clonality should become diversified. In our experiment, the early stage might fit the epitope-spreading model. The recruitment of T cells with other specificity proceeded independently in each joint. Since the time of onset of the arthritis is different among the four feet, it is also possible that the degree of autoimmune response propagation differs in each foot, resulting in the accumulation of different clones in each joint. The autoantigens driving the autoimmune response in each joint may differ in this stage. However, epitope spreading may not explain the T cell behavior in our arthritis model throughout the entire course of the disease, since the total number of T cell clones

Table 3. The TCR VDJ region of T cell clonotypes common to donor and recipient in the transfer experiments[a]

	V_β	N-D_β-N	J_β	
(A)				
Exp. 1				
V_β8.2	TGTGCCAGC	GGTGATTCCGGGACAG	CAAACTCCGACTAC	J_β1.2
	C A S	G D S G T A	N S D Y	
V_β10	TGTGCCAGCAGC	TTACTGGGGGGGCGC	AGTGCAGAAACGCTG	J_β2.3
	C A S S	L L G G R	S A E T L	
	TGTGCCAGCAGC	TTGGGGACT	AGTGCAGAAACGCTG	J_β2.3
	C A S S	L G T	S A E T L	
Exp. 2				
V_β8.1	TGTGCCAGCAG	CCGACAGTTT	TCCAACGAAAGATTA	J_β1.4
	C A S S	R Q F	S N E R L	
V_β9	TGTGCTAGCAGT	AGACAGGGG	TATGAACAG	J_β2.6
	C A S S	R Q G	Y E Q	
(B)				
V_β2	TGCAGTG	CTCAGGGGTGG	GAACAG	J_β2.6
	C S A	Q G W	E Q	
V_β9	TGTGCTAGCAGT	AGAACAGGGAGAGGT	GAACAG	J_β2.6
	C A S S	R T G R G	E Q	
	TGTGCTAGCAG	GGGCACAGGGGCAA	ACACCCAG	J_β2.5
	C A S	R G T G A N	T Q	
V_β10	TGTGCCAGCAGC	TACCAGGGGGCACTT	AACCAAGACACCCAG	J_β2.5
	C A S S	Y Q G A L	N Q D T Q	
V_β11	TGTGCAAGCAGC	ATACAGGGA	ACAGAAGTC	J_β1.1
	C A S S	I Q G	T E V	

[a]The similar electrophoretic mobility bands in donor and recipient indicated by arrows in Figs 5 and 7 were cut out and subcloned after PCR amplification. Their sequences were determined. (A) Transfer experiment in nude mice. (B) Transfer experiment in young Tg.

decreased and the identity of clonality increased with disease progression.

It is shown that the TCR repertoire of memory cells which expand selectively during the recall response are rather restricted (38). Thus, such clones might be increasing while an autoantigen repetitively stimulates and drives the (pathogenic) immune response. Adding to this, some autoreactive T cell clones might be inactivated or deleted by regulatory mechanisms. Therefore, we may reason that the number of accumulating clones in the lesion of an autoimmune disease might gradually decrease, leaving clones which are able to expand more efficiently or are less susceptible to cell death.

Alternatively, the clonal restriction in the joints might reflect avidity maturation of a T cell population. Amrani *et al.* demonstrated that a shift towards a high-avidity pathogenic T cell population may be the key event in the progression of benign insulitis to overt diabetes using NOD mice (21). Applying this hypothesis, we tend to prefer that some of the remaining common clonotypes have an advantage in clonal expansion due to their higher avidity for certain autoantigens and are related to the inflammation of the joints. However, it is too early to conclude that the remaining common clonotypes are pathogenic, since other clones might also be involved in the exacerbation of young Tg arthritis after the transfer.

Nevertheless, some of them are at least arthrotropic since they migrated to the joints of the recipients, implying that they recognize a certain autoantigen in the joint. Since it has indeed been demonstrated that dominant T cell clones in Tg recognize collagen type II (25), Tax and Env of the pX products (39), it would be of great interest to investigate whether these common clones respond to those antigens.

Taken together, during the perpetuation of arthritis, T cell clonality decreased in variety and was restricted to the clones, some of which are arthrotropic and might be related to the pathogenesis of arthritis. This 'clonal restriction' phenomena should be verified in other autoimmune diseases and its models to develop antigen-specific immunotherapy targeting certain common T cell clonotypes.

Acknowledgements

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Abbreviations

HTLV-I	human T cell leukemia virus type I
RA	rheumatoid arthritis
SSCP	single-strand conformation polymorphism
Tg	transgenic mouse

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