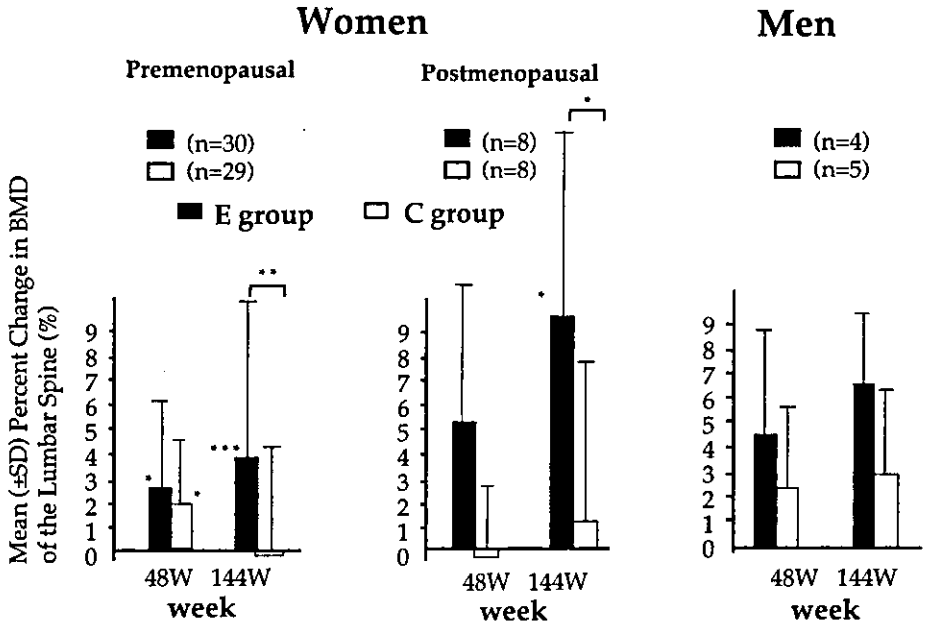

Figure 2. The mean (± SD) percentage change in BMD of the lumbar spine in analysis of subgroups (premenopausal women, postmenopausal women, men). The postmenopausal women of Group E showed the greatest improvement in mean percentage change of lumbar spine BMD at 144 weeks. *p < 0.05, **p < 0.01, ***p < 0.005.

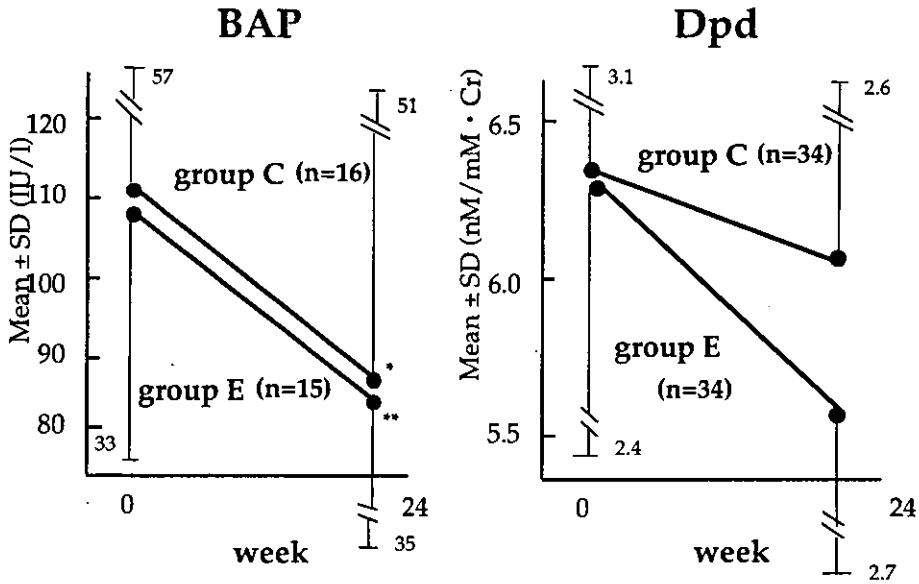

Figure 3. The change (mean ± SD) of serum bone-specific alkaline phosphatase (BAP, left) and urinary deoxypyridinoline (Dpd, right) between baseline and 24 weeks. The mean (± SD) percentage change in serum BAP concentration was significantly decreased at 24 weeks in Group E (p < 0.01). *p < 0.05, **p < 0.01.

A reduction of vertebral fractures was observed, but this did not reach statistical significance.

When we compared the lumbar spine BMD at baseline in 88 women enrolled in the study to the average of age matched normal individuals, 71 (81%) were below the average. In particular, among women younger than 40 years of age, 6 out of 30 (20%) met the criteria for osteoporosis and 10 (33%) met the criteria for osteopenia. These findings suggest that the decrease in BMD was mainly due to the corticosteroid therapy in patients with connective tissue diseases. Because such cases are thought to have a greater risk of future fractures, urgent intervention is required.

Table 2. Incidence of vertebral fractures. Data are number of patients with new vertebral fractures/number of patients who could be evaluated.

	Group E, n = 30	Group C, n = 31
Men	0/3	0/5
Women		
Premenopausal	0/20	0/18
Postmenopausal	0/7	2/8
Total no. of vertebrae fractured	0	3

Bisphosphonates are analogs of inorganic pyrophosphate that inhibit osteoclastic activity, potentially resulting in improved BMD. Etidronate, a first-generation bisphosphonate, has been widely used in various metabolic bone diseases, including Paget's disease of bone[18], hypercalcemia due to malignancy[19], and osteoporosis. It has been reported that etidronate increases the BMD in senile osteoporosis, postmenopausal osteoporosis, and corticosteroid induced osteoporosis[1-7,10,11,13,20,21].

Continuous etidronate therapy has been shown to lead to a mineralization defect in bone, and only inhibits the decrease in BMD and does not produce a distinct increase in BMD. Therefore, it has been suggested that intermittent cyclical etidronate therapy is more effective. A randomized, double-blind, multicenter study to determine the ability of intermittent cyclical etidronate therapy in Japanese patients with involutional osteoporosis[20] indicated prevention and treatment efficacy in both groups that received 200 mg and 400 mg of etidronate. The current approved dose of intermittent cyclical etidronate for treatment of osteoporosis is 200 mg daily in Japan. For this reason, we chose intermittent cyclical therapy, 200 mg of etidronate for 2 weeks with 3.0 g calcium lactate and 0.75 μg alphacalcidol daily for 10 weeks. For ethical purposes, we used 3.0 g calcium lactate and 0.75 μg alphacalcidol daily instead of placebo for our control group. As studies have shown, 0.75 to 1.0 μg alphacalcidol daily was effective in maintaining BMD in patients with osteoporosis compared with placebo[22].

Several randomized and nonrandomized trials have indicated that intermittent cyclical etidronate therapy improved the decrease in BMD in patients with corticosteroid induced osteoporosis[1-7,10,11,13,20,21,23]. Our study is consistent with these findings and demonstrated that etidronate is effective in Japanese patients with corticosteroid induced osteoporosis, as well as in Caucasians. Moreover, this is the first comprehensive study of the efficacy of etidronate 200 mg taken cyclically for 3 years in Japanese patients with corticosteroid induced bone loss.

In our study, the mean (± SD) percentage change in BMD of the lumbar spine increased in Group C at 48 weeks and 144 weeks. This result is significant, as it may show the effect of activated vitamin D_3 in patients with corticosteroid induced osteoporosis, although further longterm observation is needed as the mean percentage change of BMD decreased at 144 weeks compared to 48 weeks in Group C.

Recently, there has been increasing interest in activated vitamin D therapy as monotherapy for corticosteroid induced osteoporosis[24-27]. However, the combination therapy of activated vitamin D with etidronate seemed to be useful because this therapy will minimize the capacity for stimulating bone resorption, while leaving the beneficial effects on intestinal calcium absorption intact. Some reports describe that the combination of activated vitamin D with

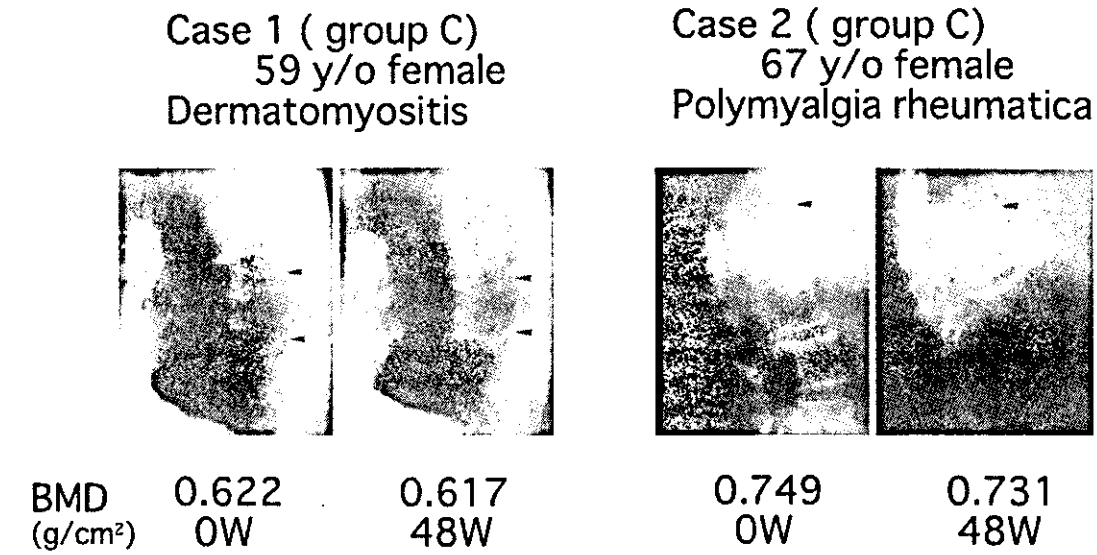

Figure 4. Radiographic change in 2 patients in Group C who had new vertebral compression fractures: in Case 1 in 6th and 8th thoracic vertebrae; in Case 2 in 12th thoracic vertebra.

bisphosphonate is better than bisphosphonate alone[23]. Our results support the combination therapy of activated vitamin D with etidronate as more effective for corticosteroid induced osteoporosis than monotherapy with activated vitamin D preparations.

In analysis of our subgroups, the groups of postmenopausal women and those with osteoporosis + osteopenia of Group E showed the greatest improvement in mean percentage change of BMD. Although the greatest increases in BMD occurring in the osteoporosis + osteopenia subgroup may be accounted for in part by the fact that their baseline BMD is lower, there was a significant increase in bone turnover, as bone absorption was very fast in these groups; and the inhibitory effect of etidronate on bone resorption would correct the bone turnover in a greater degree, causing an increase of BMD. A comparison of BMD in female patients with osteoporosis or osteopenia between Group E and Group C showed an increase in lumbar spine BMD in Group E.

Markers of bone turnover, such as serum BAP and urinary Dpd, were decreased at 24 weeks and correlated with previous reports of intermittent cyclical etidronate therapy[3,5,13]. The change of the urinary Dpd value was of lesser magnitude in Group C than in Group E, and it was thought that the effect of inhibition on bone turnover was mild in Group C.

It was notable that no new vertebral fractures were seen in Group E, although the number of patients was very limited, and there was no significant difference in this respect between groups E and C. The patients who had new vertebral fractures in Group C showed a decrease in lumbar spine BMD at 48 and 144 weeks, suggesting some correlation between the decrease of BMD and the incidence of vertebral fractures.

The trend towards reduction in the incidence of vertebral fractures in this study is comparable with that previously seen[3,10,20] for etidronate therapy in corticosteroid induced osteoporosis and greater than that seen[16,20] in the prevention of vertebral fractures with vitamin D therapy alone. The frequency of vertebral fractures was low in Group C compared to that of placebo groups in previous studies[3,10], and there might be the possibility of the effect to prevent vertebral fractures of lumbar spine occurring in Group C in our study.

We chose to administer a 200 mg daily dose of etidronate. Etidronate caused few adverse effects. In Group E, the headache and facial rash were improved when the patients discontinued etidronate. No upper GI symptoms were experienced by either group. The incidence rate of upper GI events was thought to be dose-dependent[20] and the incidence of upper GI adverse experiences seemed to be low with patients receiving 200 mg etidronate. However, further study will be required to compare the differences in the effect of the prevention of lumbar spine bone loss and vertebral fracture and in the frequency of adverse events between etidronate 200 mg daily and 400 mg daily.

In conclusion, the intermittent administration of etidronate in patients taking corticosteroids significantly increased BMD of the lumbar spine, especially for postmenopausal women and patients with osteoporosis + osteopenia, and the increase was maintained over 3 years. There was a nonstatistically significant reduction in the incidence of vertebral fractures at 3 years between the control and etidronate groups, and no patient in the etidronate group had new fractures. Thus intermittent cyclical etidronate therapy was effective and safe for the prevention and treatment of corticosteroid induced osteoporosis in Japanese patients with connective tissue diseases.

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Pseudoscleroderma associated with transforming growth factor β1–producing advanced gastric carcinoma: comment on the article by Varga

To the Editor:

We read with interest the review article by Varga (1), in which modulation of transforming growth factor β (TGFβ)–mediated profibrotic action by the Smad protein family was discussed. In systemic sclerosis (SSc), inflammatory cells infiltrating lesional tissues secrete TGFβ, which also stimulates connective tissue growth factor (CTGF) secretion in lesional fibroblasts (1). TGFβ, however, is also secreted from other cells, such as tumor cells. In particular, expression of TGFβ1 is detected in 22.8–35.9% of patients with gastric carcinoma (2,3). Here we discuss a patient in whom severe proximal scleroderma, esophageal dysmotility, and bibasilar pulmonary fibrosis developed along with development of TGFβ1–producing gastric carcinoma.

In December 2000, an 83-year-old Japanese man noticed sclerotic skin changes in his neck and upper/lower extremities and visited the dermatology clinic of Keio University Hospital. A skin biopsy specimen obtained from his right thigh showed hyperproliferation of swollen, homogeneous collagen fibers. The histologic skin changes were identical to those of SSc but not of scleredema. He did not present with Raynaud's phenomenon, sclerodactyly, digital pitting scar, or telangiectasia. Because the sclerotic skin changes subsequently spread to his upper chest, abdomen, back, and buttocks, he was admitted to the rheumatology section in our hospital. His total skin thickness score (TSS) (4) was 66 (based on a possible total score of 104). A blood test revealed no evidence of diabetes mellitus. Serum antinuclear antibodies and rheumatoid factor were not detected, but a slight increase in the white blood cell count (10,000/mm^3), the erythrocyte sedimentation rate (18 mm/hour), the C-reactive protein level (0.5 mg/dl), and the IgG level (2,290 mg/dl) was observed. Serum levels of interleukin-6 (IL-6) (5.41 pg/ml; normal <4.62) and plasma TGFβ1 (2.15 ng/ml; normal <1.80) as determined by enzyme-linked immunosorbent assay were also elevated. Gastrointestinal examination detected severe esophageal dysmotility and gastric carcinoma. An endoscopic biopsy of the gastric carcinomatous lesion showed poorly differentiated adenocarcinoma. A full-body computed tomography scan indicated bibasilar pulmonary fibrosis but no evidence of lung or abdominal metastasis. Two months after total gastrectomy, the patient's esophageal dysmotility and pulmonary fibrosis had not changed, but his TSS improved to 29. The serum level of IL-6 was normalized (1.30 pg/ml), and plasma TGFβ1 in post-tumor resection was decreased (1.88 ng/ml).

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of samples from the resected stomach was performed (5). Total RNA from specimens obtained from both carcinomatous and normal sites (Figure 1A) was isolated by guadidinium thiocyanate–phenol–chlorform extraction. Complementary DNA (cDNA) amplification for β-actin (541 bp) was performed for 30 cycles, with an annealing temperature of 58°C. Amplification of cDNA for TGFβ1 (247 bp) was performed for 35 cycles, with an annealing temperature of 65°C. Primer sequences for β-actin and TGFβ1, respectively, were as follows: forward 5′-GTG-GGG-CGC-CCC-AGG-CAC-CA-3′, reverse 5′-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3′; forward 5′-AAG-TGG-ATC-CAC-GAG-CCC-AA-3′, reverse 5′-GCT-GCA-CTT-GCA-GGA-GCG-CA-3′. Aliquots of the PCR products (7.5 μl) were separated and visualized with ethidium bromide staining after electrophoresis on a 1.5% agarose gel in Tris–acetate–EDTA buffer at 100V for 20 minutes (Figure 1B). TGFβ1 messenger RNA (mRNA) was detected in the carcinomatous site but not in the normal gastric site.

Sclerotic skin diseases resembling SSc and occurring in patients with malignant tumors or other diseases are sometimes referred to as pseudoscleroderma or pseudosclerosis (6,7). Because the distribution of skin sclerosis in our patient was different from that of SSc, he was diagnosed as having pseudoscleroderma associated with an advanced gastric carcinoma. Querfeld et al described a patient with pseudoscleroderma associated with lung cancer, in whom expression of collagen α1 and CTGF mRNA were markedly increased in fibroblasts scattered throughout the dermis (7). Although acrosclerosis and nailfold changes were observed in that patient, TGFβ1 expression in the lesional skin was not detected, and which tissue secreted collagen α1– or CTGF-stimulating factors such as TGFβ was not determined. The patient described by Querfeld et al had high titers of antinuclear antibodies and IgM anticardiolipin antibodies but not scleroderma-specific autoantibodies. Our experience together with that of Querfeld et al suggests that certain tumor cells secrete soluble factors that induce SSc-mimicking skin changes and organ involvement.

In our patient, removal of TGFβ1-producing tumor cells resulted in the amelioration of sclerotic skin changes. This is the first report of pseudoscleroderma associated with

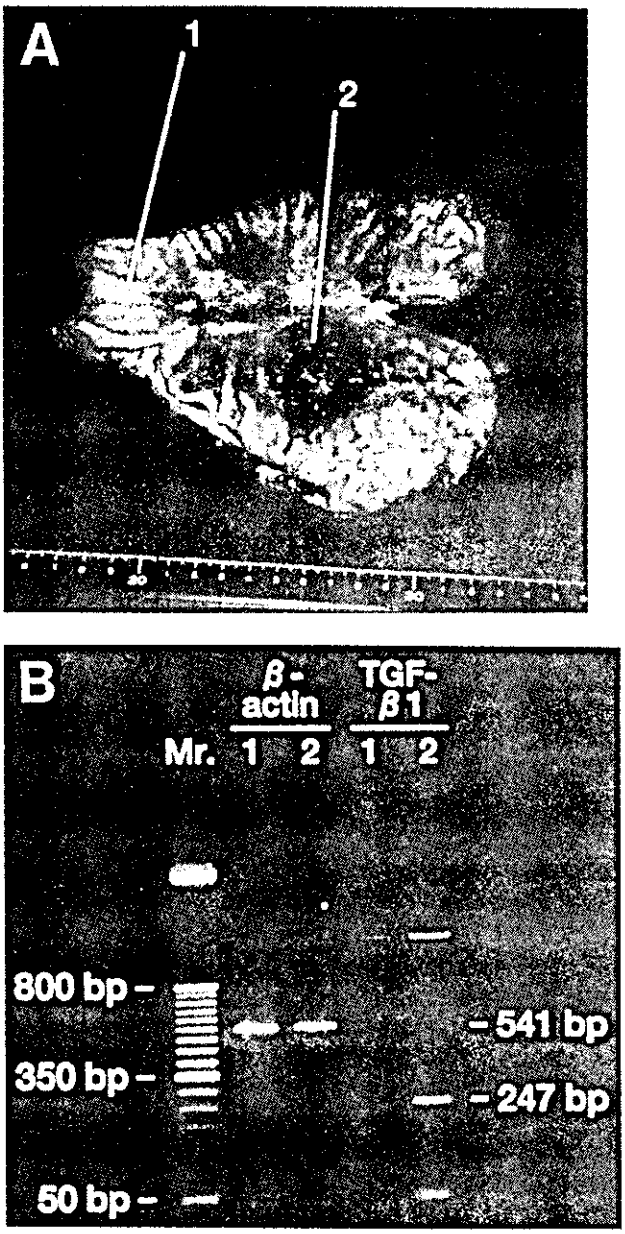

Figure 1. A. Normal (1) and carcinomatous (2) sites in the resected stomach. **B,** Reverse transcription–polymerase chain reaction analysis. Transforming growth factor β1 (TGFβ1) was detected (247 bp) in the sample obtained from the carcinomatous site but not in that obtained from the normal site. β-actin (541 bp) was amplified in both samples.

TGFβ1-secreting gastric carcinoma. Further study of the abnormal up-regulation of TGFβ1 (including the latent form of TGFβ1) in malignant tumors is needed to clarify this association.

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DOI 10.1002/art.11146

Reply

To the Editor:

The report by Fujii et al describes an elderly man with pseudoscleroderma who was found to have adenocarcinoma of the stomach. The diagnosis of pseudoscleroderma was based on the atypical distribution of skin changes (involving the neck, trunk, and extremities, but sparing the fingers), histopathologic findings in the skin, and the absence of Raynaud's phenomenon or serologic features of SSc. Following gastrectomy, the extent of skin involvement decreased by half, and serum TGFβ levels declined from 2.15 ng/ml to 1.88 ng/ml. Because TGFβ mRNA was detected by RT-PCR in the resected gastric tissue, the authors propose the intriguing hypothesis that tumor-derived TGFβ1 may have contributed to the development of widespread skin fibrosis in this patient, and that clinical improvement was attributable to a decline in TGFβ1 secretion. A previous report described concurrent pseudoscleroderma and lung cancer (1), but the tumor was not examined for TGFβ secretion, and Gruber et al reported a case of SSc associated with TGFβ-secreting adenocarcinoma of the lung (2).

TGFβ is preeminent among the multiple cytokines and growth factors implicated in the pathogenesis of fibrosis. In cancer, TGFβ has 2 paradoxical roles: as a tumor suppressor in the early stages but as a tumor promoter in later stages (3). Could tumors that secrete TGFβ induce pseudoscleroderma

and similar disorders? Despite the fact that 20% of gastric adenocarcinomas and other tumors have been shown to produce TGFβ (4), their association with scleroderma-like conditions appears to be rare. Thus, the literature does not support an association between TGFβ-producing tumors and scleroderma, although it is possible that in some cancer patients the skin changes are overlooked. In addition to pseudoscleroderma, palmar fasciitis has been described as a paraneoplastic complication (5). It is intriguing to speculate that tissue fibrosis in some patients with malignancies may be attributable to TGFβ production by tumors.

In summary, a causal relationship between pseudoscleroderma and TGFβ1 production by adenocarcinomas is proposed but remains unproven. It is tempting to consider that TGFβ secreted by certain tumors may induce scleroderma or related fibrotic disorders in some individuals. An important lesson is that patients who present with atypical features of scleroderma merit careful screening and continued monitoring for cancer.

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Emerging clinical spectrum of tumor necrosis factor receptor–associated periodic syndrome: comment on the articles by Hull et al and Dodé et al

To the Editor:

We read with interest the recent reports by Hull et al (1) and Dodé et al (2). We herein describe a family with the R92Q *TNFRSF1A* mutation, thereby widening the debate initiated by Hull and colleagues regarding the pathologic cause of myalgia in tumor necrosis factor receptor–associated periodic syndrome (TRAPS). Our report also demonstrates a wide spectrum of inflammation manifestations compatible with the proposed enlarging clinical spectrum of TRAPS reported by Dodé et al. However, we suggest the need to clarify diagnostic criteria for TRAPS, particularly in patients with low-penetrance *TNFRSF1A* mutations.

The patient, a 37-year-old man (index case), presented with a 15-year history of inflammation symptoms of unknown cause. At age 22 years he developed bilateral synovitis of the knees. Seven years later, he developed recurrent abdominal pain, testicular pain, breathlessness, myalgia, and fevers, followed by an erythematous rash. Analysis of the *TNFRSF1A* gene revealed the R92Q mutation, confirming the diagnosis of TRAPS. Muscle biopsy revealed thickening of small blood vessel walls only, without evidence of myositis or fasciitis. Corticosteroid treatment was ineffective, although twice-weekly administration of subcutaneous etanercept improved the patient's recorded symptoms.

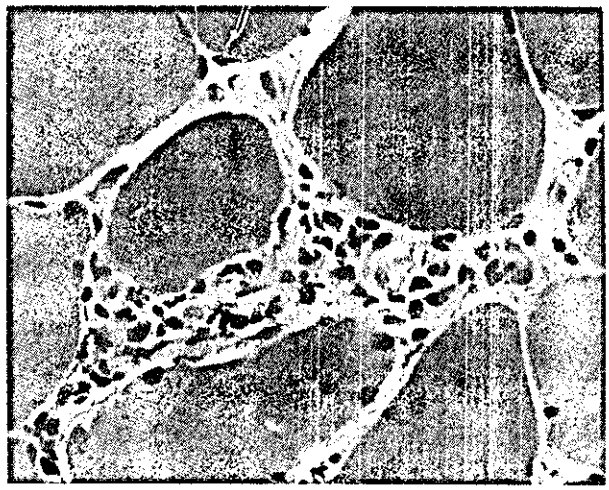

Figure 1. Lymphocytic vasculitis of muscle with the *TNFRSF1A* mutation.

The patient's 64-year-old father, who also had the R92Q *TNFRSF1A* mutation, was subsequently evaluated. He reported a 16-year history of increasingly severe but constant lower limb muscle cramps and pain, but no history of recurrent fever. Clinical examination revealed tense hard quadriceps muscles and difficulty with hand grip and walking. A complete blood cell count, the erythrocyte sedimentation rate, the C-reactive protein level, serologic evaluation of autoimmunity, and levels of antineutrophil cytoplasmic antibody, immunoglobulins (including IgD), and creatine kinase were normal. Electromyographic studies and magnetic resonance imaging (MRI) of the quadriceps were also normal, but muscle biopsy revealed lymphocytic vasculitis (Figure 1). A dramatic clinical response was achieved with intravenous methylprednisolone, but symptoms were unresponsive to both intravenous cyclophosphamide and etanercept. Repeat muscle biopsy revealed foci of interstitial lymphocytes, one of which was centered around a blood vessel. Subsequent treatment with infliximab was unsuccessful, although oral etoposide produced symptom relief and improved the patient's mobility.

The sister of the index case patient was a 36-year-old female with the R92Q *TNFRSF1A* mutation; she had no history of recurrent fever. At age 14 years, she developed bilateral tenosynovitis of the tibialis anterior that responded to tendon release but was followed by tenosynovitis of the extensor tendons in the foot. By age 17 years, she had developed intermittent cramps in her toes and arthralgia in her

Membrane-bound form of fractalkine induces IFN-γ production by NK cells

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Natural killer (NK) cells participate in both innate and adaptive immunity, in part by their prompt secretion of cytokines including IFN-γ, a pro-inflammatory cytokine with an important role in Th1 polarization. To assess the involvement of fractalkine in inflammatory processes, we examined the effect of fractalkine on IFN-γ production by NK cells. Although soluble chemokines, including MCP-1 and RANTES as well as fractalkine, had a negligible effect on IFN-γ production, immobilized fractalkine markedly induced IFN-γ production by NK cells in a dose-dependent manner. Pretreatment of NK cells with the phosphatidylinositol 3-kinase (PI 3-K) inhibitor, wortmannin, completely inhibited the production of IFN-γ induced by fractalkine, and pretreatment with the protein tyrosine kinase inhibitor, herbimycin A, partially suppressed the response, suggesting that augmentation of IFN-γ production in response to fractalkine treatment of NK cells involves signaling through PI 3-K and protein tyrosine kinases. Furthermore, co-culture of NK cells with fractalkine-transfected 293E cells markedly enhanced IFN-γ production by NK cells compared with co-culture with control 293E cells. These findings may indicate a paracrine feedback loop system in which endothelial cells may be activated to produce more fractalkine, and also suggest a role for fractalkine expressed on endothelial cells in Th1 polarization through the stimulation of IFN-γ production by NK cells.

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1 Introduction

Natural killer (NK) cells, which express cytolytic activity without prior antigenic stimulation, are thought to mediate immunity against virus infection and neoplastic transformation [1, 2]. NK cells are also believed to contribute to immune regulation through the production of pro-inflammatory cytokines such as IFN-γ and TNF-α, which activate various types of cells including T cells, macrophages and NK cells [3, 4]. IFN-γ also enhances expression of VCAM-1 and ICAM-1 on endothelial cells, resulting in consequent leukocyte trafficking across endothelial surfaces [5, 6]. Furthermore, IFN-γ induces a variety of cell types to produce chemokines, which can regulate immune responses, for example, through Th1 polarization [7–9].

Chemokines play an important role in the recruitment of various types of cells to the inflammatory sites [10, 11]. Chemokines are divided into four subfamilies depending on the arrangement of cysteine residues in the N-terminal region: CXCL, CCL, CL and CX3CL [9, 12]. Fractalkine is the only known CX3CL chemokine and is structurally distinct from other chemokines. Fractalkine exists as a membrane-bound molecule with the chemokine

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Abbreviations: MCP: Monocyte chemotactic protein **RANTES:** Regulated upon activation, normal T cell expressed and secreted **VCAM-1:** Vascular cell adhesion molecule-1 **PI 3-K:** Phosphatidylinositol 3-kinase

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domain at the end of a mucin-like stalk, and is released in soluble form through cleavage at a membrane proximal site [13, 14]. We have previously reported that CX3CR1, a receptor for fractalkine, is expressed on NK cells [15] and that fractalkine increases NK cell activities, such as granule exocytosis and cytotoxicity [16]. Fractalkine is expressed on endothelial cells activated by TNF-α, IFN-γ and IL-1, and rapidly captures cells expressing its receptor, CX3CR1 [13–15]. Since the endothelium plays an important role in the recruitment and emigration of circulating leukocytes into sites of inflammation, fractalkine expressed on inflamed endothelium may function as the vascular gatekeeper for immune responses [17]. In the present study, we report that immobilized and membrane-bound fractalkine markedly induced IFN-γ production by NK cells, data that suggest a role for fractalkine in Th1 cell polarization as well the possible existence of a paracrine loop of endothelial and NK cell activation.

2 Results and discussion

2.1 Effects of fractalkine on interferon-γ production by NK cells

NK cells are important in the early phases of immune responses against microbial infections through production of cytokines, including IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5, IL-10, and IL-15 [18, 19]. However, chemokine-induced cytokine production by NK cells remains to be defined. Since NK cells express fractalkine receptor CX3CR1 and adhere to immobilized full-length fractalkine [16], we examined whether the soluble or immobilized form of fractalkine induces production of IFN-γ by NK cells. NK cells (5×10^5/ml) were stimulated with soluble (10 nM) or immobilized (polybeads pretreated with 10 nM) fractalkine, and supernatants were collected and examined for production of IFN-γ by ELISA. The effect of immobilized fractalkine on IFN-γ production by NK cells was dose-dependent (Fig. 1A). Time-course studies revealed that IFN-γ production was observed after 24-h stimulation with the maximum response achieved at 48 h (Fig. 1B). Although some variability in magnitude was observed among individuals, immobilized fractalkine induced IFN-γ production from NK cells in repeated tests using cells from seven different donors (Fig. 1C).

Several CC chemokines, including macrophage inflammatory protein-1α (MIP-1α), IFN-γ-inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, and regulated upon activation, normal T cell expressed and secreted (RANTES), have been reported to activate NK cells, resulting in increased cytosolic free Ca^{2+}, chemotaxis, granule exocytosis and cytotoxicity [20, 21]. Therefore, we examined whether either the soluble or immobilized forms of MCP-1 and RANTES induce IFN-γ production by NK cells. NK cells (5×10^5/ml) were cultured in the presence of soluble forms of fractalkine, MCP-1 and RANTES (each 10 nM) or stimulated with polybeads pretreated with 10 nM of each chemokine at 37°C for 48 h. After incubation, supernatants were collected and IFN-γ production was examined. Although NK cells express CCR2 and CCR3 [22], MCP-1 and RANTES of either soluble or immobilized form did not induce more than marginal enhancement of IFN-γ production. However, immobilized fractalkine, but not MCP-1 nor RANTES, markedly enhanced IFN-γ production by NK cells (Fig. 1D). Fractalkine contains an extended mucin-like stalk, a transmembrane domain and intracellular domain, and fractalkine itself functions as an adhesion molecule to support cell adhesion [15, 23, 24]. In this regard, results of transfection studies in which the fractalkine mucin-like stalk was replaced with a rod-like segment of E-selectin or the chemokine domain of fractalkine was replaced with other soluble chemokines, such as MCP-1 and RANTES, revealed that the mucin-like stalk extends the chemokine domain to support cell adhesion [25, 26]. Thus, immobilized fractalkine-mediated NK cell activation leading to IFN-γ

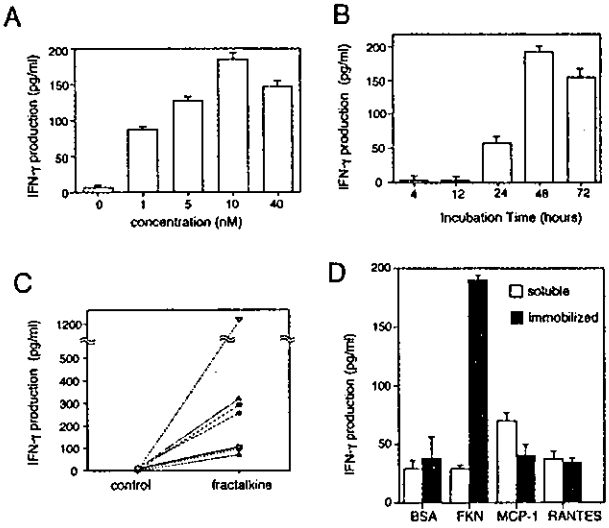

Fig. 1. Effect of immobilized fractalkine on IFN-γ production by NK cells. (A) NK cells were incubated for 24 h in the presence of various concentrations of immobilized fractalkine. (B) NK cells were incubated for the indicated period with fractalkine immobilized on polybeads. (C) NK cells were isolated from seven different healthy donors and cultured in the absence or presence of immobilized fractalkine for 24 h. (D) NK cells were incubated for 24 h with soluble or immobilized fractalkine (FKN), MCP-1 or RANTES. Supernatants were collected and IFN-γ was measured by specific ELISA.

production may be explained by the unique architecture of fractalkine, which may efficiently aggregate signal components associated with CX3CR1, consistent with a model proposed by Rodriguez-Frade et al. [27].

2.2 Effects of inhibitors on fractalkine-mediated IFN-γ production

Pretreatment of NK cells with 10 μg/ml of cycloheximide for 60 min nearly abolished fractalkine-induced IFN-γ production (Fig. 2A), suggesting that fractalkine transduces signals for *de novo* synthesis of IFN-γ. Chemokine receptors identified to date, including CX3CR1, all manifest a seven transmembrane G protein-linked architecture and transduce signals that lead to Ca^{2+} influx, cytoskeletal reorganization, integrin activation, and other functions leading to increased adhesion and migration of the cells [9, 12]. Al-Aoukaty et al. [28] have reported that fractalkine activates Gαi and Gαz types of G proteins. We have also observed that a Gαi protein inhibitor, pertussis toxin, completely blocked granule exocytosis from fractalkine-activated NK cells [16]. In addition to G protein activation, fractalkine has been reported to transduce cell activation signals through extracellular signal-related kinases (ERK1 and ERK2), stress-activated protein kinases (SAPK1/JNK1 and SAPK2/p38), Akt [down stream of phosphatidylinositol 3-kinase (PI 3-K)], src and syk kinases [22, 29, 30]. Therefore, we examined the effects of the protein tyrosine kinase inhibitor, herbimycin A, and PI 3-K inhibitor, wortmannin, on fractalkine-mediated NK cell activation leading to IFN-γ production.

The results of the experiment depicted in Fig. 2B show that herbimycin A significantly inhibits fractalkine-induced IFN-γ production, suggesting that tyrosine kinases are involved, at least in part, in fractalkine-mediated NK cell activation. We also confirmed that wortmannin completely suppressed IFN-γ production. Although further investigations to delineate the signaling pathway of CX3CR1 are required, the proposed model for a signaling pathway through G protein-coupled receptors may explain the different roles of tyrosine kinase and PI 3-K in fractalkine-mediated signal transduction [31].

2.3 IFN-γ production by co-culture of NK cells with fractalkine-expressing cells

Finally, we examined whether membrane-bound fractalkine activates NK cells. To examine this possibility, we transfected fractalkine cDNA into 293E cells, producing transient expression of membrane-bound fractalkine without up-regulation of other adhesion molecules including ICAM-1 and VCAM-1 (data not shown). Freshly isolated NK cells were incubated with confluent monolayers of 293E (control 293E) cells or fractalkine-transfected 293E (FRK-293E) cells for 24 h. After incubation, supernatants were collected and examined for IFN-γ production. As shown in Fig. 3, FRK-293E cells strongly enhanced IFN-γ production by NK cells compared to control 293E cells. This enhancement was partially but significantly inhibited with anti-fractalkine Ab. Both control 293E cells and FRK-293E cells did not produce IFN-γ spontaneously.

There is growing evidence that the chemokine-cytokine network is crucial for inflammatory responses, and the association of chemokines with the Th1 or Th2 phenotypes has been well established [32, 33]. It has been reported that CX3CR1 is preferentially expressed in Th1

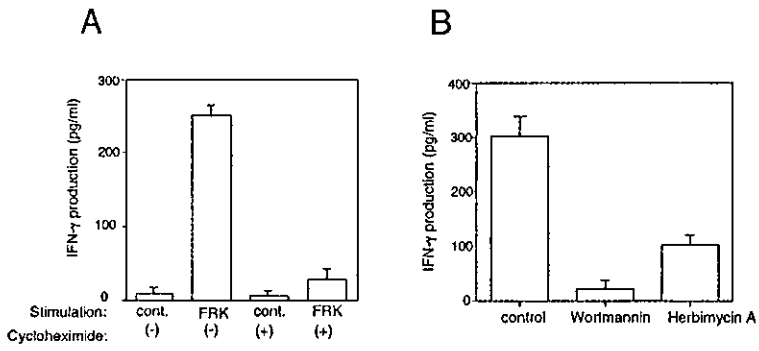

Fig. 2. Effects of inhibitors on IFN-γ production by NK cells. NK cells were pretreated with cycloheximide (A), wortmannin and herbimycin A (B). After pretreatment, NK cells were washed extensively and incubated with immobilized fractalkine (FRK) for 24 h. Then supernatants were collected and IFN-γ was measured by ELISA. The data are presented as means of three independent experiments and error bars indicate SD.

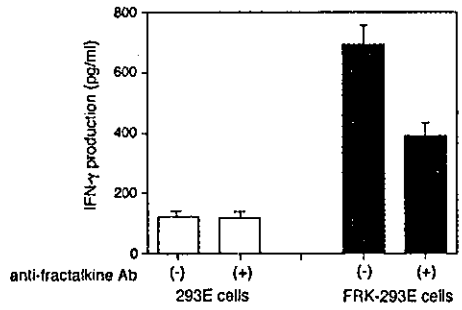

Fig. 3. Effect of membrane-bound fractalkine on IFN-γ production. Freshly isolated NK cells were co-cultured with control 293E cells or fractalkine-transfected (FRK-293E) cells in the absence or presence of anti-fractalkine neutralizing Ab for 24 h, and IFN-γ production was examined. The data are presented as means ± SD from three independent experiments.

compared with Th2 cells, and that Th1 but not Th2 cells respond to fractalkine [34], suggesting that expression of fractalkine may mediate Th1 polarization directly at the inflammation sites. In this regard, it has been reported that fractalkine is expressed on endothelial cells in cases of psoriasis, a Th1-dominated skin disorder, but not in cases of Th2-driven atopic dermatitis [34]. Overall, the Th1/Th2 balance is primarily controlled by two antagonistic cytokines, IFN-γ and IL-4 [8, 35]. Since IFN-γ activates dendritic cells to produce IL-12, which is a strong activator of NK cells and plays a selective role in driving Th1 responses [36, 37], a positive feedback loop between fractalkine and IFN-γ may accelerate Th1-based immune responses each other.

Very recently, we have reported that surface expression of CX3CR1 clearly defines cytotoxic lymphocytes commonly possessing high levels of intracellular perforin and granzymes B, which include NK cells, γδ T cells and terminally differentiated $CD8^+$ T cells [38]. Furthermore, soluble fractalkine preferentially attracts cytotoxic lymphocytes, and membrane-bound fractalkine enhances their migration in response to secondary chemokines, resulting in increased transmigration of cytotoxic lymphocytes regardless of their lineage and mode of target cell recognition [38]. Thus, fractalkine and CX3CR1 are likely to function as a vascular gatekeeper for cytotoxic lymphocytes at the inflammation sites and may be involved in the pathophysiology of vascular and tissue injuries of various clinical diseases [17].

Since IFN-γ is known to activate endothelial cells [39], the increased IFN-γ produced by NK cells may further augment expression of fractalkine on endothelial cells, contributing to a paracrine positive feedback loop. Taken together, a chemokine-to-cytokine-to-chemokine cascade may be involved in various immune responses [40], and our results presented here suggest a role for fractalkine in the Th1-based immune response, anti-viral and anti-tumor immune response, as well as its direct toxicity to endothelium.

3 Materials and methods

3.1 Monoclonal antibodies and reagents

Monoclonal antibody (mAb) against CD3 (OKT3) was purified from ascites as previously described (hybridoma from the American Type Culture Collection, Manassas, VA) [41]. Anti-fractalkine mAb was made by immunizing mice with recombinant human fractalkine expressed in a baculovirus expression system [15]. Anti-CD14 and anti-CD19 mAb were purchased from Immunotech (Marseilles, France). FITC-conjugated goat anti-mouse Ab were purchased from Becton Dickinson (Mountain View, CA). Recombinant human fractalkine, RANTES and MCP-1 were purchased from Genzyme (Cambridge, MA). Polybeads, polystyrene microspheres (2.5% solid-latex, diameter 6 μm) were purchased from Polysciences (Warrington, PA). Wortmannin and herbimycin A were purchased from Wako Chemicals (Osaka, Japan), and cycloheximide from Sigma (St. Louis, MO).

3.2 Cells and NK cell purification

293/EBNA-1 (293E) cells were purchased from Invitrogen (Carlsbad, CA). For transient expression of membrane-bound fractalkine, the expression plasmid pCAGG-Neo-fractalkine-1 [15] was transfected into 293E cells (FRK-293E) by electroporation using GENE-PULSER II (Bio-Rad, Japan). Cells were maintained with G418 contended Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal calf serum and were used for assay after 24-h incubation.

PBMC were isolated from peripheral venous blood from consenting healthy volunteers by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation. NK cells were isolated by negative selection using a mixture of anti-CD3, anti-CD14 and anti-CD19 mAb and immunomagnetic beads (PerSeptive Diagnostics, Cambridge, MA) to deplete T cells, monocytes and B cells, respectively, as described previously [41]. NK cell populations used in all experiments were >85% pure as confirmed by flow cytometric analysis for the presence of CD56 and the absence of CD3 (FACS Caliber, Becton Dickinson) [16].

3.3 Cell stimulation and measurement of cytokines

Freshly isolated NK cells (5×10^5) were cultured with soluble RANTES, MCP-1 and fractalkine for 24 h at 37°C in 96-well plates (IWAKI Glass, Osaka, Japan). For NK cell stimulation with the immobilized chemokines, polybeads were pre-coated with the indicated concentration of RANTES, MCP-1 or fractalkine in PBS, pH 8 at 4°C overnight, then treated with 1% BSA/PBS, pH 7.4 at room temperature for 2 h prior to use. Purified NK cells (5×10^5) were cultured in 96-well plates with chemokine-coated polybeads (cell:beads 1:4) for 24 h at 37°C. For the stimulation with membrane-bound fractalkine, NK cells were co-cultured with control 293E or transfected FRK-293E cells (1×10^5) for 24 h. In some experiments, NK cells were pretreated with wortmannin, herbimycin A (1 μM each for 20 min), or cycloheximide (10 μg/ml for 60 min) at 37°C. After incubation, the plates were centrifuged, and cell-free supernatants were collected and assessed for cytokine production using ELISA kits (Bio-Source International, CA) according to the manufacturer's protocols. The detectable range of each ELISA kit was 5–1,000 pg/ml and the optical density of individual wells was determined at 450 nm using microplate reader (Bio-Rad).

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Potential preventive effects of follistatin-related protein/TSC-36 on joint destruction and antagonistic modulation of its autoantibodies in rheumatoid arthritis

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Keywords: autoimmunity, autoantigens, c-*fos*, epitopes

Abstract

We previously reported that follistatin-related protein (FRP)/TSC-36 was one of the target antigens of autoantibodies in rheumatoid arthritis (RA) and that the appearance of serum autoantibodies to FRP correlated to disease activity in RA. However, the significance of FRP in autoimmunity remained to be explained due to the unknown function of FRP. Here, we disclose in part the function of FRP. Transforming growth factor (TGF)-β augmented FRP gene expression in synovial cells. FRP reduced synovial production of matrix metalloproteinase (MMP)-1, MMP-3 and prostaglandin E_2, potent agonists of joint destruction in RA. In contrast, autoantibodies to FRP from patients with RA increased their production by blocking FRP activity, probably in the autocrine system. Moreover, FRP down-regulated synovial expression of *FOS* (c-*fos*), which seemed responsible for the reduction in MMP-1 and MMP-3 caused by FRP. Therefore, FRP and its autoantibody can be regarded as defensive and offensive factors respectively in rheumatoid arthropathy. The major epitope of autoantibodies to FRP was mapped to the sequence LKFVEQNE (residues 169–176) and homologous sequences were found in proteins from *Escherichia coli*, Epstein–Barr virus, etc. FRP and its autoantibody may provide some clues to elucidate the process of disease development and a new approach to the design of therapeutics in RA.

Introduction

Follistatin-related protein (FRP) was discovered as the product of the transforming growth factor (TGF)-β1-inducible gene, TSC-36, in a murine osteoblastic cell line, MC3T3-E1 (1). FRP was named after follistatin, an inhibitor of activin, for having an amino acid sequence similar to a characteristic structure unit in follistatin, called the follistatin (FS) domain (2). The chick counterpart gene and protein of FRP are known as the follistatin-like (Flik) gene and protein (3). FRP is a secreted protein with a mol. wt of 50–55 kDa and is expressed in all organs except peripheral blood leukocytes. Human FRP is registered in the databases of Online Mendelian Inheritance in Man (OMIM; Johns Hopkins University, http://www3.ncbi.nlm.nih.gov/omim), and its entry name is FOLLISTATIN-LIKE 1 (FSTL1). There is another reported FS domain-bearing protein encoded by the gene named follistatin-related gene (FLRG), registered as FOLLISTATIN-

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LIKE 3 (FSTL3) in OMIM, which was overexpressed in a B cell leukemia (4). Recently, to our confusion, the product of FLRG has also been called 'follistatin-related protein (FSRP)' (5). It is not FRP but FSRP that has been demonstrated to have activin-binding activity (6). It is still unknown which molecules associate with FRP. FRP is an orphan molecule. Although the physiological function of FRP remains to be clarified, it is being partially disclosed. In human lung cancer cells, FRP had negative regulatory effects on growth (7). In development, a reduction in FRP gene expression brought about deficient axial patterning and holoprosencephaly, suggesting a modulating effect on signaling through bone morphogenetic protein or TGF-β-related ligands (8).

We cloned FRP as an autoantigen in rheumatoid arthritis (RA) and other systemic autoimmune diseases, and disclosed that the appearance of serum autoantibodies to FRP was predominant in RA (30%) and correlated with disease activity in patients with RA (9). However, we could not explain the significance of the production of autoantibodies against FRP in RA because of lack of information about the physiological role of FRP. TGF-β is the key to a breakthrough here, because as an inducer of FRP it is the only molecule known at present that has a functional association with FRP.

The definite pathophysiological role of TGF-β in RA has not been settled as yet. On one hand, TGF-β is thought to have protective effects on destructive arthritis in RA by antagonizing pro-inflammatory cytokines such as IL-1, tumor necrosis factor (TNF)-α and platelet-derived growth factor, which induce inflammation, enzyme release, synovial cell proliferation and destruction of joint structure (10–14). On the other hand, TGF-β is thought to have synergistic effects on arthritis with the pro-inflammatory cytokines and to recruit leukocytes to synovial tissues resulting in synovial hyperplasia (15–17). This contradiction may originate from experiments using different culture conditions or animal models.

In arthropathy of RA, matrix metalloproteinases (MMP) have a direct effect on joint destruction (18). MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1), in particular, are thought to play a major role, because MMP-1 is the only enzyme capable of efficiently degrading interstitial collagens by cleaving their triple helical domains, and MMP-3 has a wide spectrum of substrates including non-collagenous proteins, gelatin and laminin (19). It is reported that TGF-β suppresses synovial production of MMP and may take part in the prevention of extracellular matrix degradation (20,21). Prostaglandin E_2 (PGE_2) is another factor responsible for the destruction of rheumatoid joints and the most potent stimulator of bone resorption among the prostaglandins (21).

The pivotal role of TGF-β in rheumatoid arthropathy led us to speculate that FRP would have agonistic or antagonistic functions for or against TGF-β in the disease process. Therefore, we investigated the functions of FRP by estimating the effect it and its antibody has on synovial expression of MMP-1, MMP-3 and PGE_2, and moreover the effects of FRP on *FOS* (*c-fos*) (22), a candidate for the gene responsible for the tumor-like growth of synovium (23). Based on the results of those experiments, we attempted to elucidate the pathophysiological significance of autoimmunity to FRP in RA.

Methods

Preparation of recombinant FRP

We produced two types of recombinant human FRP. *Escherichia coli*-expressed FRP, GST–FRP, was prepared as previously described (9). The GST tag was removed from GST–FRP and the protein was purified by a perfusion chromatography method with a POROS HE column (Applied Biosystems, Foster City, CA). Since it was easy to prepare a large quantity of *E. coli*-expressed FRP, we used this type mainly in Northern blotting analyses. On the other hand, COS-7-expressed FRP, FLAG–FRP, was created as Pati and Chubet described (24,25). A DNA cassette with 5′ *Sal*I and 3′ *Sma*I ends encoding preprotrypsinogen signal peptide and FLAG epitope tag sequentially was produced by annealing synthetic complementary oligonucleotides (sense: 5′-TC GAC ACC ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT GCT GAC TAC AAA GAC GAT GAC GAC AAA GGA TCC C-3′; anti-sense: 5′-G GGA TCC TTT GTC GTC ATC GTC TTT GTA GTC AGC AAC TGC AGC TCC AAC AAG AGC TAG GAT CAG AAG TGC AGA CAT GGT G-3′). This DNA cassette was cloned in a *Sal*I + *Sma*I-digested pSL1180 vector (Amersham Pharmacia Biotech, Uppsala, Sweden), and ligated with signal sequence-minus FRP cDNA bearing 5′ *Sma*I and 3′ *Xho*I ends in the vector (9). The resultant FLAG–FRP cDNA was isolated by digestion with *Sal*I and *Xho*I, ligated into mammalian expression vector pCXN2 at the *Xho*I site, and transfected into COS-7 cells. pCXN2 vector was kindly provided by J. Miyazaki (Department of Nutrition, Osaka University, Osaka, Japan). FLAG–FRP in culture medium was purified with an Anti-FLAG M2 affinity gel (Eastman Kodak, New Haven, CT) according to the manufacturer's protocol.

Antibodies

Mouse mAb to human FRP (ANOC9703) was produced with GST–FRP by standard methods. $F(ab')_2$ fragments of ANOC9703 mAb were prepared with an ImmunoPure $F(ab')_2$ preparation kit (Pierce, Rockford, IL). Affinity-purified autoantibodies to FRP were derived from three patients with RA (RA21, RA22 and RA23). They were purified with a HiTrap Protein G column (Amersham Pharmacia Biotech) and an FRP-affinity column. The FRP-affinity column was prepared by coupling GST tag-removed GST–FRP with NHS-activated Sepharose-4B (Amersham Pharmacia Biotech). The deduced percentages of purified autoantibodies to FRP in serum total IgG from RA21, RA22 and RA23 were 0.21, 0.14 and 0.25 respectively. Mouse control IgG was purchased from Serotec (polyclonal mouse IgG, PMP01; Oxford, UK). Human control IgG was purified from a pooled serum mixture of six normal healthy subjects with the HiTrap Protein G column.

Analysis of MMP-1 and MMP-3 production in synovial cells

Cells (3×10^3) of the human synovial cell line SF-1 (26) or cells (5×10^3) of the rabbit synovial cell line HIG-82 (CRL-1832; ATCC, Rockville, MD) were inoculated into the wells of 96-well plates filled with 200 μl of RPMI 1640 or F12 in triplicate respectively. They were cultured in medium with 10% FCS for 72 h. After the medium was exchanged for fresh medium without FCS but with rFRP and/or antibodies, or fresh medium with 0.1% FCS, which is necessary for cytokine response, and

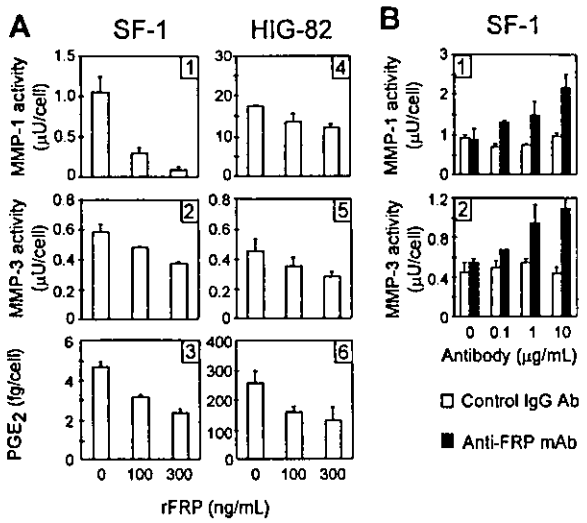

Fig. 1. Suppression of MMP-1, MMP-3 and PGE_2 production by rFRP, and their augmentation by mouse mAb to human FRP in synovial cells. (A) rFRP reduced the amount of MMP-1, MMP-3 and PGE_2 secreted from human and rabbit synovial cells, SF-1 and HIG-82 ($P < 0.001$ in graphs 1, 2 and 3, $P < 0.005$ in graphs 4, 5 and 6, one-factor ANOVA). (B) Mouse mAb to human FRP (ANOC9703) augmented the production of MMP-1 and MMP-3 from SF-1 human synovial cells, probably by blocking autocrine FRP activities ($P < 0.0001$ in graphs 1 and 2, compared to controls by two-factor factorial ANOVA).

rFRP plus an aliquot of cytokine, they were cultured for the next 72 h. The cytokines used were IL-1β (Strathmann Biotech, Hannover, Germany), TNF-α (PeproTech, London, UK), IL-6 and soluble IL-6 receptor in pairs (R & D Systems, Minneapolis, MN), and oncostatin M (PeproTech). Thereafter the culture supernatant was collected and the cells were enumerated. Activities of MMP-1 and MMP-3 in 50 μl aliquots of the culture supernatant were assayed with commercially available kits (YU-16001 and YU-26001; Yagai, Yamagata, Japan). MMP in the samples were activated by *p*-aminophenylmercuric acid (A-9563; Sigma, St Louis, MO) according to the manufacturer's protocol. The fluorescence of the reactants was measured with a FluorImager and its software ImageQuaNT (Molecular Dynamics, Tokyo, Japan).

Evaluation of PGE_2 production in synovial cells

Concentrations of PGE_2 in the culture supernatant were measured with a Correlate-EIA PGE_2 enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

Northern blotting studies of synovial cells

SF-1 cells were cultured in an FCS-free medium with 0.1% BSA for 72 h and then stimulated with 2 ng/ml of TGF-β1 (Genzyme, Cambridge, MA) or 300 ng/ml of rFRP in fresh FCS-free 0.1% BSA medium. After the stimulation, RNA was isolated from cells with TRIzol reagent (Gibco/BRL, Gaithersburg, MD). The probe for FRP was described previously (9). The probes for MMP-1 and MMP-3 were cloned from RT-PCR products of SF-1 RNA with an Original TA cloning kit (Invitrogen, Carlsbad, CA). The 5′ and 3′ primers for MMP-1 were 5′-TGG ATC CAG GTT ATC CCA AA-3′ and 5′-GGT GAC ACC AGT GAC TGC AC-3′ respectively. Those for MMP-3 were 5′-TCA GAA CCT TTC CTG GCA TC-3′ and 5′-GCT GAC AGC ATC AAA GGA CA-3′ respectively. The annealing temperature for both the primer sets was 62°C. The probe for *FOS* was prepared from human FOS cDNA (27) (CO054; Health Science Research Resources Bank, Osaka, Japan). Signal densities were measured by Image software (Scion, Frederick, MD).

Fine epitope mapping

Generally, fine epitope mapping is performed with a set of overlapping peptides designed to scan 10- to 15-amino-acid sequences of the protein by residues from the N- to C-terminus. To investigate T cell epitopes of the FRP molecule, however, we prepared peptide fragments of FRP including potential binding motifs for DRB1*0401 (DR4), DRB1*0405 (DR4) and DQ4 molecules, which V. Brusic kindly designed by computer analysis (28,29). We successfully applied these peptides to epitope mapping (B cell epitope mapping) of the FRP molecule. Methods of peptide synthesis and ELISA with antigen peptide were described previously (26).

Homology search of proteins

Homologous protein sequences were found using the FASTA program on the GenomeNet service (http://www.genome.ad.jp).

Results

The effect of FRP and its autoantibody on synovial cells

To investigate the effects of FRP and its antibody on rheumatoid arthropathy, we treated synovial cells with rFRP, its mAb or autoantibodies from patients with RA. Indeed, for the purpose of these studies, synovial cells should be fresh from synovial tissue, but it was difficult to prepare such cells abundantly and to get fixed conditions in a different series of studies. Thus, we used synovial cell lines. rFRP suppressed MMP-1, MMP-3 and PGE_2 production in the human and rabbit synovial cell lines SF-1 and HIG-82 in a dose-dependent manner (Fig. 1A). In contrast, mAb to human FRP, ANOC9703, increased MMP-1 and MMP-3 production in SF-1 cells in a dose-dependent manner (Fig. 1B): PGE_2 could not be estimated because mouse mAb to human FRP in the sample medium prevented mouse mAb to PGE_2 from reacting with plate-coated anti-mouse IgG antibodies in this ELISA system. Similarly, autoantibodies to FRP from patients with RA raised MMP-1, MMP-3 and PGE_2 production in a dose-dependent manner in both SF-1 and HIG-82 (Fig. 2). To confirm that the inhibitory effect of rFRP was indeed mediated by rFRP, but not contaminants, we examined whether the preincubation of rFRP with anti-FRP mAb can abolish the observed inhibitory effect. Anti-FRP mAb diminished the inhibitory effect by neutralizing rFRP activity in a dose-dependent manner (Fig. 3A). Thus, rFRP exerted a significant inhibitory effect on synovial cells. In addition, rFRP inhibited the induced production of MMP-1 from synovial cells activated by IL-1β, TNF-α, IL-6 and oncostatin M (Fig. 3B). Next, to exclude the possibility that the promotion of synovial MMP and PGE_2 production by antibodies to FRP was due to the Fcγ receptor-mediated

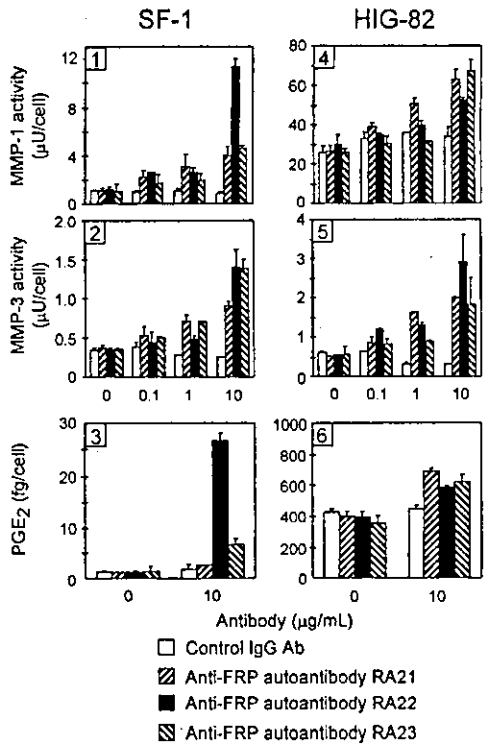

Fig. 2. Enhancement of MMP-1, MMP-3 and PGE_2 production by affinity-purified autoantibodies to FRP in RA. Affinity-purified anti-FRP autoantibodies from patients with RA increased MMP-1, MMP-3 and PGE_2 production both in SF-1 and HIG-82 cells, probably due to their inactivation of autocrine FRP ($P < 0.005$: RA21, RA22 and RA23 in graphs 1, 2 and 5, RA21 and RA22 in graph 4; $P < 0.05$: RA22 and RA23 in graph 3, and RA21 in graph 6; compared to controls by two-factor factorial ANOVA).

activation of synovial cells following FRP–anti-FRP immune complex formation, we treated synovial cells with anti-FRP mAb $F(ab')_2$ lacking the Fc portion. Anti-FRP mAb $F(ab')_2$ also increased MMP-1 production in SF-1 cells in a dose-dependent manner (Fig. 3A). Northern blotting analysis showed that FRP was intrinsically expressed in non-treated synovial cells (lane 0 h of Fig. 4A). Therefore, antibodies to FRP seemed to neutralize endogenous FRP activity probably in the autocrine system to exert a reversing effect. Down-regulation of MMP-1 and MMP-3 by rFRP was observed also at the mRNA level (Fig. 4B). *FOS* is an immediate early gene whose expression is regulated rapidly (30). rFRP also down-regulated *FOS* expression in 60 min (Fig. 4B). This suggested that the suppressive effects on MMP-1 and MMP-3 of FRP originated from its negative regulation of *FOS*. In this series of experiments, we did not observe any apparent growth inhibition of SF-1 cells by rFRP (data not shown).

FRP gene regulation by TGF-β in synovial cells

FRP was originally cloned as a TGF-β1-inducible protein in the murine osteoblastic cell line MC3T3-E1 (1). Thus, we studied the regulation of the FRP gene by TGF-β in synovial cells. As in MC3T3-E1 cells, TGF-β1 up-regulated the expression (Fig. 4A).

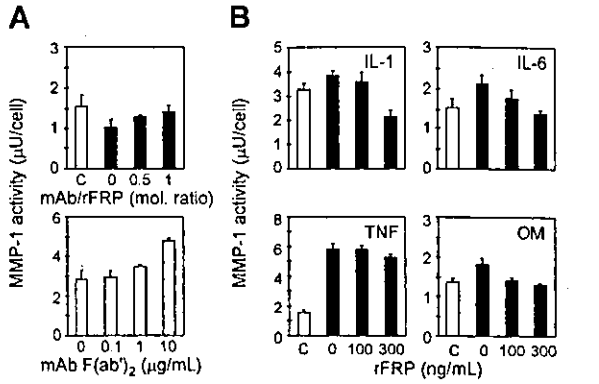

Fig. 3. Verification of rFRP activity and activation without Fcγ receptor by anti-FRP antibodies and demonstration of inhibitory effect of rFRP on activated synovial cells. (A) Top: recovered MMP-1 activity represents rFRP activity abolished by anti-FRP mAb (ANOC9703 mAb) ($P < 0.05$, one-factor ANOVA). rFRP (100 ng/ml) was preincubated for 30 min with different amounts of ANOC9703 mAb in molecular ratios 1:0, 1:0.5 and 1:1. C: control culture without rFRP or mAb. Bottom: ANOC9703 mAb $F(ab')_2$, like intact mAb, induced MMP-1 from SF-1 cells, excluding the possible role of Fcγ receptor-mediated activation ($P < 0.001$, one-factor ANOVA). (B) rFRP inhibited the induced production of MMP-1 from synovial cells activated by IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-6 plus soluble IL-6 receptor (100 and 10 ng/ml) and oncostatin M (10 ng/ml), as shown in graphs IL-1, TNF, IL-6 and OM, respectively ($P < 0.0005$, IL-1; $P < 0.005$, OM; $P < 0.01$, IL-6, tested among rFRP-treated sample values by one-factor ANOVA). C: control culture without cytokine or rFRP.

Epitope mapping of FRP recognized by autoantibodies in RA

We previously found the autoantigenic epitope of FRP to be located in the EC domain between the FS domain and the EF-hand by a crude epitope mapping method (9). To clarify this epitope further, we performed ELISA with the 14–24mer peptide fragments of FRP described in Methods. The results are shown in Fig. 5. All of the autoantibodies from three representative patients with RA reacted to the peptide with the sequence FDNGDSRLDSSEFLKFVEQNE (no. 10 peptide). However, they reacted only slightly to the peptide with the sequence YFKNFDNGDSRLDSSEF (no. 9 peptide), part of which was included in the no. 10 peptide. Thus, we concluded that the sequence LKFVEQNE (residues 169–176), a part of no. 10 peptide not shared by no. 9 peptide, composed the epitope of FRP. This result supports that of crude epitope mapping (9). Interestingly, the sequence LKFVEQNE was included in another predictive DRB1*0401-binding motif LKFVEQNET (residues 169–177), which was also designed by V. Brusic as described in Methods, but was too short to use in the peptide ELISA. The significance of this potential coincidence is unknown, although a similar coincidence has been demonstrated in other protein antigens (31,32). The T cell response to the FRP molecule and T cell epitope mapping are under investigation.

Homology search of the epitope sequence

Amino acid sequences homologous to the LKFVEQNE epitope were found in proteins from infectious microbes; —FVEQNE in

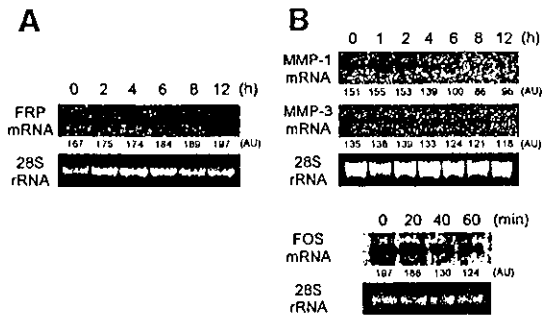

Fig. 4. Augmentation of FRP gene expression by TGF-β1, and suppression of MMP-1 and MMP-3 gene and *Fos* expression by FRP in SF-1 cells. (A) TGF-β1 (2 ng/ml) up-regulated FRP mRNA expression. FRP mRNA was expressed in non-stimulated SF-1 cells (lane 0 h). (B) FRP (300 ng/ml) down-regulated MMP-1, MMP-3 and FOS mRNA expression. Each lane contained 20 μg of total RNA. 28S rRNA bands show the amount of sample RNA. Arbitrary unit (AU) values under the bands stand for signal densities standardized by those of 28S rRNA bands.

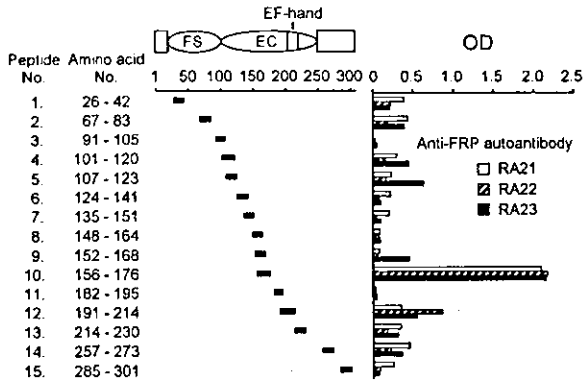

Fig. 5. Epitope mapping of FRP with peptide ELISA. ELISA was performed with sera from three patients with RA, RA21, RA22 and RA24. All of the three sera reacted strongly to no. 10 peptide, but hardly at all to no. 9 peptide, although their sequences partially overlapped. This showed that the part of no. 10 peptide not shared by no. 9, LKFVEQNE, composed the epitope of FRP. The left scale shows the amino acid numbering of the FRP molecule. The right scale shows optical density standing for the amount of IgG antibodies bound to sample peptides.

E. coli 4-hydroxybenzoate synthetase (S25660), LKFVE–N– in *Rickettsia prowazekii* 3-deoxy-*d*-manno-octulosonic-acid transferase RP089 (H71717), –KFVE–NE in Epstein–Barr virus DNA-directed DNA polymerase (DJBE2L), etc. (PIR accession numbers in parentheses).

Discussion

We previously reported that FRP is an autoantigen whose autoantibodies appear in association with disease activity in RA (9). FRP is a secreted protein with a similar structure to follistatin, an inhibitor of activin (2). Its function, however, remained to be clarified. Here, we disclosed part of its function. FRP suppresses MMP-1, MMP-3 and PGE_2 production in synovial cells, and autoantibody to FRP from patients with RA increases their synovial production by blocking FRP activity, probably in the autocrine system. This result suggests that, *in vivo*, FRP may be a synovial defensive factor together with protease inhibitors such as tissue inhibitor of MMP (33) and that the autoantibody to FRP may be an offensive factor for articular matrix degradation. TGF-β up-regulates synovial expression of FRP. Therefore, it is possible that the reported anti-arthritic effect of TGF-β may be mediated in part by the functions of FRP.

In these experiments, we used synovial cell lines derived from spontaneous transformants, SF-1 and HIG-82, in order to ensure the reproducibility of assays and to perform many experiments efficiently. Since cells in synovial tissue are quite heterogeneous, test results could differ depending on the source and culture period. HIG-82, a rabbit cell line, was also used because it was well studied and thought to stand for synovial tissue cells (34–36). We found that SF-1 preserved the features of cytokine responses among synovial cells as well as HIG-82 cells (data not shown). However, these *in vitro* studies cannot conclude the functions of FRP in cytokine-rich rheumatoid joint milieus. We are now investigating FRP effects on arthritis *in vivo* by using animal models.

FRP down-regulates the expression of *FOS* in synovial cells. *FOS* is transcribed in rheumatoid synovial cells and thought to be one of the genes responsible for the tumor-like phenotypes of synovium in RA (23). FOS (c-Fos), the protein encoded by *FOS*, forms a heterodimer complex, activation protein (AP), with JUN and related proteins, and acts as a transcription factor by binding to specific DNA sequences, AP-1 sites (37). Many of the promoter DNA sequences for MMP including MMP-1 and MMP-3 contain AP-1 sites, and the AP-1-forming proteins, especially FOS, regulate their transcriptional control (38). Therefore, the reduction in the expression of MMP-1 and MMP-3 caused by FRP may originate from the down-regulation of *FOS*.

In terms of the actual pathophysiological role of FRP in RA, its negative regulation of MMP-3 seems to have more importance than that of MMP-1. The serum concentration of MMP-3 is not only significantly correlated with disease activity indices such as C-reactive protein and IL-6 levels, but also a promising marker for predicting bone damage in the early stage of RA (39,40). Suppression of PGE_2 production by FRP is also a notable phenomenon. PGE_2 is the most potent stimulator of bone resorption among the prostaglandins (21). In addition, PGE_2 is associated with collagenase production in rheumatoid synovial cells (41). Therefore, the down-regulation of MMP-3 and PGE_2 by FRP suggests that FRP plays no small part in the prevention of rheumatoid arthropathy, and that the neutralizing autoantibody to FRP is a potential promoter of joint destruction.

We previously reported that a considerable number of patients with RA had autoantibodies to a soluble form of the IL-6 signal transducer gp130, named gp130-RAPS, and that gp130-RAPS inhibited IL-6 activity and autoantibodies to gp130-RAPS neutralized such inhibition (26). Autoantibodies to calpastatin, an inhibitor of calpain, were also reported in the sera of some patients with RA (42,43) and to neutralize calpastatin activity *in vitro* (42). Calpain can degrade the matrix components of articular cartilage as well as nuclear proteins, cytokines and structural proteins (44). Menard *et al.* proposed that autoantibodies to calpastatin have promoting effects on the

progression of RA (44). Therefore, antagonistic autoantibodies to potential negative regulators of disease progression, including FRP, may play a role through the modulation of disease development in RA. To our surprise, it was reported that neutralizing autoantibodies to IL-1α were present in some patients with RA and serum titers tended to have a negative correlation with disease activity indices (45). The autoantibody to IL-1α can be considered, in a sense, as a disease-ameliorating autoantibody. It is possible that such 'autoantibody antagonism' reflects or is responsible for the stage of disease development or disease subtype. To address this point, it is necessary to analyze extensively the effects of autoantibodies to such defensive or offensive factors on the disease development *in vivo* by using animal models, and to investigate the relationship between the appearance of those autoantibodies and disease phenotypes. Interestingly, new therapies utilizing mAb to such disease-promoting factors as TNF-α, IL-6, IL-6 receptor, etc., in cases of RA have been shown to be beneficial (46–48). This supports the idea that antibodies to causative factors can change the stage of disease.

Molecular mimicry is one hypothesis that explains the cause of autoantibody production (49,50). However, it has not yet been demonstrated by experiments. Nevertheless, to obtain a hint as to the origin of anti-FRP autoantibody production, we performed a homology search with the epitope sequence in FRP. Epitope analysis with the restricted set of antigen peptides disclosed that the sequence LKFVEQNE (residues 169–176) composed the epitope of FRP. The minimum number of amino acid residues in the liner epitope is generally ~15 (51). Thus, the sequence LKFVEQNE may well be necessary but not sufficient for immunoreactivity of FRP. The sequences homologous to LKFVEQNE were found in proteins from *E. coli*, Epstein–Barr virus, etc. It has been discussed that infection with various organisms including viruses, bacteria and mycoplasmas could be the trigger of disease development (52). The induction of autoantibodies to FRP may be associated with some infection.

The present studies disclosed part of the function of FRP. FRP is an orphan molecule as yet and the signaling pathway of FRP is still unknown. Blocking effects on FRP activity by its antibodies suggest that FRP seems to have some cell surface receptor. It is probable that FRP can affect FOS/AP-1 target genes and regulate their products to exert their related activities. To clarify the definite function of FRP, its receptor should be identified and its intracellular signal transduction should be elucidated.

In conclusion, in the pathophysiology of RA, FRP can be regarded as a preventive factor of joint matrix degradation and autoantibodies to FRP promote joint destruction by blocking FRP activity. It is not known to what extent the interaction of FRP and its autoantibodies modulates actual inflammation in joint spaces. However, FRP and its autoantibodies may provide some clues as to the process of disease development, and a new approach to the design of therapeutics in RA.

Acknowledgements

We wish to thank Dr Vladimir Brusic for the design of peptides used in this work and Dr Yasukazu Ohmoto (Otsuka Pharmaceutical) for preparing mouse mAb to FRP.

Abbreviations

AP	activation protein
FRP	follistatin-related protein
MMP	matrix metalloproteinase
OMIM	Online Mendelian Inheritance in Man
PGE_2	prostaglandin E_2
RA	rheumatoid arthritis
r	recombinant
TGF	transforming growth factor
TNF	tumor necrosis factor

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