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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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 E₂, 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://www.3.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-
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 E2 (PGE2) 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 PGE2, 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′ SalI and 3′ Smal 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 TGT GTA GTC AGC AAC TGC AGC TCC AAC AAG AGC GCT GCA GTT GCT GAC TGC TAC AAA GAC GAT GAC GAC AAA GGA GCC C-3′; anti-sense: 5′-G GGA TCC TTT GTC GTC ATC GTC TTG GTA TGT AGC AAC TGC AGC TGC TAC GCC ATC AGA AGC TAG GAT CAG AAG TGC AGA CAT GGT G-3′). This DNA cassette was cloned in a SalI + Smal-digested pSL1180 vector (Amersham Pharmacia Biotech, Uppsala, Sweden), and ligated with signal sequence-minus FRP cDNA bearing 5′ Smal and 3′ Xhol ends in the vector (9). The resultant FLAG–FRP cDNA was isolated by digestion with SalI and Xhol, ligated into mammalian expression vector pCXN2 at the Xhol 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 auto-antibodies 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 × 10⁶ of the human synovial cell line SF-1 (26) or cells (5 × 10⁹ 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
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).

**Fig. 1.**

**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-$\beta$1 (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 were 5\'-TGG 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).

**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-$\beta$, TNF-$\alpha$, 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 Fc$\gamma$ receptor-mediated...
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). 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).

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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...
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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 PGE₂ 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 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.

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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 PGE₂ production by FRP is also a notable phenomenon. PGE₂ is the most potent stimulator of bone resorption among the prostaglandins (21). In addition, PGE₂ is associated with collagenase production in rheumatoid synovial cells (41). Therefore, the down-regulation of MMP-3 and PGE₂ 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 point, it is necessary to analyze extensively the effects of disease development or disease subtype. To address this point, it is necessary to analyze extensively the effects of 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 linear 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

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AP</td>
<td>activation protein</td>
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<tr>
<td>FRP</td>
<td>follistatin-related protein</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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References


