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Follistatin-Like Protein-1 Is a Novel Proinflammatory Molecule¹

Takako Miyamae,* Anthony D. Marinov,* Dawn Sowders,‡ David C. Wilson,* Jason Devlin,‡ Robert Boudreau,§ Paul Robbins,¶ and Raphael Hirsch²

While analyzing gene expression in collagen-induced arthritis, we discovered that a poorly characterized gene, follistatin-like protein 1 (FSTL-1), is highly overexpressed in mouse paws during early arthritis, especially at the interface of synovial pannus and eroding bone. In this study, we show that FSTL-1 is a novel proinflammatory molecule with a previously unrecognized role in inflammation. Transfection of FSTL-1 into macrophages and fibroblasts leads to up-regulation of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6. Overexpression of FSTL-1 in mouse paws by gene transfer results in severe paw swelling and arthritis. The Journal of Immunology, 2006, 177: 4758–4762.

Follistatin-like protein 1 (FSTL-1)³ is a poorly characterized protein that has never previously been associated with inflammation. FSTL-1, also known as FRP and TSC-36, is an extracellular glycoprotein belonging to the BM-40/SPARC/osteonectin family of proteins containing both extracellular calcium-binding and follistatin-like domains. FSTL-1 was originally cloned from an osteoblastic cell line as a TGF-β-inducible gene (1). The protein occurs in two isoforms resulting from differential splicing. FSTL-1 was detected in the medium of all osteosarcoma and chondrosarcoma cell lines tested and in medium of some cells of the fibroblast lineage. In mice, the highest expression of FSTL-1 was observed in the lung (2).

The action of FSTL-1 is unclear, although a number of putative functions have been suggested. Both proliferative and antiproliferative effects have been reported. FRP may play a role in neuralization during embryogenesis (3), and its expression is up-regulated by estrogen (4). In contrast to other BM-40 family members, the extracellular calcium-binding domain of FSTL-1 is nonfunctional (5), suggesting that, despite its sequence homology to BM-40, it has evolved clearly distinct properties. Analysis of prostate cancers revealed that overexpression of FSTL-1 was associated with higher metastatic potential (6). In contrast, FSTL-1 expression was extinguished in v-ras-transformed rat fibroblasts, and transfection of FSTL-1 into these cells inhibited in vitro invasion (7) and led to growth inhibition in human lung cancer cells (8). In 1998, Tanaka et al. (9) cloned FSTL-1 from rheumatoid arthritis (RA) synovial tissue, demonstrated anti-FSTL-1 Abs in the serum and synovial fluid of RA patients, and suggested that FSTL-1 was an autoantigen.

Materials and Methods

Adenoviral vectors

A recombinant, E1a-E3-deleted replication defective adenovirus type 5 vector encoding the mouse FSTL-1 gene (National Center for Biotechnology Information Nucleotide Database accession number BC028921) was generated through Cre-lox recombination as described by Hardy et al. (10). The control vector, Ad-BgI II, is an E1a/E3-deleted replication-defective adenovirus type 5 lacking an insert. The vectors were grown in 293 cells and purified by CsCl gradient ultracentrifugation, dialyzed at 4°C against sterile virus buffer, aliquoted, and stored at −80°C.

Cell transfection

U937 and COS-7 cells were electroporated with pRcCMV-hFSTL-1, using a Bio-Rad Gene Pulser (Bio-Rad) according to the manufacturer’s protocol. Cells were maintained in G418 selection medium for a period of 3–4 wk, and clones were isolated.

Histologic analysis

Mouse knee joints were fixed in 10% neutral buffered formalin, decafluid, dehydrated in a gradient of alcohols, paraffin embedded, sectioned, mounted on glass slides, and stained with HE. For immunohistochemistry, knees were fixed in 2% paraformaldehyde, cut longitudinally with a scalpel blade, mounted onto a cork disk with a small drop of Cryogel (Cancer Diagnostics), and placed in liquid nitrogen-cooled isopentane for 45 s. Sections of 7 μm were obtained using a cryostat. Slides containing knee sections were fixed with 2% paraformaldehyde for 20 min and washed with PBS followed by BSA buffer (0.5% BSA and 0.15% glycine in PBS). Slides were blocked for 45 min with a 1/20 dilution of normal donkey serum (Sigma-Aldrich) in BSA buffer and washed three times with BSA buffer. The primary Ab goat anti-mouse FSTL-1 (R&D Systems) diluted to 10 μg/ml in BSA buffer was added, incubated for 2 h, and washed three times with BSA buffer. The secondary Ab Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes) diluted 1/500 in PBS was added, incubated for 2 h, and washed three times with PBS buffer. The nucleus stain DRAQ5 (Biostatus) diluted 1/1000 in PBS was added, incubated for 30 min, and washed three times with PBS. A coverslip containing a drop of gelvatol was added, and slides were stored at 4°C until observation with confocal microscope.
FIGURE 1. FSTL-1 is overexpressed in fibroblast-like synoviocytes in early CIA. Mouse knee joints were harvested from mice before immunization with CII (day 0), during acute arthritis (day 28), or during late arthritis (day 49). Tissues were dissected and stained for FSTL-1 and observed under a confocal microscope at ×40 (A–C). Higher magnification (×100) views of day 28 synovium (D–F) show CD90+ fibroblasts in red (D), FSTL-1 in green (F), and double staining of CD90 and FSTL-1 (E). Colocalization of FSTL-1 with CD90+ fibroblasts is indicated by the arrows in E.

Quantitative RT-PCR

Total RNA was isolated from frozen liver using the ToTALLY RNA Isolation Kit (Ambion) according to the manufacturer’s instructions. To remove possible genomic DNA contamination, RNA was treated with DNase I (Ambion). cDNA was synthesized with random hexamer oligonucleotides using the SuperScript II Reverse Transcriptase Kit (Invitrogen Life Technologies). PCR was performed in a LightCycler (Mx3000P; Stratagene) according to the protocol (95°C hot start for 10 min followed by 40 amplification cycles, denaturation at 95°C, primer annealing at 59°C, and amplicon extension at 72°C) using oligonucleotide primer sets for IL-1β, IL-6, and TNF-α (11). The copy number (number of transcripts) of amplified products was calculated from a standard curve obtained by plotting known input concentrations of plasmid DNA and normalizing to 18S rRNA.

Cytokine analysis

Titers of IL-1β, TNF-α, and IL-6 were determined using commercial ELISAs according to the manufacturer’s instructions (R&D Systems).

Induction and assessment of collagen-induced arthritis (CIA)

Male DBA/1 mice, 6–10 wk of age, were purchased from Harlan Sprague Dawley and The Jackson Laboratory and housed in the animal resource facility at the Children’s Hospital of Pittsburgh Rangos Research Center (Pittsburgh, PA). The study was approved by the Children’s Hospital of Pittsburgh’s Animal Research and Care Committee. Mice were treated by i.v. injection with 1010 particles of adenoviral vectors in 200 µl of PBS. CIA was induced by intradermal immunization with bovine type II collagen (CII; Elastin Products), and a booster was given 21 days later as previously described (12). Mice were evaluated for arthritis several times weekly using a macroscopic scoring system ranging from 0 to 4 (0 = no detectable arthritis; 1 = swelling and/or redness of paw or 1 digit; 2 = two joints involved; 3 = three to four joints involved; and 4 = severe arthritis of entire paw and digits). The arthritic index for each mouse was calculated by adding the score of the four individual paws. The statistical significance of difference was determined using the exact Wilcoxon test. p < 0.05 was considered significant. Mice were sacrificed at various times, and joints were used for histopathology, whereas livers were snap frozen and stored in a liquid nitrogen freezer.

Results

FSTL-1 is overexpressed in acute CIA by fibroblast-like synoviocytes

While analyzing gene expression profiles in CIA using DNA microarrays, we discovered that FSTL-1 mRNA is highly up-regulated in mouse paws during early arthritis, most prominently at the interface of synovial pannus and eroding bone (13), suggesting an active role in joint destruction. We also observed FSTL-1 expression in RA synovium. To further explore the role of FSTL-1 in arthritis, we examined its cellular distribution in the joints of mice by immunohistochemistry at various times during the course of arthritis (Fig. 1). An increase in expression was observed in early CIA (day 28), whereas only minimal expression was observed in late CIA (day 49). The expression of FSTL-1 localized to fibroblasts, as evidenced by CD90 staining. No expression was observed in macrophages, neutrophils, or T cells (data not shown).

FSTL-1 induces secretion of proinflammatory cytokines from fibroblasts and macrophages

Arthritic synovium contains large numbers of fibroblasts and macrophages that are centrally involved in joint destruction. Having

FIGURE 2. FSTL-1 induces secretion of proinflammatory cytokines from fibroblasts and macrophages. A, Monkey COS-7 fibroblasts were stably transfected by electroporation with a plasmid (pReCMV) encoding the neomycin resistance gene with or without human FSTL-1. After selection for 4 wk, cells were plated for 3 days, and supernatants were assayed for IL-6. B–D, Human U937 monocyte cells were similarly transfected. After selection for 4 wk, cells were stimulated with 10 ng/ml PMA in the presence or absence of 100 ng/ml LPS and assayed for IL-1β, TNF-α, and IL-6. Bars, mean and SEM of triplicate samples, ∗, p < 0.05.
demonstrated that fibroblast-like synoviocytes overexpressed FSTL-1 during acute arthritis, we wished to examine the downstream effects of such overexpression on this cell phenotype. We were unsuccessful at transfecting primary mouse or human synovial fibroblasts; however, we were able to stably transfect the monkey kidney fibroblast cell line, COS-7, by electroporation. After transfection with human FSTL-1, a spontaneous increase in IL-6 expression was observed (Fig. 2A). We also stably transfected the human monocyte cell line, U937, with FSTL-1. Although these cells did not spontaneously produce inflammatory cytokines, addition of PMA to induce differentiation into macrophages led to a significant increase in secretion of IL-1β, TNF-α, and IL-6 (Fig. 2, B–D). A substantial synergistic effect was observed on addition of LPS, suggesting that FSTL-1 utilizes a distinct signaling pathway different from the LPS receptor.

**FSTL-1 induces production of proinflammatory cytokines in vivo**

To determine the in vivo effects of overexpression of FSTL-1, DBA/1 mice were given an adenovirus encoding either mouse FSTL-1 (Ad-mFSTL-1) or a control virus lacking a transgene (Ad-BglII) by i.v. injection. Administration of adenovirus i.v. predominantly targets the liver. Seven days after adenovirus administration, livers were removed and analyzed for expression of TNF-α, IL-1β, and IL-6 by real time PCR. A pronounced increase was observed in mRNA for all 3 cytokines (Fig. 3), demonstrating that FSTL-1 up-regulates their expression in vivo.

**FSTL-1 exacerbates CIA**

To demonstrate that FSTL-1 could function as a proinflammatory mediator in arthritis, CIA was induced in DBA/1 mice by immunization with CII. Mouse FSTL-1 was overexpressed before onset of arthritis by i.v. administration of Ad-mFSTL-1. Control mice received Ad-BglII. Severity of arthritis, as measured by the arthritic index (12), was significantly greater in mice receiving Ad-mFSTL-1, compared with controls (Fig. 4A). There was also a significant increase in the percent of mice developing severe arthritis in the Ad-mFSTL-1 group (Fig. 4B). Histologic analysis correlated with the arthritic index scores (Fig. 5). Substantially more synovial inflammation, with infiltration of neutrophils and mononuclear cells, and cartilage destruction was observed in mice treated with FSTL-1.

**FSTL-1 induces spontaneous inflammation and synovitis**

To determine whether the observed proinflammatory effects of FSTL-1 required an inflammatory costimulus, such as immunization with CII, Ad-mFSTL-1 was injected intradermally into paws of unimmunized DBA/1 mice. To our surprise, severe paw swelling and erythema was observed, beginning 8 days after injection (Fig. 6, A and B) and persisting for 1 week before subsiding. Histologic analysis revealed synovitis, with infiltration of inflammatory cells into the synovium and surrounding tissue (Fig. 6, C and D). A substantial synergistic effect was observed on addition of LPS, suggesting that FSTL-1 utilizes a distinct signaling pathway different from the LPS receptor.
FIGURE 5. FSTL-1 exacerbates synovitis and joint destruction in CIA. Arthritic mice treated with either Ad-mFSTL-1 (A) or with a control virus, Ad-BgIII (B) were sacrificed on day 28, and knee joints were sectioned and stained with H&E. C, An age-matched healthy control. A. Severe inflammation of the synovium with invasion of cartilage and bone (arrow). F, femur; S, synovium (magnification, ×200). D. Protein homogenates from these paws had a significant increase in IL-6 and IL-1β, compared with control paws (Fig. 6, E and F).

Discussion
These findings demonstrate that FSTL-1 is a previously unrecognized proinflammatory mediator capable of inducing spontaneous inflammation. In the context of arthritis, FSTL-1 overexpression in the joints might lead to secretion of IL-6 by fibroblast-like synoviocytes which serves as a proinflammatory signal. In addition, FSTL-1 secreted by these cells might stimulate resident macrophages to secrete proinflammatory cytokines. Efforts are currently under way to identify an FSTL-1 receptor on these cells.

Tanaka et al. recently reported that administration of human FSTL-1 to BALB/c mice with Ab-induced arthritis ameliorated disease (14), possibly by reducing synovial production of matrix metalloproteinases (15). Although seemingly contradictory to our findings, the effect was mild and may be a consequence of using the xenogeneic mouse protein or using a mouse with a predominant Th2 phenotype (BALB/c). We have not been able to reproduce this finding in the CIA model, and polymorphisms in FSTL-1 were not found to be associated with genetic susceptibility to RA (16). However, we cannot rule out the possibility that FSTL-1 might have additional effects, including immunosuppressive effects in certain circumstances.

Based on their ability to bind various cell growth factors and extracellular matrices, it has been proposed that follistatin modules may function as regulators of growth factors and/or cytokines (8). Our observations therefore suggest a model for FSTL-1 activity in the context of inflammation. FSTL-1 secreted by fibroblasts acts on macrophages to increase production of TNF-α and IL-1β, which in turn increase activin production (17). Activin normally inhibits the activity of IL-6 (18), but FSTL-1, by binding activin, leads to increased IL-6 activity. Neutralizing FSTL-1 may represent a novel approach to treating arthritis and other inflammatory conditions.

Disclosures
The authors have no financial conflict of interest.

References


