Small GTPases : emerging targets in rheumatoid arthritis

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The Ras guanine nucleotide exchange factor Ras-GRF1 promotes MMP-3 production in rheumatoid arthritis synovial tissue

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Abstract

Introduction

Fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients share many similarities with transformed cancer cells, including spontaneous production of matrix metalloproteinases (MMPs). Altered or chronic activation of proto-oncogenic Ras family GTPases is thought to contribute to inflammation and joint destruction in RA, and abrogation of Ras family signaling is therapeutic in animal models of RA. Recently, expression and post-translational modification of Ras guanine nucleotide releasing factor 1 (RasGRF1) was found to contribute to spontaneous MMP production in melanoma cancer cells. Here, we examined the potential relationship between RasGRF1 expression and MMP production in RA, inflammatory osteoarthritis (OA), and reactive arthritis (ReA) synovial tissue and FLS.

Methods

Expression of RasGRF1, MMP-1, MMP-3, and interleukin (IL)-6 was detected in synovial tissue by immunohistochemistry and stained sections were evaluated by digital image analysis. Expression of RasGRF1 in FLS and synovial tissue was also assessed by immunoblotting. Double staining was performed to detect proteins in specific cell populations, and cells producing MMP-1 and MMP-3. RasGRF1 expression was manipulated in RA FLS by cDNA transfection and gene silencing, and effects on MMP-1, TIMP-1, MMP-3, IL-6, and IL-8 production measured by enzyme-linked immunosorbent assay (ELISA).

Results

Expression of RasGRF1 was significantly enhanced in RA synovial tissue, and detected in FLS and synovial macrophages in situ. In cultured FLS and synovial biopsies, RasGRF1 was detected by immunoblotting as a truncated fragment lacking its negative regulatory domain. Production of MMP-1 and -3 in RA but not non-RA synovial tissue positively correlated with expression of RasGRF1 and colocalized in cells expressing RasGRF1. RasGRF1 over-expression in FLS induced production of

MMP-3, and RasGRF1 silencing inhibited spontaneous MMP-3 production.

Conclusions

Enhanced expression and post-translational modification of RasGRF1 contributes to MMP-3 production in RA synovial tissue and the semi-transformed phenotype of RA FLS.

Introduction

Inflammation of affected joints in rheumatoid arthritis (RA) is characterized by infiltration of the synovial sublining by macrophages, lymphocytes, and other immune cells, and intimal lining layer hyperplasia due to increased numbers of intimal macrophages and fibroblast-like synoviocytes (FLS)[1] . Initial in situ and in vitro studies of invasive RA FLS revealed striking similarities with transformed cells expressing mutated proto-oncogene and tumor suppressor gene products[2]. Hyperplastic FLS invading the joints of RA patients resemble proliferating tumor cells and in vitro, RA FLS proliferate more rapidly than FLS from inflammatory non-RA patients or healthy individuals[3]. Characteristic of transformed cells, they spontaneously secrete autocrines and matrix metalloproteinases (MMPs), display anchorage-independent growth, and are resistant to contact inhibition of proliferation[4;5]. While transforming mutations in gene products involved in cellular transformation, such as Ras and PTEN, have not been detected in RA FLS[6;7], it is appreciated that signaling pathways regulated by proto-oncogene and tumor suppressor gene products are constitutively activated due to stimulation by inflammatory cytokines, chemokines, growth factors, and oxidative stress in RA synovial tissue[8].

Ras superfamily small GTPases are expressed throughout mammalian tissue, and play essential roles in coupling extracellular stimuli to multiple downstream signaling pathways[9]. Cellular stimulation results in the activation of guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP on inactive GTPase for GTP. The binding of GTP to Ras superfamily GTPases leads to a conformational change in the GTPase, allowing signaling to downstream effector proteins[10]. Of these small GTPases, Ras family homologues (H-, K-, and N-Ras) are important in coupling extracellular stimuli to activation of a shared set of signaling pathways regulating cell proliferation and survival, including mitogen-activated protein (MAP) kinase cascades, phosphoinositide 3-kinase (PI3K) and Ral GTPases[9;11]. The related but distinct family of Rho GTPases (including Rac, Cdc42 and Rho proteins) regulate cellular polarization and chemotactic responses, MAP kinase cascades, and oxidative burst machinery[12;13]. Specificity in GEF activation of GTPase families, and even GEF selectivity in activating different Ras homologs, as well as differential coupling of GEFs to specific types of cellular receptors, such as Son-of-sevenless to tyrosine kinase-dependent receptors, and Ras guanine nucleotide-releasing factor (RasGRF) 1 to G protein-coupled receptors, achieves specificity in Ras superfamily GTPase signaling.

Previous studies have demonstrated that Ras family homologs are present in RA

synovial tissue, and preferentially expressed in the intimal lining layer[14;15]. Activation of Ras effector pathways, including MAP kinases, PI3K, and nuclear factor (NF)-kB, is enhanced in RA compared to disease controls[16-18]. In RA synovial fluid T cells constitutive activation of Ras, in conjunction with inactivation of the related GTPase Rap1, contributes to persistent reactive oxygen species production by these cells[19;20]. In RA FLS, ectopic expression of dominant-negative (DN) H-Ras suppresses interleukin (IL)-1-induced extra-cellular signal-regulated kinase (ERK) activation and IL-6 production[21]. DN Raf kinase, which broadly binds to and inhibits Ras family members and related GTPases, suppresses epidermal growth factor-induced ERK and c-jun N-terminal kinase (JNK) activation in RA FLS, and reduces constitutive expression of MMPs[22]. Additionally, strategies which broadly inhibit Ras family function in vivo are protective in animal models of arthritis[21-23].
Evidence is now emerging that altered expression of Ras GEFs may contribute to autoimmune diseases. Mice lacking expression of the Ras GEF Ras guanine nucleotide-releasing protein 1 develop a spontaneous systemic lupus erythematosis (SLE)-like disease, and similar defects are observed in a subset of SLE patients[24-26]. Recent evidence has shown that expression levels of the GEF RasGRF1 regulate constitutive MMP-9 production in human melanoma cells[27]. RasGRF1 displays in vitro and in vivo exchange activity against H-Ras[28], as well as the Rho family GTPase Rac[29;30]. RasGRF1 activity can also be regulated by protease-dependent post-translational modification, as calpain-dependent cleavage of RasGRF1 enhances its Ras-activating capacity in vitro and in vivo[31]. Given similarities between FLS and transformed cancer cells, we examined the expression of RasGRF1 in RA and non-RA synovial tissue and FLS, providing evidence that elevated RasGRF1 expression and post-translational modification of this protein in RA synovial tissue may contribute to joint destruction by stimulating MMP-3 production.

Materials and Methods

Patients and synovial tissue samples

Synovial biopsy samples were obtained by arthroscopy as previously described[32] from an actively inflamed knee or ankle joint, defined by both pain and swelling, of patients with RA (n=10)[33], inflammatory osteoarthritis (OA) (n=4)[34], or reactive arthritis (ReA) (n=7)[35]. Patient characteristics are detailed in Table 1. All patients

Table I. Clinical features of RA, ReA and OA patients included in the study.

Diagnosis	Characteristic	Median (range)
Rheumatoid arthritis	Age (years)	55 (30-68)
	Male:female	6:4
	Disease duration (months)	84 (2-360)
	Erythrocyte sedimentation rate (mm/hour)	64 (2-107)
	Rheumatoid factor (kU/L)	21 (0-138)
Reactive arthritis	Age (years)	33 (22-39)
	Male:female	4:3
	Disease duration (months)	2.5 (1-14)
	Erythrocyte sedimentation rate (mm/hour)	5 (0-14)
	Rheumatoid factor (kU/L)	0 (0-1)
Osteoarthritis	Age (years)	72.5 (54-83)
	Male:female	2:2
	Disease duration (months)	66 (6-180)
	Erythrocyte sedimentation rate (mm/hour)	9.5 (5-43)
	Rheumatoid factor (kU/L)	0 (0-1)

provided written informed consent prior to the start of this study, which was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, The Netherlands.

Immunohistochemical analysis

Serial sections from six different biopsy samples per patient were cut with a cryostat (5 mm), fixed with acetone, and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide in 0.1% sodium azide/phosphate-buffered saline. Sections were stained overnight at 4°C with monoclonal antibodies against MMP-1 (MAB 1346) and MMP-3 (MAB 1339) (both from Chemicon International, Temicula, CA) and rabbit polyclonal antibodies recognizing RasGRF1 (SC-863) (Santa Cruz Biotechnology,

Santa Cruz, CA), and anti-IL-6 (Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands). For control sections, primary antibodies were omitted or irrelevant immunoglobulins were applied. Sections were then washed and incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated- or swine anti-rabbit-HRP-conjugated antibodies (from Dako, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with amino-ethylcarbazole (AEC, Vector Laboratories, Buringame, CA)[36]. Sections were then counterstained with Mayer's hematoxylin (Perkin Elmer Life Sciences, Boston, MA) and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Digital image analysis

For quantitative analysis of protein expression, stained slides were randomly coded by an independent observer, blinded to antibodies used and clinical diagnosis. Stained sections were analyzed by computer-assisted image analysis using the Qwin analysis system (Leica, Cambridge, UK) as previously described in detail[37]. Values of integrated optical densities (IOD)/mm^2 and number of positive cells/mm^2 were obtained for both the intimal lining layer and the synovial sublining, and corrected for total number of nucleated cells/mm^2.

Immunohistochemical double staining

To detect potential cell-specific expression of RasGRF1 in synovial tissue, tissue sections were incubated with anti-RasGRF1 antibodies overnight at 4^{o}C, followed by serial incubation with swine anti-rabbit-HRP antibodies, biotinylated tyramine, and streptavidin-HRP. Sections were then labeled for one hour at room temperature with FITC-conjugated antibodies to detect T lymphocytes (anti-CD3, clone SK7, Becton Dickinson, San Jose, CA), FLS (anti-CD55, mAB67, Serotec, Oxford, UK), and macrophages (anti-CD68, clone DK25, Dako), followed by incubation with alkaline phosphatase (AP)-conjugated goat anti-mouse antibody (Dako). HRP staining was developed as above, and AP staining was developed using an AP Substrate III kit (SK-5300, Vector Laboratories) according to the manufacturer's instructions.

FLS culture and transfection with cDNA and locked nucleic acids (LNA)

RA FLS and OA FLS were cultured as previously described[38]. FLS were used between passages 4 and 9 and cultured in medium containing 10% fetal calf serum (FCS). To examine the influence of RasGRF1 overexpression on FLS MMP production, 2 x 10^5 RA FLS were plated overnight in 6-well plates and then transfected with 7.5 mg control pCDNA3 or pCDNA3 encoding full-length human RasGRF1 (provided by Dr. R. Zippel, University of Milan, Milan, Italy) using Lipofectamine 2000 transfection reagent (Invitrogen, Verviers, Belgium) as per the manufacturer's instructions. Culture medium was replaced with medium containing 1.0% FCS after 24 hours, and cells harvested 48 hours post-transfection.

RasGRF1 expression in FLS was silenced using RasGRF1-specific and control LNA designed with on-line software (https://rnaidesigner.invitrogen.com/rnaiexpress/design.do) (synthesized by Exiqon A/S, Vedbaek, Denmark). LNA oligonucleotides used were RasGRF1 (TTGcgttaccttTGCt – LNA nucleotides in capital letters, DNA nucleotides in lower case letters), and as a negative control, a scrambled RasGRF1 sequence (GTAcagcaagatTGGg). LNA transductions were performed with Lipofectamine 2000 transfection reagent and 50 nM LNA. Culture medium was replaced with starvation medium (1% FCS in DMEM) after 24 hours and cells harvested after an additional 24 hours.

Protein preparation and immunoblotting

FLS were lysed in Laemli's buffer. Frozen synovial biopsies were homogenized and proteins solubilized using a ReadyPrep™ Sequential Extraction Kit (BioRad, Hercules, CA) and protein content quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL). Equivalent amounts of protein were resolved by electrophoresis on NuPage 4-12% Bis-Tris gradient gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (BioRad). Proteins were detected by immunoblotting with anti- RasGRF1 (SC-863 and SC-224, Santa Cruz), actin (Santa Cruz) or tubulin (Sigma Aldrich, St. Louis, MO) antibodies, followed by extensive washing, incubation with HRP-conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (Bio-Rad) and enhanced chemiluminescence detection (Pierce). For quantitative analysis of RasGRF1 expression, staining was detected using IRDye 680 or 800 –labelled antibodies and an Odyssey Imager (LI-COR, Bad Homburg, Germany), and quantified

using Odyssey 3.0 software.

Measurement of MMP-1, MMP-3, TIMP-1, IL-6 and IL-8 production by FLS

Medium was removed from FLS 24 hours after introduction of cDNA or LNA, and replaced with starvation medium. After 24 hours, cell-free tissue culture supernatants were harvested and analyzed using ELISA kits for MMP-1, MMP-3, TIMP-1 (all from R&D Systems Europe Ltd., Abingdon, UK), IL-6 and IL-8 (both from Sanquin Reagents, Amsterdam, The Netherlands), according to the manufacturer's instructions.

Immunofluorescence staining

Synovial tissue sections were incubated with primary anti-RasGRF1 antibodies overnight at 4°C, followed by incubation for 30 minutes with Alexa-594-conjugated goat anti-rabbit antibodies (Molecular Probes Europe, Leiden, the Netherlands). Sections were then incubated with mouse monoclonal antibodies against MMP-1, MMP-3, or IL-6, followed by incubation with Alexa-488-conjugated goat anti-mouse antibody (Molecular Probes Europe), mounting in Vectashield (Vector Laboratories) and analysis using a fluorescence microscope (Leica DMRA) coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, the Netherlands).

Statistical analysis

Wilcoxon's nonparametric signed ranks test was used to compare protein expression between intimal lining layer and the synovial sublining layer within diagnostic groups. As no trend towards a difference in RasGRF1 expression was found between inflammatory OA and ReA synovial tissues, these two non-erosive groups were combined as non-RA for further analyses. The Mann-Whitney U test was used for the comparison of RasGRF1 expression between diagnostic groups. Correlations between RasGRF1 expression and MMP-1, MMP-3 and IL-6 expression in synovial tissue were assessed by Spearman's rank correlation coefficient. ELISA results were examined using Student's t-test. P values less than 0.05 were considered statistically

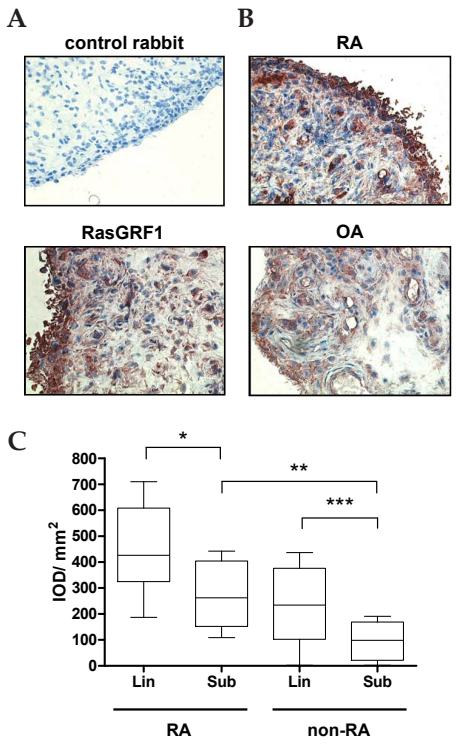

Figure 1. Detection of RasGRF1 protein expression in RA and non-RA synovial tissue. *(a) Representative stainings of RA synovial tissue with control and anti-RasGRF1 antibodies. (b) Representative stainings of RA and OA synovial tissue with anti-RasGRF1 antibodies. Stainings were developed with AEC (red), and counterstained with Mayer's hematoxyline. Magnification x 100. (c) Quantitative analysis of Ras signaling protein expression in RA and non-RA (OA and ReA) synovial tissue. Integrated optical densities (IOD)/ mm², corrected for nucleated cells, for staining of synovial sublining (sub) and intimal lining (lin) layer of 10 RA and 11-non-RA (4 inflammatory OA, 7 ReA) patients with anti-RasGRF1 antibodies. IOD were calculated by computer-assisted image analysis. Box plots represent the 25th to 75th percentiles, the lines within each box the median, and lines outside the boxes designate the 10th and 90th percentiles. Bars indicate statistically significant differences in protein expression between sublining and intimal lining layer tissues within diagnostic groups and between diagnostic groups. * P < 0.05, ** P < 0.01, *** P < 0.005.*

significant. There was no correction for multiple comparisons due to the exploratory nature of the study.

Results

Expression of RasGRF1 in RA and non-RA synovial tissue

To gain insight into potential involvement of RasGRF1 in RA, immunohistochemical staining was performed on RA synovial tissue using RasGRF-1 specific antibodies. While no specific staining was observed with irrelevant control rabbit antibodies, robust staining was observed in RA synovial tissue with anti-RasGRF1 antibodies (Figure 1A). RasGRF1 staining was most apparent throughout the intimal lining layer,

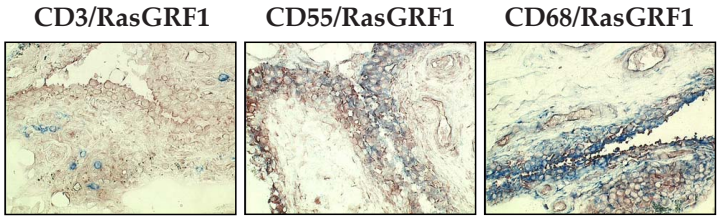

Figure 2. Representative double stainings of RA synovial tissue with antibodies against RasGRF1 and cell-specific markers. *Synovial tissue sections were stained overnight with antibodies against RasGRF1, followed by antibodies against CD3, CD55, and CD68. After biotin tyramide enhancement, staining was developed with AEC (red, RasGRF1) and Fast blue (blue, cell-specific markers). Magnification x100.*

but was also observed in infiltrating mononuclear cells found in the synovial sublining. Initial qualitative analysis of RasGRF1 expression in RA and inflammatory OA synovial tissue suggested that RasGRF1 expression was elevated in RA synovial tissue (Figure 1B). We therefore compared RasGRF1 expression in RA and non-RA (inflammatory OA and ReA) synovial tissue quantitatively, using digital image analysis (Figure 1C). Preliminary analyses indicated no differences in RasGRF1 expression between inflammatory OA and ReA synovial tissue, either in the intimal lining layer (mean integrated optical density [IOD]/mm^2 ± standard error of the mean OA, 259.0 ± 131.6; ReA, 263.4 ± 77.0) or the synovial sublining layer (OA, 113.3 ± 55.7; ReA, 135.6 ± 51.9) (data not shown). Therefore, these two non-erosive groups were combined as non-RA for further analyses. Comparing RA with non-RA synovial tissue, RasGRF1 expression was elevated in RA ($P < 0.05$) and non-RA ($P < 0.01$) intimal lining layer as compared to the synovial sublining. RasGRF1 expression was enhanced in the synovial sublining of RA tissue as compared to non-RA synovial tissue ($P < 0.01$), and a trend towards enhanced RasGRF1 expression was observed in the RA intimal lining layer. Correction of RasGRF1 expression for the number of RasGRF1-positive cells, confirmed that RasGRF1 expression was enhanced in both the synovial sublining ($P < 0.005$) and intimal lining layer ($P < 0.05$) of RA patients compared to non-RA patients (data not shown). Qualitative double labeling of RA synovial tissue with antibodies recognizing RasGRF1 and markers for T lymphocytes (CD3), FLS (CD55), and macrophages (CD68) revealed that RasGRF1 expression was restricted to FLS and macrophages (Figure 2).

RasGRF1 expression in RA and non-RA FLS

To independently confirm RasGRF1 expression in synovial tissue and FLS detected by immunohistochemistry, we performed immunoblotting experiments on lysates derived from intact RA and OA synovial biopsies, and RA and OA FLS. In protein lysates derived from intact RA and OA synovial biopsies (Figure 3), we were unable to detect full-length 140 kDa RasGRF1. However, we did observe prominent expression of a 98 kDa truncation product, and lower and variable levels of 75 and 54 kDa truncation products. These C-terminal fragments are thought to be generated by calpain-dependent cleavage, resulting in constitutive activation of RasGRF1[27;31]. In analyses of FLS lysates, full-length 140 kDa RasGRF1 was detected by immunoblotting in only one of six RA FLS lines (RA FLS5), and neither of two OA FLS lines tested (Figure 4A). In contrast, a 54 kDa RasGRF1 C-terminal fragment was detected in all RA and OA FLS lines, a 75 kDa fragment in three of five RA FLS and both OA FLS lines, and a 98 kDa C-terminal fragments in four of six RA and both OA lines. Quantitative analysis of RasGRF1 protein expression in 5 RA and 5 OA FLS lines revealed no significant difference in total RasGRF1 expression (Figure 4B). With the exception of the 74 kDa RasGRF1 fragment, which was detected at lower levels in RA FLS ($P < 0.05$), other RasGRF1 truncation fragments, as well as full-length RasGRF1, were expressed at similar levels in RA and OA FLS.

To verify that the observed truncation products were derived from RasGRF1, rather than non-specific interactions with the antibodies, we performed additional experiments. First, RA FLS were transfected with cDNA encoding full-length RasGRF1 (Figure 4C and Figure 4D). Quantitative analysis of proteins detected by immunoblotting demonstrated that transfection of RA FLS with RasGRF1 cDNA encoding

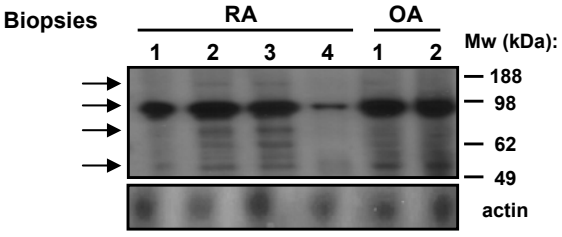

Figure 3. RasGRF1 is expressed as a truncated protein in synovial tissue. *Immunoblot analysis of RasGRF1 and actin in RA and OA synovial biopsy lysates. 98, 75 and 54 kDa proteins reacting with RasGRF1 antibodies, and expected position of full-length 140 kDa RasGRF1, are indicated on the left by arrowheads. Relative mobility of molecular weight standards (kDa) are indicated to the right.*

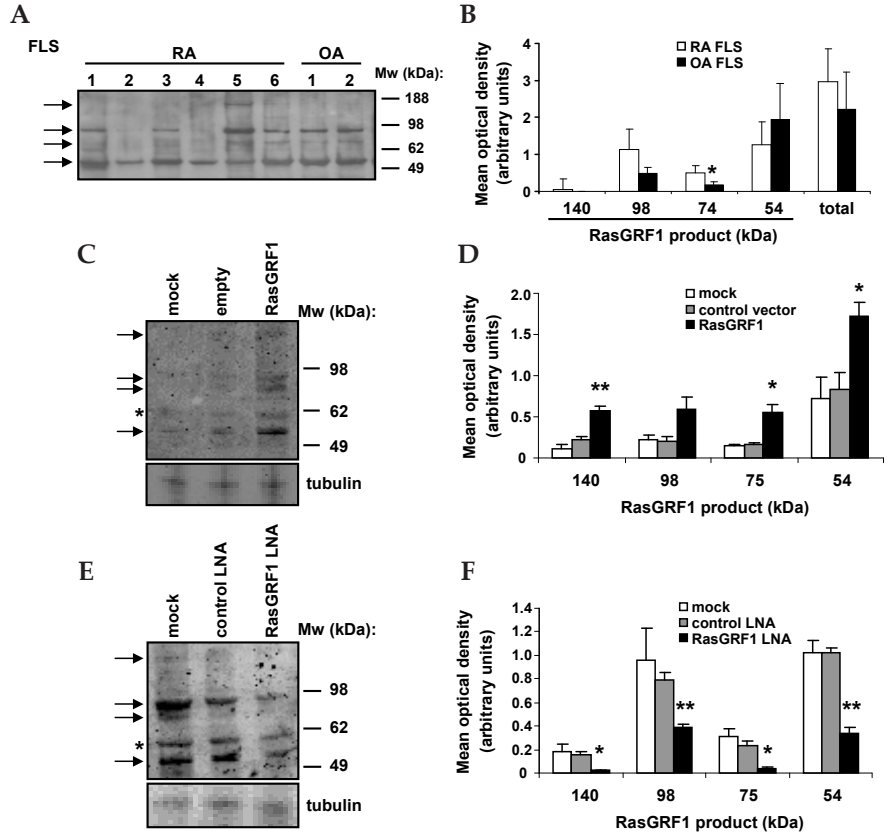

Figure 4. RasGRF1 is expressed as a truncated protein in FLS. *(a) Immunoblot analysis of RasGRF1 in RA and OA FLS. 140, 98, 75 and 54 kDa proteins reacting with RasGRF1 antibodies are indicated on the left by arrowheads. Relative mobility of molecular weight standards (kDa) are indicated to the right. (b) Expression of 140, 98, 75, and 54 kDa RasGRF1 polypeptides, as well as total RasGRF1 signal, normalized to tubulin expression was quantified in RA (n = 5) and OA (n = 5) FLS lines, and expressed as mean optical density ± SEM. (c) Overexpression of RasGRF1 in RA FLS. RA FLS were treated with transfection reagent alone (mock) or transfected with empty (control) vector or vector encoding RasGRF1, and cell lysates immunoblotted with antibodies against RasGRF1 (upper panel) and tubulin (lower panel). Expression of full-length and truncated RasGRF1 polypeptides is indicated with arrows, and a 60 kDa polypeptide with an asterisk. (d) Expression of 140, 98, 75, and 54 kDa RasGRF1 polypeptides following transfection of RA FLS with empty vector or RasGRF1, normalized to tubulin expression, was quantified and expressed as mean optical density ± SEM (middle panel) (n=4). (e) Silencing of RasGRF1 expression with LNA. RA FLS were treated with transfection reagent alone (mock) or transduced with control or RasGRF1 LNA and lysates assessed for expression of RasGRF1 (upper panel) and tubulin (lower panel) by immunoblotting and (f) quantitative analysis as in (d). * P < 0.05, ** P< 0.01 compared to controls.*

full-length RasGRF1 resulted in the enhanced expression of the 140 ($P < 0.01$), 98, 75 ($P < 0.05$), and 54 kDa ($P < 0.05$) forms of RasGRF1. Second, we silenced RasGRF1 expression by transduction of RA FLS with RasGRF1-specific LNA. LNA are antisense nucleotide analogs containing methylene bridges which mimic RNA monomer structure, disrupt gene expression by promoting mRNA degradation and/or preventing gene product translation[39]. RasGRF1-specific LNA decreased RasGRF1 expression in RA FLS compared to control scrambled LNA (Figure 4E), while leaving tubulin expression unaffected. Significant decreases in the expression of full-length 140 kDa RasGRF1 ($P < 0.05$), and 98 ($P < 0.01$), 75 ($P < 0.05$) and 54 kDa ($P < 0.01$) forms were achieved (Figure 4F). Exposure of FLS to transfection reagent alone resulted in the generation of an additional 60 kDa polypeptide (mock-treated FLS in Figures 4C and 4E, noted by an asterisk next to figures) not observed in synovial biopsies or untreated FLS, possibly due to activation of an unidentified cellular protease.

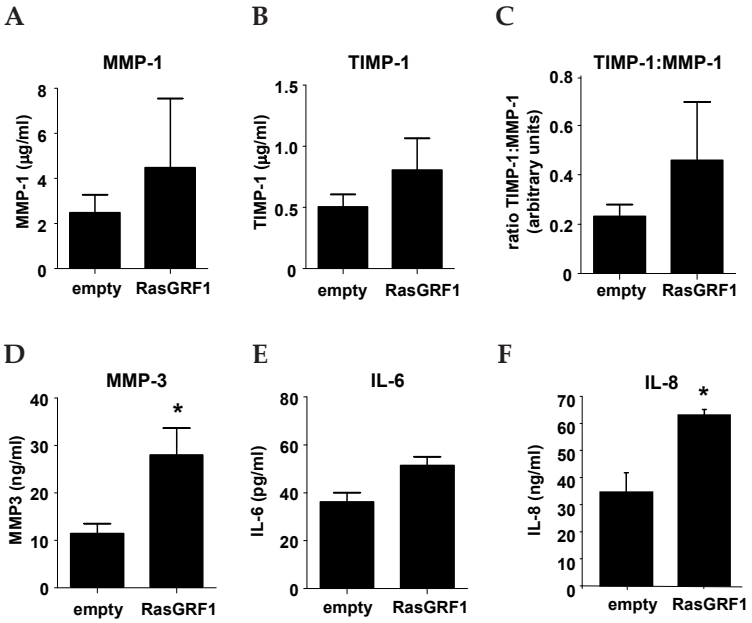

Figure 5. Effect of RasGRF1 over-expression on RA FLS MMP and cytokine production. *Tissue culture supernatants from RA FLS transfected with empty vector or RasGRF1 were harvested and assessed for production of (a) MMP-1, (b) TIMP-1, (c) the ratio of TIMP-1 to MMP-1, (d) MMP-3, (e) IL-6 (n = 4 each) and (f) IL-8 (n = 3) by ELISA. * $P < 0.05$ compared to controls.*

Effects of changes in RasGRF1 expression on RA FLS MMP-3 production in vitro

As RasGRF1 expression levels regulate MMP production in cancer cell lines[27], we examined if modulation of RasGRF1 expression in RA FLS might also regulate constitutive MMP and cytokine production. Quantitative analysis of FLS tissue culture supernatants demonstrated that RasGRF1 over-expression had no effect on FLS production of MMP-1 (Figure 5A) or TIMP-1 (Figure 5B). Additionally, the ratio of TIMP-1 expression relative to MMP-1 was unaffected (Figure 5C). However, forced expression of RasGRF1 induced an approximately 150% increase in MMP-3 production (27.99 ng/ml ± 5.62) (mean ± SEM) compared to FLS transfected with empty control vector alone (11.47 ng/ml ± 2.02) ($P < 0.05$) (Figure 5D). Enhancing RasGRF1 expression had no effect on spontaneous IL-6 production by RA FLS (Figure 5E),

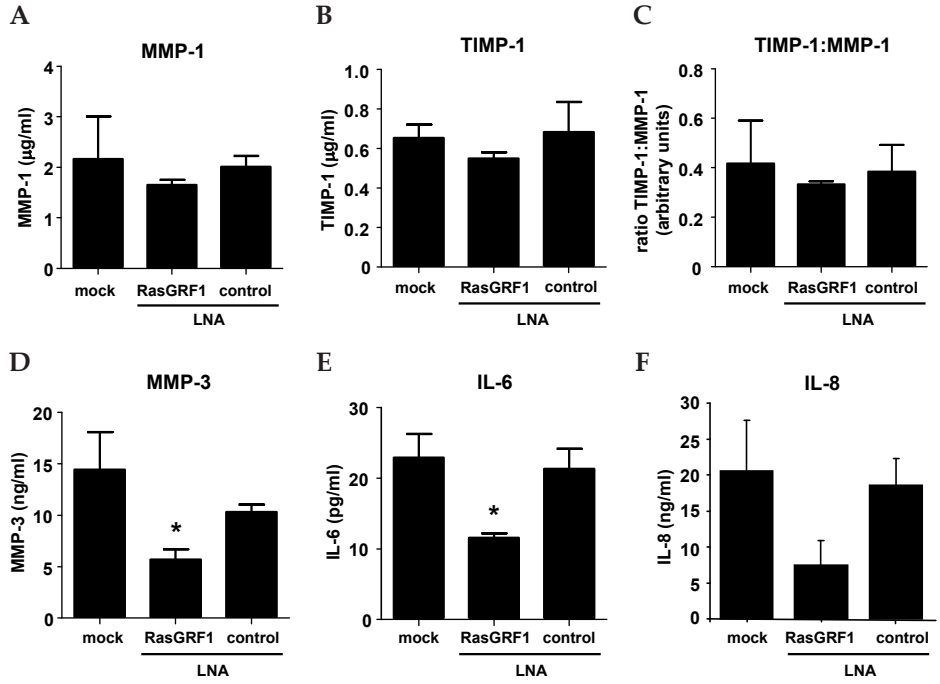

Figure 6. Effect of RasGRF1 gene silencing on RA FLS MMP and cytokine production. *Tissue culture supernatants from RA FLS treated with transfection reagent alone (mock) or transfected with control or RasGRF1 LNA were harvested and assessed for production of MMP-1 (a), TIMP-1 (b), the ratio of TIMP-1 to MMP-1 (c), MMP-3 (d), IL-6 (e) (n = 4 each) and (f) IL-8 (n = 3) by ELISA. * $P < 0.05$ compared to controls.*

but did increase spontaneous IL-8 secretion by approximately 2-fold ($P < 0.05$) (Figure 5F). To determine if RasGRF1 was required for spontaneous MMP or cytokine production, we silenced RasGRF1 gene expression using LNA. Again, modulation of RasGRF1 expression failed to influence MMP-1 and TIMP-1 production, or the ratio of TIMP-1 relative to MMP-1 (Figure 6A-C). A significant suppression of spontaneous MMP-3 production was observed in tissue culture supernatants of FLS transduced with RasGRF1-specific LNA (Figure 6D) ($P < 0.05$), as compared to FLS treated with transfection reagent alone or in combination with control scrambled LNA. Although over-expression of RasGRF1 in RA FLS failed to enhance basal IL-6

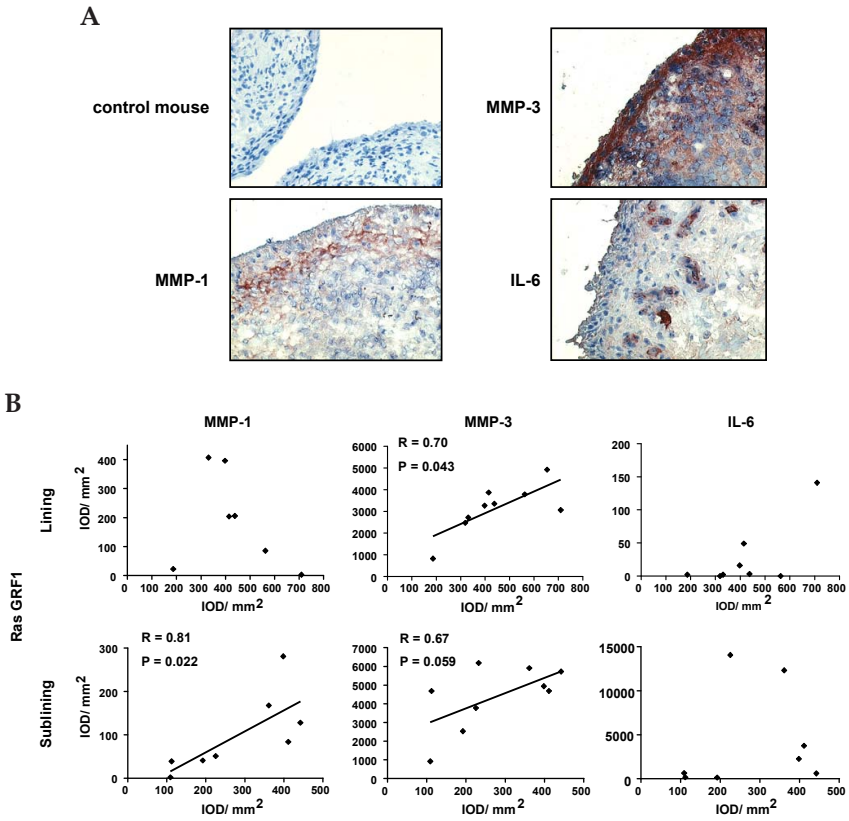

Figure 7. Association of RasGRF1 expression with MMP production in RA synovial tissue. *(a) Representative stainings of RA synovial tissue with control and anti- MMP-1, MMP-3, and IL-6 antibodies (magnification x 100). (b) Correlation of Ras signaling protein expression with MMP-1 and MMP-3 production in RA synovial tissue. Pearson R values (R) and P values are indicated.*

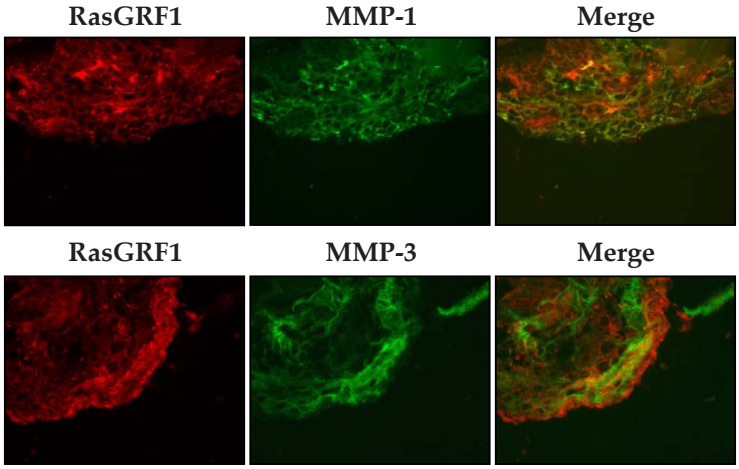

Figure 8. Double immunofluorescence labeling of RasGRF1, MMP-1 and MMP-3 in RA synovial tissue. *RA synovial tissue was stained with combinations of anti-RasGRF1 and either anti-MMP-1 (upper panels) or anti-MMP-3 (lower panels). Sections were then stained with fluorochrome-conjugated anti-rabbit Ig (red) and anti-mouse IgG (green) antibodies to visualize RasGRF1 and MMP expression, respectively. Colocalization of RasGRF1 with MMPs is visualized by yellow staining in merged images (right panels).*

production (Figure 5E), IL-6 levels were significantly decreased following silencing of RasGRF1 expression (Figure 6E) ($P < 0.05$). An apparent 67% reduction in spontaneous IL-8 production was also noted, but this did not reach statistical significance ($P = 0.069$) (Figure 6F).

Relationship between RasGRF1 expression and MMP production in RA synovial tissue

Our in vitro data indicated an important role for RasGRF1 in regulating MMP-3 expression in RA FLS. We therefore examined if expression of RasGRF1 was associated with MMP-3 production in RA synovial tissue. Immunohistochemical analysis demonstrated that MMP-1, MMP-3, and IL-6 were readily detected in RA synovial tissue (Figure 7A). RasGRF1 expression demonstrated a strong positive correlation ($R= 0.81$, $P = 0.022$) with MMP-1 in the RA synovial sublining, but not in the intimal lining layer (Figure 7B). Instead, a positive correlation between RasGRF1 and MMP-

3 expression was observed in the intimal lining layer (R = 0.70, P = 0.043). In non-RA patients, no association between RasGRF1 and MMP-1 (synovial sublining: R = 0.17, P = 0.703; intimal lining layer: R = -0.89, P = 0.083) or MMP-3 (synovial sublining: R = 0.83, P = 0.058; intimal lining layer: R = -0.20, P= 0.917) expression was observed (data not shown). No correlation was observed between RasGRF1 expression and IL-6 expression in either RA or non-RA patient cohorts (Figure 7B, and data not shown). Double immunofluorescent staining revealed colocalization of RasGRF1 with MMP-1 and MMP-3 in RA synovial tissue (Figure 8). Colocalization of RasGRF1 with MMP-1 was observed in the synovial sublining (Figure 8, upper panels), while RasGRF1 colocalization with MMP-3 was restricted to the intimal lining layer (Figure 8, lower panels). Together, these data indicate that RasGRF1 may contribute to RA FLS MMP-3 production in vivo.

Discussion

Our results demonstrate that RasGRF1 regulates spontaneous MMP-3 production in RA FLS, and suggest that over-expression of RasGRF1 sensitizes signaling pathways promoting MMP-3 production and joint destruction in RA. RasGRF1 specifically activates H-Ras, but not other Ras homologues in vivo[28], and RasGRF1 activation of H-Ras induces constitutive MMP-9 production in human melanoma cells[27]. RasGRF1 can also activate the Rho family GTPase Rac1[29;30], and a role for Rac1, potentially via activation of JNK, has been recently shown in the regulation of RA FLS proliferation and invasiveness[40]. Data has been reported indicating that RasGRF1 can also stimulate GTP exchange on R-Ras in vitro, although this GEF activity has yet to be verified in vivo[41;42].

Our data raise the possibility that changes in expression of GEFs, such as RasGRF1, or negatively regulatory GAPs, may be more relevant to the pathology of RA than GTPase expression levels. We observe a strong positive correlation between RasGRF1 expression in RA synovial tissue on one hand, and production of MMP-1 and MMP-3 on the other. Such an association is not clearly observed in non-RA synovial tissue. Consistent with the notion that RasGRF1 is involved in the regulation of MMPs, we find that RasGRF1 expression colocalizes to synovial cells producing MMP-1 and MMP-3 in situ, and modulation of RasGRF1 in RA FLS in vitro regulates spontaneous MMP-3 production by these cells. The inability of RasGRF1 modulation to regulate MMP-1 production in RA FLS, despite the positive association

of expression of these proteins in the synovial sublining in vivo, may indicate that other RasGRF1-expressing cells, namely macrophages, are a more important source of MMP-1 in vivo. Consistent with this, we observe a relationship between RasGRF1 and MMP-1 in the synovial sublining, rather than the intimal lining layer where FLS predominate. Additionally, co-localization of cells expressing RasGRF1 and MMP-1 is most apparent in the synovial sublining layer. Further direct studies will be needed to examine if RasGRF1 regulates MMP-1 production in synovial macrophages. Alternatively, RasGRF1-dependent secretion of IL-8 or other as yet unidentified inflammatory cytokines may indirectly promote MMP-1 production in vivo through the recruitment and/or activation of leukocytes.

We provide additional in vitro evidence that although many FLS stimuli regulate both MMP-1 and MMP-3 expression, regulation of these two proteases is not requisitely coupled. For instance, adhesion of RA FLS to laminin-111 in the presence of tumor growth factor-β induces expression of MMP-3 but not MMP-1[43]. Inhibition of JNK can partially block TNF-α -induced MMP-1 production by RA FLS, MMP-3 production is independent of JNK[44]. Reciprocally, mitogen-activated protein kinase-activated protein kinase 2 (MK2) regulates MMP-3 secretion, but not MMP-1, in OA chondrocytes[45]. That regulation of MMP-1 is uncoupled from that of MMP-3 likely reflects differential utilization of NF-kB, AP-1, Ets, and hypoxia-inducible factor-1α transcription factors by the promoters of the MMP-1 and MMP-3 genes[43;46;47]. Similarly, we find that RasGRF1 is necessary for spontaneous IL-6 production by RA FLS, but over-expression of RasGRF1 is not sufficient to augment IL-6 secretion. This may reflect a necessary coordination of RasGRF1 signaling with other signaling pathways, such as previously reported cooperative effects between Ras GTPase and c-myc pathways in the regulation of RA FLS activation[22]. Further definition of pathways by which RasGRF1 modulates MMP and cytokine production will require identification of the immediate downstream target(s) of this GEF in FLS.

While RasGRF1 expression is sufficient and required for spontaneous MMP-3 production in RA FLS, similar effects of RasGRF1 on MMP-1, TIMP-1 and IL-6 are not observed. Stimuli which activate RasGRF1 include ligands for both tyrosine kinase receptors and G-protein-coupled receptor[48]. Examples of receptors known to regulate RasGRF1 and expressed in RA synovial tissue include those for lysophosphatidic acid and muscarinic acid, NMDA, and nerve growth factor[49-52]. In preliminary studies, we have found that silencing of RasGRF1 in RA FLS has no effect on TNF-α or IL-1b -induced MMP-3 production (data not shown). RasGRF1 activity can also

be regulated by post-translational modification, as calpain-dependent cleavage of RasGRF1 enhances Ras-activating capacity in vitro and in vivo[27;31]. Enhanced expression of RasGRF1 in RA compared to non-RA may sensitize RA FLS to produce MMPs in response to extracellular stimuli. This would result from disease-specific extracellular stimuli activating full-length RasGRF1, as well as constitutive signaling from post-translationally modified RasGRF1, such as the predominantly expressed 96 kDa carboxy-terminal fragment we observe in synovial tissue. Identification of the protease(s) responsible for RasGRF1 cleavage in vivo may lead to new therapeutic strategies in the treatment of arthritis.

Conclusions

RasGRF1 expression and post-translational modifications regulate spontaneous MMP-3 production in RA FLS, and is associated with MMP-3 production in RA synovial tissue. Contributions of RasGRF1 to MMP-3 production in RA and other forms of arthritis will likely depend upon 1) RasGRF1 expression levels, 2) the extent of activating post-translational modifications of RasGRF1, and 3) the strength of extracellular stimuli leading to activation of residual full-length RasGRF1. Our data suggest a molecular mechanism by which Ras signaling pathways might contribute to the semi-transformed and invasive phenotype of RA FLS in the absence of oncogenic mutations in Ras superfamily GTPases.

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