Ras family GTPase signaling contributions to inflammation and joint destruction in rheumatoid arthritis

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chapter 3
Silencing expression of Ras family GTPase homologues decreases inflammation and joint destruction in experimental arthritis
chapter 3

Silencing expression of Ras family GTPase homologues decreases inflammation and joint destruction in experimental arthritis

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Abstract

Changes in the expression and activation status of Ras proteins are thought to contribute to the pathologic phenotype of stromal fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA), a proto-typical immune-mediated inflammatory disease. Broad inhibition of Ras and related proteins has shown protective effects in animal models of arthritis, but each of the closely related Ras family homologues, H-Ras, K-Ras, and N-Ras, can make distinct contributions to cellular activation. Here we examined the expression of each Ras protein in synovial tissue and FLS obtained from patients with RA and other forms of inflammatory arthritis. Each Ras protein was expressed at similar levels in synovial tissue and cultured FLS, regardless of disease diagnosis. Each homolog was also activated following FLS stimulation with TNF-α or IL-1β. Constitutively active mutants of each Ras protein enhanced IL-1β-induced FLS MMP-3 production, while only active H-Ras enhanced IL-8 production. Gene silencing demonstrated that each Ras protein contributed to IL-1β-dependent IL-6 production, while H-Ras and N-Ras supported IL-1β-dependent MMP-3 and IL-8 production, respectively. The overlap in contributions of Ras homologues to FLS activation suggested that broad targeting of Ras GTPases in vivo might suppress global inflammation and joint destruction in arthritis. In this study, we show that simultaneous silencing of H-Ras, K-Ras, and N-Ras expression significantly reduces inflammation and joint destruction in murine collagen-induced arthritis.
Introduction

Inflammation of affected joints in rheumatoid arthritis (RA) is characterized by infiltration of the synovial sublining by innate and adaptive immune cells, and intimal lining layer hyperplasia [1]. Initial in situ and in vitro studies of invasive RA stromal fibroblast-like synoviocytes (FLS) revealed striking similarities with transformed cells expressing mutated proto-oncogene and tumor suppressor 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]. In analogy to transformed cells, RA FLS also 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 family GTPase homologues (H-, K-, and N-Ras) are expressed throughout mammalian tissue, and play essential roles in coupling extracellular stimuli to multiple downstream signaling pathways [9]. Ras homologues share high sequence identity, especially in their effector domain, which directly couples GTPases to downstream signaling proteins. Under certain experimental conditions, Ras homologues can redundantly activate a shared set of signaling pathways, including mitogen-activated protein (MAP) kinase cascades, phosphoinositide 3-kinase (PI3K) and Ral GTPases. However, genetic and cell biology studies have provided strong evidence that each Ras homologue has distinct signaling properties. Specificity in Ras homolog signaling is conferred by differential subcellular localization of each homolog, differences in selectivity for downstream effector proteins, differential expression of Ras effectors in tissues, and utilization of specific GEFs to activate Ras proteins [9,10].

Previous studies have demonstrated that Ras family homologues are present in RA synovial tissue, and preferentially expressed in the intimal lining layer [11,12]. Activation of Ras effector pathways, including MAP kinases, PI3K, and nuclear factor (NF)-κB, is enhanced in RA compared to disease controls [13-15]. In RA synovial fluid T cells, constitutive activation of Ras proteins, in conjunction with inactivation of the related GTPase Rap1, contributes to persistent reactive oxygen species production by these cells [16]. A role for de-regulated H-Ras signaling in contributing to the aggressive phenotype of RA FLS has been suggested by the recent finding that Ras guanine nucleotide releasing factor 1 (RasGRF1), a GEF specific for H-Ras, is over-expressed in an activated form in these cells, contributing to spontaneous MMP-3 production [17]. Conversely, ectopic expression of dominant-negative H-Ras suppresses interleukin (IL)-1β-induced extra-cellular signal-regulated kinase (ERK) activation and
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IL-6 production in RA FLS [18]. Dominant-negative c-Raf kinase, which broadly binds to and inhibits Ras homologues and related Ras family members, suppresses epidermal growth factor-induced ERK and c-jun N-terminal kinase (JNK) activation in RA FLS, and reduces constitutive expression of MMPs [19]. Supporting this in vitro evidence that Ras protein signaling can contribute to pathogenic cellular behavior in RA, strategies which broadly inhibit the function of Ras and related protein in vivo are protective in animal models of arthritis [18-20]. However, the involvement and requirement of specific Ras homologues in RA has not been examined. In this study, we find that H-Ras, K-Ras, and N-Ras are widely expressed in the synovium and FLS of patients with RA and other forms of inflammatory arthritis. Using ectopic expression of constitutively active Ras mutants and gene silencing strategies, we demonstrate that each Ras protein makes distinct but overlapping contributions to basal and IL-1β-induced FLS production of IL-6, IL-8, and MMP-3. These results suggest the potential suitability of therapeutic strategies broadly targeting Ras family function in RA, and we observe that combinatorial silencing of H-, K-, and N-Ras in vivo reduces disease severity and joint destruction in murine collagen-induced arthritis (CIA).
Material and Methods

Patients and Synovial Tissue Samples

Synovial biopsy samples were obtained from an actively inflamed knee or ankle joint from two independent cohorts of patients by arthroscopy as previously described [21]. Cohort I included 10 patients with RA, 4 with inflammatory osteoarthritis (OA), and 7 with reactive arthritis (ReA), and characteristics of these patients have been previously described in detail [17]. Cohort II included patients with RA (n = 20) and psoriatic arthritis (PsA) (n = 19). Patient characteristics of Cohort II are detailed in Table 1. All patients met established criteria for RA, inflammatory OA, ReA, and PsA, respectively [22-25]. Written informed consent was provided by all patients prior to participation in the study, and the study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, The Netherlands.

Table 1 / Characteristics of study patients

<table>
<thead>
<tr>
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<th>RA (n=20)</th>
<th>PsA (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>52.0 (38-68)</td>
<td>54.5 (35-70)</td>
</tr>
<tr>
<td>No. male/no. female</td>
<td>11/9</td>
<td>8/11</td>
</tr>
<tr>
<td>Disease durations, years</td>
<td>12.0 (3-44)</td>
<td>12.5 (4-22)</td>
</tr>
<tr>
<td>Swollen Joint Count</td>
<td>12 (3-26)</td>
<td>8 (1-32)</td>
</tr>
<tr>
<td>Tender Joint Count</td>
<td>8 (2-18)</td>
<td>10 (2-48)</td>
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<tr>
<td>No. receiving MTX</td>
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<td>18</td>
</tr>
<tr>
<td>Dosage MTX, mg/wk#</td>
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<td>10 (2.5-20)</td>
</tr>
<tr>
<td>No. erosive/No. non erosive</td>
<td>14/5</td>
<td>7/12</td>
</tr>
<tr>
<td>% RF positive</td>
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<td>0</td>
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<tr>
<td>ESR, mg/hour</td>
<td>19 (4-101)</td>
<td>29 (4-59)</td>
</tr>
<tr>
<td>CRP, mg/liter</td>
<td>4 (1-98)</td>
<td>11 (3-51)</td>
</tr>
<tr>
<td>DAS28</td>
<td>6.0 (3.7-8.0)</td>
<td>7.8 (3-12.6)</td>
</tr>
</tbody>
</table>

* RA= Rheumatoid Arthritis; PsA= Psoriatic Arthritis; MTX= methotrexate; No.= number; RF= rheumatoid factor; ESR= erythrocyte sedimentation rate; CRP= C-reactive protein; DAS28= Disease Activity Score 28. Unless indicated otherwise, values are expressed as the median (range).

#Difference in values between RA and PsA patient cohorts is statistically significant (P < 0.05).
Immunohistochemical Analysis
Serial sections from six different biopsy samples per patient were cut with a cryostat (5 μm), fixed with acetone, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide and 0.1% sodium azide in phosphate-buffered saline (PBS). Sections were stained overnight at 4°C with murine monoclonal antibodies recognizing Ras proteins (pan-Ras, Cell Signaling, Beverly, MA), H-Ras (F235), K-Ras (F234), N-Ras (F155) (all from Santa Cruz Biotechnology, Santa Cruz, CA). 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 antibodies (from Dako, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with amino-ethylcarbazole (AEC, Vector Laboratories, Buringame, CA) [26]. Sections were then counterstained with Mayer's hematoxylin (Perkin Elmer Life Sciences, Boston, MA) and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) for analysis.

Immunohistochemical Double Staining
To detect potential cell-specific expression of Ras homologues in RA synovial tissue, tissue sections were incubated with Ras proteins overnight at 4°C, followed by incubation with goat anti-mouse-HRP. Sections were then labeled for one hour at room temperature with FITC-conjugated antibodies to detect T lymphocytes (anti-CD3, clone SK7, Beconton Dickinson, San Jose, CA), FLS (anti-CD55, mAB67, Serotec, Oxford, UK), and macrophages (anti-CD68, clone DK25, Dako), followed by rabbit anti-FITC antibodies (Dako) and swine anti-rabbit Ig-alkaline phosphatase (AP) conjugate (Dako) for 30 minutes each. HRP staining was developed as above, and AP staining was visualized using an AP Substrate III kit (SK-5300, Vector Laboratories) according to the manufacturer's instructions.

Digital Image Analysis
Stained slides were randomly coded by an observer blinded to antibodies used and clinical diagnosis, and analyzed by computer-assisted image analysis using the Qwin analysis system (Leica, Cambridge, UK) as previously described in detail [27]. Values of integrated optical densities (IOD)/mm2 were obtained for both the intimal lining layer and the synovial sublining, and corrected for total number of nucleated cells/mm2.

Cell Culture
The human Jurkat T cell leukemia line, RA FLS and PsA FLS were cultured as previously described [16,28]. FLS were isolated from patient synovial biopsy samples by enzymatic digestion. FLS were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Paisley, Scotland, U.K) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Breda, The Netherlands). Experiments were conducted using fourth to ninth passage FLS, and all experiments were conducted with FLS plated in 6-well tissue culture dishes.
Immunoblotting
Jurkat and RA FLS cell lines were lysed in Laemli’s 1x sample buffer, and the cellular extracts were resolved by electrophoresis on NuPage 4-12% Bis-Tris gradient gels (Invitrogen, Verviers, Belgium). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen), followed by blocking of membranes with 1% milk (BioRad, Hercules, CA) in Tris-buffered saline, pH 8.0 containing 0.05% Tween-20 (BioRad) (TBS/T). Membranes were then incubated overnight at 4˚C with primary antibodies in TBS/T. Primary antibodies used in these studies included anti-Ras antibodies (as described above), and antibodies recognizing actin (Santa Cruz Biotechnology) and tubulin (Sigma Aldrich, St. Louis, MO). Immunoblots were developed with secondary HRP-conjugated antibodies (BioRad) and enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

Detection Of Activated Ras Proteins
To measure Ras activation in FLS, cells were serum-starved overnight, and then stimulated in the absence or presence of TNF-α, IL-1β, or PMA/l for 5 minutes. Cells were washed in cold PBS, lysed, and active GTP-bound Ras proteins were precipitated using c-Raf-Ras binding domain (RBD) immobilized on glutathione-agarose beads as previously described [16]. Bound Ras proteins were eluted with Laemmli’s sample buffer and subjected to gel electrophoresis and immunoblotting as above.

Quantitative Measurement Of Ras Homolog mRNA
RNA from samples was obtained using Trizol reagent according to the manufacturer’s instructions (Invitrogen). cDNA was then synthesized using oligoT primer and SuperScriptII enzyme (Invitrogen). Quantitative (q) PCR was performed using a Lightcycler 480 as instructed by the manufacturer (Roche, Woerden, The Netherlands). Sequences of primers used for qPCR were: H-Ras forward, ttgaggacatccaccagctaca; H-Ras reverse, gccgagattccacagtgc; K-Ras forward, tgttcacaaaggtttgtctcc; K-Ras reverse, ccttataatgttctgctgttg; N-Ras forward, cagaggcagtggagcttga; N-Ras reverse, gcttttcccaacaccacct; hATPase 6 Uprobe 23 forward, cataatgacccaccaatcaca; hATPase 6 Uprobe 23 reverse, gagagggcccctgttagg (Roche). qPCR reactions for each sample were performed in quadruplicate and values corrected using control hATPase 6 to calibrate.

Locked Nucleic Acid (LNA) Synthesis
LNA oligonucleotides were synthesized by Santaris Pharma A/S (Harsholm, Denmark) using β-D-LNA monomers (Santaris Pharma A/S). Syntheses were synthesized with complete phosphorothioate backbones using the phosphoramidite approach [29] on an ÄKTA Oligopilot (GE Healthcare) and polystyrene primer support. LNA were purified by ion exchange, desalted, characterized by liquid chromatography/mass spectrometry (Agilent), and the molecular mass of LNA verified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) on a Biflex III MALDI (Brucker Instruments, Leipzig, Germany). The oligonucleotide sequences of LNA used in this study were (5’-3’; capital
letters, LNA; lowercase letters, DNA; C, methylcytosine): H-Ras, TCCgtcagctCCTc; K-Ras, CACaagt-tatattCAGt; N-Ras, GGAAttgtcagtGCGc; scrambled, ACTagctatactagCTAt; pan-Ras, CATGgcactgtacTCCt.

Cell Transfection and Gene Silencing
Jurkat cells were transfected by electroporation (250 V, 950 μF) with 40 μg empty pEF-BOS vector, or empty vector plus increasing quantities (3, 10, and 30 μg) of pEF-BOS encoding H-Ras, K-Ras, or N-Ras (kindly provided by Dr. R. Mareis, ICRF, London, UK). Jurkat cells were harvested and lysed 48 hours post-transfection. RA FLS transfection was performed using 5 μg empty pEF-BOS plasmid or plasmid encoding active Ras homologs, using 12.5 μl Lipofectamine 2000 transfection reagent (Invitrogen). 24 hours post-transfection, medium was replaced with DMEM containing 1% FCS, and cells cultured for 24 hours in the absence or presence of additional stimuli prior to analysis of cells and culture supernatants. LNA transductions were performed in six-well culture plates using increasing concentrations of LNA (up to 50 nM) mixed with Lipofectamine 2000 and serum-free DMEM medium, as per the manufacturer’s instructions. After overnight incubation, transduction medium was replaced with fresh DMEM containing 1% FCS.

Cytokine and MMP-3 Measurement
Transfected and transduced RA FLS were cultured 24 hours in the absence or presence of 10 ng/ml IL-1β and cell-free supernatants were collected. ELISA kits for IL-6, IL-8 (both from Sanquin Reagents, Amsterdam, The Netherlands) and MMP-3 (R&D Systems Europe, Ltd., Abingdon, UK) were used according to the manufacturers’ directions.

Animals, Induction and Assessment of Collagen-Induced Arthritis (CIA)
C57BL/6 mice (Harlan, Horst, The Netherlands) were housed under conventional conditions and fed ad libitum. All animal experiments were approved by the animal ethical committee of the Academic Medical Center, University of Amsterdam. Mice (8–11 weeks of age) were immunized with chicken collagen type II (cCII, Chondrex, Inc., Redmond, WA), dissolved at 2 mg/ml in 0.05M acetic acid by gently stirring overnight at 4°C, mixed with complete Freund’s adjuvant (CFA) (2 mg/ml of Mycobacterium tuberculosis, Chondrex, Inc.). The immunization was performed by intradermal injection at the base of the tail on Day 0 using a 100 μl emulsion containing 100 μg cCII and 250 CFA. Starting on Day 14, mice were randomly assigned to one of four groups (n =8 per group), and treated intraperitoneally with 1 or 4 mg/kg/day with control or pan-Ras LNA. On Day 21, mice received a repeat immunization with cCII in CFA. Mice were monitored 3 times per week in a blinded manner for clinical signs of arthritis, using a semi-quantitative scoring system (0 to 4): 0, normal; 1, redness and/or swelling in one joint; 2, redness and/or swelling in more than one joint; 3, redness and/or swelling in the entire paw; and 4, deformity and/or ankylosis. Hind paw ankle thickness was measured using a dial caliper (POCO 2T 0- to 10-mm test gauge; Kroeplin Längenmesstechnik, Schlüchtern, Germany).
Histological and Radiological Analyses
Hind paws were fixed in 10% buffered formalin for 48 hours, decalcified in 15% ethylenediaminetetraacetic acid (EDTA), and then embedded in paraffin. Sagittal serial sections of the paws (5 μm) were stained with hematoxylin and eosin, or Safranin O, as previously described (30). Inflammation was graded on a scale from 0 (no inflammation) to 3 (severely inflamed joint) based on infiltration of the synovium by inflammatory cells. Cartilage erosion was scored using a semi-quantitative scoring system from 0 (no erosions) to 3 (extended erosions). Two observers without knowledge of the treatment groups scored viewed X-rays and scored joint destruction based on a scale from 0 to 4: 0, no damage; 1, minor bone destruction observed in one enlightened spot; 2, moderate changes, two to four spots in one area; 3, severe erosions afflicting the joint; and 4, complete destruction of the joints.

Determination of Anti-collagen Antibodies by ELISA
Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml bovine collagen type II (bCII) in 0.1 M sodium carbonate buffer (pH 9.7) overnight at 4°C. Plates were then blocked for 1 hour with 2% milk in PBS at room temperature. Mouse sera were added in serial dilutions in 2% milk/PBS, and incubated overnight at 4°C, followed by washing and incubation with 1 μg/ml biotinylated rat anti-mouse IgG1 or IgG2α (Southern Biotechnology Associates, Birmingham, AL) in 2% milk/PBS for 1 hour at room temperature. After washing, plates were incubated with streptavidin-conjugated AP (Jackson ImmunoResearch, Newmarket, Suffolk, UK) for 1 hour at room temperature, washed, developed with p-nitrophenyl phosphate substrate (Sigma-Aldrich, St Louis, MO), and the reaction quenched with 2M H₂SO₄. The resulting optical density (OD) was measured at 415 nm.

Statistical analyses
Wilcoxon’s nonparametric signed ranks test was used to compare protein expression between intimal lining layer and the synovial sublining layer within diagnostic groups. The Mann-Whitney U test was used for the comparison of Ras homolog expression between diagnostic groups. ELISA results were examined using Student’s t-test. In murine arthritis experiments, the change in clinical arthritis scores for each mouse following booster immunization was determined and the areas under the curve calculated. The significance of the differences between the means of delta paw swelling, radiological, and histologic scores between groups was determined by using the unpaired Student’s t-test. P values ≤ 0.05 were considered statistically significant.
Remmers van Ras GTPases zijn goede targets voor de behandeling van artritis
**Results**

**Immunoreactivity of Ras antibodies**

Previous studies of Ras protein expression in RA synovial tissue have been limited to semi-quantitative analysis of positive staining cells using pan-Ras antibodies which do not discriminate between Ras homologues [11,12]. To gain more insight into which Ras homologues were recognized by the pan-Ras antibody, we transfected Jurkat T cells with increasing amounts of cDNA encoding H-, K-, or N-Ras (Figure 1A). Immunoblotting of lysates demonstrated that the pan-Ras monoclonal antibody recognized primarily H- and K-Ras. Of importance, immunoblotting with monoclonal antibodies specific for H-, K-, and N-Ras validated the specificity of these antibodies. We next qualitatively addressed the question of Ras signaling protein expression levels in RA synovial tissue. Consistent with previous studies [12], pan-Ras antibodies weakly but specifically stained RA synovial tissue (Figure 1B). Each of the signaling proteins examined, H-Ras, K-Ras, and N-Ras, were expressed in both RA and inflammatory OA synovial tissue (Figure 1B). In contrast, no staining was observed with negative control antibodies (Figure 1C). Qualitative double labeling of RA synovial tissue with antibodies recognizing Ras homologues and markers for T lymphocytes (CD3), FLS (CD55), and macrophages (CD68) (Figure 1D) revealed that H-Ras and N-Ras were detected in all cell types studied. K-Ras expression was more restricted, detectable only in FLS and macrophages.
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Figure 1 / Ras family homologues are expressed in the synovial tissue of patients with inflammatory arthritis.
(a) Jurkat cells were transfected with control cDNA (mock) or increasing concentrations (μg) of cDNA encoding active H-, K-, and N-RasV12 cDNA as indicated. The specificity of pan-Ras and homologue-specific Ras antibodies was examined by immunoblotting of cell lysates. (b) Expression of Ras proteins in synovial tissue sections of patients with RA and inflammatory OA was examined with pan-, H-, K-, and N-Ras antibodies by IHC (n=5, a representative picture is shown for each staining). (C) RA patient synovial tissue was stained by IHC with control mouse IgG. Magnification: x100. (d) Ras homologues are widely expressed in synovial tissue cells. Immunohistochemical double staining was performed on RA synovial tissue sections to detect T lymphocytes (CD3), FLS (CD55), and macrophages (CD68) (all blue) expressing H-Ras, K-Ras and N-Ras (red). Arrows indicate regions shown in insets. Magnification: x100; x400 (Inset).
Ras homolog expression patterns in RA and non-RA synovial tissue

No differences were noted in pan–Ras antibody staining between RA synovial sublining and intimal lining layers. However, staining in the synovial sublining of RA patients was significantly higher than that observed in non-RA (inflammatory OA and ReA) patients (p < 0.05) (Figure 2A). H–Ras expression was significantly enhanced in the intimal lining layer of both RA (p < 0.01) and non-RA (p < 0.005) as compared to synovial sublining tissue. No differences in expression of H-, K-, or N-Ras were observed between diagnostic groups. We also examined reactivity of pan–Ras and Ras homolog –specific antibodies in a second cohort of 20 RA and 19 PsA patients (Figure 2B). Again, pan–Ras antibody staining in RA synovial sublining tissue was significantly enhanced (p < 0.05). In this cohort, H-Ras expression was also significantly higher in the intimal lining layer of RA and PsA patients (p < 0.01) than in the synovial sublining layer, and H–Ras expression in this synovial region was elevated in RA compared to PsA (p < 0.05). No differences between diagnostic groups or synovial intimal lining and sublining layers were noted for N-Ras or K-Ras expression.

Figure 2 / Ras homologues are expressed at similar levels in the synovial tissue of patients with RA and other forms of inflammatory arthritis.
Quantitative analysis of immunoreactivity of pan–Ras, H–Ras, K–Ras, and N–Ras antibodies in the synovial lining (Lin) and sublining (Sub) of patients as determined by immunohistochemistry and digital imaging. (a) Ras antibody reactivity in patients with RA (n = 10), non-RA inflammatory arthritis (inflammatory OA, n = 4; ReA, n = 7). (b) Ras antibody reactivity in patients with RA (n = 20) and PsA (n = 19). Data is expressed as the integrated optical density (IOD)/ mm² (arbitrary units). 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. *p < 0.05, **p < 0.01.
Expression of Ras homologues in FLS

To gain more insight into the potential participation of each Ras homolog in FLS activation, we first verified in vivo evidence of FLS H-, K-, and N-Ras protein expression in vitro. Real-time PCR was performed on mRNA obtained from cultured RA (n = 5) and PsA (n = 4) FLS lines (Figure 3A). H-Ras, K-Ras and N-Ras mRNA was expressed at similar levels in both RA and PsA lines. At the protein level, each of the proteins could also be detected, albeit at highly variable levels, in both RA and PsA FLS lines (Figure 3B).

**Figure 3** Each of the three Ras homologues is expressed in RA and PsA FLS.
(a) q-PCR analysis of H-Ras, K-Ras, and N-Ras expression in RA (white bar, n = 5) and PsA (gray bar, n = 4) FLS lines, normalized to hATPase 6 expression. Data are expressed in arbitrary units as the mean and standard error of the mean. (b) RA and PsA FLS lysates were analyzed by immunoblotting with antibodies recognizing pan-Ras, H-Ras, K-Ras, N-Ras and actin proteins.

Ras homologues participate in the inflammatory activation of RA FLS and support cytokine and MMP production

We next examined if the Ras homologues expressed in RA FLS participated in the inflammatory activation of these cells. RA FLS were left unstimulated, or stimulated with TNF-α or IL-1β. Active Ras proteins were precipitated from cell lysates and detected by immunoblotting with specific antibodies (Figure 4a). Cytokine-dependent activation of Ras proteins was readily detected with pan-Ras antibodies. Use of homolog-specific antibodies demonstrated that H-Ras, K-Ras, and N-Ras were each activated following cytokine stimulation. In unstimulated FLS, active H-Ras was most easily detected.
Given that each of the Ras homologues could be activated in FLS, we determined whether H-, K-, and N-Ras might make specific contributions to FLS activation. RA FLS were transfected with control vector, or vector encoding active mutants of H-Ras, K-Ras, and N-Ras. Enhanced expression of each of the transfected Ras homologues could be detected in RA FLS lysates by immunoblotting (Figure 4B). Analysis of tissue culture supernatants (Figure 4C, upper panels) revealed that active mutants of either H-Ras or K-Ras enhanced basal production of IL-6 by FLS (H-Ras, 260.09% of control, p < 0.05; K-Ras, 299.45%, p < 0.05). Each of the active Ras mutants also increased basal IL-8 production (H-Ras, 331.82%, p < 0.0005; K-Ras, 244%, p < 0.05; N-Ras, 206.65%, p < 0.05). In contrast, only H-Ras stimulated basal MMP-3 production (134.9%, p < 0.05). When transfected RA FLS were stimulated with IL-1β (Figure 4C, lower panels, IL-6 production was robustly increased, but no cooperative or additive effects were observed by ectopic expression of active Ras mutants. However, H-Ras (421.9%, p < 0.0005) further enhanced IL-1β-induced IL-8 production. Each of the active Ras mutants also enhanced IL-1β-induced MMP-3 secretion (H-Ras, 178.32%, p < 0.05; K-Ras, 166.35%, p < 0.05; N-Ras, 198.5%, p < 0.005).

Figure 4 / Each of the Ras homologues responds to RA FLS stimulation and contributes to inflammatory parameters of FLS activation.

(a) GTP-bound Ras proteins were precipitated with Raf-RBD protein from lysates of RA FLS treated for 5 minutes with medium alone, TNF-α, or IL-1β. Activated proteins were detected by immunoblotting of precipitates with pan-Ras, H-Ras, K-Ras, and N-Ras antibodies. Immunoblotting with actin antibodies was performed on whole cell lysates. (b) RA FLS were transfected with empty control vector or vector encoding active H-Ras, K-Ras, and N-Ras V12 mutants. Immunoblotting of lysates of transfected cells was performed with antibodies recognizing H-Ras, K-Ras, N-Ras and tubulin. (c) Tissue culture supernatants of transfected RA FLS lines (n=5), in the absence (medium) or presence of overnight IL-1β stimulation, were analyzed by ELISA for IL-6, IL-8 and MMP-3 content. *p < 0.05. **p < 0.01.
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Effects of Ras homolog gene silencing on RA FLS cytokine and MMP production

Our studies with active Ras mutants suggested that each of the Ras homologues was sufficient to stimulate FLS secretion of one or more cytokines or MMPs, but not indicate whether or not there was requisite participation of the Ras proteins in IL-6, IL-8 and MMP-3 production. To address this, we transduced RA FLS with increasing concentrations of control scrambled LNA, and pan-Ras LNA. Treatment of cells with pan-Ras LNA resulted in a specific, dose-dependent reduction in expression of each of the three Ras homologues (Figure 5A). Treatment of cells with homolog-specific Ras LNAs resulted in selective suppression of H-, K-, and N-Ras (Figure 5B). Treatment of FLS with 50 nM H-Ras LNA did result in slight reductions in K- and N-Ras expression, but as this higher concentration of LNA was required for efficient H-Ras knockdown, all further experiments were performed with 50 nM control and Ras LNAs.

Although active forms of each of the Ras proteins could enhance basal IL-6 and IL-8 production (Figure 4C) only pan-Ras and H-Ras LNA significantly reduced IL-6 production (Figure 6). In contrast, N-Ras expression was required to support basal IL-8 production. Significant reductions in basal MMP-3 production were observed only in the presence of H-Ras LNA, consistent with our previous observations that RA FLS MMP-3 production is regulated by the H-Ras-specific GEF RasGRF1 [17]. In the presence of IL-1β, suppression of H-Ras resulted in significant decreases in the production of IL-6 (61.07% inhibition) and MMP-3 (37.52%) (p < 0.05). K-Ras knockout only influenced IL-1β-dependent IL-6 production (52.19% decrease, p < 0.05). N-Ras LNA inhibited IL-1β-dependent production of both IL-6 (54.51%) (p < 0.05) and IL-8 (71.62%) (p < 0.05).
Figure 5 / LNA allows gene silencing of specific Ras homologues in RA FLS. (a) Pan-Ras LNA suppresses expression of H-Ras, K-Ras and N-Ras. RA FLS were left untreated, exposed to transfection (Tx) reagent alone, or transfected with control scrambled LNA (50 nM) or increasing concentrations (5, 10 and 50 nM) pan-Ras LNA. (b): RA FLS were transfected with control scrambled LNA (50 nM) or increasing concentrations (5, 10 and 50 nM) H-Ras (left column), K-Ras (middle column) or N-Ras (right column) LNA. Whole cell lysates of transfected cells were analyzed by immunoblotting with antibodies recognizing pan-Ras, H-Ras, K-Ras, N-Ras, and actin proteins. Each column represents results from one of at least three independent transfection experiments.

Figure 6 / Ras homologues make differential but overlapping contributions to basal and IL-1β-induced cytokine production. RA FLS lines (n=5) transfected with scrambled control, pan-Ras, H-Ras, K-Ras, and N-Ras LNA (50 nM each) were incubated overnight in medium alone or medium containing IL-1β. Tissue culture supernatants were analyzed for IL-6, IL-8, and MMP-3 content by ELISA. *p < 0.05.
Pan-Ras LNA reduces disease severity in murine collagen-induced arthritis (CIA)
As our *in vitro* studies indicated distinct but highly overlapping contributions of Ras homologues to the inflammatory activation of RA FLS, we examined the influence of pan-Ras LNA treatment on CIA in mice. C57BL/6 mice were immunized with chicken collagen type II (cCII) in CFA, and the immunization was repeated on Day 21. Starting on Day 14, mice were treated with control or pan-Ras LNA (1 or 4 mg/kg/day). At the time of animal sacrifice on Day 43, mRNA was isolated from the livers of mice to monitor the efficiency of Ras homolog knockdown (Figure 7A). H-Ras, K-Ras, and N-Ras mRNA levels in mice treated with control LNA did not differ significantly from untreated control mice. In contrast, H-Ras mRNA levels were suppressed by approximately 90% in pan-Ras LNA–treated mice, and significant but more modest decreases in K-Ras and N-Ras mRNA expression were observed.

Compared to control LNA, pan-Ras LNA had no influence on disease incidence following induction of CIA (data not shown). However, administration of pan-Ras LNA significantly reduced disease severity during the course of the experiment (*p* < 0.005) (Figure 7B). Increasing the dose of pan-Ras LNA to 4 mg/kg had no further significant effect on disease severity. Effects of pan-Ras LNA on paw swelling were more modest, and only transient nature, occurring between Day 25 and Day 32 (*p* < 0.05) (Figure 7C). Pan-Ras LNA treatment resulted in decreased synovial infiltration (*p* < 0.05) (Figures 8A and 8B) and cartilage destruction (*p* < 0.05) (Figures 8A and 8C) as evidenced by HE and safranin O staining, respectively. Similar partial protection against erosive bone disease was also provided by pan-Ras LNA (Figure 8D). Finally, mice treated with pan-Ras LNA displayed significant reductions in serum levels of anti-bCII IgG1 (28.5% of control; *p* < 0.05) and IgG2a (30.7% of control; *p* < 0.005) antibodies (Figure 9A). The ratio of anti-bCII IgG2a to anti-bCII IgG1 was also severely reduced in mice treated with pan-Ras LNA (*p* < 0.01), indicating that suppression of Ras signaling interfered with antibody isotype class switching (Figure 9B).
Figure 7 / Pan-Ras LNA decrease disease severity and paw swelling in murine CIA. (a) mRNA expression of H-Ras, K-Ras and N-Ras was measured by q-PCR in livers of healthy untreated wild-type (WT) mice, and arthritic mice treated with scrambled control and pan-Ras LNA. Data was normalized relative to hATPase 6 expression. (b) Disease severity was assessed by clinical scoring and expressed as the area under the curve for the course of the experiment. (c) Paw joint swelling in mice was measured using a caliper and data expressed as the change in paw thickness (Δ paw swelling) relative to paw size at the induction of arthritis. *p < 0.05. ***p < 0.005.
Silencing expression of Ras family GTPase homologues decreases inflammation and joint destruction in experimental arthritis

Figure 8 / Pan-Ras LNA decrease synovial infiltration, cartilage destruction, and bone erosion in CIA.

(a) Knee joints of arthritic mice treated with scrambled control LNA and pan-Ras LNA were stained with HE and safranin O. (b) Synovial infiltration in mice was assessed by semi-quantitative scoring of HE-stained tissue sections. (c) Cartilage destruction in mice was assessed by semi-quantitative scoring of safranin O-stained tissue sections. (d) X-Rays were obtained from paws of mice treated with scrambled control LNA and pan-Ras LNA. E: Bone erosions were assessed by semi-quantitative scoring of radiographs. *p < 0.05.
Figure 9 / Silencing of Ras homologue expression reduces anti-collagen antibody production in murine CIA.
(a) Serum was obtained from arthritic mice and the concentration of anti-bovine collagen type II (anti-bCII) immunoglobulins (Ig) G1 and G2a determined by ELISA. (b) Ratio of anti-bCII IgG2a to anti-bCII IgG1 concentrations. 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. *p < 0.05. **p < 0.01.
Discussion

Recent advances have provided us with an unparalleled ability to design and develop highly selective pharmacological inhibitors which distinguish between closely related proteins. A prototypical example of this is the recent identification of compounds which specifically inhibit distinct isoforms of PI3K catalytic subunits, allowing the selective targeting of isoforms primarily involved in inflammatory immune cell trafficking and activation [31]. However, the design of compounds and their clinical application in the treatment of RA and other diseases can outpace our understanding of the specific biological and pathological contributions of their targets. This is observed in recent clinical trials involving the treatment of RA patients with inhibitors of p38 MAPK, which have reported a disappointing lack of clinical efficacy [32;33]. One possible contributing factor to this may be that while the compounds used in these trials targeted primarily p38\(\alpha\), ongoing fundamental studies have demonstrated prominent activation of not only p38\(\alpha\) in RA synovial tissue, but p38\(\delta\) as well [34].

Ras proteins represent a newly emerging set of targets in the treatment of chronic inflammatory diseases, playing a central role in coupling the ligation of antigen, cytokine, growth factor, and TNF family receptors to cellular responses of gene regulation, proliferation and survival. Receptor-dependent activation of GEFs converts Ras proteins to their GTP-bound form, allowing interaction with and signaling to downstream effector pathways, such as MAPK cascades, PI3K, and other small GTPases [35]. In RA, enhanced expression and or activation of Ras proteins has been proposed to cooperate with other proto-oncogene products, such as c-Myc, to promote FLS activation and proliferation [19]. Although a small number of in vivo experiments have suggested a therapeutic potential in targeting Ras proteins, a lack of knowledge about the contributions of specific Ras homologues to RA currently precludes hypothesis-based development and application of compounds. Initial characterizations using a monoclonal antibody recognizing H- and K-Ras reported expression of Ras proteins in the intimal lining layer and vasculature of RA, but not OA, synovial tissue [11]. An independent study using a distinct pan-Ras antibody observed weak expression of Ras throughout the synovium in RA and other forms of inflammatory arthritis, which was lower than that observed in OA [12]. In our study, a pan-Ras antibody primarily recognizing H- and K-Ras, and to a lesser extent N-Ras, displays quantitatively enhanced immunoreactivity in the synovial sublining of RA patients compared to non-RA patients. Discrepancies between these three studies can likely be attributed to variation in the cross-reactivity of pan-Ras antibodies used. Using homolog-specific antibodies, we find that H-Ras is most readily detectable in synovial tissue, at significantly higher levels in the intimal lining layer than in the synovial sublining layer, in both RA and non-RA patients. H-Ras and N-Ras are widely expressed in all cell types studied (T cells, macrophages, and FLS), while weak K-Ras expression is restricted to macrophages and FLS. However, we were unable to detect any difference in expression of specific H-Ras, K-Ras, or N-Ras homologues between RA and non-RA patients. Thus, the repertoire of inflammatory stimuli in
synovial tissue, or changes in the functional expression of Ras regulatory proteins, such as RasGRF1, rather than differential expression of Ras homologues, are more likely to determine involvement of Ras proteins in inflammatory arthritis [17].

Specificity in Ras protein signaling is achieved at several levels [35]. First, although widely expressed, different homologues are enriched in specific tissues. For example, we detect primarily H-Ras and N-Ras in synovial T lymphocytes. Even within FLS lines established from different RA and PsA patients, we note wide variation in the expression of each Ras homologue. Whether this is maintained in vivo, and whether the in vitro variation is a consequence in the differentiation status of the FLS, prior inflammatory pressures in vivo, or epigenetic mechanisms is currently unknown. A second level of specificity is obtained by differential expression and utilization of GEFs which activate Ras proteins. Some GEFs, such as RasGRF1, demonstrate fine specificity for activation of H-Ras [36]. We have previously found that RasGRF1 is over-expressed in RA synovial tissue, in an active form, and that this protein is sufficient and required for constitutive MMP-3 production by RA FLS [17]. Consistent with this, we observed in this study that only H-Ras, but not other Ras homologues, can regulate basal MMP-3 secretion in RA FLS. Other Ras GEFs, such as Sos (son of sevenless), can activate each of the Ras homologues, and we observe that both TNF-α and IL-1β efficiently activate H-, K-, and N-Ras in RA FLS. A third variable which will affect the cellular output of Ras signaling is the array of potential downstream effectors expressed in a given cell type [9]. In a given cell type, H-, K-, and N-Ras each display different efficiencies in activating downstream effector pathways (c-Raf, PI3K and Rac pathways) and cellular functions (focus formation, anchorage-independent growth, and cell migration) [37-39]. Finally, cells may express one or more related downstream effectors, such as c-Raf, B-Raf, and Raf-A, or the α, β, γ and δ isoforms of the PI3K catalytic subunit, which in turn differentially interact with a given Ras homolog [40].

These complexities in Ras signaling are underscored by our analyses of the effects of Ras activation and silencing on RA FLS. While activation of any of the Ras homologues is sufficient to enhance IL-1β-induced MMP-3 production, only H-Ras cooperates with IL-1β to produce IL-8. In contrast, while each of the Ras homologues participates in IL-1β signaling and contributes to optimal IL-6 production, only N-Ras is required for IL-8 synthesis, and H-Ras for MMP-3. Retroviral introduction of dominant-negative H-Ras to the joints of rats reduces clinical disease severity and radiological damage in the adjuvant-induced model of RA [18]. However, our analyses of RA synovial tissue and RA FLS indicate that due to redundancies in Ras signaling in RA, clinically beneficial strategies may need to broadly target Ras family members. Previous studies have shown that transfection of RA FLS with DN c-Raf slows their invasiveness following implantation into SCID mice [19]. Additionally, farnesylation inhibitors, which disrupt post-translational modifications of Ras family members needed for protein function, suppress IL-1β and TNF-α-induced activation of RA FLS in vitro, and are protective in CIA
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[20,41]. However, it is unclear if inhibition of Ras family proteins was responsible for clinical benefits observed in these studies. For example, DN c-Raf can also bind to other Ras-related proteins, including R-Ras and the Rap1 family members, each of which make unique contributions to cellular biology [42]. Farnesylation inhibitors, in turn, will target not only Ras homologues, but all G protein-coupled receptors, such as chemokine receptors, as well [43]. Here, we demonstrate that broad but specific silencing of Ras family homologues provides protective effects in experimental arthritis, a strategy which may be further explored using gene therapy and pharmacological approaches.
References


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