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

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Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis

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Abstract

Introduction: Animal studies suggest important roles for the mitogen-activated protein kinases (MAPKs) p38, extracellular signal regulated kinases (ERK) 1/2 and c-Jun N-terminal kinase (JNK) in the perpetuation of inflammation and disease progression in rheumatoid arthritis (RA). The objective of this study was to determine the expression and activation of MAPKs, and their potential relationship with clinical and cellular parameters of disease, in patients with clinically active RA and psoriatic arthritis (PsA).

Methods: Expression and phosphorylation of p38, ERK, and JNK was examined in arthroscopic synovial biopsies from 20 RA and 19 PsA patients by immunohistochemical analysis, using quantitative computer-assisted image analysis. Phosphorylated (p)-MAPK levels were related to patient clinical parameters and synovial expression of tumor necrosis factor α (TNFα), matrix metalloproteinase (MMP)-1, MMP-3, and the angiogenic receptor Tie2. Localization of p-MAPKs and Tie2 in distinct cell populations within synovial tissue was determined by immunofluorescent double staining.

Results: No differences in the number of cells expressing p-p38, p-ERK, or p-JNK were observed between RA and PsA synovial tissue. However, the percentage of cells expressing JNK that had detectable p-JNK was elevated in PsA synovial tissue (p< 0.05). The relative phosphorylation of p38 (p < 0.05) and ERK (p< 0.05), but not JNK, compared to total expression of each MAPK, was significantly higher in RA synovial tissue than in PsA. MAPK phosphorylation status was not associated with clinical parameters of disease activity in RA or PsA, or with expression of MMP-1 or MMP-3. In RA, p-ERK was associated with increased Tie2 expression (R = 0.583, p < 0.01), while in PsA p-JNK was associated with expression of TNFα (R = 0.713, p < 0.01). In RA synovial tissue, Tie2 and p-ERK prominently colocalized to synovial macrophages.

Conclusion: In patients with clinically active arthritis, relative participation of p38 and ERK signaling in synovial tissue distinguishes RA from PsA. However, there is no clear association of MAPK activation with clinical parameters of disease activity in these forms of arthritis. ERK phosphorylation is intimately associated with Tie2 expression in RA, primarily in synovial macrophages, while in PsA, JNK phosphorylation is associated with TNF production. Our results suggest that activation of specific MAPKs is differentially linked to angiogenesis and inflammation in RA and PsA.
Introduction

Joint destruction in inflammatory arthritis is mediated by infiltration of activated immune cells, the release of matrix metalloproteinases (MMPs) and other proteinases by activated macrophages and fibroblast-like synoviocytes (FLS), activation of osteoclasts, and angiogenesis in the synovium of affected joints. Recent attention has focused on the role of widely expressed mitogen-activated protein kinases (MAPK) in mediating cytokine production and tissue damage in rheumatoid arthritis (RA) [1,2]. MAPKs, consisting of p38 kinases (α, β, γ and δ isoforms), extracellular signal-regulated kinases (ERKs) 1 and 2, and c-Jun N-terminal kinases (JNK) 1-3, are activated by many extracellular stimuli, including growth factors and inflammatory cytokines. Activation of each MAPK is achieved by specific upstream serine/threonine protein kinase cascades, which are initiated by extracellular signals and result in MAPK phosphorylation [2].

In the synovial tissue of RA patients, each MAPK is detected in its phosphorylated, active form [3,4]. Activation of p38 plays an important role in coupling FLS by IL-1β, TNFα, toll-like receptors (TLR) and pattern-recognition receptors to synthesis of pro-inflammatory cytokines, chemokines, and MMPs [3,5-8]. The same extracellular stimuli, as well as growth factors, also promote ERK activation [3]. Although ERK can regulate activation of AP-1 and Elk transcription factors, involved in cytokine and MMP synthesis, potential contributions of ERK to inflammation and joint destruction in RA have yet to be indentified. In RA FLS, ERK inhibition has no influence on TNFα-induced IL-6, IL-8 or MMP-3 production, but can partially prevent MMP-1 transcription [7,9]. Additionally, several studies have indicated that ERK activation appears to be important in chondrocyte and osteoclast-mediated joint destruction [2]. As is the case with ERK, the consequences of JNK activation in RA synovial tissue are also largely unknown. However, inhibition of JNK activity in RA FLS can suppress induction of MMP-1 by IL-1β [9].

In animal models of arthritis, pharmacological or genetic inhibition of MAPK signaling suppresses inflammatory cytokine production, paw swelling and joint destruction [1,2]. Specificity in the involvement of each MAPK in RA is suggested by studies in mice over-expressing human TNFα, where arthritis development is associated with selective activation of p38 and ERK, but not JNK [10]. Inhibition of p38, but not JNK1, blocks arthritis in this model [11,12]. Collectively these studies have suggested a therapeutic benefit in the targeting of MAPKs in RA, and compounds targeting MAPKs are in pre-clinical and clinical assessment [1,2,13].

Despite this, synovial MAPK phosphorylation status has only been assessed in RA patients with destructive end-stage arthritis, and not in patients most likely to be participating in
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clinical trials. RA synovial tissue obtained during arthroscopy and tissue obtained during surgery display differences in both cellular composition and cytokine profiles [14]. Additionally, clinical parameters or cellular biomarkers associated with MAPK activity in RA or other types of inflammatory arthritis have yet to be identified. These questions are particularly relevant given initial disappointing results of p38 inhibitors in clinical trials [13,15,16]. Here, we quantitatively examined the phosphorylation and expression of MAPKs in the synovial tissue of patients with clinically active RA. In comparison, we also studied the synovial tissue of patients with PsA, an inflammatory arthritis which, although clinically distinct from RA, has similarities in regard to level of inflammatory cell infiltration, inflammatory cytokine expression, and contribution of angiogenesis to joint destruction [17-20].
Materials and Methods

Patients and synovial tissues

Synovial biopsies were obtained from the active joints of 20 RA and 19 PsA patients by needle arthroscopy as previously described [21]. All RA and PsA patients fulfilled the 1987 American College of Rheumatology criteria for RA [22] and the classification criteria for psoriatic arthritis (CASPAR) [23], respectively. Patient characteristics are described in Table 1. All patients supplied prior written informed consent, and this study was approved by the medical ethics committee of the Academic Medical Center, University of Amsterdam.

Table 1 / Characteristics of study patients

<table>
<thead>
<tr>
<th></th>
<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>
</tr>
<tr>
<td>No.receiving MTX</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Dosage MTX, mg/wk#</td>
<td>15 (7.5-30)</td>
<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>
<td>85</td>
<td>0</td>
</tr>
<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).

Immunohistochemistry

Biopsy sections were cut with a cryostat (5 μm) and mounted on glass slides (Starfrost; Knittelglaser, Braunschweig, Germany). The tissues were then fixed with acetone, and endogenous peroxidase activity blocked by immersion in 0.3% hydrogen peroxide and 0.1% sodium azide in phosphate-buffered saline (PBS). Slides were incubated overnight at 40 °C with primary antibody diluted in 1% bovine serum albumin (BSA)/PBS. Primary antibodies used in this study were polyclonal rabbit antibodies specific for p38, ERK, JNK (all from Cell Signaling, Beverly, MA) and Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and murine monoclonal antibodies recognizing phosphorylated (p)- p38, ERK, and JNK (Santa Cruz Biotechnology), MMP-1, MMP-3 (both from Chemicon International, Temecula, CA), and anti-
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TNFα (Monosan, Amersfoort, The Netherlands). Equivalent concentrations of control rabbit polyclonal or mouse monoclonal antibodies were used as negative controls. Sections were washed with PBS and incubated with goat anti-mouse or swine-anti-rabbit -horseradish peroxidase (HRP)-conjugated antibodies (Dako, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with aminoethylcarbazole (Sigma, St. Louis, MO) [24]. Slides were counterstained with Mayer’s hematoxylin and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).

Digital image analysis
Stained sections were analyzed in a random order by two independent observers blinded to the clinical diagnosis. Expression and/or phosphorylation of proteins in synovial tissue was determined using computer-assisted image analysis as previously described (Qwin analysis system, Leica, Cambridge, UK) [25]. Expression and/or phosphorylation of proteins was calculated for both the intimal lining layer and synovial sublining as the number of positive cells/mm² or the median integrated optical density (IOD) per mm² tissue, and normalized for tissue cellularity. Relative phosphorylation values were obtained by dividing p-MAPK IOD by total MAPK IOD.

Immunofluorescence staining
Synovial tissue sections were incubated with primary anti- p-ERK antibodies overnight at 4°C in 1% BSA/PBS, blocked with normal goat serum, and incubated with Alexa-594-conjugated goat anti-mouse antibodies (Molecular Probes Europe, Leiden, The Netherlands). Sections were then incubated with Alexa-488-conjugated mouse antibodies specific for CD3, CD20, CD55, CD68, CD163, and vWF (Molecular Probes Europe). To visualize potential colocalization of Tie2 in specific cell populations, or with p-p38, p-ERK, and p-JNK, Alexa-594-conjugated goat anti-rabbit antibodies were used to detect Tie2, Alexa-488-conjugated goat anti-mouse antibodies for p-MAPKs, and directly labeled antibodies as above for cellular markers. Slides were mounted in Vectashield (Vector Laboratories) and analysed using a fluorescence microscope (Leica) which was coupled to a CCD camera and Image-Pro Plus software (Media Cybernetics, Dutch Vision Components, Breda, The Netherlands).

Statistics
Statistical analysis was performed using Windows Graphpad Prism 4 software. For comparison of values between the synovial sublining and intimal lining layers within patient cohorts, Wilcoxon’s nonparametric signed rank test was used. The Mann-Whitney U test was used to compare differences in expression or phosphorylation of markers between cohorts. Correlations between markers or between markers and clinical parameters were examined by Spearman’s rank correlation coefficient. Results were considered significant if p < 0.05.
Results

Patient characteristics
The characteristics of RA and PsA patients with clinically active disease included in this study are summarized in Table 1. Between RA and PsA patient cohorts, no significant differences were observed in regard to disease duration, swollen joint counts (SJC), tender joint counts (TJC), DAS28, erythrocyte sedimentation rate (ESR), or serum C-reactive protein concentrations (CRP). Dosages of methotrexate were slightly higher in RA patients (median 15 mg/wk, range 7.5-30) than in PsA patients (median 10 mg/wk, range 2.5-20) (p < 0.05).

Expression and phosphorylation of MAPKs in RA and PsA patient synovial tissue
Representative photomicrographs of p-MAPK and MAPK staining in RA and PsA patient synovial biopsies are shown in Figure 1. The number of cells expressing phosphorylated (p)-p38 (positive cells/mm²) was significantly higher in the synovial sublining tissue than in the intimal lining layer in both RA (p < 0.005) and PsA (p < 0.01) (Figure 2A). In both RA and PsA similarly enhanced numbers of cells expressing p-ERK (RA, p < 0.01; PsA, p < 0.01) and p-JNK (RA, p < 0.005; PsA, p < 0.05) were also observed in the synovial sublining tissue as compared to the intimal lining layer. However, increased numbers of p-MAPK-positive cells observed in the synovial sublining tissue were mirrored by significant increases in the frequencies of cells in the synovial sublining tissue expressing p38 (RA, p < 0.005; PsA, p < 0.005), ERK (RA, p < 0.01; PsA, p < 0.005), and JNK (RA, p < 0.005; PsA, p < 0.05) (Figure 2B). Between patient cohorts, no significant differences in the numbers of cells expressing p-MAPKs were observed, but the number of cells expressing p38 (p < 0.01) and ERK (p < 0.01) were significantly enhanced in PsA synovial sublining tissue compared to RA. Thus, engagement of each MAPK in synovial tissue, as measured by the presence of cells expressing p-MAPK, appears to be a reflection of the number of cells in the synovial tissue expressing each MAPK, rather than region- or disease-specific activation of MAPKs. The only exception to this was observed in PsA patient intimal lining layer tissue, where the percentage of JNK-expressing cells demonstrating p-JNK (32.2% ± 7.6, mean ± SEM) was elevated compared to RA (17.2% ± 4.5) (Figure 2C, p < 0.05).
Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis.

Figure 1 / Expression and phosphorylation of MAPKs in RA and PsA synovial tissue. Representative stainings of RA and PsA synovial tissue with antibodies recognizing phosphorylated (p-) and total p38, ERK and JNK. Stainings were developed with biotin tyramide enhancement, horseradish peroxidase and aminoethylcarbazole, followed by counterstaining with Mayer's hematoxylin.
Figure 2 / Quantitative analysis of number of cells expressing MAPKs and phosphorylated MAPKs in RA and PsA synovial tissue.

(a) Quantitative analysis of number of cells staining positive for phosphorylated (p-) p38, ERK and JNK. (b) Quantitative analysis of number of cells staining positive for p38, ERK and JNK. Values indicated are the number of positive cells/mm² corrected for cellularity as calculated by digital image analysis in RA and PsA synovial sublining (SL) (gray boxes) and intimal lining (L) layers (white boxes). Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. (c) The percentage of cells expressing p-MAPK relative to the number of cells expressing MAPKs ([number p-MAPK-positive cells/number MAPK-positive cells] x100). Data is presented as mean and SEM. Statistically significant differences are indicated above lines connecting relevant data sets. * p < 0.05, ** p < 0.01, *** p < 0.005.
Relative phosphorylation levels of MAPKs in RA and PsA synovial tissue
To examine the relative engagement of each MAPK in RA and PsA synovial tissue, we measured the integrated optical density of stainings with antibodies recognizing phospho-specific and total MAPKs (Figure 3). Phosphorylation of p38 was significantly higher in PsA than RA (sublining, $p < 0.05$; intimal lining layer, $p < 0.05$), as was phosphorylation of ERK (sublining, $p < 0.0005$; intimal lining layer, $p < 0.005$), and JNK (intimal lining layer, $p < 0.01$) (Figure 3A). However, as observed in analysis of the numbers of cells expressing p-MAPKs and MAPKs (Figure 2), many of the differences in MAPK phosphorylation levels between RA and PsA synovial tissue reflected differences in expression of each MAPK between cohorts (Figure 3B), rather than changes in the relative phosphorylation levels of each MAPK (Figure 3C). For example, p38 and JNK expression was significantly higher in PsA synovial sublining layers than in RA (Figure 3B). When normalized to patient variation in total MAPK expression (Figure 3C), relative p38 phosphorylation was significantly higher in the synovial sublining of RA compared to the intimal lining layer ($p < 0.005$), and greatly enhanced in RA compared to PsA ($p < 0.005$). Relative phosphorylation of ERK was also elevated in the intimal lining layer compared to the synovial sublining in both RA ($p < 0.005$) and PsA synovial tissue ($p < 0.01$). Comparing RA to PsA, the relative phosphorylation of both p38 ($p < 0.05$) and ERK ($p < 0.05$) was elevated in RA synovial sublining compared to PsA. We observed no differences in relative JNK phosphorylation between the synovial sublining and intimal lining layers in RA or PsA, nor between RA and PsA.
**Figure 3** / Quantitative analysis of expression levels of MAPKs and phosphorylated MAPKs in RA and PsA synovial tissue.

(a) Quantitative analysis of phosphorylated (p-) p38, ERK and JNK staining intensity. (b) Quantitative analysis of p38, ERK, and JNK staining intensity. Values indicated are the integrated optical density (IOD)/mm² corrected for cellularity. Calculated by digital image analysis in RA and PsA synovial sublining (SL) (gray boxes) and intimal lining (L) layers (white boxes). (c) Relative MAPK phosphorylation in RA and PsA synovial tissue. Values were calculated by determining the ratio of the IOD of p-MAPK to the IOD of total MAPK for each patient. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Statistically significant differences are indicated above lines connecting relevant data sets. * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.001.
Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis.

Relationship between MAPK phosphorylation and clinical and cellular markers of disease activity in RA and PsA

No correlation was observed between the relative phosphorylation levels of p38, ERK or JNK, and patient serum concentrations of CRP or RF, patient ESR, or patient DAS28, SJC or TJC (data not shown). Additionally, we observed no significant relationship between relative MAPK phosphorylation and TNFα, MMP-1, or MMP-3 synovial tissue expression in RA (data not shown). In PsA however, we observed a strong positive relationship between relative p-JNK and TNF-α expression in the intimal lining layer (R= 0.7133; p = 0.0092), but no association of MAPK phosphorylation with MMP-1 or MMP-3 production (data not shown).

Examining potential relationships between MAPK activation and Tie2, a biomarker for angiogenesis, we observed that Tie2 was expressed at equivalent levels in RA and PsA synovial tissue (Figure 4A and Figure 4B). Both in RA (p < 0.005) and PsA (p < 0.05), Tie2 expression was elevated in the synovial sublining compared to the intimal lining layer (Figure 4B). Strikingly, relative phosphorylation levels of ERK, but not other MAPKs, displayed a strong positive correlation with Tie2 expression in RA synovial sublining tissue (R= 0.583, p < 0.01) (Figure 5). In PsA, no correlations were observed between relative MAPK phosphorylation levels and Tie2 expression (Figure 6).

Figure 4: Quantitative analysis of Tie2 expression in RA and PsA.
(a) Representative stainings of RA synovial tissue with anti-Tie2 (upper panel) and control rabbit IgG (lower panel) antibodies. (b) Expression of Tie-2 in the intimal lining layer (gray bars) and synovial sublining (white bars) of RA and PsA synovial tissue as determined by digital image analysis. Statistically significant differences between data sets are indicated. *** p< 0.005.
**Figure 5** / Relationship between MAPK phosphorylation and Tie2 expression in RA synovial tissue. Potential correlations between p38, ERK, and JNK phosphorylation with Tie2 expression in RA synovial tissue intimal lining (lining) and sublining layers were calculated by Spearman’s rank correlation coefficient. Squares indicate individual patient values. Statistically significant R and p values are indicated.
Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis.

Figure 6 | Relationship between MAPK phosphorylation and Tie2 expression in PsA synovial tissue. Potential correlations between p38, ERK, and JNK phosphorylation with Tie2 expression in PsA synovial tissue intimal lining (lining) and sublining layers were calculated by Spearman’s rank correlation coefficient. Squares indicate individual patient values. Statistically significant R and p values are indicated.

PsA synovial tissue

Localization of Tie2 and phosphorylated MAPKs in RA and PsA synovial tissue

due to the size of our study and number of comparisons assessed, we could not rule out that the association we observed between ERK phosphorylation and Tie2 expression was due to chance. To gain further insight into the potential relationship between ERK activation and Tie2 expression, we performed immunofluorescent doublestaining experiments on RA and PsA synovial tissue, examining Tie2 expression in relationship to p-MAPKs and specific cell populations. When examined in relationship to MAPK phosphorylation, Tie2 colocalized in RA synovial tissue with cells expressing p-ERK, but not p-p38 or p-JNK (Figure 7), while in PsA synovial tissue, Tie2 colocalized only in cells expressing p-JNK. In RA synovial tissue, Tie2 was not expressed in CD3+ T lymphocytes, and rarely in endothelial cells and CD55+ FLS (Figure 8). In contrast, Tie2 was readily detected in CD68+ synovial macrophages. A qualitatively identical cellular distribution of Tie2 was observed in PsA synovial tissue. In RA synovial tissue ERK phosphorylation was most prominently observed in RA T lymphocytes,
synovial tissue macrophages and endothelial cells (Figure 9). In contrast, ERK phosphorylation in PsA synovial tissue was restricted primarily to synovial macrophages. Together, these results suggest that in RA synovial tissue, p-ERK is found primarily in Tie2-bearing macrophages, providing a cellular basis for the observed correlation between these two markers.

**Figure 7 / Colocalization of phosphorylated MAPKs with Tie2 in RA and PsA synovial tissue.** RA and PsA synovial tissue sections were stained with primary antibodies and fluorochrome-conjugated secondary antibodies to detect phosphorylated (p-) p38, ERK, or JNK (all green) and Tie2 (red). Colocalization of proteins is visualized by yellow labeling in merged images.
Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis.

Figure 8 | Cellular distribution of Tie2 expression in RA and PsA synovial tissue. RA and PsA synovial tissue sections were stained with primary antibodies and fluorochrome-conjugated secondary antibodies to detect Tie2 (red) and T lymphocytes (CD3), FLS (CD55), macrophages (CD68 and CD163) or endothelial cells (vWF) (all in green). Localization of Tie2 in specific cell populations is visualized by yellowing labeling in merged images.
Figure 9 | Cellular distribution of phosphorylated ERK in RA and PsA synovial tissue.

RA and PsA synovial tissue sections were stained with primary antibodies recognizing phosphorylated (p-) ERK and secondary fluorochrome-conjugated anti-mouse IgG antibodies (red), followed by fluorochrome-conjugated antibodies recognizing T lymphocytes (CD3), FLS (CD55), macrophages (CD163), or endothelial cells (vWF) (all in green). Localization of p-ERK in specific cell populations is visualized by yellow labeling in merged images.
Phosphorylation of extracellular signal regulated kinases 1 and 2 is specifically associated with angiogenic Tie2 expression in the synovium of patients with active rheumatoid arthritis but not psoriatic arthritis.
Discussion

Given that each of the MAPKs is activated in RA synovial tissue, their central role in coupling extracellular stimuli to proliferation and cytokine gene transcription, and a large literature identifying protective effects of MAPK inhibitors in animal models, a strong therapeutic potential for targeting MAPKs in RA and other chronic inflammatory diseases has been suggested [1-3]. Particularly, elegant studies showing prominent and requisite involvement of p38 activation in mediating synovial inflammation and joint destruction in the human TNFα transgenic mouse model of arthritis have indicated that strategies targeting this MAPK may be effective in treating RA [10,11]. However, despite the clinical promise observed in experimental systems, recently reported trials in which RA patients have been treated with p38 pharmacological inhibitors have failed to demonstrate efficacy [15,16]. Particularly in regard to p38, evidence is emerging that inhibition of upstream MAPK kinases may be more effective in preventing inflammation and joint destruction in animal models [26]. Additionally, meticulous evaluation of experimental arthritis in mice has indicated model-specific involvement and kinetics of activation of each of the MAPKs during the induction of disease [10,11,27]. These new developments have collectively underscored our current lack of knowledge regarding the activation status of MAPKs in patients with active arthritis, and the contributions of each MAPK to the persistence of inflammation and disease progression in RA.

MAPK phosphorylation and expression in patients with clinically active arthritis is subtly different than the localization of these enzymes and their activity in the synovium of patients undergoing joint replacement surgery, which may be important clinically. In previous studies, p-p38 was primarily detected in the intimal synovial lining layer, as well as sublining endothelial cells, p-ERK in the synovial sublining, and p-JNK at low levels in synovial sublining macrophages and intimal lining layer FLS [3]. In synovial specimens from RA and PsA patients with active disease, we detect cells engaging each of the MAPKs most readily in the synovial sublining. However, many of these differences simply reflect the number of cells expressing each MAPK, and taking this into consideration, there are few differences in the synovial distribution of cells in which MAPKs are activated. Exceptions include increased frequencies of p-p38-positive cells in the intimal lining layer of PsA, compared to the synovial sublining, and of p-ERK in the synovial sublining of RA tissue compared to the intimal lining layer.

The presence of a given phosphorylated MAPK in a cell, in and of itself, provides little information regarding the signaling output of the MAPK. In T cells, antigen receptor-dependent
activation of ERK is a digital, "all-or-nothing" signaling event [28]. However, MAPK activation can be regulated in a digital or graded analog manner, dependent upon the cell type, external stimulus, the intracellular compartment in which the MAPK is activated, and the repertoire of guanine nucleotide exchanges factors available to initiate the MAPK signaling cascade [28-31]. Given the heterogeneity of cellular composition and inflammatory stimuli present in synovial tissue, MAPK output is likely to be regulated by both digital and analog signaling networks. Therefore, we also determined the relative levels of MAPK activation in synovial tissue, comparing the total intensity of p-MAPK and total MAPK signals. Using this strategy, we found that enhanced activation of synovial sublining p38 and ERK distinguished RA from PsA synovial tissue.

Despite the efficacy of MAPK pharmacological inhibitors in preventing arthritis in animal models, in this study we were unable to establish an association between localized MAPK phosphorylation and clinical parameters of disease activity in RA or PsA, including SJC, TJC, ESR, CRP, and DAS28. Rather than indicating that MAPK activation does not contribute to pathology in inflammatory arthritis, our data likely suggest that multiple signaling pathways, including MAPKs, make redundant contributions to the disease process. In line with this, RA patients treated with p38 inhibitors display a transient decrease in CRP levels, which later recover to baseline levels [15,16]. Additionally, MAPK activity may make pleiotropic contributions to both inflammatory and reparatory processes in arthritis. Although data in patients is lacking, kinetic analyses of collagen-induced arthritis in mice has revealed a bimodal wave of p38 activation, the second of which occurs while clinical disease is diminishing [27]. Substantial p38 and ERK activity is also maintained following successful anti-TNF-α treatment of established arthritis in the human TNF-α transgenic model [10]. We similarly fail to find an association between MAPK activation and TNF-α or MMP production in RA synovial tissue. In PsA, an association was found between JNK activation and synovial TNF-α production, possibly suggesting a more central role for JNK in this disease. This possibility is also suggested from prospective studies of PsA patients treated with etanercept, where decreases in ERK and JNK activity were observed, while p38 phosphorylation status was unaffected [32].

In this study, we observe a specific and strong positive correlation between phosphorylation of ERK and expression of the angiogenic marker Tie2, a receptor tyrosine kinase, in the synovial sublining layer of RA, but not PsA patients. Previous studies of RA synovial tissue have indicated that Tie2 is expressed by endothelial cells, macrophages, and FLS [33-35]. In RA, we find that both p-ERK and Tie2 are prominently localized to synovial macrophages. The molecular basis of this association remains to be determined. One trivial possibility is
that ERK is most highly activated in RA synovial macrophages, and Tie2 merely an unexpectedly robust marker for synovial macrophages. Tie2 protein is expressed on a subset of human peripheral blood monocytes and tumor-infiltrating monocytes, but is downregulated following macrophage differentiation [36,37]. Alternatively, Tie2 signaling in RA synovial macrophages might selectively activate ERK. The ligands for Tie2, angiopoietin (Ang) -1 and -2, differentially activate endothelial cell MAPKs in vitro. While Ang-1 activates p38, ERK, and JNK, Ang-2 activates only JNK [38,39]. The differential association of Tie2 expression with p-ERK in RA and PsA might thus mirror the relatively enhanced production of Ang-1 compared to Ang-2 in RA synovial tissue [40]. While future studies will need to test these possibilities, our data suggest that specific targeting of ERK MAPKs may be therapeutically beneficial in preventing angiogenesis in RA.
Conclusions

This study provides our first insights into the relationships between activation of MAPKs and clinical and cellular parameters of disease activity in RA and PsA. Despite elevated p38 phosphorylation in RA synovial tissue compared to PsA synovial tissue, no relationship of p38 phosphorylation with clinical parameters of disease activity, or cellular markers of inflammation (TNF-α), joint destruction (MMP) or angiogenesis (Tie2) is observed in RA of PsA. This may explain in part the lack of sustained efficacy of p38 inhibitors in the clinical setting. JNK phosphorylation is closely associated with TNF-α production in PsA, suggesting a potential clinical benefit of JNK inhibitors in this disease. ERK phosphorylation is enhanced in RA compared to PsA, and intimately associated with expression of Tie2. Although the molecular association between ERK phosphorylation and Tie2 expression requires further elucidation, strategies targeting ERK may be useful in suppressing angiogenesis in RA.

List of Abbreviations

Ang = angiopoietin; BSA = bovine serum albumin; CRP = serum C-reactive protein; ERK = extracellular regulated kinase; ESR = erythrocyte sedimentation rate; FLS = fibroblast-like synoviocyte; HRP = horseradish peroxidase; IOD = integrated optical density; JNK = c-jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MMP = matrix metalloproteinase; p = phospho-, or phosphorylated; PBS = phosphate-buffered saline; PsA = psoriatic arthritis; RA = rheumatoid arthritis; SJC = swollen joint counts; TJC = tender joint counts; TNF-α = tumor necrosis factor-α.

Competing Interests

DT is employed by Array BioPharma, a for-profit biotechnology company in the business of developing small molecule therapeutics for the treatment of human disease. As an employee of Array BioPharma, DT has an option to purchase shares of Array common stock, as part of a compensation package. Array BioPharma helped support this work through a collaborative service agreement. Patents from this work may be filed by the company and/or the institution performing this study.
Authors' Contributions

DdL and MES performed and evaluated the experiments, and DdL conducted statistical analyses and drafted the manuscript. DMG collected patient material, and assembled and assessed patient demographic and clinical data. DT contributed to the design of the experiments. PPT contributed to the design of the study and evaluation of data. KAR contributed to the design of the study, evaluation of data, and drafting of the manuscript. All authors read and approved the final manuscript.

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