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

de Launay, D.

Citation for published version (APA):
Summary and General Discussion
chapter 8

Summary and General Discussion
Summary and General Discussion

In rheumatoid arthritis (RA), stromal fibroblast-like synoviocytes (FLS) play a central role in orchestrating the influx of immune cells needed to perpetuate inflammation, activating osteoclasts to mediate bone erosion, as well as directly invading cartilage and bone tissues [1]. FLS display many behavioral properties that are similar to transformed cancer cells: they proliferate quickly in vitro in an anchorage-independent manner, spontaneously secrete autocrines and matrix metalloproteinases (MMPs), and in vivo, can "metastasize" to unaffected joints to initiate pathology [1-3]. Members of the Ras family of small GTPases are widely expressed intracellular signaling proteins, which when activated act as potent oncogenes [4]. Given previous reports that Ras proteins were over-expressed or potentially mutated in RA synovial tissue, and that multiple downstream signaling pathways of Ras proteins were activated in RA synovial tissue, in this thesis we examined the potential contributions of Ras signaling pathways in RA [5-8].

Increases in the expression of wild-type (non-mutated) Ras family GTPases in a cell usually has neutral functional consequences, as Ras activation is tightly and catalytically regulated by activating guanine nucleotide exchange factors (GEFs) and inactivating GTPase activating proteins (GAPs) [9]. Potential changes in Ras GEF and GAP expression in RA synovial tissue had not been previously examined. In chapter 2 we examined the potential role of the Ras GEF Ras guanine nucleotide releasing factor 1 (RasGRF1) in RA, as recent evidence had suggested that post-translational modification of this protein contributed to spontaneous MMP production in cancer cells [10,11]. We found that protein expression of RasGRF1 was significantly enhanced in the synovial tissue of RA patients compared to osteoarthritis (OA) and reactive arthritis patients. Remarkably, visualization of RasGRF1 expressed in synovial tissue, as well as RA FLS lines cultured in vitro by immunoblotting revealed that RasGRF1 was expressed in a truncated form which should render it constitutively active, as previously observed in melanoma cells [11]. Consistent with this, RasGRF1 transfected into FLS was expressed in truncated forms, and increased the spontaneous production of MMP-3 production by RA FLS. The molecular mechanisms leading to RasGRF1 cleavage were not addressed in this study, but RasGRF1 is known to be sensitive to calpain-dependent cleavage [10]. In line with this possibility, calpain levels are increased in the synovial fluid and tissue of patients with RA [12]. We inferred from our studies that RasGRF1 mediates its effect on MMP-3 production via H-Ras, as previous studies have indicated that of the Ras homologues, RasGRF1 specifically targets H-Ras [13]. However, RasGRF1 also displays exchange activity against the Rho family GTPase Rac [14,15]. The role of Rac in RA FLS biology has not been extensively studied, but interference with Rac function can decrease RA FLS invasiveness in vitro [16].

Although each of the closely related Ras homologues, H-Ras, K-Ras, and N-Ras, with the exception of one splice variant of K-Ras, make redundant contributions to mammalian development, gene array
and immunology studies from knock-out mice, as well as in vitro studies have indicated discreet roles for each protein in biology [17-21]. In chapter 3, we examined the contribution of each of the Ras homologues to pathology in RA. Previous studies examining whether Ras proteins were overexpressed in the synovial tissue of RA patients had reach disparate conclusions, likely a result of using different Ras antibodies which recognized multiple Ras homologues with different selectivities [5,6]. Studying multiple RA and non-RA patient cohorts with homolog-specific antibodies, we found that H-Ras, K-Ras and N-Ras were expressed at similar levels in RA synovial tissue compared to disease controls, although elevated H-Ras expression was elevated in RA compared to psoriatic arthritis (PsA). Each of the three homologues was expressed at variable levels in RA FLS, and each was activated following stimulation with TNFα or IL-1β. Over-expression of active Ras mutants in RA FLS revealed that H-Ras, K-Ras, and N-Ras could all enhance spontaneous IL-8 production, H-Ras and K-Ras had similar effects on IL-6, and only H-Ras could stimulate MMP-3 production. Gene silencing experiments demonstrated specific requirements for N-Ras in IL-1β-induced IL-8 production, while H-Ras was required for MMP-3 production. The clear involvement of H-Ras in MMP-3 regulation was consistent with our findings in chapter 2 that RasGRF1, an H-Ras GEF, could regulate RA FLS production of MMP-3. However, the overall picture which emerged from our in vitro studies was that there was extensive redundancy in RA FLS regarding the regulation of global inflammation parameters. This combined with the widely variable expression of each Ras homolog in RA FLS, suggested that strategies broadly targeting Ras function would be beneficial in protecting against arthritis in vivo. In support of this, we found that simultaneous silencing of H-Ras, K-Ras, and N-Ras expression in mice using a pan-Ras locked nucleic acid, protected mice against inflammation and joint destruction in collagen-induced arthritis (CIA). These results are generally in agreement with studies intending to abrogate Ras family function with less specific compounds, such as dominant-negative Raf and farnesyltransferase inhibitors [22,23], and validate the broad targeting of Ras proteins as a potential therapeutic approach. However, future studies with homologue-specific LNA and knock-out mice will be needed to examine the specific contributions of each Ras protein to pathology.

As our studies in chapter 3 clearly demonstrated involvement of Ras homologues in the inflammatory activation of RA FLS, we next examined in chapter 4 the involvement of one downstream arm of Ras signaling, mitogen-activated protein (MAP) kinase signaling cascades, in RA. Previous independent studies of synovial tissue in patients undergoing joint replacement surgery had indicated that members of each of the families of MAP kinases, p38 kinases, extracellular-regulated kinases (ERKs) and C-jun N-terminal kinases (JNKs) were expressed and activated in RA synovial tissue [24,25]. This observation, in combination with numerous studies showing that genetic deletion or pharmacological inhibition of MAP kinases prevented inflammation and joint destruction in animal models of RA, has initiated extensive investment of academic, pharmaceutical, and clinical resources to the development of specific MAP kinase inhibitors which might be used to treat RA [8,26]. However, no studies had
yet investigated the activation status of MAP kinases in patients which will eventually be enrolled in clinical trials, those with active RA, and no cellular or clinical markers associated with MAP kinase activation had been identified. Using digital imaging techniques, we found by immunohistochemical analyses that there were no differences between RA and PsA patients in regard to the percentages of synovial cells with detectable phosphorylated p38, ERK or JNK. However, the percentages of cells with detectable MAP kinase phosphorylation is of questionable (patho)physiological significance, as even in healthy tissue MAP kinases would be activated by normal exposure to growth factors and cell-cell contacts. When we measured the intensity of staining for phosphorylated MAP kinases in relationship to total MAP kinase protein present in the synovial tissue, we found that relative activation of p38 and ERK was enhanced in RA synovial tissue, suggesting a specific role for these MAP kinases in RA. We were unable to establish an association between relative MAP kinase activation and any patient clinical parameters in RA or PsA, nor association with expression of MMP-1 and MMP-3, often used as readouts in in vitro studies examining the effects of MAP kinase inhibitors on RA FLS activation [27,28]. Remarkably however, we found a clear positive correlation between ERK activation and expression of the angiogenic marker Tie2 in RA synovial tissue. This may suggest either a selective role for ERK signaling pathways in promoting angiogenesis in RA or, alternatively, reflect selective activation of ERK proteins by angiogenic (Tie2) signaling in RA. Curiously, using immunofluorescent double labeling techniques, we found that activated ERK proteins and Tie2 co-localized in RA synovial macrophages. Previous studies had demonstrated Tie2 to be expressed on synovial endothelial cells, FLS, and macrophages [29]. The effects of Tie2 ligation by angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) on endothelial cells has been best characterized. Ang-1 promotes vascular stabilization, while Ang-2 can promote vascular remodeling and sensitizes endothelial cells to TNFα-induced inflammatory gene transcription [30,31]. Also, Ang-1 stimulation induces MMP-3 production by RA FLS [32]. Potential effects of angiopoietin signaling on myeloid lineage cells, especially in regard to inflammation, are not well-studied, but numerous recent reports have identified an essential role for Tie2-bearing monocytes in promoting tumorigenesis [33,34], and demonstrated a chemotactic effect of Ang-2 on monocytes [35]. Therefore, it is possible that the relationship between Tie2 and ERK activation in synovial macrophages reflects angipoietin-dependent signaling in RA, and this possibility was further studied in chapter 7.

In chapter 5, we extended our analysis of synovial MAP kinase activation to a distinct cohort of early arthritis patients. This study was initiated for three reasons. First, patients with established RA are similar to patients with PsA in regard to synovial cellular composition and cytokine expression, as well as responses to TNF blockade, and little is known at the cellular or molecular level regarding how distinct forms of inflammatory arthritis develop from initial synovitis [36-38]. Second, recent analyses of the kinetics of MAP kinase activation in murine models of RA demonstrated that each MAP kinase participated differently in the initiation, destructive and repair phases of disease, dependent upon the
animal model used [39]. Third, in analyses of data obtained in chapter 4, we noted that although activation of p38 and ERK was significantly elevated in RA compared to PsA, ERK was selectively activated in those RA patients with erosive disease (data not shown). However, since this was a cross-sectional study, this data could not be interpreted as we could not establish a relationship between ERK activation at the time of arthroscopy and initiation or progression of bone erosions. Therefore, we conducted a prospective study in which synovial biopsies were obtained from disease-modifying antirheumatic drug (DMARD)-naïve early arthritis patients at baseline. These patients were diagnosed with either RA, spondyloarthritis (SpA) or undifferentiated arthritis (UA) at baseline, and followed for 2 years. A definitive diagnosis was made at the end of two years as RA, SpA, or UA, and X-rays obtained at baseline and at two years follow-up. Strikingly, we found that ERK and JNK, but not p38, activity at baseline was elevated in patients receiving a final diagnosis of RA compared to other diagnostic outcomes. Additionally, JNK activation was elevated in UA patients later diagnosed with RA compared to patients who remained UA, and JNK activation was predictive for eventual diagnosis as RA. JNK and ERK were also elevated in RA patients who developed erosive disease. Of interest, JNK activation could predict erosive disease in the within the total early arthritis cohort. Together, these findings suggest a specific role for JNK in the development of RA, and a general role for this protein in the development of erosive disease. These findings are significant as they generate, to our knowledge, the first biochemical “fingerprint” of the development of RA and erosive disease. Understanding the gene targets of JNK and ERK in these early arthritis patients will further our knowledge of the molecular processes by which RA develops.

An additional important finding in chapter 5 was the comparative non-involvement of p38 in early arthritis. Previous studies in patients undergoing joint replacement [25] and patients with active disease (chapter 4, this thesis) readily detected active p38 in the synovial tissue of patients with RA, but we observed very little in patients with early UA, RA or SpA, even though p38 itself was present. This finding will need to be validated in independent cross-sectional studies of patients from these cohorts, as well as future analyses of patients in our early arthritis cohort as they participate in follow-up arthroscopies. However, the result is intriguing in light of kinetic studies performed in animal models of RA. In murine CIA, p38 activation is most evident late in disease, when clinical parameters and cytokine biomarkers of disease activity are declining [39]. This raises the possibility that p38 activation observed in established RA might drive repair mechanisms, albeit ineffectively, rather than make pro-inflammatory contributions to disease. Such a possibility may explain the lack of clinical efficacy observed in recent clinical trials treating RA patients with p38 inhibitors [40,41]. Activation of p38 can suppress ERK and JNK signaling pathways, promote anti-inflammatory IL-10 production, and destabilize pro-inflammatory cytokine mRNA [42]. Unfortunately, none of these parameters have been currently assessed in these p38 inhibitor clinical trials. Additionally, it may be useful to test the possible extension of findings in cancer biology, where p38 activation has been demonstrated to be an
important brake on Ras-induced oncogenesis, which may be relevant to FLS biology in RA [43].

In chapter 6, we examined the involvement of forkhead box O (FoxO) transcription factors in RA. FoxO transcription factors regulate expression of genes involved in cellular proliferation, cell cycle arrest, apoptosis, and protection against environmental stress [44-46]. Activation of PI3 kinases, three of which (p110α, p110β, and p110δ) are Ras effectors, results in activation of protein kinase B (PKB, also known as Akt). PKB in turn phosphorylates FoxO family members FoxO1, FoxO3a, and FoxO4. Phosphorylation of FoxO proteins by PKB disrupts FoxO association with DNA, and promotes association with 14-3-3 proteins and nuclear export [44,45]. Previous studies had reported depressed expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a negative regulator of PI3 kinase signaling, and enhanced PKB phosphorylation in RA synovial tissue [47-49]. This indicated to us that enhanced PI3 kinase-dependent inactivation of FoxO family proteins may promote cellular proliferation and survival in RA synovial tissue. We found that FoxO1, FoxO3a and FoxO4 were expressed and phosphorylated on PKB-dependent residues in RA and inflammatory OA synovial tissue. Of the three FoxO proteins, only FoxO4 phosphorylation was significantly higher in RA than in OA, and FoxO4 phosphorylation was primarily detected in synovial macrophages. We could find no relationship between phosphorylation of FoxO4, or FoxO1 in FLS, or FoxO3a in T lymphocytes, and expression of PTEN in synovial tissue. Moreover, no correlations were observed between the FoxO proteins in regard to phosphorylation levels. Together, this indicated that local cytokine environments, rather than defects in PTEN expression drive PI3-kinase signaling pathways in RA. Intriguingly, phosphorylation of each of the FoxO proteins inversely correlated with clinical parameters of disease activity in RA patients. Expecting PI3 kinase activity to drive cellular proliferation and survival, we had expected, if anything, a positive correlation. However, it is now known that inflammatory stimuli and oxidative stress induce JNK-dependent phosphorylation of FoxO proteins, which drives FoxO nuclear import and counterbalances PKB effects [46,50]. This would provide a relatively straightforward interpretation of the data. Additionally, it is now also clear that active FoxO proteins can stimulate transcription of genes directly involved in promoting inflammation and joint destruction, including chemokine receptors, adhesion molecules, and MMPs [51-53]. Thus, the need for transcriptional activity of FoxO proteins to promote survival and inflammatory gene expression may outweigh the needs for cellular proliferation in RA synovial tissue. A relevant experimental example of this may be found in FoxO3a knockout mice, which are resistant to autoantibody-induced arthritis because neutrophils are unable to suppress expression of pro-apoptotic Fas ligand [54]. Further studies will be needed to determine specific contributions of each FoxO family member to RA, especially in regard to the gene targets regulated by these transcription factors.

Finally, in chapter 7, we again extend on unexpected findings in chapter 4, which indicated a strong association between ERK activation and Tie2 expression in RA synovial tissue. Active ERK and Tie2 colocalized to the cell same population, raising the possibility that Tie2 may be differentially activated
in RA compared to PsA. To test this we examined the relationship between expression of Ang-1 and Ang-2, the ligands of Tie2, and relative phosphorylation of Tie2, in RA and PsA synovial tissue. Tie2 is a receptor tyrosine kinase which is phosphorylated on several cytoplasmic tail tyrosine residues following activation [30]. At least in endothelial cells, Ang-1 stimulation prominently activates p38, ERK, JNK and phosphatidylinositol 3-kinase (PI3 kinase), while Ang-2 activates primarily JNK [55,56]. In our study, we observed expression patterns of Ang-1 and Ang-2 that were similar to previous independent reports [57,58]. Ang-1 expression was significantly higher in RA patients compared to PsA, while Ang-2 levels were similar. However, the ratio of Ang-1/Ang-2 was much higher in RA patients, and a strong inverse correlation between Ang-1 and Ang-2 expression was observed in RA, but not PsA, indicating that in RA expression of these two cytokines is mutually exclusive. The reason for this is uncertain, and will require additional studies to identify the cell populations producing Ang-1 and Ang-2 in each cohort. Possibly, different cytokines regulate Ang-1 and Ang-2 production in each disease, or specific gene polymorphisms regulating Ang-2 protein expression [59,60] may be involved in RA.

Relative phosphorylation of Tie2 was enhanced in RA, and correlated strongly with Ang-1/Ang-2 ratios. In PsA, an inverse correlation was observed, suggesting that Ang-2 plays a more prominent role in Tie2 activation, while Ang-1 predominates in RA. In light of our finding that Tie2 is associated with ERK activation in RA, this interpretation would be consistent with observations that Ang-1 more robustly stimulates ERK activation than Ang-2, at least in endothelial cells. Ongoing studies are examining if Ang-1 and Ang-2 similarly regulate Tie2 downstream signaling pathways in macrophages.

In conclusion, studies presented in this thesis have provided our most comprehensive insight into the contributions of Ras family protein signaling to pathology in RA. We find that activation of Ras family proteins, rather than changes in their expression, are most relevant to FLS activation in RA. However, redundancies in the signaling capacity of each Ras homologue may preclude the development of effective strategies targeting single Ras proteins. We also demonstrate selective involvement of the MAP kinases p38 and ERK in established active RA, and ERK is tightly associated with Tie2 angiogenic signaling. In early arthritis, ERK and JNK play a more prominent role, and JNK activation is predictive of eventual diagnosis of RA, as well as erosive disease. Lack of involvement of p38 early in the disease process may indicate that it is not playing a strong pro-inflammatory role in RA. Finally, we show that PKB-dependent inactivation of FoxO proteins is enhanced in RA compared to disease controls, but that this occurs primarily in patients with decreased disease activity. This points to a role of FoxO-regulated transcriptional targets in the perpetuation of inflammation in RA. Future studies designed to identify the molecular mechanisms regulated by Ras-dependent signaling pathways will are likely to provide important insights into the etiology and perpetuation of disease in RA.
References


