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

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Angiopoietins -1 and -2 make distinct contributions to synovial Tie2 engagement in patients with rheumatoid arthritis and psoriatic arthritis

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Abstract

**Objectives:** The angiogenic factors angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are differentially expressed in the serum and synovium of patients with different forms of arthritis, but the consequences of this for engagement of their common receptor Tie2, and Tie2 contributions to inflammation and joint destruction are unknown. Here we examined relationships between synovial Ang-1 and Ang-2 expression and Tie2 activation in patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA) in vivo.

**Patients and methods:** The expression of Ang-1, Ang-2, Tie2, and phosphorylated (p) active Tie2 was examined by immunohistochemistry and digital imaging analysis in synovial biopsies from 20 RA patients and 19 PsA patients. Relationships between angiopoietin expression and Tie2 engagement were compared. RA synovial biopsies were cultured in the absence or presence of Ang-1 or Ang-2, alone or in combination with tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS), and tissue culture supernatants examined for IL-6 production by enzyme-linked immunosorbent assay (ELISA).

**Results:** Synovial Ang-1 expression was elevated in patients with RA (p < 0.05), while Ang-2 expression was similar in RA and PsA. As a result the ratio of Ang-1 expression to Ang-2 was increased in RA (p < 0.001). Reciprocal regulation of Ang-1 and Ang-2 expression was observed in RA synovial tissue (r = 0.784, p < 0.005) but not PsA. Relative engagement of Tie2 was enhanced in RA compared to PsA (p < 0.05), and a strong positive correlation was observed between Tie2 engagement and the ratio of Ang-1/Ang-2 expression (r = 0.786, p < 0.01). In contrast, PsA synovial Tie2 engagement inversely correlated with the ratio of Ang-1/Ang-2 expression (r = -0.752, p < 0.01). Ex vivo, both Ang-1 and Ang-2 induced IL-6 production in RA synovial culture explants.

**Conclusion:** Expression of Ang-1 and Ang-2 is differentially regulated in RA and PsA. In RA, synovial Tie2 engagement is driven primarily by Ang-1, while Ang-2 promotes Tie2 activation in PsA. Both Ang-1 and Ang-2 can directly contribute to inflammation in RA synovial tissue by stimulating IL-6 production, but potential distinct contributions of Ang-1 and Ang-2 to inflammatory arthritis remain to be defined.
Ras Family GTPase Signaling Contributions to Inflammation and Joint Destruction in Rheumatoid Arthritis

Introduction

Joint destruction in inflammatory arthritis is mediated by the release of matrix metalloproteinases (MMPs) and other proteinases by activated stromal fibroblast-like synoviocytes (FLS) and macrophages, differentiation and activation of osteoclasts, and angiogenesis in the synovial membrane of inflamed joints. Angiogenesis in the synovial membrane increases inflammatory white blood cell access to affected joints, sustains the enhanced nutritional requirements of invasive, hyperplasic synovial tissue, and stimulates osteoclast-mediated bone resorption [1,2]. In both rheumatoid arthritis (RA) and psoriatic arthritis (PsA), expression of angiogenic factors and markers of endothelial activation are closely associated with joint destruction [1,3]. For example, in recently diagnosed RA, elevated serum concentrations of both vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-1 predict the development and rate of erosive joint damage [4,5]. Serum VEGF levels similarly predict the development of erosive disease in PsA [4]. Reciprocally, RA and PsA patients treated with tumor necrosis factor α (TNFα)-blocking compounds display decreases in both serum and synovial VEGF concentrations, synovial expression of angiogenic markers, and vascular endothelial deactivation [6-10]. Signaling via the tyrosine kinase receptor Tie2 makes contributions to angiogenesis and blood vessel remodeling that are critically distinct from VEGF receptor ligation [11]. Like VEGF, Tie2 and its ligand Ang-1 are required for embryonic angiogenesis [12,13]. Early studies indicated that Ang-2, a second ligand of Tie2, may act as an endogenous antagonist of Ang-1, as transgenic over-expression of Ang-2 in mice resulted in embryonic lethality similar to that observed following genetic deletion of Ang-1 or Tie2 [14]. However, subsequent analysis of Ang-2 knock-out mice revealed that this growth factor, while not required during embryogenesis, is essential for post-natal remodeling of vascular and lymphatic structures [15]. The distinct roles of Ang-1 and Ang-2 in angiogenesis might reflect differences in the ability of these growth factors to stimulate Tie2-dependent intracellular signaling pathways. Ang-1 activates phosphatidylinositol 3-kinase, p38, extracellular signal regulated kinase (ERK) and c-Jun Nterminal kinase (JNK) signaling pathways in endothelial cells [16,17]. Ang-2 can also activate each of these pathways, with the exception of JNK, but is far less potent than Ang-1. Moreover, while Ang-1 synergizes with VEGF to stimulate endothelial cell ERK activation, Ang-2 is antagonistic [18]. Ang-1, Ang-2 and Tie2 are all expressed in the synovial tissue of patients with RA and PsA [19-22]. Tie2 activation on endothelial cells and FLS, respectively, promotes angiogenesis and cartilage destruction in synovial tissue [21,23]. Blockade of Tie2 signaling in vivo prevents angiogenesis in the murine collagen-induced arthritis model [21], and forced over-expression of Tie-2 in vivo promotes murine disease resembling psoriasis [24]. While these studies collectively suggest a therapeutic benefit in the targeting of Tie2, little is known regarding how or if interplay between Ang-1 and Ang-2 regulates synovial Tie2 activation, or the pathological consequences of synovial Tie2 engagement. Here, we quantitatively examined the engagement of Tie2 in RA and PsA, and the relationship between angiopoietin expression and Tie2 phosphorylation in each disease.
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 [25]. All RA and PsA patients fulfilled the 1987 American College of Rheumatology criteria for RA [26] and the classification criteria for psoriatic arthritis (CASPAR) [27], respectively. Patient characteristics for both cohorts have been previously described in detail (de Launay et al, submitted, Chapter 3 in this thesis). Clinical characteristics of the patient cohorts were similar, with no significant differences between RA and PsA patients in regard to disease duration, swollen joint counts, tender joint counts, DAS28, erythrocyte sedimentation rate (ESR), or serum C-reactive protein concentrations (CRP). Dosages of MTX received by RA patients (median 15 mg/wk, range 7.5-30) were higher than those received by PsA patients (median 10 mg/wk, range 2.5-20) (p<0.05). All patients supplied written informed consent prior to inclusion in the study, and this study was approved by the medical ethics committee at the Academic Medical Center, University of Amsterdam. Six samples of synovial tissue from each joint was embedded en bloc in Tissue Tek OCT (Miles Diagnostics, Elkhart, IN) and immediately snap-frozen in methylbutane (−70°C). The biopsies were subsequently stored in liquid nitrogen until processing.

Immunohistochemistry

Biopsy sections from each patient 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). The slides were incubated overnight at 40°C with optimized dilutions of primary antibody diluted in 1% bovine serum albumin/PBS. Primary antibodies used in this study were polyclonal rabbit antibodies specific for FITC (Invitrogen, Breda, The Netherlands), Tie2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho (p)-Tie2 rabbit hyperimmune serum (Calbiochem/Merck Chemicals Ltd., Nottingham, UK). Goat polyclonal antibodies specific for Ang-1 and Ang-2 were from Santa Cruz. Equivalent concentrations of irrelevant goat or rabbit polyclonal antibodies were used as negative controls for angiopoietin and Tie2 stainings, respectively. For p-Tie2 stainings, the concentration of IgG in anti-p-Tie2 hyperimmune serum was determined and normal rabbit serum supplemented with an equivalent concentration of rabbit anti-FITC antibodies used as a negative control. Sections were washed with PBS and incubated with mouse anti-goat or swine anti-rabbit–horseradish peroxidase (HRP)–conjugated antibodies (Dako, Glostrup, Denmark), followed by sequential incubation with biotinylated tyramide and streptavidin–HRP, and development with aminoethylcarbazole (Sigma, St. Louis, MO) [28,29]. Slides were counterstained with Mayer’s hematoxylin and mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany).
Digital image analysis
Expression and/or phosphorylation of proteins in synovial tissue were determined using computer-assisted image analysis of 18 high-power fields from different parts of each section (Qwin analysis system, Leica, Cambridge, UK) [30,31]. Expression and/or phosphorylation of proteins was calculated as the median integrated optical density (IOD) per mm² tissue, and normalized for tissue cellularity. Relative phosphorylation of Tie2 was calculated by dividing the IOD p-Tie2 by the IOD Tie2 for each patient and expressed in arbitrary units. Data for Tie2 stainings was previously obtained in Chapter 4, this thesis.

Synovial biopsy explant culture
Intact synovial biopsies were obtained from three RA patients and cultured, three biopsies per condition, for 48 hours in complete DMEM medium supplemented with 10% FCS. Biopsies were left unstimulated or stimulated with Ang-1 or Ang-2 (both 200 ng/ml, R&D Systems Europe, Ldt., Abingdon, UK), alone or in combination with TNF-α (10 ng/ml, Biosource International, Camarillo, CA) or LPS (1 μg/ml, Sigma). Cell-free tissue culture supernatants were harvested for cytokine analysis.

Measurement of cytokine production
IL-6 concentrations in cell-free tissue culture supernatants from synovial biopsy explants were examined by ELISA (Sanquin Reagents, Amsterdam, The Netherlands) as per the manufacturer’s instructions.

Statistics
Statistical analysis was performed using Windows Graphpad Prism 4 software. The Mann-Whitney U test was used to compare differences in expression or phosphorylation of markers between cohorts. Correlations between markers were examined by Spearman’s rank correlation coefficient. Results were considered significant if p < 0.05.
chapter 7  Angiopoietins -1 and -2 make distinct contributions to synovial Tie2 engagement in patients with rheumatoid arthritis and psoriatic arthritis

Results

Reciprocal expression of Ang-1 and Ang-2 in RA and PsA synovial tissue
To better understand the relationship between angiopoietin expression and synovial Tie2 activation, we performed quantitative immunohistochemical analysis of synovial tissue from arthroscopic biopsies obtained from 20 RA and 19 PsA patients. We first examined synovial expression of Ang-1 and Ang-2. In control experiments, no visible staining was observed in synovial tissue incubated with irrelevant polyclonal goat antibodies (Figure 1A). Ang-1 was expressed throughout the synovium of RA patients (Figure 1B). Intense staining was noted in the intimal lining layer, but expression was also noted in the synovial sublining and perivascular regions (Figure 1B upper left panel, and data not shown). Ang-2 expression in RA synovial tissue was distributed similarly to that observed for Ang-1, although staining intensity was consistently lower (Figure 1B, upper right panel). RA synovial tissue Ang-1 and Ang-2 expression patterns observed in our studies were consistent with previous independent observations [20-22]. In PsA, Ang-1 was most highly expressed in the intimal lining layer, with only weak expression in the synovial sublining (Figure 1B, lower left panel). Ang-1 staining patterns contrasted sharply with Ang2, which was strongly expressed in both the intimal lining layer and synovial sublining cellular infiltrates (Figure 1B, lower right panel). Again, qualitative staining patterns of Ang-1 and Ang-2 in PsA synovial tissue were comparable to previous independent observations [22]. Quantitative analysis of Ang-1 and Ang-2 expression in RA and PsA synovial tissue revealed that Ang-1 expression was significantly higher in RA (p < 0.05) (Figure 2A). Additionally, a trend toward enhanced Ang-2 expression was observed in PsA synovial tissue, although this did not reach statistical significance (p = 0.061). Both in vitro and in vivo observations have suggested that under certain conditions, Ang-2 can antagonize Ang-1-dependent activation of endothelial cell Tie2, suggesting that relative differences in Ang-1 and Ang-2 expression may be biologically relevant [14,15,32]. Comparing Ang-1 expression relative to Ang-2 in each patient, we also noted that the ratio of expression of synovial Ang-1 to Ang-2 was elevated in RA (IOD Ang-1/IOD Ang-2, median ± SEM, arbitrary units, 4.873 ± 1.022) compared to PsA (0.573 ± 0.203) (p < 0.001) (Figure 2B). Remarkably, Ang-1 and Ang-2 expression in RA synovial tissue appeared to mutually exclusive of each other, as a strong negative correlation was observed between Ang-1 and Ang-2 expression within this cohort of patients (R = 0.784, p < 0.005) (Figure 2C). In contrast, no relationship was observed between Ang-1 and Ang-2 expression in PsA (R = 0.001, p = 0.907) (Figure 2D). These data indicate not only that Ang-1 and Ang-2 are differentially expressed in RA and PsA synovial tissue, but that different cellular or genetic mechanisms may drive angiopoietin production in each disease.
**Figure 1** / Expression of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) in rheumatoid arthritis (RA) and psoriatic arthritis (PsA) synovial tissue. RA tissue sections were stained with negative control goat antibodies (a) and RA and PsA tissue sections stained with goat anti-Ang1 and anti-Ang-2 antibodies. (b) Stainings were developed with biotin tyramide enhancement, horseradish peroxidase and aminoethylcarbazole, followed by counterstaining with Mayer’s hematoxylin.
Angiopoietins -1 and -2 make distinct contributions to synovial Tie2 engagement in patients with rheumatoid arthritis and psoriatic arthritis

Figure 2 | Quantitative comparison of Ang-1 and Ang-2 expression in RA and PsA synovial tissue. Synovial sections from 20 RA and 19 PsA patients were stained with antibodies against Ang-1 and Ang-2 as in Figure 1 and the integrated optical density (IOD)/mm² corrected for cellularity calculated by digital image analysis (a). (b) Ratios of Ang-1 expression relative to Ang-2 (IOD Ang-1/IOD Ang-2, arbitrary units) were calculated for RA and PsA patients. Data is 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. P values (p) of statistically significant differences are indicated above lines connecting relevant data sets. (c) Correlations between Ang-1 and Ang-2 expression in RA (c) and PsA (d) patient cohorts. Correlations were calculated by Spearman’s rank correlation coefficient. Circles indicate individual patient values and R and P values are indicated.

Relative engagement of synovial Tie2 is enhanced in RA compared to PsA

Activation of Tie2 is accompanied by phosphorylation of multiple tyrosine residues on the intracellular tail of the receptor, generating binding sites for downstream signaling proteins [33]. To determine the relative participation of Tie2 signaling in RA and PsA synovium, synovial tissue sections were stained with purified rabbit polyclonal antibodies recognizing rabbit hyper-immune serum generated against
Tie2 peptide phosphorylated at tyrosine residue 1111 (p-Tie2). Normal rabbit serum supplemented with rabbit anti-FITC antibodies, which served as negative control antibodies for anti-p-Tie2, failed to stain RA synovial tissue (Figure 3A). Tie2 phosphorylation was observed in the intimal lining layer, perivascular regions, and cellular infiltrates within the synovial sublining of RA patients (Figure 3B). Similar variable staining patterns were observed in PsA synovial tissue (data not shown). Quantitative analysis demonstrated similar amounts of Tie2 and p-Tie2 in RA and PsA synovial tissue (Figure 3C). However, when p-Tie2 levels were adjusted to account for patient variation in Tie2 expression, it was evident that engagement of expressed Tie2 was elevated in RA (IOD p-Tie2/IOD Tie2, median ± SEM, arbitrary units, 0.623 ± 0.098) compared to PsA (0.184 ± 0.107) (p < 0.05) (Figure 3D).

**Figure 3** Expression and phosphorylation of Tie2 in RA and PsA synovial tissue. Representative stainings of RA synovial tissue with negative control (a) rabbit antibodies diluted in normal rabbit serum. (b) Representative stainings of synovial tissue from 3 RA patients with anti-phospho (p)-Tie2 antibodies. Quantitative analysis of Tie2 expression and phosphorylation (p-Tie2) (c) and relative Tie phosphorylation (IOD p-Tie2/IOD Tie2, arbitrary units) (d) in RA and PsA. Data is 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. * p < 0.05.
Angiopoietins make specific contributions to synovial Tie2 activation in RA and PsA

If Ang-2 were to act as an antagonist of Ang-1 in synovial tissue, we would expect Tie2 phosphorylation status to be dependent upon relative local expression levels of Ang-1 and Ang-2. We therefore examined how Ang-1 and Ang-2 expression might influence engagement of Tie2 in RA and PsA synovial tissue. In RA, we observed a strong positive correlation between the ratio of Ang-1/Ang-2 expression and the relative phosphorylation of Tie2 ($R = 0.786$, $p < 0.01$) (Figure 4A). Such a relationship was not apparent in PsA synovial tissue. Indeed, synovial Tie2 engagement was inversely correlated with the ratio of Ang-1/Ang-2 expression in PsA ($R = -0.752$, $p < 0.01$) (Figure 4B). These data indicate that while Ang-2 may antagonize Tie2 phosphorylation in RA synovial tissue, paradoxically, Ang-2 is largely responsible for Tie2 engagement in PsA synovial tissue.

**Figure 4 / Relationship between Tie2 phosphorylation and Ang1/Ang2 expression in RA and PsA synovial tissue.**

Potential correlations between relative Tie2 phosphorylation (IOD p-Tie2/IOD Tie2) and the ratio of Ang-1/Ang-2 expression (IOD Ang-1/IOD Ang-2) in (a) RA and (b) PsA patients were calculated by Spearman’s rank correlation coefficient. Circles indicate individual patient values and $R$ and $P$ values are indicated.

Both Ang-1 and Ang-2 stimulate synovial IL-6 production in RA

Although angiopoietins have been largely studied within the context of their ability to maintain or restructure vascularization, recent studies have indicated that they might promote inflammation and joint destruction through direct influences on endothelial cell and FLS cytokine and matrix metalloproteinase production [23,34]. To examine if angiopoietins might contribute directly to synovial activation in RA, freshly isolated intact synovial biopsies from RA patients were cultured ex vivo in the absence or presence of Ang-1 or Ang-2. To examine potential effects of angiopoietins on synovial TNFα receptor or toll-like receptor signalling, parallel cultures were also stimulated with TNFα or LPS. In all three patients examined, Ang-1 or Ang-2 alone could stimulate IL-6 production in RA synovial...
tissue (Figure 5, upper panel). Little effect of either Ang-1 or Ang-2 was observed on RA synovial explant IL-6 production in response to TNFα (Figure 5, middle panel), while in two of three patients, both angiopoietins enhanced LPS-induced IL-6 production (Figure 5, lower panel). These pilot experiments provide initial indications that angiopoietins can directly stimulate inflammatory cytokine production in RA synovial tissue.

Figure 5 / Ang-1 and Ang-2 enhance RA synovial tissue IL-6 production ex vivo.
Synovial biopsy explants from 3 RA patients were cultured in triplicate for 24 hours in medium alone (upper panel), TNFα (TNF, middle panel) or LPS (lower panel) in the absence (–) or presence of 200 ng/ml Ang-1 or Ang-2. Cell-free tissue culture supernatants were harvested and analyzed for IL-6 content by ELISA.
Het netwerk van de NS verschilt niet veel van de signaaltransductie in RA
Discussion

In this study we confirm and extend previous reports indicating RA and PsA differ significantly in regard to the relative synovial expression of Ang-1 and Ang-2 [22]. Remarkably, our data provide evidence that Ang-1 and Ang-2 synthesis is regulated by distinct mechanisms in RA and PsA. This has consequences for functional engagement of synovial Tie2 in each form of arthritis, as in RA, Tie2 engagement is largely dependent upon Ang-1 expression. In contrast, Ang-2 expression drives Tie2 phosphorylation in PsA. These data are consistent with morphological observations that vascularization in RA and PsA displays Ang-1 and Ang-2 phenotypes, respectively [22]. However, we also provide preliminary evidence that both Ang-1 and Ang-2 can redundantly promote IL-6 production in RA synovial tissue, indicating a proinflammatory role for angiopoietins independent of their effects on vascular stabilization and remodeling.

The importance of angiogenesis in supporting inflammation and bone destruction in RA and other forms of inflammatory arthritis has been well-documented in the clinic. Serum levels of VEGF, the best characterized pro-angiogenic growth factor, are elevated in the serum of RA patients compared to disease controls [7]. In studies of patients with early arthritic symptoms, serum VEGF levels were higher in patients with RA than those with self-limiting arthritis or OA. In these early RA patients, a strong correlation was observed between serum VEGF levels and eventual development of bone erosions [4]. VEGF contributes to joint destruction via several mechanisms. A primary mechanism is likely the induction of endothelial cell proliferation and blood vessel formation, required for delivering nutrients and inflammatory white blood cells to synovial tissue [1]. Additionally, VEGF may promote bone resorption through direct stimulation of osteoclasts or indirectly via RANKL induction on endothelial cells [35,36]. Studies in animal models of RA have previously demonstrated a protective value in direct targeting of neovascularization [37,38]. Based on differences in synovial expression levels, it is thought that Ang-1 and Ang-2 make distinct contributions to RA and PsA, particularly in regard to stabilizing or remodeling synovial vascularization [22]. However, it is uncertain why Ang-1 expression is elevated relative to Ang-2 in RA. Additionally, we note that Ang-1 and Ang-2 expression appear to be reciprocally regulated in RA but not PsA. One possibility is that Ang-1 and Ang-2 expression is restricted to cell populations which differentially contribute to synovial tissue composition in RA and PsA. This explanation seems unlikely however, as RA and PsA are remarkably similar in regard to the magnitude of inflammatory cell infiltration, cellular composition, contribution of angiogenesis to joint destruction, and responses of these parameters to anti-TNF treatment [1,3,4,8,9,39,40]. In RA synovial tissue, Ang-1 is produced by endothelial cells, macrophages and FLS [20,21], while the same cell populations, as well as smooth muscle cells produce Ang-2 [20]. Similar studies have not yet been performed in PsA synovial tissue, and thus direct comparison of cellular localization of angiopoietins in RA and PsA will need to be conducted. An alternative possibility is that differences in Ang-1 and Ang-2 expression could be related to the activation status of cells within the synovial membrane or disease-specific
cytokine networks contributing to inflammation in RA and PsA. TNFα can stimulate Ang-1 production in RA FLS [21,41]. Tumor growth factor β, but not IL-1β can also induce Ang-1 production by RA FLS, but Ang-2 expression is not altered in response to any of these cytokines [41,42]. Again, formal experiments examining how angiopoietin expression is regulated in cells derived from PsA synovial tissue are needed. A final possibility which might explain differential expression of angiopoietins in RA and PsA is the contribution of genetic polymorphisms, as single nucleotide polymorphisms (SNPs) have been detected in the ANG2 gene [43]. Although these SNPs have not been well-characterized in regard to their effects on Ang-2 expression or function, at least one has been identified as making potential contributions to acute respiratory distress syndrome [44]. A second intriguing finding in our study is that we observe a strong positive correlation between Tie2 phosphorylation and the ratio of synovial Ang-1/Ang-2 expression in RA. In contrast, an inverse relationship is observed in PsA synovial tissue. How might we reconcile these phenomena? The idea that Ang-1 and Ang-2 might act as mutual antagonists was raised by initial observations that mice transgenically expressing Ang-2 died during embryonic development, displaying phenotypic characteristics of Ang-1 and Tie2 knockout mice [14]. However, eventual analysis of Ang-2 knock-out mice demonstrated that Ang-2 plays a critical post-natal role in promoting remodeling of the vascular and lymphatic systems [15]. Similarly, initial reports that Ang-2 could inhibit Ang-1-dependent endothelial cell Tie2 activation were not widely reproducible and more likely represented use of biologically inactive Tie2 rather than a physiological property of Ang-2. [3,6,8-10]. However, the intracellular signaling pathways initiated by Ang-1 and Ang-2, though highly overlapping, are distinct, which by inference might mean that each angiopoietin differentially promotes phosphorylation of distinct tyrosine residues on Tie2 [17,45]. We have used an antibody recognizing tyrosine-phosphorylated residue 1111 of Tie2 in our studies. The development of antibodies suitable for immunohistochemistry recognizing phosphorylated residues 1100 and 1106, currently unavailable, will allow us to consider if the differential relationships we observe between angiopoietin expression and Tie2 phosphorylation manifest themselves as differences in Tie2 engagement or differences in Tie2-dependent signaling pathways initiated by angiopoietins. An additional possibility which bears consideration is the influence of the cellular environment on Tie2 signaling, as recent studies have indicated that the ability of Ang-1 and Ang-2 to stimulate Tie2 activation is dependent upon whether cells bound to other or to extracellular matrix components [32,46]. A final important observation is our initial evidence that both Ang-1 and Ang-2 can stimulate IL-6 production in RA synovial biopsies ex vivo. Although potential roles of Tie2 signaling in inflammatory arthritis have focused primarily on vascular remodeling [1], it is now evident that angiopoietins have pro-inflammatory effects on many cell types relevant to RA. Ang-1 can directly stimulate MMP-3 production in RA FLS, and Ang-2 can sensitize activation of endothelial cells by TNFα as well as act as a chemoattractant for monocytes [23,34,47]. Together, these data suggest that strategies targeting Ang-1 and Ang-2 in RA and other forms of arthritis might suppress synovial inflammation by mechanisms distinct from direct effects on angiogenesis.
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