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Signal transduction pathways in rheumatoid arthritis with emphasis on the p38 mitogen-activated protein kinase (MAPK) pathway

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1. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, primarily located in the synovial joints, leading to destruction of the cartilage and bone as a result of the chronic disease activity. RA affects 0.5 - 1% of the population in the industrialized world is two to three times more frequent in women than men and can lead to disability and reduced quality of life.

1.1. Pathogenesis

The cause of RA is still unknown, but both genetic and environmental factors appear to play a role. The association with certain human leukocyte antigen (HLA) DR4 subtypes and RA has been recognized for a long time, and now it is found that rather specific amino acid sequences, the so-called shared epitope (SE) confer the highest risk for developing RA. RA is characterized by the presence of autoantibodies, especially rheumatoid factors and antibodies to citrullinated proteins (anti-CCP) in the majority of the patients. Recently in a study in blood bank donors it was demonstrated that approximately half of patients with RA had specific serologic abnormalities (rheumatoid factor and anti-CCP antibodies) several years before the onset of symptoms. The earliest events in RA might involve activation of the innate immune system, which triggers a T-cell response possibly directed towards citrullinated proteins.

Infiltrating T-cells in the synovial membrane may, by cell-cell contact, and activation by different cytokines, such as TNF-α, IFN-γ and IL-17, activate monocytes, macrophages and synovial fibroblasts. These cells than produce pro-inflammatory cytokines, mainly TNF-α, IL-1 and IL-6. As the disease progresses multiple cytokine networks enter a state of persistent activation, triggering the production of matrix metalloproteinases, ultimately resulting in irreversible damage of cartilage and bone.

1.2. Therapy

DMARDs (disease modifying anti-rheumatic drugs) are drugs that are intended to inhibit both the inflammatory and destructive processes in RA. Of these DMARDs methotrexate (MTX) is the most commonly used and is regarded as the gold standard of DMARD therapy. At doses used in the treatment of RA, MTX is likely to act via a number of intracellular pathways. Upon transport into cells, MTX is converted to polyglutamated forms, which promote intracellular retention. This results eventually in induced release of adenosine, which has an anti-inflammatory effect on neutrophils and mononuclear cells. MTX modulates cytokine responses at a number of levels and may promote apoptosis of activated lymphocytes. Since five years a new DMARD has become available, Leflunomide, which is an active metabolite that inhibits dihydro-orotate-dehydrogenase, an enzyme involved in de novo pyrimidine synthesis. Inhibition of this enzyme affects various signal-transduction mechanisms, the generation of cytokines, and cell proliferation and migration.

However, these DMARDs still have limited efficacy and may lead to toxicity problems. The pro-inflammatory role of cytokines and the involvement of different cell types led to the development of therapeutics to selectively target cytokines. The key role of TNF-α in the pathogenesis of RA was discovered both in experimental animal models and in RA patients by Feldmann, Maini and others.
Chapter 2

Three drugs clinically available for treatment that block the activity of TNF-α: Infliximab (chimaeric monoclonal antibody to human TNF), Adalumimab (human monoclonal antibody to TNF) and Etanercept (soluble TNF receptor construct) and one to block IL-1 activity: Anakinra (IL-1 receptor antagonist). The efficacy and possible toxic effects of these new drugs have been reviewed by Olsen and Stein. Although TNF blockade has been a major breakthrough in the therapy of RA in the last ten years, there are some drawbacks. About half of the patients in clinical trials do not achieve adequate clinical responses expressed as ACR50 responses, remission is rare and these drugs also have side effects, for instance increased risk of tuberculosis.

New drugs to inhibit the production and activity of inflammatory cytokines may be found in inhibitors of intracellular signal transduction pathways. These pathways are involved in the primary production of these cytokines, as well as in the responses generated by these cytokines. TNF-α and IL-1 induce a variety of signal transduction cascades that lead to the activation of transcription factors and next to the transcription and translation of genes, coding for inflammatory mediators. The cascades that are important in RA and the potential inhibitors will be discussed below.

2. SIGNAL TRANSDUCTION PATHWAYS IN RA

Intracellular signal transduction pathways are intracellular mechanisms by which cells can react to extracellular stimuli such as stress and inflammatory cytokines. In short, intracellular signal transduction is the process by which a cell converts an extracellular signal into a response. A key role thereby is for proteins called kinases, which act as key regulators of cell function by catalyzing (facilitating) the addition of a negatively charged phosphate group to proteins. These signalling cascades ultimately lead to induction of gene transcription and translation into specific proteins. These inflammatory proteins, including cytokines, matrix metalloproteinases, and cyclooxygenase (COX-2) are involved in the pathogenesis of RA.

The main intracellular signal transduction pathways implicated in RA include the mitogen-activated protein kinase (MAPK) pathways, nuclear factor-kappa B (NF-κB) and the Janus kinase (JAK-STAT) pathway.

2.1. Mitogen-activated protein kinase (MAPK) pathways (figure 1)

There are four well-characterized families of MAPKs, acting by phosphorylation of specific serine (Ser), threonine (Thr) and tyrosine (Tyr) residues of target substrates thereby controlling important cellular functions, such as gene expression, mitosis, movement, metabolism and apoptosis. The MAPK isoforms themselves are phosphorylated by dual-specificity serine-threonine MAPK-kinases (MAPKK or MEK) which in turn are phosphorylated by upstream MAPK-kinase-kinases (MAPKKKs or MEKKs).

The MAPK family include the extracellular signal-regulated kinases ERK1 and ERK2 (also known as the p42/p44 MAPK pathway), the c-jun NH2-terminal kinases JNK 1, JNK 2 and JNK 3, the four p38 enzymes, p38α, p38β, p38γ and p38δ, and the ERK5 or big MAP kinase 1 (BMK1). All MAPKs share the amino-acid sequence Thr-Xxx-Tyr in which X differs: X is glutamic acid (Glu), proline (Pro) and glycine (Gly) for the ERK, JNK and p38 MAPK respectively.
2.1.1. The ERK 1/2 signaling pathway is a major determinant in the control of cell growth, cell differentiation and cell survival. Growth factors, cytokines, viral infection, and carcinogens are the main activators of the proto-oncogene Ras, a small GTP-binding protein, which is the common upstream molecule of the MEKKs Raf (Raf1, A-Raf or B-Raf) 17. Activated Rafs phosphorylate MEK1/2, which in turn activate ERK1 and ERK2. ERKs can directly phosphorylate a set of transcription factors including Ets-1, c-Jun and c-myc, or activate RSK (ribosomal S6 kinase), which leads to the activation of the transcription factor CREB (cyclic AMP-responsive element-binding protein). MEK1 and MEK2 activity has been detected in a significant number of primary human tumor cells. Inhibitors of the ERK pathway (Ras-, Raf- and Src-inhibitors) are entering clinical trials as potential anti-cancer agents. PD98059 and U0126 are non-ATP competitive MEK 1/2 inhibitors, which block phosphorylation and activation of ERK1 and 2 by MEK 18.

2.1.2. JNKs were discovered to bind and phosphorylate the DNA-binding protein c-Jun (a component of the AP-1 (activator protein) transcription complex) and to increase its transcriptional activity. Regulation of the JNK pathway is extremely complex and is influenced by many kinases. In general, stress or cytokines can initiate a series of events in which GTP-binding proteins such as Cdc42, Rac or Ras can activate protein kinases such as GCK (germinal centre kinase), which in turn can activate ASK (apoptosis stimulating kinase), TAK (TGFβ-activated kinase) and the MEKKs 1,2 and 3 19. Then MKK4 and MKK7 are activated, which are the direct activators of the JNK MAPKs. Targets of this pathway include c-Jun, ATF2 (activating transcription factor...
2) and ELK-1. JNK is one of the primary MAPKs required for expression of matrix metalloproteinases and joint destruction in models of inflammatory arthritis. The best known JNK2 inhibitor is SP600125, while another JNK pathway inhibitor CEP1347, has been reported to inhibit members of the MLK (mixed lineage kinase) family, which are upstream activators of the JNK pathway.

2.1.3. p38 MAPKs are, like the JNKs, stress-activated protein kinases, that mediate responses to cellular stress factors, such as UV light, osmotic shock and cytokines. The main activation route for p38 MAPK is through phosphorylation by MMK3 / MKK6 and possibly by MKK4, which in turn are activated by the MAPKKKs (figure 2). Members of the MAPKKK superfamily include MEKK 1-4, MLK, Tpl2 (tumor progression locus-2), TAK-1 and ASK-1. The MAPKKKs themselves are activated by small GTP-binding proteins, including Rac and Cdc42, partly involving p21-activated kinases (PAKs). In inflammation the most important route for activation is via TNF-α and IL-1 by ligation of their respective membrane receptors and recruitment of intracellular adaptor molecules. Activated p38 MAPKs can phosphorylate downstream kinases (MAPKAPK-2, MSK-1, PRAK and MNK) and transcription factors such as ATF-2, CHOP (C/EBP homologous protein) and ELK-1. Lipopolysaccharide (LPS) signal transduction is also one of the activation routes for

Figure 2. p38 mitogen-activated protein kinase (MAPK) pathway.
p38 MAPK. LPS is a common constituent of Gram-negative bacterial outer membranes and is the principal initiator of septic shock. Signalling of LPS involves a receptor complex with Toll-like receptor 4 (TLR-4), CD14 and the adaptor molecules MyDD88 and IRAK (interleukin-receptor associated kinase). The route then resembles the IL-1 route: via TRAF6 and TAK1 p38 MAPK can be activated as well as the JNK and the NF-κB pathways.

The p38 MAPK signal transduction pathway will be discussed in detail below.

2.1.4. The fourth and least studied MAP kinase pathway, BMK1 or ERK5, is activated in response to growth factors and stress. This pathway has not only been implicated in cell survival, proliferation and differentiation, but also in pathologic processes such as carcinogenesis, cardiac hypertrophy and atherosclerosis. MEK5 is the upstream kinase of BMK1, and can be inhibited by inhibitors of MEK 1/2 such as PD98059 and U0126. The MEK5 kinases identified thus far are MEKK2 and MEKK3, which are also known to regulate p38 MAPK and JNK activity through the activation of MKK3/6 and MKK4/7 respectively.

2.2. NF-κB pathway (figure 3)
The transcription factor Nuclear Factor κ-B (NF-κB) is a key factor in the transcription of many inflammatory genes. NF-κB is a complex group of heterodimeric and homodimeric transcription factors, consisting of five members: c-Rel, RelA (p65 or NF-κB3), RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). Normally these dimers bind to the specific inhibitors of NF-κB, known as IκB proteins. Upstream kinases, including members of the MAPKKK family and NF-κB-activating-kinase (NAK) can induce phosphorylation and degradation of IκB, thereby liberating the NF-κB dimers, which translocate to the nucleus and regulate gene transcription. The modification of the IκB proteins depends on the IKK (IκB kinase) complex, which is composed of three subunits: the catalytic subunits IKK-α(1) and IKK-β(2), and the regulatory subunit IKK-γ (also known as NEMO). These subunits have specific roles in the regulation of NF-κB activity. The activation of NF-κB has been implicated in the pathogenesis of RA. Translocation of nuclear p50 and p65 was demonstrated in RA synovial lining cells and in mononuclear cells of the sublining. It has been demonstrated that NF-κB suppression is beneficial in many models of inflammatory disease. Moreover, for many therapeutic agents it has been shown that at least some of their effects are due to NF-κB blockade. Efforts to block this pathway have led to the development of small-molecule inhibitors of various kinases and regulatory proteins and also to research in gene therapy. In arthritis IKK-β seems an attractive target for therapy, because it regulates cytokine production in many cell types including synovial fibroblasts. Although it is obvious that NF-κB plays a key role in inflammatory diseases, there are major safety concerns about inhibition of this transcription factor, because of the major role that NF-κB plays in host defence, homeostasis, cell survival and response to stress.
2.3. JAK/STAT pathway (figure 4)
The Janus kinase (JAK) family and the signal transducer and activator of transcription (STAT) transcription factors play an important role in cytokine signal transduction. Type I cytokine receptors (for colony stimulating factors, and several interleukins) and type II cytokine receptors (for interferons) lack intrinsic kinase activity and rely on Jak proteins to initiate signalling. In the case of IL-6 an association with the IL-6 receptor and gp130 subunits takes place that activates JAKs. This is followed by the phosphorylation of the tyrosine-based docking sites on the receptor and recruitment of STATs. They form homo-hetero-dimers and translocate to the nucleus, where they bind target sequences. Dimerization of the IL-6-type cytokine receptors does not only lead to activation of the JAK/STAT signalling pathway, but also of the MAPK cascade.

Negative regulation of IL-6 signalling via the JAK/STAT pathway may occur in different ways. Suppressor of cytokine signalling (SOCS) proteins are induced in response to IL-6 binding and can bind directly to the JAKs. SH2-domain-containing tyrosine phosphatase-1 (SHP-1) either can dephosphorylate JAKs or activated receptor subunits. Protein inhibitors of activated STATs (PIAS) family members inactivate STAT dimers.
THE p38 MAPK SIGNAL TRANSDUCTION PATHWAY

3.1. Identification

The human p38α MAPK were first identified as the molecular target of pyridinylimidazole class of compounds, that were known to block TNF-α and IL-1 release from lipopolysaccharide (LPS)-stimulated human monocytes 32. Originally these proteins were designated cytokine-suppressive anti-inflammatory drug-binding proteins (CSBP) 33. Until now, five isoforms of p38 MAPK have been identified: p38β1 and p38β2 have more than 70% identity to p38α, whereas p38γ and p38δ have approximately 60% identity to p38α. Functional differences between the isoforms are related to their differential expression, activation, and substrate specificity 34. p38α, p38β and p38δ are widely produced in various tissues, while p38γ is expressed primarily in skeletal muscle. Inflammatory cells synthesize predominantly p38α and p38δ protein, but endothelial cells also produce p38β 35,36.

ERK, JNK and p38 MAPK activation were almost exclusively found in synovial tissue from RA, but not osteoarthritis patients. p38 MAPK activation was observed in the synovial lining layer and in synovial endothelial cells 37.

3.2. Downstream effects of p38 MAPK activation

3.2.1. Gene expression

One of the main downstream effects of the p38 MAPK pathway is regulation of gene expression, which can be at the transcriptional level, but also at the translational level,
leading to protein synthesis. p38 MAPK has been implicated in the activation of various transcription factors including: ATF-2, MEF-2C, CHOP, and NF-κB \(^{38-40}\). Other transcription factors are phosphorylated by downstream protein kinases that are themselves activated by phosphorylated p38 MAPK, such as MAPKAPK-2 (MAPK activated protein kinase). Furthermore MAPKAPK-2 deficient mice show diminished production of IL-6 and TNF-α \(^{41}\). These effects are thought to be mediated by a mechanism involving mRNA turnover and protein translation. The p38 MAPK/MAPKAPK-2 pathway is crucial to inflammatory cytokine production.

### 3.2.2. Post transcriptional regulation of mRNA stability

As will be discussed later, several p38 MAPK inhibitors have been developed which were shown to block the production of inflammatory cytokines. This inhibition seems to be a combined effect at the level of transcription and translation. Inducible cytokines usually have short lived mRNAs and contain an AU-rich element (ARE) in their 3’ untranslated region responsible for their high turn-over rate \(^{42}\). These AREs contain repeats of the motif AUUUA, and were discovered as instability elements. In the case of inflammatory response mRNAs, the instability is countered by signalling in the p38 MAPK pathway \(^{43}\).

Under normal conditions, these AU-rich elements are occupied by AU-binding proteins, thereby blocking translation or transcription. It has been demonstrated that upon stimulation these AU-binding proteins are phosphorylated in a p38 MAPK-dependent manner, resulting in their release, and allowing translation of these mRNAs \(^{43,44}\). Inhibitors of p38 MAPK can target these events directly or via MAPKAPK-2. It has been demonstrated that both translation and stability of TNF-α mRNA are regulated by the p38MAPK pathway \(^{45}\), whereas IL-6 and IL-8 mRNA stability is regulated by p38 MAPK, but the extent of inhibition of protein production varies with cell type \(^{46,47}\). Activation of p38 MAPK has also been shown to enhance the mRNA stability of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) \(^{48}\). Finally, Lasa et al demonstrated that the gluco-corticoid dexamethasone destabilizes COX-2 mRNA by inhibiting p38 MAPK. This effect was induced by expression of MAPK-phosphatase-1 (MKP-1) \(^{49}\).

### 3.3. Inactivation by phosphatases

For control of the MAPK signal transduction pathways dephosphorylation is necessary. Over the last decade a family of endogenous negative regulators of MAPK, the MAPK phosphatases (MKPs), also known as DUSPs (dual-specificity phosphatases) have been described \(^{50}\). The MKPs dephosphorylate the tyrosine and threonine motifs of MAPK to deactivate MAPK-dependent signalling. At least 10 mammalian MKPs have been cloned and characterized, which have different subcellular distribution, substrate specificity, and expression patterns. MKP-1 was the first isolated MKP, and is a 39.5 kD protein that is preferentially localized in the cell nucleus. It is capable of dephosphorylating all MAPK families, although a preference for dephosphorylating p38 MAPK and JNK has been described \(^{51}\). MKP-1 is an early response gene that is induced by the same stimuli that activate the MAPKs, such as cytokines, osmotic shock and UV radiation \(^{52,53}\). Recently the expression of MKP-1 in
rheumatoid synovial fibroblasts was demonstrated as well as a role for glucocorticoid dependent upregulation of MKP-1\textsuperscript{32}.

4. p38 MAPK INHIBITORS (figure 5)

4.1. Development

Already in 1988 the inhibition of IL-1 production by the anti-inflammatory compound SK&F 86002 was reported\textsuperscript{54}, but it took until 1994 to discover that the molecular target of the pyridinyl imidazole class of compounds indeed proved to be p38 MAPK, originally known as CSBP \textsuperscript{32}. Since the original report of the efficacy of these compounds, they have become the most widely studied inhibitors of this kinase. The compounds have been used as framework for further synthetic work and have been utilized to elucidate the role of p38 MAPK in the immune system. Crystallographic and kinetic experiments have shown that the pyridinyl imidazole family of p38 MAPK inhibitors bind at the ATP binding site of p38 MAPK and compete with ATP for binding to active, phosphorylated p38 MAPK \textsuperscript{55}. When p38 MAPK is in the unactivated form, ATP is non-competitive with many p38 MAPK inhibitors. After the structure-activity relationship was established SB 203580 and other 2, 4, 5-triaryl imidazoles were prepared as pharmacological tools to regulate cytokine synthesis. A large number of preclinical studies have reported that specific and selective p38 MAPK inhibitors block the production of inflammatory cytokines \textit{in vitro} and \textit{in vivo}.

Furthermore, the p38 MAPK pathway is involved in the induction of several other inflammatory molecules such as cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).

\textbf{Figure 5. p38 MAPK inhibitors.}

The p38 MAPK inhibitor used in our studies, RWJ 67657, was developed by Johnson and Johnson Pharmaceutical Research and Development and was first described in 1999\textsuperscript{36}. RWJ 67657 (4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyn-1-ol) has been shown to be effective in inhibiting the release of TNF-\(\alpha\) from LPS-treated human peripheral blood mononuclear cells with an IC\(_{50}\) of 3 nM. In comparison to the literature standard SB 203580 this new compound proved to be approximately 10-fold more potent in all p38 MAPK dependent systems tested.
Moreover the compound inhibited the enzymatic activity of p38α and β, but not of γ and δ, in vitro and had no significant activity against a variety of other enzymes.

4.2. p38 MAPK inhibitors in animal models for arthritis
Several p38 MAPK inhibitors have been evaluated in animal arthritis models. Already in 1996 the pharmacological profile of SB 203580 (GlaxoSmithKline) was investigated in adjuvant-induced arthritis in Lewis rats, whereas in 1998 the pharmacologically effects of SB 220025 (GlaxoSmithKline) were investigated in an angiogenesis and chronic disease model. RPR-200765A (Aventis) reduced incidence and progression in the rat streptococcal cell wall (SCW) arthritis model at doses given orally, while prevention of the onset and progression of collagen-induced arthritis were reported for FR167653 (Fujisawa Pharmaceuticals). Recently SB 242235 (GlaxoSmithKline) was evaluated in a new model of arthritis, pristane-induced arthritis, and demonstrated to significantly reduce all arthritis scores.

4.3. p38 MAPK inhibitors in human studies
RWJ 67657 is one of the p38 MAPK inhibitors who until now have been used in human studies. The effects on clinical and cytokine response to endotoxaemia were studied in healthy human volunteers and reported by Fijen et al. Single oral doses of RWJ 67657 dose-dependently decreased symptoms and elevated cytokine levels, induced after administration of endotoxin. Furthermore single-dose pharmacokinetics and pharmacodynamics of RWJ 67657 were investigated in healthy male subjects. RWJ 67657 was rapidly absorbed (mean tmax = 0.6-2.5 h) and there were no significant adverse effects associated with single doses of this drug. This study demonstrates that RWJ 67657 has acceptable safety and pharmacokinetics to warrant further investigation in a repeat-dose setting. Other compounds that have been investigated in rheumatoid arthritis patients are VX-745 and SCIO-469, which is now in phase II clinical trial.

5. CONCLUSIONS
The discovery of p38 MAPK inhibitors have dramatically increased the understanding of signal transduction pathways involved in inflammation. It is widely expected that p38 MAPK inhibitors will have efficacy in arthritis and other inflammatory diseases. However, clinical trials have been stopped due to safety issues. One of the reasons for these undesirable effects might be the cross-reactivity against other kinases. Solutions for these problems might lie in the development of non-ATP competitive inhibitors or inhibitors, which target other molecules in the p38 MAPK pathway.

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