Experimental studies on signal transduction pathways in rheumatoid arthritis
Bijl-Westra, Johanna

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Summary, general conclusions and future perspectives

Johanna Westra
SUMMARY, GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis the potential of signal transduction pathways as treatment target in rheumatoid arthritis (RA) was investigated.

To explain the importance of intracellular signal transduction pathways in inflammation we first outlined the pathways involved in rheumatoid arthritis in chapter 2. In particular the p38 mitogen-activated protein kinase (MAPK) pathway has been found to be an interesting target for therapy in RA. The p38 MAPK is activated in different cells by inflammatory cytokines such as TNF-α and IL-1β, but also by other stress factors. The most important downstream effect of signal transduction pathways in inflammation is the induction of gene transcription and translation into specific proteins. These proteins, including cytokines, matrix-metalloproteinases, and cyclo-oxygenase-2 (COX-2) are involved in the pathogenesis of RA. Moreover the p38 MAPK pathway has been found to play a role in stabilizing mRNA of inflammatory genes, thereby influencing the efficiency of translation.

The important advantage of inhibition of the p38 MAPK pathway is not only that the production of the major pro-inflammatory cytokines is inhibited, but also that the effects of these cytokines on other cells is inhibited.

In chapter 3 the effects of the p38 MAPK specific inhibitor RWJ 67657 on the production of pro-inflammatory mediators by rheumatoid synovial fibroblasts (RSF) was investigated. There is growing evidence that activated RSF play a major role in both initiating and driving RA. These cells can attach to the articular cartilage and invade the extracellular matrix. Especially by the production of matrix metalloproteinases (MMPs) irreversible damage to cartilage and bone is induced. In this study synovial fibroblasts isolated from RA synovium were stimulated with TNF-α and/or IL-1β, and the effects of p38 MAPK inhibition on protein and mRNA levels of MMP-1 (collagenase 1), MMP-3 (stromelysin-1), tissue inhibitor of metalloproteinase -1 (TIMP-1), IL-6 and IL-8 were determined, as well as the mRNA expression of COX-2 and aggrecanase-1 (ADAMTS-4, a disintegrin and metalloproteinase with thrombospondin-1 motif). Although TNF-α is considered to be the most important pro-inflammatory cytokine in RA, stimulation with IL-1β induced higher levels of pro-inflammatory mediators in rheumatoid synovial fibroblasts. We found that MMP-3 production was significantly inhibited at 1 µM RWJ 67657, MMP-1 production at 10 µM, whereas TIMP-1 production was not inhibited. Significant inhibition of IL-6 and IL-8 protein production was already seen at 0.1 µM of RWJ 67657. The effects on mRNA expression profiles were in concordance with the inhibition of protein production. Significant inhibition of COX-2 mRNA expression already occurred at 0.01 µM, while inhibition of ADAMTS-4 mRNA was seen at 1 µM. Effects of RWJ 67657 below 5 µM are considered relevant, since these concentrations are achieved after a single oral dose in humans.
The inhibitory effects of RWJ 67657 on MMP-3 production are important since MMP-3 is present at very high levels in RA synovial fluid, and is not only a major degradative enzyme itself, but acts as activator of other MMPs as well. Another significant effect is the strong inhibition of COX-2 mRNA expression. COX-2 is selectively induced during inflammation and leads to the enhanced production of prostaglandins. Inhibition of prostaglandin production, either with general or selective cyclo-oxygenase inhibitors, is a major goal for treatment in RA. The results from this study are promising since production of inflammatory cytokines, MMPs, and COX-2 in rheumatoid synovial cells appears to be inhibited by p38 MAPK inhibition.

The synovial lining layer consists mainly of synovial fibroblasts and macrophages. Monocytes/macrophages are the major producers of TNF-α and IL-1β, but also of other inflammatory cytokines and MMPs. The strong effects of p38 MAPK inhibitors was originally discovered by the inhibition of TNF-α production in LPS-stimulated monocytes, but the effects on macrophages were unknown. In chapter 4 we used monocyte-derived macrophages (MDM) from healthy controls and RA patients to investigate the effects of p38 MAPK inhibition on macrophages. Isolation and culture of macrophages from synovial tissue is disturbed by the overgrowth of fibroblasts, so for this study monocytes were differentiated with macrophage-colony stimulating factor (M-CSF) and low serum concentration, which generated macrophages with high HLA-DR expression, that were not activated.

Treatment of MDM with increasing concentrations RWJ 67657 resulted in a highly significant inhibition of TNF-α production at 0.01 µM and significant inhibition of IL-8 at 0.1 µM. Effects on MMP production were not seen, since MMP-1 (collagenase-1) production by MDM was below detection limit and MMP-9 (gelatinase-2) production in these cells was constitutively high, and was only moderately induced by cytokines and not inhibited by p38 MAPK treatment. Inhibition at the level of mRNA expression was seen for TNF-α, IL-1β, IL-8 and COX-2. The effects on macrophages were not as strong as for the synovial fibroblasts, since higher concentrations of the p38 MAPK inhibitor were needed to reach significant inhibitory activity.

From the previous chapter it was clear that there are differences between monocytes and monocyte-derived macrophages (MDM), for instance in regulation of IL-1β production, which is produced by monocytes but not excreted by macrophages. In chapter 5 therefore the reactivity of monocytes and MDM towards p38 MAPK inhibition was investigated, as well as the effects of p38 MAPK inhibition on differentiation of monocytes into macrophages.

We found that monocytes produced much more cytokines (TNF-α, IL-1β and IL-8) than MDM, but that MMP-9 was more abundantly produced by MDM. Furthermore it was demonstrated that p38 MAPK inhibition of cytokine production was more effective in monocytes than in MDM. Differentiation of monocytes into MDM in the presence of the p38 MAPK inhibitor reduced both...
cytokine and MMP-9 production by MDM. To determine whether signal transduction pathways were altered due to cell differentiation the activation state of intracellular signal transduction pathways was assessed using a kinase array on both stimulated and unstimulated monocytes and MDM. Results showed that all kinases involved in the p38 MAPK pathway were activated in the stimulated cells. However there were a large number of kinases that were differentially activated in monocytes compared to MDM.

Another group of cells involved in inflammation are the endothelial cells (EC), which line the blood vessels. EC are not just passive bystanders but are active responders to stimuli like activated leukocytes and cytokines. Activated EC can produce a number of inflammatory mediators, such as chemokines and they express cellular adhesion molecules (CAMs), which play a major role both in recruitment as well as migration of leukocytes into the inflamed area. In chapter 6, the effects of p38 MAPK inhibition on expression of the adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) was investigated and the effects on the production of the chemokines IL-8 and monocyte chemo-attractant protein-1 (MCP-1).

p38 MAPK inhibition at a relevant concentration (1 µM) significantly reduced protein and mRNA levels of IL-8, MCP-1 and of IL-6. The effects on CAM expression were negligible. We could only demonstrate an insignificant reduction of E-selectin protein and mRNA expression. Since chemokines play an essential role in maintaining the leukocyte-endothelial interactions after the initial interaction regulated by the selectins, the significant downregulation of IL-8 and MCP-1 could have an important effect on leukocyte attraction and infiltration in inflammatory disease.

Since the effects of p38 MAPK inhibition on CAM expression by endothelial cells were only modest the involvement of another inflammatory pathway was investigated in chapter 7. Overexpression of mutant IκB protein leading to continuous blocking of NF-κB mediated signalling confirmed a major role of this pathway in controlling both TNF-α and IL-1β induced expression of most of the genes studied. MOL-294, which inhibits thioredoxin involved in NF-κB signalling, inhibited adhesion molecule expression in contrast to pyrrolidine dithiocarbamate (PDTC) and dexamethasone (DEX), which both exerted limited effects at 1 µM. In the previous chapter we demonstrated that 1 µM RWJ 67657, an inhibitor of p38 MAPK activity, diminished TNF-α and IL-1β induced expression of IL-6, IL-8, and E-selectin, but had little effect on VCAM-1 and ICAM-1. Combined treatment of HUVEC with MOL-294 and RWJ 67657 resulted in significant blocking of the expression of the majority of genes studied. The inhibitory effects were much stronger than those observed by single drug treatment, indicating that the use of combinations of drugs affecting multiple targets in activated endothelial cells may offer new therapeutic possibilities.
Finally in chapter 8 a study was designed to investigate the influence of p38 MAPK inhibition on acute phase protein (APP) production, which is dependent on both JAK/STAT and p38 MAPK pathways. In diseases like RA acute phase proteins such as C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR), which is largely determined by the concentration of fibrinogen, are used as markers of disease activity. We investigated the effects of p38 MAPK inhibition on APP production and mRNA expression in four human hepatoma cell lines, after stimulation with IL-6 and/or IL-1β or TNF-α. These effects were also investigated in human liver slices, a model to mimic the liver *in vivo*. Concluding we found that production and mRNA expression of CRP and fibrinogen, but not SAA (serum amyloid A) were significantly inhibited by a p38 MAPK specific inhibitor in hepatoma cell lines. In liver slices increased production of APP was detected after stimulation, but p38 MAPK inhibition reduced only fibrinogen production. The consequences of a differential inhibition of the acute phase protein production may well be that in the case of p38 MAPK inhibitor therapy in rheumatoid arthritis SAA will be a better marker for disease activity than CRP and fibrinogen, because SAA is not directly affected by p38 MAPK inhibition.

**CONCLUSIONS**

In this thesis the potential of signal transduction pathways as treatment target in rheumatoid arthritis was investigated. We found that in cells, which are essential players in the inflammatory process in RA, p38 MAPK inhibition had important effects. In rheumatoid synovial fibroblasts MMP-3, IL-6, and IL-8 production and mRNA expression were significantly inhibited. The mRNA expression of COX-2 was dramatically reduced at very low p38 MAPK inhibitor concentrations.

Previously it was shown that TNF-α production by LPS-stimulated monocytes was inhibited by p38 MAPK inhibition. We demonstrated that also TNF-α production by macrophages, is effectively reduced by p38 MAPK inhibition. Endothelial cells which are actively involved in recruitment of inflammatory leukocytes, have display a different response to p38 MAPK inhibitors. Chemokine expression is effectively inhibited by p38 MAPK inhibition, while cell adhesion molecule expression is regulated by the NF-κB pathway. Finally we showed that there is cross-talk between the p38 MAPK pathway and the JAK-STAT pathway in hepatoma cells, by demonstrating that C-reactive protein and fibrinogen are reduced in hepatoma cell lines by p38 MAPK inhibition.

Summarizing we conclude that indeed p38 MAPK is one of the most important signal transduction pathways in RA and that inhibition has important effects on inflammatory cells. Because the NF-κB pathway also plays a role in this inflammatory process a combination of drugs that affect both pathways could even be more effective. Because however p38 MAPK inhibitors are not in clinical practice at the moment due to side effects, the search for new therapeutic targets is still going on.
FUTURE PERSPECTIVES

The research described in this thesis confirms the important role of the p38 MAPK pathway in pro-inflammatory responses. However p38 MAPK is not the only pathway involved in inflammation. There seem to be a large number of other kinases and transcription factors that might be promising therapeutic targets. Progression in this field has been hampered by the complexity of the different intracellular signal transduction pathways involved in chronic inflammatory diseases such as RA.

Over the years the interest in studying synovial tissue has increased because, the synovium is recognized as the primary site of inflammation in RA. Synovial biopsies can be used to investigate the autoinflammatory response for diagnostic purposes and pathogenetic studies. Serial biopsies can be used to evaluate the effects of novel treatment modalities. A new approach to study the synovium would be the mapping of intracellular signaling systems and to investigate their linkage to normal and disease-related processes. A significant step forward has been made by the development of kinase arrays. Kinase arrays enable to study the activity of all (activated) kinases in whole tissue or cell lysates. One array may include over 1000 peptides, which are known substrate sequences for kinases. This new technique offers the opportunity to determine the intracellular signal transduction profile (IST) of the synovium of patients with RA at different stages of the disease and during different therapies.

We recently started to investigate the IST routes (or profiles) in RA patients. We think it will be important to make such a profile from each RA patient, because the IST profile could be an important predictor for the individual response to the installed therapy.

For this study the kinase array will be used, which makes it possible to investigate all involved IST routes at the same time. This kinase array already has been used in cultured cells, intestinal tissue and mice. We now want to extrapolate the findings towards synovial tissue. The findings as obtained by the kinase array will next be compared with findings on activation routes in more conventional and well validated assays.

We hope that by knowing the involved IST routes in RA, we will be able to treat a RA patient with the right therapy at the right time and make a basis for the development of new therapeutics. This could also mean that combinations of signal transduction inhibitors or combinations of signal transduction inhibitors with other drugs, for instance MTX, could be effective therapies in RA.