Differential influence of p38 mitogen-activated protein kinase (MAPK) inhibition on acute phase protein synthesis in human hepatoma cell lines.

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ABSTRACT
Inhibition of intracellular signal transduction is considered to be an interesting target for therapy in inflammation. Especially p38 MAPK inhibitors have been developed and are now in phase II clinical trials for rheumatoid arthritis. This 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. 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.

Two out of four cell lines produced C-reactive protein (CRP), especially after combined IL-6 and IL-1β stimulation. CRP production could be significantly inhibited by the p38 MAPK specific inhibitor RWJ 67657 at 1 µM, which is pharmacologically relevant. Fibrinogen production was also inhibited at 1 µM in all cell lines. Serum amyloid A (SAA) was produced in all four lines. In contrast to CRP, SAA production was not inhibited at 1 µM RWJ 67657. Concluding we found that production and mRNA expression of CRP and fibrinogen, but not SAA production and mRNA expression, significantly were inhibited by p38 MAPK specific inhibitor in hepatoma cell lines. We think that in the case of p38 MAPK inhibitor therapy in rheumatoid arthritis SAA might be a better marker for disease activity than CRP and fibrinogen, because SAA is not directly affected by p38 MAPK inhibition.
INTRODUCTION.
Rheumatoid arthritis (RA) is a chronic inflammatory disease, which leads to the destruction of cartilage and bone in the joints. Cytokines like interleukin (IL)-1 and tumour-necrosis factor (TNF)-α are major players in the pathogenesis of RA. The liver is an important target of systemic inflammatory mediators leading to the production of many components for defence and of the acute phase plasma proteins (APP). Measurement of the APP C-reactive protein (CRP) is useful in managing disease, since the concentration reflects the inflammatory status of a patient. In RA, serial measurements of CRP are of prognostic value.

IL-6 is recognized as the principal regulator of most APP genes (the so-called type-2 or IL-6 like APPs), including the three chains of fibrinogen, haptoglobin, and the protease inhibitors α1-antichymotrypsin, α1-antitrypsin and α2-macroglobulin. Type-1 APPs are regulated by the IL-1like cytokines and include α1-acid glycoprotein (AGP), complement C3, serum amyloid A (SAA) and CRP. The IL-6 like cytokines bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp 130 (glycoprotein 130). Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. The importance of the JAK/STAT pathway in RA has not completely been established yet, but STAT-1 and STAT-3 seem to have both protective and pathogenic properties, with a regulating role for the suppressors of cytokine signalling (SOCS).

Dimerization of IL-6-type cytokine receptor not only activates the JAK/STAT pathway, but may also stimulate the mitogen-activated protein kinase (MAPK) cascade through activation of Ras, a GTP-binding protein. Simultaneous activation of the JAK/STAT and MAPK pathways in a rat hepatoma cell line has also been described for IL-22, an IL-10-related cytokine. A role for one of the MAPK homologues, p38 MAPK, in IL-6 induced functions has been established in different studies. Zaubermann et al. reported that p38 MAPK activity was required for biological functions including acute phase protein production from HepG2 hepatoma and proliferation of B9 hybridoma cells, an IL-6-dependent cell line.

p38 MAPK plays a dominant role in signal transduction pathways in inflammatory diseases and in the last years several specific inhibitors have been developed (for review see Kumar et al). The p38 MAPK inhibitor RWJ 67657 has been shown to significantly inhibit the release of TNF-α from lipopolysaccharide-treated human peripheral blood mononuclear cells, but also from macrophages. Since p38 MAPK inhibitors are now in phase II clinical trial for RA, it is important to know whether these inhibitors have a direct effect on the production of acute phase proteins, due to cross talk between the JAK/STAT and p38 MAPK cascades. This will elucidate whether these APP still are a valuable marker for the disease activity during treatment with p38 MAPK inhibitors.

In this study, we investigated the effect of p38 MAPK inhibition on IL-6, IL-1β and TNF-α induced acute phase protein production in four different hepatoma cell lines both at the level of mRNA expression and at the level of protein production. The effects on CRP, SAA, complement factor 3 (C3), and fibrinogen were studied. For mRNA analysis we studied the SAA-1 gene. Fibrinogen was studied by analyzing both fibrinogen-β and fibrinogen-γ genes. Fibrinogen-β chain synthesis is considered to be rate-limiting for assembly and secretion of mature fibrinogen. The promoter regions of both β- and γ-genes share the IL-6 responsive element, but the γ-gene lacks the C/EBP response element.
MATERIALS AND METHODS.

Reagents.

RWJ 67657 was provided by Johnson and Johnson (R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ). The human hepatocellular carcinoma cell lines HepG2 and Hep3B, and the hepatoma cell line PLC/PRF/5 were purchased from the ATCC (American Type Culture Collection, Manassas, VA). The hepatoma cell line HuH7 was a kind gift from Dr. R. Kleemann (TNO, Leiden, the Netherlands). Recombinant human IL-1β, IL-6 and TNF-α were from R&D Systems (Minneapolis, MN). Foetal calf serum (FCS) and DMEM (Dulbecco’s-modified Eagle Medium) were obtained from Biowhittaker (Verviers, Belgium).

Specific antibodies to p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, STAT-3 and phospho-STAT3 were purchased from Cell Signalling Technologies (Beverly, MA) and detecting antibody peroxidase-swine-anti-rabbit IgG was from DAKO (Glostrup, Denmark). Antibodies for CRP and fibrinogen ELISA were obtained from DAKO, capture-antibodies for C3 ELISA were from Calbiochem (San Diego, CA) and detecting antibodies were from ICN/Biomedicals (Irvine, CA). Antibodies and ELISA for SAA were developed in our laboratory 16. All reagents for RNA isolation and reverse transcriptase reaction were purchased from Invitrogen, Life Technologies (Gaithersburg, MD). Reagents for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA).

Culture of hepatoma cell lines.

Cell lines were maintained in DMEM supplemented with 10% FCS and gentamycin in a humidified atmosphere of 5% CO2/95% air. Hep3B and PLC/PRF/5 were passaged twice a week in a 1:3 ratio, HuH7 in a 1:10 ratio. HepG2 was passaged weekly in a 1:3 ratio. For experiments, the hepatoma cells lines were grown to confluence in 12 wells (1 ml) or 24 wells (0.5 ml) tissue culture plates (Corning, Schiphol, the Netherlands). Activation of the cells with 50 ng/ml IL-6 and/or 10 ng/ml IL-1β or 10 ng/ml TNF-α was performed in DMEM with 1% FCS and 0.1 µM dexamethasone.

Activation of p38 MAPK and STAT-3.

Phosphorylation of MAPKAPK2, p38 MAPK and STAT-3 in hepatoma cell lines was analyzed by western blotting. Confluent cells were stimulated with (combinations of) IL-6, IL-1β or TNF-α for 30 minutes in DMEM with 1% FCS and 0.1 µM dexamethasone with or without pre-treatment with 1 µM RWJ 67657. Cell extracts were prepared and semidry blotting was performed as described previously 13. Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) after the first detection for second or third immunodetection.

Production of acute phase proteins by hepatoma cell lines.

Cells were stimulated with IL-6, IL-1β and TNF-α alone or in combination during 48 hours. The effect of p38 MAPK inhibition was investigated by pre-incubation for 1 hour with a concentration range of RWJ 67657 (0, 0.01, 0.1, 1 and 10 µM, diluted from stock solution of 10 mM in DMSO-dimethylsulfoxide). Production of acute phase proteins was determined in the culture supernatants by ELISAs. The detection limits of the assays were 0.1 ng/ml and 40 ng/ml for CRP and fibrinogen respectively. The detection limit for the C3 assay was 80 ng/ ml. The SAA ELISA was performed as described before 16. Shortly, a capture monoclonal antibody (Reu.86.5, which reacts with all subtypes of SAA) was coated (1: 1000), followed by incubation with diluted supernatants or standards. Detection was done with
a peroxidase-conjugated monoclonal antibody (Reu.86.1, specific for SAA-1), followed by the substrate reaction. The detection limit of the assay was 2 ng/ml.

**mRNA analysis of acute phase proteins.**

Hepatoma cells (PLC/PRF/5 and Hep3B) were grown to confluence in 12 wells plates and stimulated for 24 hours as described above. One hour pre-treatment with 0, 0.1, 1 and 10 µM RWJ 67657 was done for all stimulations. Total RNA was isolated from the cells with TRizol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). DNAse treatment (Ambion, Huntingdon, Cambridgeshire, UK) was performed and subsequently cDNA was synthesized from 2.0 µg of total RNA using M-MLV Reverse Transcriptase and oligo (dT)_{14-18} (Invitrogen, Life Technologies). For the measurement of mRNA for albumin, CRP, SAA, C3, fibrinogen-β, fibrinogen-γ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1 µl of cDNA in duplicate was used for amplification by the Taqman real-time PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes. Amplification was performed using standard conditions: denaturation at 95°C for 15 seconds, 40 cycles of amplification with annealing at 60°C for 1 minute, and extension at 50°C for 2 minutes. According to the comparative Ct (threshold cycle value) method described in the ABI manual, the resulting mRNA amount of the gene of interest was normalized to the housekeeping gene GAPDH, yielding the ΔCt value. The ΔCt value of unstimulated cells was subtracted from the average ΔCt value of each sample, yielding the ΔΔCt. The amount of target, normalized to an endogenous reference (GAPDH) and relative to the control sample, is given by: $2^{-\Delta\Delta C_T}$.

**Statistics.**

One-way ANOVA with Dunn's Multiple Comparison Test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA).

**RESULTS.**

**Activation of p38 MAPK and STAT-3.**

Phosphorylation of p38 MAPK and STAT-3 in the cell lines Hep3B and PLC/PRF/5 was analyzed after stimulation with (combinations of) IL-6, IL-1β and TNF-α for 30 minutes. In figure 1 phosphorylation of p38 MAPK in HEP3B was demonstrated with all stimuli including IL-6, and inhibition of phosphorylation due to the p38 MAPK inhibitor was shown for MAPKAPK2, the downstream substrate of p38 MAPK. Strong activation of STAT-3 was demonstrated after IL-6 stimulation, which was not affected by p38 MAPK inhibition.

**Production of acute phase proteins by hepatoma cell lines.**

In the supernatants of the 4 different hepatoma cell lines the production of the acute phase proteins CRP, SAA, fibrinogen and C3 was measured by ELISA. CRP (type-1 APP) production could not be detected in HepG2 and HuH7 cell lines. In PLC/PRF/5, highest CRP production was seen after combined IL-1β and IL-6 stimulation, and to a lesser extent after TNF-α stimulation alone or together with IL-6. In Hep3B, CRP production was found exclusively after simultaneous stimulation with IL-1β and IL-6.
In both cell lines, CRP production was significantly inhibited by RWJ 67657 both at 1 and 10 µM.

High production of SAA was detected in all hepatoma cell lines after combined stimulation with IL-1β and IL-6 (figure 2B). Inhibition due to p38 MAPK inhibition was seen only at 10 µM in Hep3B and HuH7. No significant inhibition was seen in PLC/PRF/5 and HepG2.

Results of fibrinogen production are shown in table 1. Fibrinogen is constitutively produced in the liver and in cultured hepatoma cell lines as well. Fibrinogen is a type-2 APP, and after stimulation with IL-6 a maximal fourfold increase of production is seen. In Hep3B fibrinogen production was not constitutive and could only be detected after IL-6- or combined stimulation. Significant inhibitory effects of p38 MAPK at 10 µM were seen in all 4 cell lines after stimulation with IL-6 alone or in combination with IL-1β. In Hep3B, HepG2 and HuH7 at inhibition was seen at 1 µM, but not in PLC/PRF/5.

In table 2, we show the results of C3 production in the hepatoma cell lines. Like fibrinogen, C3 is also constitutively produced, and production is increased after stimulation with all cytokines and combinations. Significant inhibition at 10 µM RWJ 67657 was seen in all 4 cell lines after IL-1β stimulation and in Hep3B and HepG2 after IL-1β + IL-6 stimulation. In HepG2, there was also significant inhibition of IL-6 induced C3 production at 1µM. Pre-treatment of hepatoma cells with 0.1% DMSO had no significant effect on any acute phase protein production (data not shown).

mRNA analysis of acute phase proteins.

With quantitative RT-PCR mRNA expression of CRP, SAA-1, fibrinogen-β, fibrinogen-γ, complement C3 and albumin was measured in Hep3B and PLC/PRF/5. In HepG2 and HuH7 no CRP mRNA could be detected, as was seen for CRP protein production. Figure 3 shows the results of mRNA expression of 3 different experiments in Hep3B cells after 24-hour stimulation. Results of mRNA analysis in PLC/PRF/5 were comparable. CRP and SAA-1 mRNA expressions were abundantly induced, especially after combined stimulation with IL-6 and IL-1β. Pre-treatment with 0.1, 1 or 10 µM RWJ 67657 reduced CRP mRNA expression in a dose dependent way but not SAA-1 mRNA expression. Reduction of CRP mRNA at 10 µM was significant both after IL-6 and combined IL-6/IL-1β stimulation. Fibrinogen and C3 mRNA are constitutively expressed, but can be increased by cytokine stimulation. Fold inductions after IL-6, combined IL-6/IL-1β and combined IL-6/TNF-α stimulation for fibrinogen-β and fibrinogen-γ were 45.0, 21.0, 7.7 and 13.8, 5.3, 2.5 respectively. mRNA induction for both fibrinogen-chains was significantly reduced at 10 µM RWJ 67657 treatment after IL-6 stimulation. C3 mRNA expression was induced after all stimulations, but there was no inhibitory effect detectable due to pre-treatment with RWJ 67657. Albumin mRNA expression was reduced in all stimulated cells, and was further reduced with 10 µM p38 MAPK inhibitor in IL-6-stimulated cells (data not shown).
Table 1. Effect of RWJ 67657 pre-treatment on fibrinogen production (µg/ml) in 4 hepatoma cell lines (mean of 6 experiments). Cells were unstimulated or stimulated with (combinations of) cytokines for 48 hours, with or without pre-treatment of 0.01, 0.1, 1 and 10 µM RWJ 67657. Fibrinogen levels were measured in cell supernatants by ELISA and expressed in µg/ml. The table shows the mean and SEM (* p<0.05, ** p<0.01, *** p<0.001, One-way ANOVA with Dunn’s Multiple Comparison Test, tested against the stimulated control). (BD = below detection limit, ND = not determined)

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DISCUSSION.
In this study, the effect of p38 MAPK inhibition on the acute phase response was investigated in hepatoma cell lines. We found significant inhibition of CRP- and fibrinogen production at 1 µM RWJ 67657, which is considered a pharmacologically relevant concentration.

Knowledge of the central role of IL-1 and TNF-α in inflammation has led to the successful treatment by cytokine blocking agents in RA. Also the use of anti-IL-6 antibody intended to block the JAK/STAT pathway seems promising 17. The interest in the regulation of cytokine production by signal transduction pathways and transcription
factors has led to new therapeutic targets. For RA several p38 MAPK inhibitors have been designed, which have been tested pre-clinically and in clinical studies. Recent studies investigating prognostic factors in RA, have demonstrated that increased levels of CRP especially are predictive for ongoing joint damage. It is important to know whether the levels of acute phase proteins reflect disease-activity during treatment, or that the production itself is influenced by treatment.

By western blotting it was demonstrated that p38 MAPK could be activated after IL-6 stimulation and that MAPKAPK2 phosphorylation effectively was blocked by p38 MAPK inhibition. A study by Zauberman *et al.* demonstrated that the p38 MAPK inhibitor SB 203580 reduced IL-6 induced MAPKAPK2 activation in HepG2 cells and also that haptoglobin and α1-acid glycoprotein levels were reduced by p38 MAPK inhibition.

In contrast to others it was not possible for us to detect CRP production or mRNA expression by HepG2 and HuH7 cells, making these cell lines less valuable as model to study acute phase response reactions. Induction of CRP production and mRNA expression however was found in Hep3B and PLC/PRF/5 cells, which could be reduced significantly by pre-treatment with the p38 MAPK inhibitor at 1 µM. The study by Parasrampuria *et al.* demonstrated that after a single oral dose of RWJ 67657 ranging from 0.25 to 30 mg/kg a plasma concentration of 0.01 to 6 µM of the p38 MAPK inhibitor could be reached in humans, so 1 µM is a pharmacologically relevant concentration. All 4 cell lines showed high production of SAA, and inhibition by RWJ 67657 was seen only at 10 µM. Strong induction of SAA-1 mRNA was demonstrated in PLC/PRF/5 and Hep3B after combined IL-6 and IL-1β stimulation. Our results confirmed the data reported by Hagihara *et al.* concerning the critical role of IL-6 in the synergistic induction of SAA gene expression in hepatoma lines. Inhibition by RWJ 67657 pre-treatment could not be detected.

Fibrinogen synthesis is mainly mediated by IL-6, as confirmed in our studies in the 4 hepatoma cell lines. Combined stimulation induces lower fibrinogen production, due to cross-talk between IL-6 and IL-1β signalling pathways leading to IL-1β dependent down-regulation of STAT-1 phosphorylation. p38 MAPK treatment additionally reduced fibrinogen production independent of which stimulus was used, apparently by another mechanism. Fibrinogen plays an important role in the coagulation cascade, but is also an important acute phase protein, and the often-used ESR largely depends on the fibrinogen concentration. The fact that indeed fibrinogen production is reduced by p38 MAPK treatment could have important implications for the use of ESR as marker of disease activity.

CRP and SAA are the major acute phase reactants in rheumatoid arthritis, but they have a different function and are differentially regulated, although both CRP and SAA are potently induced by combined IL-6 and IL-1β stimulation. The fact that CRP production is inhibited at the pharmacological relevant concentration of 1 µM RWJ 67657, while SAA production is not, might be explained by differences in the acute phase responsive elements in the promoter regions of the two APP. Cunnane *et al.* demonstrated that SAA is the best marker for the assessment of inflammatory joint disease, while Rau *et al.* demonstrated that in acute pancreatitis SAA had a wider dynamic range, but measurement of CRP provided an earlier differentiation between patients. The results presented in this study may implicate that SAA measurement should be included in clinical trials when p38 MAPK inhibitors are used.
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FIGURE LEGENDS.

Figure 1.
Western blot of p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, phospho-STAT-3 and STAT-3 after stimulation with (combinations of) cytokines, with or without 1 hour pre-treatment with 1 μM RWJ 67657.
Hep3B cells were stimulated with 10 ng/ml IL-1β, TNF-α, 50 ng/ml IL-6 or combinations there-of in DMEM with 1% FCS and 0.1 μM dexamethason for 30 minutes. Phosphorylation was measured by Western blot using specific antibodies against p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK2, STAT3 and phospho-STAT3.

Figure 2.
Effect of RWJ 67657 pre-treatment on CRP (A) and SAA (B) production in hepatoma cell lines after stimulation with (combinations of) cytokines.
Cell were unstimulated (○) or stimulated with IL-1β (▲), IL-6 (●), TNF-α (△), IL-1β + IL-6 (■) or TNF-α + IL-6 (□) for 48 hours, with or without pre-treatment of 0.01, 0.1, 1 and 10 μM RWJ 67657. CRP and SAA levels were measured in cell supernatants by ELISA and expressed in ng/ml. In the graphs the mean and SEM is shown. ( * p<0.05, *** p<0.001, One-way ANOVA with Dunn’s Multiple Comparison Test, tested against the stimulated control).

Figure 3.
Effect of RWJ 67657 pre-treatment on mRNA expression of CRP, SAA-1, fibrinogen-β, fibrinogen-γ, and complement C3 in Hep3B cells after stimulation with (combinations of) cytokines.
Cells were stimulated with IL-1β, IL-6, IL-1β + IL-6, TNF-α, and TNF-α + IL-6 for 24 hours with or without pre-treatment with 0.1, 1 and 10 μM RWJ 67657. mRNA expression was determined with real-time RT-PCR, results are expressed as fold induction compared to unstimulated cells (fold induction = 1). Bars show mean (n=3) and SEM. ( * p<0.05, One-way ANOVA with Dunn’s Multiple Comparison Test, tested against the stimulated control).
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Figure 2A

Tendon Function Vs. Sollerman scores at 8 years

\[ r = -0.51 \]

Figure 2B

Tendon Function Vs. HAQ scores at 8 years

\[ r = 0.53 \]
Figure 3.

Box Plot of Baseline and 1 Year MRI Bone Erosion Score for all RA-Related Surgery and Non-Surgery Patients
Differential influence of p38 mitogen-activated protein kinase (MAPK) inhibition on acute phase protein synthesis in human hepatoma cell lines

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