Chemokine production and E-selectin expression in activated endothelial cells are inhibited by p38 MAPK (mitogen activated protein kinase) inhibitor RWJ 67657

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ABSTRACT
Endothelial cells play an important role in inflammatory diseases like rheumatoid arthritis by recruitment of inflammatory cells. The cytokines TNF-α and IL-1β are major inducers of endothelial cell activation and are stimulators of inflammatory signal transduction pathway involving p38 MAPK (mitogen-activated protein kinase). The present study investigated the effects of p38 MAPK inhibition on cell adhesion molecule (CAM) expression and chemokine production by endothelial cells both on mRNA and protein level. Pre-treatment of endothelial cells with the pharmacologically relevant concentration of 1 μM of the p38 MAPK inhibitor RWJ 67657 reduced TNF-α and IL-1β induced mRNA and membrane expression of E-selectin. Moderate inhibitory effects on ICAM-1 and VCAM-1 expression were found. Significant reduction of mRNA expression and protein production of the inflammatory cytokine IL-6 and the chemokines IL-8 and MCP-1 was demonstrated. Treatment with RWJ 67657 could lead to reduced leukocyte infiltration by the reduction of E-selectin expression and chemokine production.

Key words. chemokine, endothelial cell, E-Selectin, MAPK inhibitor
INTRODUCTION
Rheumatoid arthritis (RA) is an inflammatory disease characterized by hyperplasia, increased vascularity, and infiltration of inflammatory cells into the synovial membrane. It is now known that the endothelial cells (EC), which line the lumen of the blood vessels, are not passive bystanders but are active responders to stimuli like activated leukocytes and cytokines. After stimulation EC can produce a number of inflammatory mediators and express cellular adhesion molecules (CAMs). Most CAMs involved in endothelial activation belong to three families, i.e., the integrins, selectins, and immunoglobulin superfamilies. The adhesion of leukocytes to EC takes place in four steps. The first step is a weak adhesion (tethering and rolling) mediated by E-, P- and L-selectins. The next step is leukocyte activation and is a consequence of interaction between chemokines and their receptors on leukocytes. Then firm adhesion takes place, which is mediated mostly by interaction between integrins on the leukocytes and vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on the EC. The final step is the transendothelial migration, which is directed by secreted chemokines bound to endothelial heparan sulphate glycosaminoglycans. Essential chemokines are interleukin (IL)-8 which attracts neutrophils, and monocyte chemo-attractant protein (MCP)-1, the main attractant for mononuclear cells.

The cytokines TNF (tumour necrosis factor)-α and IL-1β, produced by activated macrophages in the synovium, have been identified as the major inducers of EC activation in RA. Blockade of these cytokines has proven to be effective in the treatment of RA, although Redlich et al recently concluded that targeting individual molecules such as TNF-α may not be sufficient in interfering with both inflammation and joint destruction. Successful drug treatment of RA is associated by a decrease in cytokine production, but also by a decrease in E-selectin and ICAM-1 expression.

In vitro studies demonstrated that in TNF-α and/or lipopolysaccharide (LPS) stimulated human umbilical vein endothelial cells (HUVEC) the nuclear factor-κB (NF-κB), and p38 mitogen-activated protein kinase (MAPK) pathways are important in controlling adhesion molecule expression. Activation of all three stress- and mitogen-activated protein kinases (SAPK/MAPK) was found throughout the RA synovial tissue, whereas activated p38 MAPK predominantly was located in the synovial lining layer and in synovial endothelial cells. Interest in protein kinases and in particular in p38 MAPK as drug targets has increased in the last years as recently reviewed by Kumar et al. The p38 MAPK inhibitor RWJ 67657 (4-[4-(4-fluorophenyl)-1-(3-phenylpropyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]-3-butyln-1-ol) has been shown to be effective in inhibiting the release of TNF-α from LPS-treated human peripheral blood mononuclear cells with an IC₅₀ of 3 nM. This compound was approximately 10-fold more potent than the reference standard p38 MAPK inhibitor SB 203580 in all p38 dependent in vitro systems tested. RWJ 67657 specifically inhibited the enzymatic activity of recombinant p38 α and β, but not of γ and δ in vitro, and had no significant activity against a variety of other kinases. We recently demonstrated that RWJ 67657 significantly inhibited IL-6, IL-8, MMP-3 and COX-2 mRNA expression by IL-1β and TNF-α stimulated rheumatoid synovial fibroblasts. Furthermore Fijen et al showed that this compound induced a dose-dependent suppression of leukocyte and endothelial cell response after endotoxin challenge in
humans. Recent pharmaco-kinetic and pharmaco-dynamic studies of RWJ 67657 in humans showed that the compound has an acceptable safety profile to warrant further investigations.

In the present study the effects of p38 MAPK inhibition in IL-1β or TNF-α stimulated HUVEC concerning adhesion molecule expression and chemokine production were studied at the level of mRNA expression as well as protein production. Pre-treatment of activated endothelial cells with 1 µM RWJ 67657 reduced E-selectin mRNA and protein expression, and significantly reduced IL-8, MCP-1 and IL-6 production and mRNA expression. These results indicate that treatment with RWJ 67657 could lead to reduced leukocyte infiltration and therefore could have an important therapeutic benefit.

MATERIALS AND METHODS
Reagents
RWJ 67657 was provided by Johnson and Johnson (R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ). Recombinant TNF-α and IL-1β and ELISA antibodies for the detection of IL-8 and MCP-1 were obtained from R&D Systems (Minneapolis, MN). Antibodies for the detection of IL-6 were purchased from Sanquin (Amsterdam, The Netherlands). Specific antibodies to p38 MAPK, phospho-p38 MAPK and phospho-MAPK activated protein kinase (MAPKAPK)-2 were purchased from Cell Signalling Technologies (Beverly, MA) and detecting antibody peroxidase - swine-anti-rabbit was from DAKO (Glostrup, Denmark). Mouse anti-human E-selectin antibody (H18/7) was kindly provided by Dr. M. Gimbrone Jr., Harvard Medical School. Monoclonal anti-human VCAM-1 (CD106) and anti-human ICAM-1 (CD54), both labelled with phycoerytrin (PE) were from Beckton Dickinson (Bedford, MA). Monoclonal anti-human PECAM-1 (platelet endothelial cell adhesion molecule, CD31) was obtained from DAKO and PE-labeled goat-anti-mouse was from SBA (Southern Biotechnology Associates, Birmingham, AL). RNA isolation kit was from Stratagene (La Jolla, CA), quantitation of RNA was performed with Ribogreen RNA quantitation kit (Molecular Probes Europe BV, Leiden, The Netherlands).

Other reagents for RNA isolation and reverse transcriptase reaction were purchased from Invitrogen (Breda, The Netherlands). Reagents, primers and probes for real-time RT-PCR were obtained from Applied Biosystems (Nieuwkerk a/d IJssel, The Netherlands).

Endothelial cell culture and stimulation
HUVEC were obtained from the Endothelial Cell Facility at the UMCG (The Netherlands) as described previously. Primary isolates combined from 2 or 3 donors were cultured on 1% gelatin (Sigma-Aldrich, Zwijndrecht, The Netherlands) - precoated plastic tissue culture plates or flasks (Corning, Schiphol, The Netherlands) at 37° C under 5% CO₂/95% air. The culture medium constisted of RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 5 U/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 µg/ml endothelial cell growth factor, extracted from bovine brain according to the procedure described by Maciag. After attaining confluence, cells
were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml) and split at a 1:3 ratio. In the experiments presented here HUVEC were used up to passage 4. In the experimental set-up HUVEC were grown to confluence in gelatin-coated 6-well plates. Fresh medium was added before cells were stimulated for 6 or 24 hours with 10 ng/ml TNF-α or IL-1β, with or 1 hour without pre-treatment with 1μM RWJ 67657 (stock solution 10 mM in DMSO = dimethylsulfoxide).

**Phosphorylation of p38 MAPK and MAPKAPK-2 in HUVEC**

HUVEC were pre-treated with 0, 0.1, 1, and 10 μM RWJ 67657 for 1 hour and stimulated for 30 minutes as mentioned above. Cell extracts were prepared by lysing the cells with 1X SDS sample buffer (containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 62.5 mM Tris-HCl (pH=6.8) and 0.01% brome-phenol blue). Cells were scraped off the wells and the lysates were subsequently sonicated for 5-10 seconds and boiled for 5 minutes. After centrifugation the samples were loaded onto a 10% SDS-PAGE gel and resolved by running at 200 V and 15 Watt constant. Semidry-blotting was performed onto a nitrocellulose membrane after which immunodetection with anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-MAPKAPK-2 and peroxidase labelled swine-anti-rabbit was performed. Enhanced chemi-luminescence (ECL) detection was performed according to the manufacturers guidelines (Lumi-Lightplus, Roche Diagnostics, Mannheim, Germany).

**RNA isolation and real-time RT-PCR**

HUVEC with or without 1 hour pre-treatment with 1μM RWJ 67657 were stimulated for 6 and 24 hours with 10 ng/ml TNF-α or IL-1β. Total RNA was isolated using the Absolutely RNA Microprep Kit according to the manufacturers guidelines. RNA was analysed qualitatively by gel electrophoresis and quantitatively by Ribogreen RNA Quantitation Kit. One microgram of total RNA was used for the synthesis of first strand cDNA using Superscript III RNase H Transcriptase in 20 μl final volume containing 250 ng random hexamers and 40 units Rnase OUT inhibitor. For the measurement of mRNA for CD31, E-selectin, VCAM-1, ICAM-1, IL-8, MCP-1, IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1 μl of cDNA in triplicate was used for amplification by the Taqman real-time PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with specific Taqman primers/probes. In our experiments GAPDH and other genes of interest were always determined in the same RT-PCR run. 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 HUVEC 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: $2^{-\Delta\Delta Ct}$. 
Flow cytometric analysis

HUVEC were stimulated for 6 and 24 hours as mentioned above and detached from the wells by short treatment with trypsin, and subsequently resuspended in FCS to neutralize the trypsin. After washing with PBS supplemented with 5% FCS, HUVEC were incubated with PE-labeled monoclonal antibodies against VCAM-1 (dilution 1:10) and ICAM-1 (1:25) for 45 minutes on ice or with monoclonal antibodies against CD31 (1:25) and E-selectin (undiluted) followed by incubation with PE-labeled goat-anti-mouse antibodies. Cells were fixed with 0.5% paraformaldehyde/PBS and adhesion molecule expression was detected by flow cytometric analysis in an Epics-Elite Flow cytometer (Coulter Electronics, Mijdrecht, The Netherlands). Non-specific staining was assessed by staining with irrelevant isotype-matched monoclonal antibodies. The effects of RWJ 67657 on adhesion molecule expression was determined in 3 experiments with different HUVEC cultures.

ELISA based determination of IL-8, MCP-1 and IL-6 in cell culture supernatants

HUVEC were pre-treated with 1 μM RWJ 67657 for 6 and 24 hours and stimulated with 10 ng/ml TNF-α or IL-1β. IL-6 levels in cell supernatants were measured as described previously. IL-8 and MCP-1 were measured by ELISA, using matched antibody pairs for ELISA and recombinant proteins as standards from R&D Systems. In short, Corning high-binding ELISA plates were coated overnight with monoclonal antibodies in PBS. After blocking with 2% BSA (bovine serum albumin)/PBS diluted supernatants were added. Bound chemokines were detected with biotinylated polyclonal antibodies followed by incubation with peroxidase labelled Streptavidin (Sanquin, Amsterdam, The Netherlands). Colour reaction was performed with TMB (3’3’5’5’-tetramethylbenzidin, Roth, Karlsruhe, Germany) and concentration of protein was determined with the SOFTmax PRO software (Molecular Devices, Sunnyvale, CA). Detection limits for ELISAs was 20 pg/ml for IL-6 and IL-8 and 50 pg/ml for MCP-1.

STATISTICS

Paired T-tests were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Effect of RWJ 67657 on phosphorylation of p38 MAPK and MAPKAPK-2

Upon stimulation with 10 ng/ml TNF-α and/or IL-1β p38 MAPK is rapidly phosphorylated within minutes, while after 60 minutes the level of phosphorylation is decreased (data not shown). In figure 1 the effect of RWJ 67657 on phosphorylation of p38 MAPK and its downstream substrate MAPKAPK-2 after 30 minutes of stimulation is shown. RWJ 67657 does not inhibit phosphorylation of p38 MAPK, but it does inhibit its activity as can be seen from the inhibition of MAPKAPK-2 phosphorylation at 0.1 μM. Complete inhibition was demonstrated at a concentration of 1 μM RWJ 67657. The solvent 0.01% DMSO did not affect phosphorylation of either kinase.
Figure 1. Phosphorylation of p38 MAPK and MAPKAPK-2 in stimulated HUVEC after pre-treatment with 10, 1 and 0.1 µM RWJ 67657.
HUVEC were treated with TNF-α and/or IL-1β for 30 minutes with or without 1 hour pre-treatment with RWJ 67657. Phosphorylation was measured by Western blot using specific antibodies against p38 MAPK, phospho-p38 MAPK and its direct downstream substrate MAPKAPK-2. Control incubations were performed with 0.01% dmso, the vehicle solvent for RWJ 67657.

Figure 2. Effect of RWJ 67657 pre-treatment on mRNA expression of CD31, E-selectin, VCAM-1 and ICAM-1 in HUVEC.
Cells were stimulated with TNF-α and/or IL-1β for 6 or 24 hours and pre-treated with 1µM RWJ 67657 for 1 hour. mRNA expression was determined with real-time RT-PCR and results are expressed as fold induction compared to unstimulated cells (fold induction=1). Bars show means (n=4-6) and SEM. (* p< 0.05, paired T-test calculated against the stimulated control).
**Effects of RWJ 67657 on mRNA expression of adhesion molecules**

With real-time RT-PCR mRNA expression of CD31, E-selectin, VCAM-1 and ICAM-1 was determined in HUVEC (n=4-6) after 6 and 24 hours of stimulation with 10 ng/ml TNF-α or IL-1β. The expression is measured as fold induction compared to the unstimulated sample. As can be seen from figure 2 mRNA expression of CD31 did not change during stimulation, in contrast to the strong induction of mRNA expression for E-selectin, VCAM-1 and ICAM-1. After 6 hours of stimulation the mRNA expression of these adhesion molecules was higher than after 24 hours of stimulation. Furthermore, IL-1β induced E-selectin mRNA expression to a higher extent than did TNF-α. Pre-treatment with 1 µM RWJ 67657 did not influence the expression of CD31 nor that of the induced VCAM-1 expression. E-selectin expression however was reduced to 78% and 65% respectively after TNF-α or IL-1β stimulation at 6 hours, and to 43% and 35% respectively after 24 hours of stimulation. ICAM-1 mRNA expression at 24 hours was reduced to 73% after TNF-α stimulation and 56% after IL-1β stimulation.

**Effects of RWJ 67657 on membrane expression of adhesion molecules**

Membrane adhesion molecule expression by HUVEC was determined at 6 and 24 hours after stimulation with 10 ng/ml TNF-α or IL-1β. E-selectin expression was higher after stimulation with IL-1β than with TNF-α. This was also the case for VCAM-1 expression except at 24 hours when TNF-α-induced expression was higher. Both cytokines induced high ICAM-1 expression, which increased with time (data not shown).

The effect of pre-treatment with 1 µM RWJ 67657 on adhesion molecule expression was calculated as follows: the MFI (mean fluorescence intensity) of stimulated cells for each experiment was set to 100%, and the expression due to the treatment with RWJ 67657 was calculated relative to the 100% (Table 1). Pre-treatment with RWJ 67657 led to reduced expression of E-selectin under all conditions tested, of VCAM-1 after 6 hours, and of ICAM-1 after TNF-α stimulation for 6 hours (all not statistically significant).

<table>
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<tr>
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<th>TNF-α 6 hr</th>
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<td>104 +/- 13</td>
<td>95 +/- 20</td>
<td>91 +/- 6</td>
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<td>E-selectin</td>
<td>77 +/- 22</td>
<td>59 +/- 19</td>
<td>81 +/- 14</td>
<td>67 +/- 9</td>
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<td>105 +/- 37</td>
<td>73 +/- 17</td>
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<td>ICAM-1</td>
<td>79 +/- 15</td>
<td>99 +/- 29</td>
<td>100 +/- 21</td>
<td>93 +/- 21</td>
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**Table 1. Effect of RWJ pre-treatment on adhesion molecule expression by HUVEC (n=3).**

Adhesion molecule expression on TNF-α and IL-1β stimulated HUVEC was determined 6 and 24 hours after stimulation. The effect of 1 hour pre-treatment with 1 µM RWJ 67657 was calculated as percentage compared to the MFI of stimulated cells (=100%).
In figure 3 one of the three experiments is shown for adhesion molecule expression after TNF-α and IL-1β stimulation, and after RWJ 67657 pre-treatment followed by cytokine stimulation. The MFI values and percentage bright positive cells are indicated in the figures. As can be seen from the figure there was an overall reduced expression of E-selectin after treatment with the p38 MAPK inhibitor and also the number of positive cells was decreased. This reduction however was not statistically significant. VCAM-1 and ICAM-1 were moderately reduced. Control experiments with 0.01% DMSO showed no significant effects of the solvent on adhesion molecule mRNA or protein expression (data not shown).

![Figure 3. Effect of RWJ 67657 pre-treatment on adhesion molecule expression on HUVEC after 6 and 24 hours. HUVEC were stimulated with TNF-α or IL-1β. E-selectin, ICAM-1 and VCAM-1 expression was measured by flowcytometry (thick black line) and after pre-treatment with 1µM RWJ 67657 (grey line). The mean fluorescence intensity (MFI) is depicted in the graph: first line cytokine stimulated expression, second line cytokine + RWJ 67657 treated expression. In brackets the percentage of bright positive cells is indicated. The thin black line represents the isotype control.](image)

**Effects of RWJ 67657 on chemokine and cytokine mRNA expression and production**
Quantitative mRNA expression of IL-8, MCP-1 and IL-6 was determined in HUVEC (n=4-6) after 6 and 24 hours of stimulation with 10 ng/ml TNF-α or IL-1β in the presence or absence of RWJ 67657. Stimulation with IL-1β induced higher mRNA expression of both IL-8 and IL-6 compared to stimulation with TNF-α (figure 4A). At both time points a marked inhibition of IL-8 and IL-6 mRNA expression was seen due to pre-treatment with 1 µM RWJ 67657. This inhibition was statistically significant in
nearly all cases. MCP-1 mRNA expression was induced equally by TNF-α and IL-1β, and decreased in time. The inhibition seen by the p38 MAPK inhibitor was not statistically significant.

The production of IL-8, MCP-1 and IL-6 protein by HUVEC after 24 hours of TNF-α or IL-1β stimulation is shown in figure 4B. Protein production was induced at high levels, especially IL-8 and MCP-1. Pre-treatment with 1 µM RWJ 67657 induced a significant reduction of IL-8, MCP-1 an IL-6 production. No effect of 0.01% DMSO on cytokine production was observed (data not shown).

Figure 4. (A). Effect of RWJ 67657 pre-treatment on mRNA expression of IL-8, MCP-1 and IL-6 in HUVEC.
Cells were stimulated with TNF-α and IL-1β for 6 or 24 hours in the absence or presence of 1µM RWJ 67657 pre-treatment for 1 hour. mRNA expression was determined with real-time RT-PCR. Results are expressed as fold induction compared to unstimulated cells (fold induction=1). Bars show means (n=4-6) and SEM. (* p< 0.05, ** p<0.001, paired T-test, calculated against the stimulated control).

(B). Effects of RWJ 67657 pre-treatment on protein production of IL-8, MCP-1 and IL-6 by HUVEC (n=5).
Cells were stimulated with TNF-α or IL-1β for 24 hours and pre-treated with 1µM RWJ 67657 (t=-1hr). Protein production was measured in supernatants by ELISA and expressed in ng/ml. Bars show mean and SEM. (* p< 0.05, **p<0.001, paired T-test, calculated against the stimulated control).
DISCUSSION

The effects of RWJ 67657, a p38 MAPK inhibitor, on TNF-α and IL-1β stimulated endothelial cells were investigated in this study. Complete inhibition of MAPKAPK-2 phosphorylation was demonstrated at 1 µM. At this concentration we found inhibition of E-selectin expression, both at the level of mRNA and protein production. A significant inhibition of production of the chemokines IL-8 and MCP-1 was found, and also production of the pro-inflammatory cytokine IL-6 was significant inhibited.

In inflammatory diseases the accumulation of leukocytes in a given tissue can lead to varying degrees of cell damage, extra cellular matrix disruption and organ dysfunction. Several attempts have been made to therapeutically block leukocyte adhesion to endothelium and thus control inflammation. This has been done for instance by the use of specific monoclonal antibodies to adhesion molecules in animal models 18, and also in RA patients 19. Also currently used anti-rheumatic agents, for example methotrexate, glucocorticosteroids and leflunomide interfere to varying degree with the expression or function of different CAMs 20,21. Therapeutic strategies aimed at blocking chemokines and their receptors have also been studied. Recently Haringman et al reported relevant biological effects in RA patients treated with chemokine receptor 1 antagonist 22. In rheumatoid synovial tissue p38 MAPK is predominantly expressed in the lining layer and in endothelial cells 9 and therefore we wanted to investigate the effects of the p38 MAPK inhibitor on adhesion molecule expression and chemokine production.

In our study we found a complete inhibition of phosphorylation of MAPKAPK-2, the direct downstream substrate of p38 MAPK at a concentration of 1µM RWJ 67567. The study by Parasrampuria demonstrated that after a single oral dose 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 14. We therefore decided to perform our study with 1 µM RWJ 67567, which equals a dose of 5-10 mg/kg. We did not compare our p38 MAPK inhibitor with the literature standard SB 203580 for two reasons: first, RWJ 67657 is already described to be 10-fold more potent than SB 203580 in all p38-dependent systems tested 11. Secondly, it has been demonstrated that SB 203580 can also block protein kinase B (PKB) activity at 3-5 µM 23, as well as JNK activities at 3-10 µM 24. Therefore SB 203580 is now considered not to be a specific p38 MAPK inhibitor.

Different subsets of leukocytes use different (combinations of) cell adhesion molecules (CAMs) depending on the inflammatory stimulus and the site of inflammation. Furthermore, the functional consequences of downregulating cell adhesion molecule expression on the endothelial surface can be at the level of leukocyte rolling, adhesion, and transmigration. McCafferty 25 reported that in postcapillary venules (studied in the cremaster muscle venules, size ~30 µm in diameter) E-selectin knock out could completely abrogate antigen challenge induced leukocyte recruitment: leukocyte rolling as well as adhesion and transmigration were completely abolished in the E/P-selectin k.o. mice, while in the P-selectin k.o. mice only leukocyte rolling was affected. In contrast, leukocyte recruitment induced by local TNF-α administration was neither affected in P-selectin k.o. nor in P-/E-selectin k.o.. In both the antigen induced and TNF-α induced inflammation, the number of cells infiltrating and the cell types making up the infiltrate were similar. The main difference between the two models was the expression of VCAM-1 in the venular endothelium of the TNF-α
induced inflammation, indicating that VCAM-1 is possibly able to control leukocyte recruitment to sites of inflammation, irrespective whether P- or E-selectin is present. Norman and colleagues recently reported on the role of CAMs during immune complex-dependent inflammation in the mouse cremaster muscle \(^{26}\). While E-selectin inhibition by antibody infusion could abrogate leukocyte rolling on the venular endothelium (venules were 25-40 µm in diameter), its effects on leukocyte migration were limited. In contrast, treatment with anti-VCAM-1 antibody could inhibit leukocyte adhesion more than 70%, with subsequent functional consequence being a more than 80% inhibition of leukocyte migration.

In the current study we showed that pre-treatment of HUVEC with RWJ 67657 inhibited E-selectin expression to 77%/59% (TNF-α induced, 6 hrs resp. 24 hrs) and 81%/67% (IL-1β induced, 6 hrs resp. 24 hrs). Moreover, we showed that VCAM-1 protein expression was inhibited at the 6 hours time point, 66 % (TNF-α induced) versus 73 % (IL-1β induced), while also ICAM-1 was inhibited at 6 hours after start TNF-α activation. Reports on p38 MAPK inhibition on ICAM-1 expression after TNF-α stimulation in literature are contradicting \(^{27,28}\), whereas results after IL-1β stimulation have not been reported. Our results concerning VCAM-1 mRNA and protein expression corroborate the data by Pietersma et al \(^{29}\), who stated that p38 MAPK regulates endothelial VCAM-1 expression at the post-transcriptional level.

Extensive in vivo studies will be needed to answer the question whether the anti-inflammatory effects in the ranges reported here will affect leukocyte rolling, adhesion, and subsequent recruitment to the diseased tissue.

The fact that at 1 µM RWJ 67657 phosphorylation of MAPKAPK-2 was completely blocked, while adhesion molecule expression was partly inhibited, indicates that other signal transduction routes are also important in regulating adhesion molecule expression in HUVEC. Activation through p38 MAPK and NF-κB have been described to act synergistically in inducing adhesion molecule expression in endothelial cells \(^{7,8}\). Recently Viemann et al \(^{30}\) reported that the induction of cell-surface receptor expression measured with oligonucleotide microarray was highly depended on IKK2/NF-κB activation , whereas there was additional modulation by p38 MAPK. With real-time RT-PCR they found that for instance VCAM-1 and IL-8 were dependent of both pathways, where as ICAM-1 expression was dependent of NF-κB activation alone. So both pathways play a role in endothelial activation, with CAM expression being more regulated by the NF-κB pathway, and chemokine production more by the p38 MAPK pathway. In our study we demonstrated that p38 MAPK inhibition affects E-selectin expression both at the mRNA and the protein level in HUVEC. The study by Fijen et al demonstrated that RWJ 67657 at a single dose of 5, 10 and 20 mg/kg prevented endotoxin-induced increase of circulating ICAM-1 and circulating E-selectin and also of integrins on neutrophils \(^{13}\). They demonstrated that also in vivo neutrophil and endothelial activation could be prevented by RWJ 67657.

With quantitative real-time RT-PCR we found that induction of IL-8 and IL-6 mRNA was higher after stimulation with IL-1β than with TNF-α, and a significant inhibition by RWJ 67657 was seen after both stimuli. In our study RWJ 67657 was added 1 hour before stimulation of the cells, but also administration of the compound up till 4 hours after stimulation of HUVEC induced marked reduction in IL-8 and IL-6 mRNA levels.
(unpublished results). MCP-1 mRNA and protein production was equally high after both stimuli. A significant inhibitory effect was demonstrated by RWJ 67657 treatment for IL-8, MCP-1 and IL-6 protein production. These observations corroborate data reported by others.\(^{31,32}\) 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 infiltration in inflammatory disease.

Previously we demonstrated significant inhibitory effects on inflammatory mediators produced by rheumatoid synovial fibroblasts\(^{12}\) and we also showed significant inhibition of TNF-\( \alpha \) production in monocyte-derived macrophages\(^{33}\). p38 MAPK inhibitors have effects on different cell types, which could enhance the therapeutic effects, but also enlarge the risk of side effects. One of the reasons for undesirable effects might be the cross-reactivity against other kinases, which was not the case for RWJ 67657\(^{11}\). Moreover, RWJ 67657 has been shown to have acceptable safety and acceptable pharmacokinetics to warrant further investigation\(^{14}\). p38 MAPK inhibitors like RWJ 67657 have shown to effectively inhibit pro-inflammatory mediators in different cells \textit{in vitro}, and also \textit{in vivo}. Whether kinase inhibitors will have an important role in the treatment of RA will be dependent of safety issues, and will be investigated in the near future.

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