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Role of p38 Mitogen-Activated Protein Kinase in Chemokine-Induced Emigration and Chemotaxis In Vivo

Denise C. Cara, Jaswinder Kaur, Melanie Forster, Donna-Marie McCafferty, and Paul Kubes

It has been proposed that L-selectin engagement with ligand activates p38 mitogen-activated protein kinase (MAPK) and can impact on downstream events of leukocyte rolling, including adhesion, and emigration. Using a novel chemotactic assay in vivo, we visualized slow release of chemokine from an agarose gel positioned 350 μm from a postcapillary venule, which induced directed migration (chemotaxis) of neutrophils. In this system, keratinocyte-derived cytokine induced phosphorylation of p38 MAPK, which phosphorylated a downstream protein (ATF-2). This latter event was blocked by the concentration of p38 inhibitors used in this study. Mice were treated with two different p38 inhibitors: SKF86002 and SB203580. Neither inhibitor affected rolling or adhesion in microvessels. Intravenous treatment with SKF86002 (5, 10, and 20 mg/kg) 30 min before the inflammatory stimulus inhibited the total number of emigrated cells at a dose of 20 mg/kg (62%, p < 0.05), despite the presence of many adherent cells within the vessels. A similar inhibition was observed with 20 mg/kg of a second p38 inhibitor SB203580 (67%, p < 0.05). In addition to emigration, both p38 inhibitors impaired the ability of emigrated cells to migrate through the tissue toward the chemotactic stimulus. In fact, the majority of emigrated leukocytes in p38 inhibitor-treated animals remained within 50 μm of the venule. Superfusion of the tissue with SKF86002 (0.7 mM) to impact only on emigrated and not vascular leukocytes resulted in no impairment in emigration, but in a significant reduction in chemotaxis away from the vessel wall. Again, the majority of emigrated leukocytes remained within 50 μm of the blood vessel. Our results suggest that p38 does not affect rolling or adhesion, but that it is involved in leukocyte emigration and chemotaxis through interstitium in response to keratinocyte-derived cytokine in vivo. The Journal of Immunology, 2001, 167: 6552–6558.

Leukocyte recruitment is a hallmark feature of the inflammatory response, and it involves a sequential series of molecular interactions between the leukocyte and endothelial cells. First, leukocytes in the mainstream of blood flow come into contact with the endothelium and they roll along the endothelial surface via a group of molecules termed the selectins (1). Next, rolling leukocytes are activated by pro-inflammatory molecules presented on the endothelial surface to firmly adhere to the endothelium via integrins. Once adherent, leukocytes then emigrate out of the vasculature and respond to directional (chemotactic) stimuli that guide them to the inflammatory source (2).

Selectins, integrins, and chemotactic factors show a close relationship with cytoskeleton and exert an important role as signal-transducing receptors, activating different biochemical pathways, including the mitogen-activated protein kinase (MAPK)3 cascade. It has been reported that the cross-linking of L-selectin on neutrophils (3), the exposure of neutrophils to TNF-α or LPS (4, 5), or stimulation of neutrophils by chemotactic factors such as fMLP (6) result in the phosphorylation and activation of a p38 MAPK. Consistent with a role for p38 MAPK in neutrophil recruitment have been in vivo studies showing that p38 MAPK inhibitors reduce the neutrophil infiltration in numerous inflammatory processes. Neutrophil infiltration into the peritoneal cavity induced by arachidonic acid (7), as well as carrageenan or urate crystals (8), was inhibited by SKF86002. In other p38 MAPK inhibition studies, infiltration of neutrophils into the airspace and lung induced by LPS (9) in Clostridium difficile toxin A-induced enteritis (10) and in Helicobacter pylori-induced gastritis (11) was inhibited by M39, SB203580, and FR167653, respectively.

Although these studies suggest the anti-inflammatory potential of the p38 MAPK inhibitors, they do not provide insight as to the specific mechanism(s) of action. For example, in vitro p38 MAPK inhibitors have been proposed as inhibitors of selectin function (12), integrin function as it pertains to adhesion (13), as well as chemotaxis (6), suggesting that the p38 MAPK inhibitors are general inhibitors of the recruitment cascade. However, this has never been systematically examined in the in vivo setting.

Therefore, in this study we investigated the role of p38 MAPK in leukocyte recruitment in vivo. Using intravital microscopy and a novel in vivo chemotaxis assay system, we were able to visualize the leukocyte rolling, adhesion, emigration, and directional migration toward a source of chemotactic factor in the extravascular tissue of the cremaster muscle. The latter was performed with time-lapse photography. The data reveal absolutely no effect of SB203580 and SKF86002 on selectin-dependent leukocyte rolling or integrin-dependent leukocyte adhesion, but the data do show very significant effects upon downstream events of neutrophil adhesion, i.e., a profound reduction in neutrophil emigration and chemotaxis.

1 Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; KC, keratinocyte-derived cytokine; LSP1, leukocyte-specific gene 1.
Materials and Methods

Animals
Male C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All mice weighed between 20 and 30 g and were used between 6 and 10 wk of age. The animals were anesthetized with an i.p. injection of a mixture of 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB, Montreal, Quebec, Canada). For all protocols, the left jugular vein was cannulated to administer additional anesthetic or drugs if necessary.

Intravital microscopy

The mouse cremaster preparation was used to study the behavior of leukocytes in the microcirculation and adjacent connective tissue as previously described (14). Briefly, an incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle using a cautery. The testicle and the epididymis were separated from the underlying muscle and were moved into the abdominal cavity. The muscle was then spread out on an optically clear viewing pedestal and was secured along the edges with 4-0 sutures. The exposed tissue was superfused with warm bicarbonate-buffered saline (pH 7.4). An intravital microscope (Axioskop; Carl Zeiss Canada, Don Mills, Ontario, Canada) with a ×25 objective lens (Wetzlar/L250; 35; E. Leitz Inc., Munich, Germany), and a ×10 eyepiece was used to examine the cremasteric microcirculation. A video camera (5100 HS; Panasonic, Osaka, Japan) was used to project the images onto a monitor, and the images were recorded for playback analysis using a conventional videocassette recorder or time-lapse videocassette recorder.

Single unbranched cremasteric venules (25–40 μm in diameter) were selected, and to minimize variability, the same section of cremasteric venule was observed throughout the experiment. The number of rolling, adherent, and emigrated leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling cells passing by a given point in the venule per minute. A leukocyte was considered to be adherent if it remained stationary for at least 30 s, and total leukocyte adhesion was quantified as the number of adherent cells within a 100-μm length of venule. Leukocyte emigration was defined as the number of cells in the extravascular space within 200 × 300 μm² area, as well as in each 50 μm of distance from the venule. Only cells adjacent to and clearly outside the vessel under study were counted as emigrated. Directional migration or chemotaxis was examined using time-lapse photography and was plotted as a function of distance moved toward the chemotactic source.

Induction of chemotaxis

An agarose gel containing keratinocyte-derived cytokine (KC), the murine homolog of GRO-α (R&D Systems, Minneapolis, MN), was used to induce chemotaxis in the cremaster preparation (15). The agarose gel was prepared by adding 10 ml of 2% HBSS to a boiling concentrated agarose solution (4% in 10 ml of distilled water). A 100-μl aliquot of this solution was removed and KC was added to this aliquot to achieve a final concentration of 5.2 μM. To enable visualization of the gel on the cremaster muscle, a small amount of India ink was added to each preparation. A 1-mm² piece of the mixture was punched out using the tip of a Pasteur pipette. This piece of gel was carefully placed on the surface of the cremaster in a preselected avascular area 350 μm² (two monitor screens wide) from a postcapillary venule. The gel was held in place using a coverslip, and the tissue was superfused beneath the coverslip at a minimum rate (0.7 ml/min) so as not to disrupt the chemotactic gradient established adjacent to the agarose gellet. The image was recorded for 90 min: 30 min for control without gel (in superfused beneath the coverslip at a minimum rate (0.7 ml/min) so as not to disrupt the chemotactic gradient established adjacent to the agarose gellet. The tissue was superfused for 40 min with saline then for 5 min with saline, and then superfused for 10 min with saline. The supernatant was stored at −70°C for activity assay.

For immunoprecipitation of phosphorylated p38 MAPK, 200 μl of cell lysate containing ~200 μg of total protein was incubated with 20 μl of resuspended immobilized Phospho-p38 MAPK (Thr180/Tyr182) monoclonal Ab (New England Biolabs, Beverly, MA) overnight at 4°C. The samples were then centrifuged for 30 min at 4°C, and the pellet was washed twice with 500 μl of lysis buffer and washed twice with 500 μl of kinase buffer containing 20 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na VO₃, and 10 mM MgCl₂. Immunocomplexes were resuspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg of activating transcription factor-2 (ATF-2, New England Biolabs) for 30 min at 30°C. The reaction was terminated with 25 μl of 3× SDS sample buffer containing 187.5 mM Tris- HCL (pH 6.8 at 25°C), 6% (w/v) SDS, 30% glycerol, 150 mM DTT, and 0.03% (w/v) bromophenol blue. The samples were boiled for 5 min and loaded on 10% SDS-PAGE gels. Gels were transferred onto polyvinylidene difluoride membranes using a semidybd electrophoretic transfer apparatus (Bio-Rad, Hercules, CA) for 55 min at 180 mA. Following transfer, the membranes were incubated for 1 h at room temperature in 25 ml of blocking buffer (2% skim milk/TBS with 0.1% Tween 20). After washing three times for 5 min each with 15 ml of TBS with 0.1% Tween 20, the membrane and the primary Ab were incubated in 10 ml of Ab dilution buffer with gentle agitation overnight at 4°C. After incubation, the membranes were washed and incubated with HRP-conjugated anti-rabbit secondary Ab and HRP-conjugated anti-biotin Ab to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 h at room temperature. Then the membranes were washed and incubated with 10 ml of LumiGLO (0.5 ml of 20% LumiGLO, 0.5 ml of 20% hydrogen peroxide, and 9.0 ml of water) with gentle agitation for 1 min at room temperature. Finally, the membranes were drained of excess developing solution, wrapped in plastic wrap, and exposed to x-ray film.

Histology At the end of each intravital microscopy experiment, the cremaster muscles were removed and fixed in 10% neutral buffered formalin. The tissues were dehydrated gradually in ethanol, embedded in paraffin, cut into 4-μm sections, stained with H&E, and examined under direct light microscopy.

Statistical analysis

The results were expressed as means ± SEM. Student’s t test was applied with a Bonferroni correction where necessary. A value of p < 0.05 was considered statistically significant.

Results

p38 MAPK inhibitor does not affect leukocyte rolling or adhesion

Fig. 1 demonstrates the impact of systemic administration of p38 MAPK inhibitor on the first two steps of the multistep cascade of leukocyte recruitment (leukocyte rolling and adhesion). With time, rolling decreases in untreated sham preparations (just gel without KC). KC administration maintained rolling cells at ~100 cells/min. Treatment of animals with p38 MAPK inhibitor did not affect the number of rolling leukocytes associated with KC (Fig. 1A). KC caused a significant increase in the number of adherent leukocytes. The p38 MAPK inhibitor (SB203580) had no inhibitory effect on the number of adherent cells (Fig. 1B). Simply placing the gel without KC onto the preparation does not induce any adhesion (sham group).

p38 MAPK inhibitors block downstream of leukocyte adhesion

The p38 MAPK inhibitor impacted upon downstream events of leukocyte adhesion. The slow release of KC from the gel induced very significant emigration in cremaster muscle (Fig. 2A). Without KC (just gel), fewer than 10 cells are seen within the field of view. The addition of KC induces emigration of >70 cells/field of view. The cells move toward the KC source. Intravenous treatment with
p38 MAPK inhibitor (SKF86002) reduced the total number of emigrated cells in a dose-response fashion. Significance was achieved at 20 mg/kg. Fig. 2B reveals identical results with a second inhibitor of p38 MAPK (SB203580).

*p38 MAPK inhibitors impaired the leukocyte chemotaxis induced by KC*

Aside from inhibiting emigration, the i.v. treatments with either p38 inhibitor impaired the chemotactic response induced by KC. When the cremaster muscle was divided into 25-μm sections, ~20 leukocytes were within the first 25 μm, 15 leukocytes were in the next 25 μm, and so on, with fewer and fewer cells available in each subsequent partition. SB203580 inhibited the number of chemotaxing cells found in each partition (Fig. 3A). It was apparent that the further away from the vessel, the greater the inhibition of leukocyte chemotaxis. For example, a 56–63% inhibition of the number of leukocytes was seen in the first 75 μm, whereas 83% inhibition was seen at 75–100 μm and 100% inhibition was noted at further distances. Similarly, at a dose of 20 mg/kg, SKF86002 inhibited 62% of the chemotaxis in the first 50 μm, 70% in the space from 50–100 μm, and 90% in the space from 100 to 150 μm (Fig. 3B). At lower concentrations of SKF86002, this observation was even more apparent, with inhibition of chemotaxis becoming apparent only at 100–150 μm away from the blood vessel.

*Impaired chemotaxis is not due to reduced emigration*

Although the data clearly demonstrate that both emigration and subsequent chemotaxis were affected by the p38 MAPK inhibitors, it could be argued that fewer emigrated leukocytes beget fewer cells chemotaxing. To dissociate these two events, the tissue was superfused with p38 MAPK inhibitor. This still permitted leukocytes to emigrate out of the vessels. Fig. 4A highlights that the total number of emigrated cells was quite similar during p38 MAPK inhibitor superfusion of the tissue. Clearly, circulating cells within the vasculature were still able to roll, adhere, and even emigrate before the inhibitor had time to significantly affect the leukocyte biology. Fig. 4B demonstrates that once outside the vessel, fewer cells were seen chemotaxing toward the source of KC. Most cells remained closely opposed to the blood vessels (0–50 μm) in the presence of SKF86002.

*Histology analysis*

Fig. 5A is a photomicrograph intended to show the dramatic impairment in chemotaxis and that the cells are neutrophils. The micrograph is not intended to show emigration. Leukocyte behavior was quantitated using intravital microscopy (Figs. 1–4). The figure shows the presence of emigrated neutrophils polarized toward the source of KC and that the cells are neutrophils. Fig. 5B is an insert showing that these cells are polymorphonuclear leukocytes. Fig. 5C shows a decrease in the total number of neutrophils in the tissue surrounding the blood vessel, but significant adhesion of cells in the vessel in p38-treated mice. The arrow indicates the direction toward the KC source. Although it is difficult to elucidate from this photomicrograph whether the cells are adherent on the luminal or abluminal side, intravital microscopy allowed for real time detection of adhesion and emigration and revealed that following p38 MAPK treatment, the majority of cells adhered but did not emigrate.

*Assay of p38 phosphorylation and its activity in cremaster muscle*

Fig. 6A shows that the phosphorylation of p38 MAPK was increased in response to KC-treated cremaster muscle. The inhibitor
SKF86002 had no effect on phosphorylated p38 levels. The activity of the phosphorylated p38 MAPK, measured by its ability to phosphorlyate ATF-2, was also increased with KC relative to saline treatment of cremaster muscle. Intravenous administration of SKF86002 greatly reduced ATF-2 phosphorylation (Fig. 6B). These results are consistent with the inhibitor blocking p38 MAPK activity. This experiment was repeated three times with similar results.

Discussion
Many stimuli, including cytokines, growth factors, and stress factors have been shown to stimulate the p38 MAPK cascade, leading to numerous biological responses. Once activated, MAPKs such as p38 MAPK can phosphorylate and activate other kinases. This event occurs by a rapid, sequential mechanism whereby the protein or peptide binds first into the substrate pocket (peptide-binding channel) of p38 MAPK followed by ATP binding into the ATP pocket (18). The downstream targets of p38 MAPK include MAPK-activated protein kinases 2, 3, and 5 and transcription factors ATF-2, CHOP-1, and others (19, 20). Pyridinyl imidazole compounds including SB-203580 and SKF86002 are widely used as specific inhibitors of p38 MAPK. Their specificity is such that they only inhibit the p38α and p38β but not the p38δ or p38γ (21). Not surprisingly, these inhibitors do not inhibit other MAPK members even at 100 μM or five times the concentration used in this study (22). Finally, a great interest has developed in these inhibitors as they have been shown to have significant therapeutic benefit in a number of models of inflammation, including endotoxemia (17, 9), collagen-induced arthritis (17), pulmonary inflammation (9), and gastritis (11).

Although p38 MAPK inhibitors significantly reduced neutrophil recruitment in each of the aforementioned models, the complexity of the models preempts any possibility of elucidating the mechanism by which p38 MAPK may contribute to neutrophil recruitment. For example, in endotoxemia, p38 MAPK inhibitors were shown to reduce TNF production, which was likely the reason that neutrophil recruitment was reduced (9). Indeed, in vitro studies, adhesion molecule expression has been shown to be reduced and integrin activation is prevented with p38 MAPK inhibition, potentially reducing the number of rolling, adhering, emigrating, or migrating (through interstitium) leukocytes (3, 12, 13). To minimize the biological complexity, we designed a very simple model that would allow us to identify each of the steps in the neutrophil recruitment cascade, including rolling, adhesion, emigration, and directional migration (chemotaxis) through tissue in response to a single exogenously applied CXC chemokine.

Because the recruitment process is indeed a cascade of events, intervening at one step will inevitably affect downstream events. Therefore, it is possible that the reduction in emigration with the p38 MAPK inhibitors indirectly also attenuated the subsequent chemotaxis through the tissues. However, there are a number of reasons why the reduction in emigration may not account for the reduction in chemotaxis. First, the cells that did emigrate in the presence of p38 MAPK inhibitors failed to chemotax in an effective manner toward the chemokine source. This was best exemplified by the fact that proportionately, a greater inhibition of leucocyte number was seen at increasing distance from the vessel. In...
a second series of experiments, we only exposed the neutrophils to the p38 MAPK inhibitors after they emigrated out of the vasculature (superfused SKF86002). Using this approach, we were able to see a clear impairment in the ability of the neutrophils to move through the extravascular space. This is an interesting observation in that it suggests that activation of p38 MAPK is an ongoing process that does not require prophylactic treatment. Interruption of p38 MAPK activity stopped the biological endpoint—in this case chemotaxis.

It is tempting to conclude that p38 MAPK has at least two separate functions: the activation of p38 MAPK is essential for neutrophil emigration and p38 MAPK contributes to subsequent chemotaxis. However, the dual function for p38 MAPK may reflect the fact that the neutrophil uses the same molecular mechanism to migrate between endothelium and for subsequent chemotaxis and the distinction is simply the two separate microenvironments (within the microcirculation and outside the microcirculation). A second possibility is that the two events require distinct intracellular pathways, with p38 MAPK as an upstream regulator. Support for the latter is that quite distinct adhesive mechanisms are used for transendothelial migration and tissue chemotaxis. PECAM-1 (24), CD47 (25), and CD18 (26) have been postulated as important adhesion molecules that contribute to endothelial transmigration. By contrast, the β1 integrins including α5β1 have been shown to be essential for neutrophil chemotaxis in tissues (27). In fact, it appears that the endothelial transmigration process is essential for the expression of β1 integrin molecules (28). Whether p38 MAPK is important in the β1 integrin up-regulation during emigration remains unclear.

A shortcoming of in vivo experiments is the inability to inhibit p38 MAPK in a single cell type. Therefore, we cannot conclude whether the effect of the p38 MAPK inhibitors is a direct effect upon neutrophils or involves other cells such as endothelial cells. This is not trivial in as much as endothelial cell activation has been shown to be essential for subsequent neutrophil transmigration (29). For example, endothelium cell contraction with subsequent paracellular gap formation is required for neutrophil transendothelial migration in response to chemotactic factors (30). In fact, a very recent in vitro study reported that pretreatment of endothelial cells with SB203580 reduced neutrophil migration on endothelium toward endothelial cell junctions. The impairment in neutrophil

![FIGURE 5.](image-url)
migration was a result of inhibiting endothelial cytoskeleton rearrangements (31). Taken together, it is possible that this process is involved in the reduced neutrophil emigration observed in our study. Indeed, we appear to have some indirect evidence that p38 MAPK was activated in parenchymal cells (presumably endothelial cells) because in the complete absence of any neutrophil infiltration in response to KC in anti-β-selectin Ab-treated mice, p38 MAPK activation was still noted (data not shown).

The role of p38 MAPK in neutrophil chemotaxis is supported by the report that installation of KC into lungs induced neutrophil accumulation that was significantly decreased by p38 MAPK inhibition. Although colleagues postulated that this may have been a result of impaired chemotaxis (9), our data extend that work to demonstrate that both endothelial transmigration and tissue chemotaxis were impaired. Interestingly, FMLP but not IL-8 has been shown to activate p38 MAPK and induce neutrophil chemotaxis (12). Read, M. A., M. Z. Whitley, S. Gupta, J. W. Pierce, J. Best, R. J. Davis, and E. C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. J. Cell Biol. 139:1349.

It was shown that neutrophils from LSP1 knockout mice showed impaired chemotaxis in response to KC (37). Taken together with our data, we would propose that KC activates p38 MAPK, which phosphorylates downstream proteins like LSP-1 to permit neutrophil migration through tissue.

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References


