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CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration *in vivo*

[1]Xiao Wei Zhang, [1]Qing Liu, [1]Yusheng Wang & *,[1]Henrik Thorlacius

[1]Department of Surgery, Malmö University Hospital, Lund University, 20502 Malmö, Sweden

1 The purpose of this study was to examine the impact of CXC chemokines, i.e. macrophage inflammatory protein-2 (MIP-2) and KC, on leukocyte-endothelium interactions in detail and to evaluate the role of P-selectin by use of intravital microscopy in the mouse cremaster muscle.

2 Administration of MIP-2 and KC provoked a dose (5–500 ng)- and time (0–4 h)-dependent increase in leukocyte rolling, adhesion and tissue recruitment. Neutrophils comprised more than 92% of the leukocyte response. Pretreatment with an antibody directed against P-selectin (RB40.34) significantly inhibited MIP-2- and KC-induced leukocyte rolling by more than 96%. This marked decrease in rolling abolished firm adhesion and extravascular accumulation of neutrophils (>89% reduction), suggesting that CXC chemokines induce P-selectin-dependent rolling, which in turn apparently is a precondition for the subsequent stationary adhesion and extravasation of neutrophils.

3 Moreover, the extravascular recruitment of leukocytes was evaluated in whole-mounts of the cremaster muscle without preceding intravital microscopy. Using this approach, it was again observed that MIP-2- and KC-induced neutrophil accumulation was completely dependent on P-selectin function. In contrast to the CXC chemokines, administration of the classical chemoattractant formyl-methionyl leucyl phenylalanine (fMLP) did not provoke extravascular tissue accumulation of neutrophils.

4 We could not detect gene expression of CXCR2 in murine endothelial cells, whereas neutrophils were positive, indicating that the stimulatory effect of CXC chemokines on leukocyte-endothelium interactions is not a direct effect on the endothelium but rather an indirect effect *via* activation of an intermediary tissue cell. However, challenge with MIP-2 and KC did not increase the number of degranulated mast cells.

5 In conclusion, our data demonstrate that CXC chemokines induce all steps in the extravasation process of leukocytes, including rolling, adhesion and transmigration *in vivo*. Moreover, these results show that P-selectin plays a critical role in MIP-2 and KC provoked neutrophil recruitment as a critical mediator of initial leukocyte rolling. Additionally, our study suggest that a restricted action of MIP-2 and KC on neutrophils is far too simplistic to explain the complex mechanisms of action of CXC chemokines on neutrophil infiltration *in vivo*.

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Abbreviations: fMLP, formyl-methionyl leucyl phenylalanine; i.p., intraperitoneal; MIP-2, macrophage inflammatory protein-2; MNL, mononuclear leukocyte; PMNL, polymorphonuclear leukocyte; RT–PCR, reverse-transcription polymerase chain reaction; TNF-α, tumour necrosis factor-α

Introduction

Activation and trafficking of leukocytes in inflamed tissues are regulated by secreted chemokines (Zlotnik *et al*., 1999). The family of chemokines is divided into two major subfamilies, CXC and CC, on the basis of structural properties (Zlotnik *et al*., 1999). Characteristic of CXC chemokines is their ability to specifically recruit neutrophils (Rollins, 1997; Bacon & Oppenheim, 1998; Zlotnik *et al*., 1999) and it has been shown that TNF-α-induced neutrophil activation and infiltration are mediated through the induction and release of CXC chemokines (Smart & Casale, 1994; Tessier *et al*., 1997; McColl & Clark-Lewis, 1999; Liu *et al*., 2000). In the mouse, the CXC chemokines comprise MIP-2 and KC, which are considered to be murine homologues of human interleukin-8 (IL-8) and growth-related oncogene chemokines (Tekamp-Olson *et al*., 1990; Oquendo *et al*., 1989). Notably, CXC chemokine-dependent leukocyte infiltration has been implicated as a fundamental part of the pathogenesis of several important clinical conditions, such as endotoxemia-induced lung injury (Schmal *et al*., 1996), glomerulonephritis (Feng *et al*., 1995) and bacterial meningitis (Diab *et al*., 1999). Leukocyte recruitment is a multistep process (rolling–adhesion–transmigration) and supported by specific adhesion molecules, which coordinate the interactions between leukocytes and endothelial cells (Butcher, 1991; Springer, 1994). Numerous studies have shown that leukocyte rolling is mediated mainly by the selectin family (P-, E-, and L-selectin) of adhesion molecules (Butcher, 1991; Springer, 1994; Vestweber & Blanks, 1999) although subsets of integrins have been reported to support rolling under certain

*Author for correspondence; E-mail: henrikthorlacius@hotmail.com

conditions (Carlos & Harlan, 1994). The low affinity adhesive rolling interaction reduces the velocity of circulating leukocytes and this reduction may be important to allow time for these cells to detect chemotactic signals from the local environment and the endothelial surface (Jung *et al*., 1999). However, the detailed mechanisms of action of CXC chemokines on leukocyte-endothelium interactions remain elusive.

The specific effect of chemokines are mediated by seven-transmembrane spanning, G-coupled receptors and at least five receptors (CXCR1–5) have been demonstrated to selectively bind CXC chemokines (Zlotnik *et al*., 1999). CXCR2 (IL-8 receptor B) is the high affinity receptor on murine neutrophils for MIP-2 and KC (Huber *et al*., 1991; Cacalano *et al*., 1994; Jones *et al*., 1997). Interestingly, an accumulating body of evidence suggests that the expression of CXCR2 is not restricted to neutrophils but also is detected on a number of other cells, such as mast cells (Lippert *et al*., 1998), tumour cells (Reiland *et al*., 1999) and endothelial cells (Murdoch *et al*., 1999), indicating a broad spectrum of biologic activities of CXC chemokines. However, the literature on the expression of CXCR2 on human endothelial cells is complex and partly contradictory. For example Murdoch *et al*. (1999) has reported the presence of CXCR2 in endothelial cells, whereas others could not find endothelial cell expression of CXCR2 (Schonbeck *et al*., 1995; Petzelbauer *et al*., 1995; Gupta *et al*., 1998). The expression of CXCR2 on murine endothelial cells is presently unknown and it is not known if MIP-2 and KC can directly activate endothelial cells to express adhesion molecules and, thus, contribute to leukocyte-endothelium interactions and tissue accumulation.

Based on the above considerations, the objective of this study was to examine the effect of MIP-2 and KC on leukocyte rolling, adhesion and extravascular recruitment *in vivo*. In addition, we wanted to determine the role of P-selectin in CXC chemokine-induced neutrophil recruitment and the potential expression of CXCR2 on endothelial cells.

Methods

Treatment of animals and experimental protocols

Male Balb/c mice weighing ~23–28 g were maintained on 12-h dark and 12-h light cycles and given food and water *ad libitum*. Mice were anaesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight intraperitoneally (i.p.). In order to delineate the role of P-selectin in all stages (i.e. leukocyte rolling, firm adhesion and extravasation) of the leukocyte response to MIP-2 (R&D Systems Europe, Ltd., Abingdon, Oxon, U.K.) and KC (R&D Systems Europe) challenge, two separate protocols were used: (1) intravital microscopy and whole-mount analysis of leukocyte endothelium interactions and extravascular recruitment, respectively; and (2) whole-mount analysis of tissue recruitment of leukocytes without prior intravital microscopy. The basis for this separation of protocols is that previous studies have shown that preparation of tissues for intravital microscopy provokes a certain level of 'spontaneous' leukocyte rolling (Fiebig *et al*., 1991; Dore *et al*., 1993; Yamaki *et al*., 1998) and we have demonstrated previously that whole-mount analysis is an important complement to intravital microscopy when studying the *in vivo* effect of inflammatory mediators (Yamaki *et al*., 1998). This preparation-induced leukocyte rolling is due to a subtle activation of the endothelium and is completely mediated by endothelial P-selectin expression (Dore *et al*., 1993; Mayadas *et al*., 1993). Thus, in order to also study the effect of chemoattractants on leukocyte recruitment in a system without the associated preparation-induced endothelial cell activation, whole-mounts were also evaluated without initial intravital microscopic observation. Blood samples were taken from the tail artery after the experiment for analysis of systemic and differential leukocyte counts using a hemocytometer. The animal experiments were approved by the Regional Ethical Committee for Animal Experimentation.

Preparation of the mouse cremaster muscle and intravital microscopy

The cremaster muscle was prepared for intravital microscopy as described earlier (Baez, 1973). In brief, a midline incision of the skin and fascia was made over the ventral aspect of the left scrotum and the incised tissues were retracted to expose the cremaster muscle sack. The cremaster muscle was then separated from the epididymis and the testis was pushed back to the side of the preparation. The preparation was performed on a transparent pedestal to allow transillumination and microscopic observation of the cremaster muscle microcirculation. Great care was taken to avoid any bleeding from the margins of the cremaster muscle by using electrocautery. Throughout the preparation and experiment the exposed tissue was covered with a plastic membrane to prevent the tissue from drying. Intrascrotal injection of MIP-2 and KC (in 0.15 ml PBS) was performed at indicated doses and time-points prior to microscopic observation. In order to delineate the role of P-selectin in MIP-2- and KC-induced leukocyte recruitment an anti-P-selectin antibody (RB40.34, rat IgG, Pharmingen, San Diego, CA, U.S.A.) and isotype-matched control antibody (R3-34, rat IgG, Pharmingen) were given i.v. immediately prior to intrascrotal administration of CXC chemokines. Observations of the cremaster microcirculation were made using an Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with water immersion lenses ($\times$40/NA 0.75 and $\times$63/NA 0.90). The microscopic image was televised (Sony Triniton) using a charge-coupled device videocamera (FK 6990 Cohu, Pieper GmbH, Berlin, Germany) and recorded on videotape (Panasonic SVT-S3000 S-VHS recorder) for subsequent off-line analysis. After positioning under the microscope, a 10-min equilibration period preceded quantitative measurements. Analysis of leukocyte flux and leukocyte-endothelium interactions (rolling and adhesion) was made in venules (inner diameter 26–37 μm) with stable resting blood flow. Rolling leukocyte flux was determined at indicated time points by counting the number of rolling leukocytes per 30 s passing a reference point in the microvessel and expressed as cells min^{-1}. Leukocyte adhesion in venules (stationary for $>$30 s) was counted along 680 μm long segments of the endothelial lining on one side of the vessel lumen and expressed as number of adherent cells mm^{-1}. Red blood cell velocity was measured on-line by the use of an optical Doppler

velocimeter (Microcirculation Research Institute, Texas A & M University, College Station, TX, U.S.A.). Venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8 ((red blood cell velocity/1.6)/venular diameter) as described previously (House & Lipowsky, 1987).

Histology

Samples of intact cremaster muscle microvascular networks were fixed in 4% formaldehyde overnight and then stained with Giemsa stain for 1 h. After differentiation in acetic acid (0.01%), the samples were mounted on gelatin (1%)-precoated glass slides and covered with a coverglass by applying DPX after drying as described in detail in for the rat mesentery previously (Yamaki *et al*., 1998). The ratio of intravascular polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes was based on analysis in venules (inner diameter 20–50 μm) with a mean value of 2–6 vessels in each animal. Leukocyte emigration was quantified by counting the number of extravascular PMNLs and MNLs per high power field observed along a randomly selected venule in each preparation and expressed as number of cells mm^{-2}. The number of intact and degranulated mast cells were counted and expressed as the percentage degranulated cells. Challenge with compound 48/80 (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a positive control for mast cell degranulation.

Endothelial cells

The polyoma transformed murine endothelioma cell line eEnd.2 was cultured in DMEM supplemented with 10% foetal calf serum (FCS), L-glutamine, penicillin and streptomycin, and subcultured twice weekly as described previously (Williams *et al*., 1989).

Leukocytes

Polymorphonuclear leukocytes (PMNL) were freshly isolated from Balb/c mice. The bone marrow was flushed aseptically out of the femurs and humeri bones with ice-cold PBS and their neutrophils were isolated by using Ficoll-Paque™ Research Grade (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The purity of bone marrow neutrophils was more than 70% as assessed by Turk stain in a haematocytometer. Cells were resuspended in culture medium (DMEM/F12 with 10% FCS) until use in the reverse-transcription polymerase chain reaction (RT–PCR) assay.

RT–PCR

Total cellular RNA was extracted from eEnd.2 and PMNL cells using an acid guanidinium–phenol–chloroform method (TRI zol Reagent; GIBCO–BRL Life Technologies, Grand Island, NY, U.S.A.) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) in order to remove potential genomic DNA contaminants according to manufacturer's protocol. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. RT–PCR was performed with SuperScrip One-Step RT–PCR system (GIBCO–BRL Life Technologies, Grand Island, NY, U.S.A.). Each reaction contained 1000 ng (End.2) and 350 ng (PMNL) of cellular total RNA as a template and 0.2 μM of each primer in a final volume of 50 μl. Mouse β-actin served as an internal control gene. The RT–PCR profile was one cycle of cDNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, one cycle of final extension at 72°C for 10 min. After RT–PCR, aliquots of the RT–PCR products were separated on a 2% agarose gel containing ethidium bromide and photographed. The primers sequences of CXCR2 and β-actin were as follows: CXCR2 (f) TGT TCT TTG CCC TGA CCT TGC-3′, CXCR2 (r) 5′-ACG CAG TAC GAC CCT CAA ACG-3′; β-actin (f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′. The primers were designed according to the mus musculus interleukin-8 receptor gene (Lee *et al*., 1995).

Statistical analysis

Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on Ranks (Dunnett's method) for unpaired samples. The results are presented as mean values ± s.e.m. Unless stated otherwise, *n* represents number of animals.

Results

MIP-2 and KC-induced neutrophil recruitment is dependent on P-selectin-mediated rolling

It was found that challenge with MIP-2 and KC (5–500 ng) increased leukocyte rolling, adhesion and extravascular accumulation in a dose-dependent manner (data not shown) and 500 ng of MIP-2 and KC, which caused a robust leukocyte response, was used for further studies on the role of P-selectin. Differential analysis revealed the leukocyte infiltrate comprised more than 92% neutrophils while mononuclear leukocytes were rarely found (Table 1). For both chemokines, the intravascular leukocyte response (leukocyte rolling and firm adhesion) peaked at 2 h of stimulation with 500 ng of MIP-2 and KC (Figure 1a,b, $n = 5$), whereas the extravascular accumulation of neutrophils increased progres-

Table 1 Percentage of PMNL and MNL in venules and in the extravascular space

	Venular leukocytes		*Extravascular leukocytes*	
	PMNL (%)	*MNL* (%)	*PMNL* (%)	*MNL* (%)
PBS ($n = 5$)	95 ± 1	5 ± 1	95 ± 3	5 ± 3
MIP-2 ($n = 5$)	96 ± 1	4 ± 1	95 ± 1	5 ± 1
Anti-P + MIP ($n = 5$)	94 ± 1	6 ± 1	92 ± 1	8 ± 1
KC ($n = 4$)	96 ± 1	4 ± 1	95 ± 1	5 ± 1
Anti-P + KC ($n = 5$)	95 ± 1	5 ± 1	92 ± 2	8 ± 2

The percentage of polymorphonuclear (PMNL) and mononuclear leukocytes (MNL) were determined in venules and in the extravascular space of in Giemsa stained cremaster muscles. Mice were challenged intrascrotally for 2 h with 500 ng of MIP-2 and KC with or without anti-P-selectin antibody (Anti-P, 40 μg, i.v.) pretreatment. Data are mean values ± s.e.m. and *n* represents the number of animals.

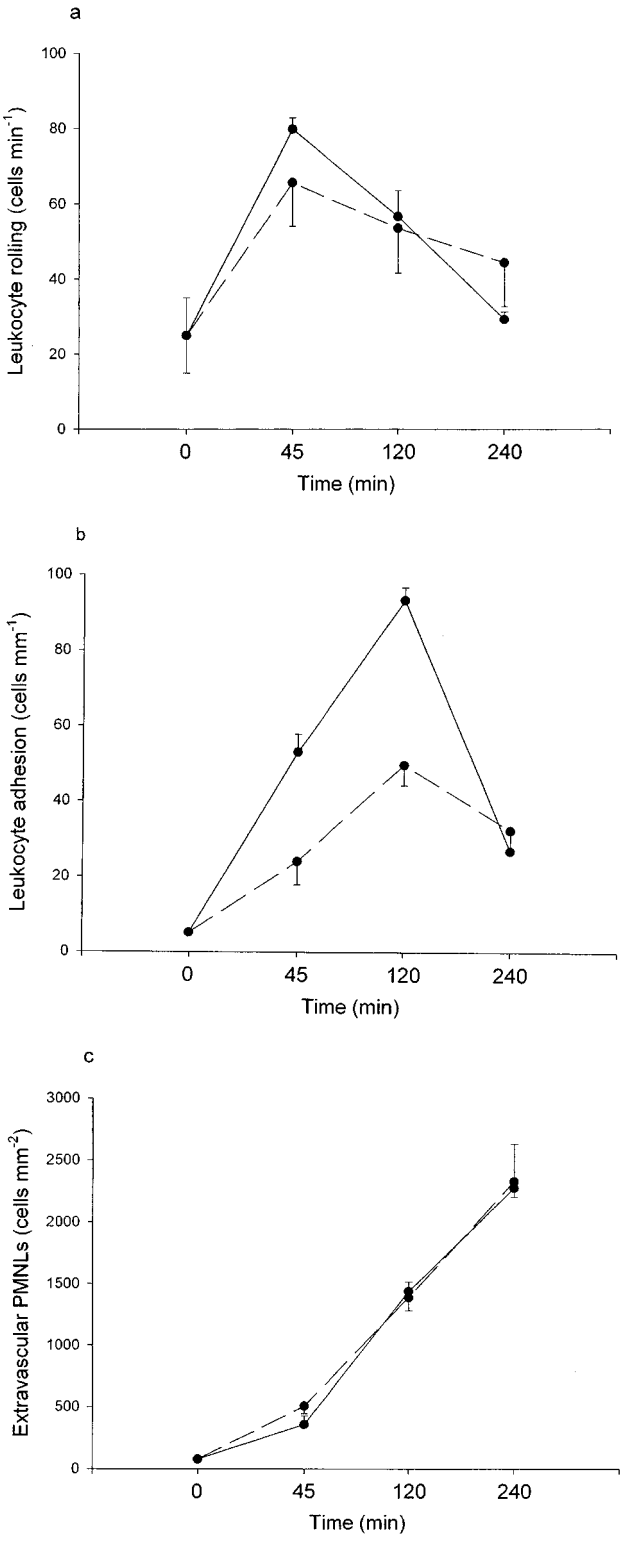

Figure 1 Time-dependent effect of intrascrotal challenge with 500 ng of MIP-2 (solid line) and KC (dashed line) on leukocyte (a) rolling (b) firm adhesion and (c) extravascular recruitment in the mouse cremaster muscle. Data represents mean ± s.e.mean and $n = 3-5$.

sively over time (Figure 1c, $n = 5$). It is noteworthy that the intravascular leukocyte response returned back to baseline values after 4 h of MIP-2 and KC challenge (Figure 1a,b, $n = 5$), illustrating the acute and reversible effect of CXC chemokines on neutrophil recruitment *in vivo*.

To evaluate the role of the P-selectin in the neutrophil response to CXC chemokine activation, we used i.v. pretreatment with a monoclonal antibody directed against mouse P-selectin (RB40.34, 40 μg per mouse). We found that pretreatment with the anti-P-selectin antibody markedly inhibited leukocyte rolling in MIP-2 and KC treated venules by more than 96% (from 76 ± 11 to 2 ± 0) and by 100% (from 94 ± 7 to zero), respectively, indicating that CXC chemokine-induced rolling is exclusively mediated by P-selectin (Figure 2a, $P < 0.05$ *vs* Control antibody, $n = 5$). Importantly, administration of the P-selectin antibody did not change systemic neutrophil count (Table 2). Interestingly, we observed that, in parallel to the reduction in rolling, inhibition of P-selectin function abolished the number of firmly adherent and extravascular leukocytes in response to MIP-2 and KC challenge (Figure 2b,c, $P < 0.05$ *vs* Control antibody, $n = 5$), suggesting that P-selectin-dependent rolling is a prerequisite in CXC chemokine-induced neutrophil adhesion and extravasation. As shown in Table 3, we observed that haemodynamic parameters were similar in all experimental groups. The level of rolling, firm adhesion and tissue recruitment of neutrophils was almost identical after a challenge with 500 ng of MIP-2 and KC (Figure 2a–c), suggesting a similar potency of the CXC chemokines in promoting inflammatory cell recruitment.

In separate experiments, histological analysis of giemsa stained whole-mounts of cremaster muscle preparations were undertaken without preceding intravital microscopic observation. Using this approach, we observed that MIP-2 and KC provoked a marked extravasation of neutrophils, i.e. 1384 ± 158 and 1050 ± 47 cells mm^{-2}, respectively (Figure 3, $n = 5$, $P < 0.05$ *vs* control). In fact, this level of extravasated neutrophils was almost identical to that observed when intravital microscopy was performed (Figure 2c). Noteworthy, immunoneutralization with the anti-P-selectin antibody (40 μg) completely abolished MIP-2- and KC-induced neutrophil recruitment (Figure 3, $P < 0.05$ *vs* PBS, $n = 5$), demonstrating that P-selectin is indeed a critical adhesion molecule supporting accumulation of neutrophils in CXC chemokine treated issues.

CXCR2 expression in endothelial cells

Next, we examined CXCR2 expression in murine endothelial cells and neutrophils. Total RNA was isolated, reverse transcribed into cDNA and PCR amplificated with specific primers for CXCR2. No detectable gene expression of CXCR2 mRNA was found in the endothelial cells (Figure 4). In contrast, neutrophils (PMNL) was found to express CXCR2 (Figure 4). As shown in Figure 4, the house-keeping gene β-actin was expressed as expected in both endothelial cells (EC) and neutrophils (PMNL).

Mast cell degranulation in MIP-2 and KC-treated mice

The percentage of mast cell degranulation was $2.2 \pm 0.6\%$ ($n = 5$) in negative control mice. It was found that, challenge with high dose (500 ng) of MIP-2 and KC for 2 h had no effect on the number of degranulated mast cells, i.e.

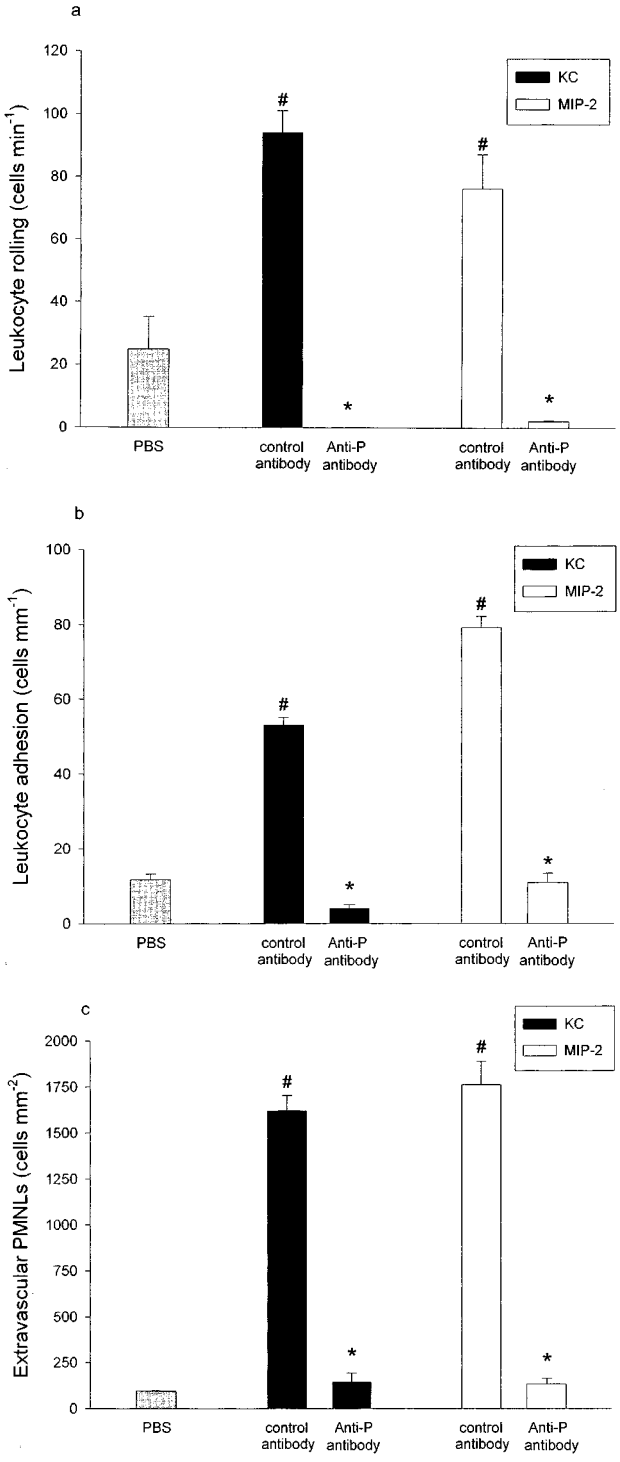

Figure 2 Effect of an anti-P-selectin antibody (Anti-P, 40 μg per mouse) and an isotype-matched control antibody (Control antibody, 40 μg per mouse) on (a) rolling (b) firm adhesion and (c) extravascular recruitment of neutrophils in the mouse cremaster muscle induced by intrascrotal challenge with MIP-2 (open bars) and KC (filled bars) for 2 h. In separate experiments, mice were treated intrascrotally with PBS alone (grey bars). Data represents mean$\pm$ s.e.mean. $^*P<0.05$ *vs* control antibody and $^{\#}P<0.05$ *vs* PBS, $n=5$.

$1.6\pm0.4\%$ ($n=5$) and $4.5\pm1.4\%$ ($n=4$), respectively. In contrast, administration of compound 48/80 (5 μg, 2 h), which was used as a positive control, provoked widespread mast cell degranulation, constituting $20.8\pm4.4\%$ ($n=4$) of the cremaster muscle mast cells.

Table 2 Systemic leukocyte counts

	PMNL	*MNL*	*Total*
Control ($n=5$)	0.8 ± 0.1	1.8 ± 0.3	2.5 ± 0.4
Control antibody + MIP-2 ($n=5$)	3.9 ± 0.2*	2.9 ± 0.2*	6.9 ± 0.2
Anti-P + MIP-2 ($n=5$)	5.9 ± 0.4*	3.8 ± 0.4*	9.7 ± 0.8*
Control antibody + KC ($n=5$)	3.1 ± 0.4*	2.7 ± 0.3	5.9 ± 0.6*
Anti-P + KC ($n=5$)	4.2 ± 0.8*	2.8 ± 0.5*	7.0 ± 1.1*

Blood was collected from the tail and analysed in a haemocytometer. The cells were defined as polymorphonuclear (PMNL) or mononuclear (MNL) leukocytes. Mice were challenged for 2 h with PBS, MIP-2 (500 ng) and KC (500 ng) after pretreatment with an anti-P-selectin antibody (Anti-P, 40 μg, i.v.) or an isotype-matched control antibody (Control antibody, 40 μg, i.v.). Data are mean$\pm$s.e.mean and represent 10^6 cells ml^{-1}. Asterisks indicate significant difference ($P<0.05$ *vs* PBS alone and n represents the number of animals).

Neutrophilia in MIP-2 and KC-treated mice

In PBS-treated mice the systemic neutrophil count was $0.8\pm0.1\times10^6$ cells ml^{-1}. Notably, intrascrotal administration of 500 ng of MIP-2 and KC caused a rapid and transient increase in the number of circulating neutrophils (Figure 5a,b). We observed that MIP-2 increased the neutrophil count to more than 4 fold at 45 min and 2 h and after 4 h the values returned to baseline. KC showed a similar systemic neutrophil response although somewhat slower than that observed after MIP-2 challenge (Figure 5a,b).

Discussion

TNF-α-induced leukocyte rolling and recruitment are dependent on endothelial cell activation and P-selectin expression (Weller *et al*., 1992; Robinson *et al*., 1999; Månsson *et al*., 2000). MIP-2 and KC are TNF-α-inducible CXC chemokines (Tessier *et al*., 1997; Schramm *et al*., 2000), which are important mediators of neutrophil activation and migration in a wide spectrum of diseases, including bacterial meningitis (Diab *et al*., 1999), glomerulonephritis (Feng *et al*., 1995) and endotoxemia-induced lung injury (Schmal *et al*., 1996). Our present investigation demonstrates that MIP-2 and KC alone induce all steps in the extravasation process, including, rolling, stationary adhesion as well as tissue recruitment of neutrophils *in vivo*. Moreover, inhibition of P-selectin function abolishes CXC chemokine-induced leukocyte rolling and the subsequent firm adhesion and tissue accumulation of neutrophils, suggesting that P-selectin-mediated rolling plays a critical role in neutrophil recruitment provoked by MIP-2 and KC. On the other hand, we observed herein that murine endothelial cells do not express CXCR2 in contrast to neutrophils. Taken together, these results demonstrate that P-selectin constitute an important part in CXC chemokine-induced neutrophil recruitment by supporting initial rolling along the microvascular endothelium. Our findings expand on previous studies by elucidating the detailed mechanisms of action of CXC chemokines on leukocyte-endothelium interactions and may help clarify the complex mechanisms of

Table 3 Haemodynamic parameters in venules of the cremaster muscle

	Diameter (μm)	*Red blood cell velocity* (mm sec^{-1})	*Wall shear rate* (s^{-1})
Control ($n=5$)	29±0.8	1.5±0.2	260±45
Control antibody + MIP-2 ($n=5$)	31±0.8	1.8±0.2	295±42
Anti-P + MIP-2 ($n=5$)	30±0.8	1.6±0.2	269±35
Control antibody + KC ($n=5$)	35±0.5	1.7±0.1	256±17
Anti-P + KC ($n=5$)	33±1.4	1.9±0.2	291±30

Data are mean±s.e.mean and n represents the number of animals.

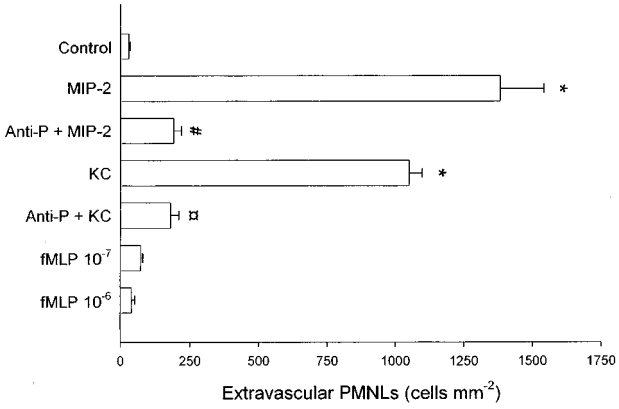

Figure 3 Number of extravascular neutrophils (PMNLs) per mm^2 in the mouse cremaster muscle whole mounts without prior intravital microscopic observation (see Methods for detailed explanation). Mice were challenged intrascrotally with PBS (Control), MIP-2 (500 ng) KC (500 ng) and fMLP (10^{-7} and 10^{-6} M) for 2 h before harvesting of the cremaster muscle tissue. In separate experiments, mice were pretreated intravenously with the anti-P-selectin antibody RB40.34 (Anti-P, 40 μg per mouse) immediately prior to challenge with 500 ng of MIP-2 and KC. Data represents mean±s.e.mean and $n=4-5$. *$P<0.05$ *vs* control, #$P<0.05$ *vs* MIP-2 alone and ¤$P<0.05$ *vs* KC alone.

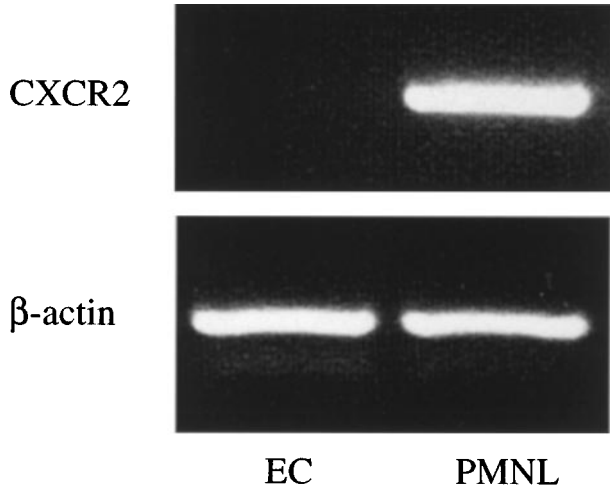

Figure 4 Expression of CXCR2 mRNA in murine neutrophils (PMNL) endothelial cells (EC). β-actin serves as an housekeeping gene. The results presented are from one experiment, which is representative of three others performed.

action of CXC chemokines in acute inflammation, which apparently is not restricted to direct activation of neutrophils.

Herein, we found that MIP-2 and KC induced all steps in the transmigration process of leukocytes, i.e. leukocyte rolling, firm adhesion and extravasation. The dose- and time-dependent effects of MIP-2 and KC on neutrophil recruitment were largely similar in potency. That CXC chemokines and classical chemoattractants, such as fMLP, platelet-activating factor and leukotriene B_4 provoke neutrophil activation and adhesion has been shown previously (Bacon & Oppenheim, 1998; Rollins, 1997; Thorlacius *et al*., 1995), however, this is the first study showing that MIP-2 and KC increase leukocyte rolling along the endothelium. Leukocyte rolling is dependent on endothelial cell activation and expression of selectins (Weller *et al*., 1992; Månsson *et al*., 2000), suggesting that administration of CXC chemokines activate the endothelium in multicellular tissues *in vivo*. In fact, we found in this investigation that leukocyte rolling in MIP-2 and KC-treated muscle is strictly mediated by P-selectin. Moreover, inhibition of P-selectin-dependent rolling completely inhibited firm adhesion and extravascular accumulation of neutrophils in response to MIP-2 and KC challenge. One previous study reported that immunoneutralization against P-selectin reduced dermal accumulation of neutrophils by 60% in response to stimulation with a rat CXC chemokine (cytokine-induced neutrophil chemoattractant) in the mouse air pouch (Harris *et al*., 1996). The reason for the relatively low effect of anti-P-selectin antibody against neutrophil recruitment in that investigation compared to the complete inhibition observed in our present study is not known at present, but may be attributable to the use of a rat CXC chemokine in a mouse model in the study by Harris *et al*. (1996). However, our findings demonstrate that P-selectin-mediated rolling is a critical precondition for the subsequent stationary adhesion and extravasation of neutrophils *in vivo*. It is noteworthy that we have recently demonstrated that TNF-α-induced neutrophil accumulation is critically dependent on P-selectin-mediated rolling (Månsson *et al*., 2000) and considered together, these observations indicate that leukocyte rolling supported by P-selectin on the vascular endothelium is a fundamental and common pathway for cytokine- and chemokine-provoked inflammatory cell infiltration. This conclusion supports the hypothesis that P-selectin is a pivotal target for pharmacological intervention in acute inflammation.

Endothelial cell activation and surface upregulation of selectins is a precondition for extravascular accumulation of neutrophils at sites of inflammation (Weller *et al*., 1992; Månsson *et al*., 2000). Our results demonstrating that MIP-2 and KC stimulate neutrophil recruitment, which was completely abolished by administration of an anti-P-selectin-antibody, indicate that CXC chemokines activate the vascular endothelial cells *in vivo*. This is in contrast to our

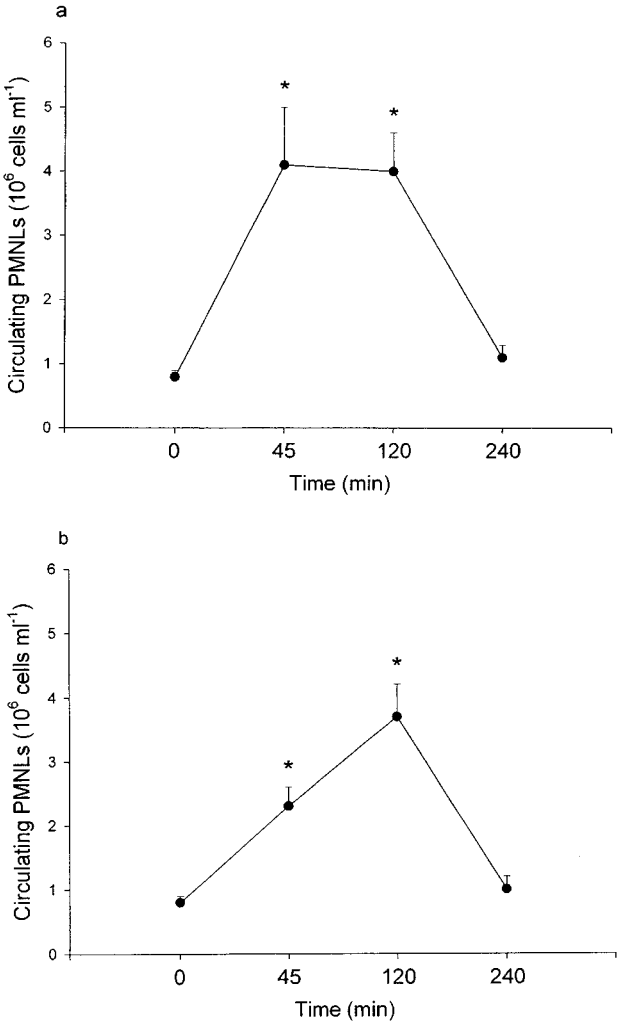

Figure 5 Changes in the number of circulating neutrophils (PMNLs) at different time-points after intrascrotal administration of 500 ng of (a) MIP-2 and (b) KC. Blood was collected from the tail and analysed in a haemocytometer. Data are mean ± s.e.mean and represent 10^6 cells ml^{-1}. $^*P<0.05$ *vs* 0 and $n=5$.

findings on fMLP, which is prototype of classical chemoattractants, which had no effect on neutrophil infiltration in undisturbed tissues (i.e. in experiments without preceding intravital microscopy). Thus, on the basis of these results, it is suggested that CXC chemokines act in a principally distinct manner from classical chemoattractants in that these chemokines are capable of activating endothelial cells in tissues *in vivo*. The mechanism of this CXC chemokine-induced endothelial cell activation and leukocyte rolling is not known at present. In this study, we did not detect expression of CXCR2 on murine endothelial cells, indicating that the effect of MIP-2 and KC *in vivo* is mediated by an intermediary cell in the extravascular tissue. This finding is in line with several previous studies reporting that CXCR2 is not expressed on human endothelial cells (Schonbeck *et al*., 1995; Petzelbauer *et al*., 1995; Gupta *et al*., 1998). One recent study has suggested that mast cells may play a role as an intermediary cell in MIP-2-provoked leukocyte recruitment in the peritoneum (Mercer-Jones *et al*., 1999). However, this concept is not supported by our present study in which we observed that mast cell degranulation was undetectable and identical in tissues stimulated with saline and CXC chemokines. Another candidate may be the tissue macrophages, which express CXCR2 (Chuntharapai *et al*., 1994) and upon activation secrete TNF-α and other inflammatory substances with the capacity to upregulate P-selectin on endothelial cells and increase leukocyte rolling (Weller *et al*., 1992; Månsson *et al*., 2000). In this context, it can not be excluded that circulating neutrophils may be activated by CXC chemokines and release TNF-α or reactive oxygen species capable of stimulating endothelial cell activation and P-selectin expression (Weller *et al*., 1992; Patel *et al*., 1991).

We observed that administration of MIP-2 and KC transiently increased the number of circulating neutrophils by 4 fold. It may be speculated that release of CXC chemokines into the circulation may be responsible for the neutrophilia observed in inflammatory diseases, such as sepsis and colitis. Previous studies on human CXC chemokines have shown that IL-8 produces a similar effect on systemic neutrophil count (Hechtman *et al*., 1991; Van Zee *et al*., 1992; Jagels & Hugli, 1992; Terashima *et al*., 1998). The mechanisms behind systemic neutrophilia triggered by IL-8 is reportedly attributable to increased release from the bone marrow and not due to demargination of neutrophils in the pulmonary circulation (Terashima *et al*., 1998). It is interesting to note that this MIP-2- and KC-induced neutrophila observed in the present study adds another character of the murine CXC chemokines to the list of similarities between murine and human CXC chemokines.

Taken together, our novel results indicate that CXC chemokines, MIP-2 and KC, are capable of inducing all steps in the extravasation process of neutrophils (rolling–adhesion–transmigration). Moreover, P-selectin plays a critical role in CXC chemokine-provoked neutrophil recruitment by supporting initial rolling adhesive interaction. Our data suggest that this P-selectin-mediated neutrophil rolling is a precondition for the subsequent firm adhesion and transmigration in MIP-2 and KC stimulated tissue. However, endothelial cells, in contrast to neutrophils, did not express CXCR2, suggesting that an intermediary cell in the extravascular tissue provoke endothelial cell activation in CXC chemokine-induced acute inflammation.

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