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CC chemokines induce P-selectin-dependent neutrophil rolling and recruitment *in vivo*: intermediary role of mast cells

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1 Based on *in vitro* chemotaxis experiments, it is widely held that CC chemokines, such as macrophage inflammatory protein-1α (MIP-1α) and macrophage chemotactic protein-1 (MCP-1) mainly support lymphocyte trafficking.

2 The objective of the present study was to examine the role of MIP-1α and MCP-1 in neutrophil recruitment *in vivo* by use of intravital microscopy of the mouse cremaster microcirculation.

3 MIP-1α and MCP-1 caused a dose-dependent increase in leukocyte rolling, adhesion and recruitment. Indeed, neutrophils comprised more than 85% of the leukocyte response to MIP-1α and MCP-1. An anti-P-selectin antibody reduced MIP-1α and MCP-1-provoked leukocyte rolling by more than 94%. Concomitantly, firm adhesion and extravasation of neutrophils in response to MIP-1α and MCP-1 challenge were significantly decreased by more than 78 and 84%, respectively. In contrast, an anti-E-selectin antibody had no influence on CC chemokine-induced neutrophil recruitment.

4 Flow cytometric analysis revealed that MIP-1α and MCP-1 had no effect on P-selectin expression on endothelial cells, suggesting that neutrophil recruitment elicited by CC chemokines *in vivo* is not mediated *via* a direct effect on the endothelium but rather *via* an indirect effect involving activation of an intermediary tissue cell. Indeed, it was found that MIP-1α-induced neutrophil accumulation was significantly decreased by 58% in mast cell-deficient mice.

5 These findings demonstrate that CC chemokines trigger P-selectin-dependent rolling and tissue recruitment of neutrophils *via* tissue mast cells *in vivo* and suggest that CC chemokines may also be important targets in neutrophil-mediated tissue damage in multicellular organs.
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Keywords: Adhesion molecules; cell trafficking; chemokines; neutrophils

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin; MIP, macrophage inflammatory protein; MCP, macrophage chemotactic protein; MNL, mononuclear leukocyte; PMNL, polymorphonuclear leukocytes; RT–PCR, reverse-transcription polymerase chain reaction; TNF-α, tumour necrosis factor-α

Introduction

Extravascular localization of leukocytes are regulated by secreted chemokines (Bacon & Oppenheim, 1998; Rollins, 1997; Zlotnik *et al*., 1999). The family of chemokines comprise two major subfamilies, CXC and CC, on the basis of structural properties (Bacon & Oppenheim, 1998; Rollins, 1997). It has been suggested that the action of CXC chemokines, such as MIP-2 and KC, is typically restricted to activate and recruit neutrophils (Bacon & Oppenheim, 1998; Rollins, 1997). On the other hand, it is generally held that CC chemokines, such as MIP-1α and MCP-1, predominantly stimulate chemotaxis of lymphocytes and eosinophils but not neutrophils (Bonecchi *et al*., 1999; McColl *et al*., 1993; Cheng *et al*., 2001). However, the majority of these studies have been performed in *in vitro* systems comprising only endothelial cells and subpopulations of leukocytes (Bonecchi *et al*., 1999; McColl *et al*., 1993; Cheng *et al*., 2001) and it is important to note that several resident cells in the extravascular matrix expressing CCR receptors, such as mast cells and macrophages, may trigger neutrophil recruitment in response to CC chemokine stimulation *in vivo*. In fact, there is an enlarging body of evidence supporting the concept that CC chemokines are involved in neutrophil trafficking *in vivo*. For example, treatment with antibodies against MIP-1α and MCP-1 have been reported to reduce extravascular accumulation of neutrophils in pathological models of inflammation (Appelberg, 1992; Shanley *et al*., 1995; Matsukawa *et al*., 1999; Diab *et al*., 1999). Moreover, MIP-1α-deficient mice exhibit clear-cut defects in neutrophil responses in complex inflammatory reactions (Domachowske *et al*., 2000). The specific effect of CC chemokines are mediated by seven-transmembrane spanning, G-coupled receptors and at least 11 receptors (CCR1-11) have been demonstrated to selectively bind CC chemokines (Proudfoot, 2002). The literature on CC chemokine receptor expression on endothelial cells is complex and partly contradictory. For example, Xu *et al*. (1995) detected binding sites for MIP-1α on endothelial cells, which could not be confirmed by others (McColl *et al*., 1993). Interestingly, two recent studies further implicate CC chemokines in neutrophil recruitment by demonstrating that surface expression of CCR1 on neutrophils may be upregulated after stimulation with IFN-γ and GM-CSF

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(Bonecchi *et al*., 1999; Cheng *et al*., 2001). Moreover, the phenotype of CCR1 gene-targeted mice implicates a role of CCR1 in neutrophil trafficking (Domachowske *et al*., 2000; Gao *et al*., 1993; Hall *et al*., 2001).

Inflammatory cell recruitment is a multistep process (rolling-adhesion-transmigration) supported by specific adhesion molecules, which coordinate the interactions between leukocytes and endothelial cells (Butcher, 1991). Numerous studies have shown that leukocyte rolling is mediated mainly by the selectin family (P-, E-, and L-selectin) of adhesion molecules (Butcher, 1991; Carlos & Harlan, 1994) although subsets of integrins have been reported to support rolling under certain conditions (Carlos & Harlan, 1994). The low affinity adhesive rolling interaction reduces the velocity of the 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). In fact, numerous studies have documented that initial rolling is a precondition for subsequent firm adhesion and tissue accumulation of neutrophils (Lindbom *et al*., 1992; Von Andrian *et al*., 1992; Mansson *et al*., 2000). However, the detailed mechanisms of action of CC chemokines on neutrophil-endothelium interactions and the specific role of selectins remain elusive.

The aim of the present study was to examine the effect of MIP-1α and MCP-1 on the extravasation process of neutrophils and the potential role of endothelial selectins (P- and E-selectin) *in vivo*. In addition, we wanted to determine surface expression of P-selectin on endothelial cells in response to CC chemokine challenge.

Methods

Animals and intravital microscopy

Male Balb/c and WBB6F1 (Jackson Laboratory, Bar Harbor, ME, U.S.A) mice weighing $\sim$23–26 g were maintained on 12-h dark and 12-h light cycles and given food and water *ad libitum*. WBB6F1 mice are mast cell deficient due to a disruption in the Kit oncogene on chromosome 5, encoding the tyrosine kinase receptor for stem cell factor, which is essential for the development of mast cells. The animal experiments were approved by the Regional Ethical Committee for Animal Experimentation. Mice were anaesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutical, Beerse, Belgium) per 100 g body weight intraperitoneally. Blood samples were taken from the tail artery after the experiment for analysis of systemic and differential leukocyte counts using a haemocytometer. The cremaster muscle was prepared for intravital microscopy as described earlier (Mansson *et al*., 2000; Kanwar *et al*., 1998; Ley *et al*., 1995). 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 observations of the cremaster muscle microcirculation were undertaken after a 15-min equilibration time. Intrascrotal injection of MIP-1α, MCP-1 and MIP-2 (R & D Systems Europe, Ltd., Abingdon, Oxon, U.K.) at indicated doses, diluted in 0.15 ml phosphate-buffered saline (PBS) was performed at 3 h prior to microscopic observation. In order to delineate the role of the selectins in CC chemokine-induced neutrophil recruitment, monoclonal antibodies directed against P-selectin (40 μg per mouse, RB40.34, rat IgG, Pharmingen, San Diego, CA, U.S.A) and against E-selectin (40 μg per mouse, 10E9.6, rat IgG, Pharmingen) were given i.v. immediately prior to intrascrotal administration of chemokines. In separate experiments, the role of histamine in MIP-1α-induced leukocyte recruitment was evaluated by pretreating mice with the histamine-1-receptor antagonist diphenhydramine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A) at 10 mg kg^{-1}, a dose that abolishes histamine-induced leukocyte rolling (Asako *et al*., 1994; Yamaki *et al*., 1998b). 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. Analysis of leukocyte flux and leukocyte-endothelium interactions (rolling and adhesion) were made in venules (inner diameter 30–38 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 over night and then stained with Giemsa stain for 1 h. After differentiation in acetic acid (0.01%) for 10 min, the samples were mounted on gelatin-precoated glass slides and covered with a cover glass as described in detail previously for the rat mesentery (Yamaki *et al*., 1998a). Leukocyte emigration was quantified by counting the number of extravascular polymorphonuclear (PMNL) and mononuclear (MNL) leukocytes per high power field observed along a randomly selected venule in each preparation and expressed as the number of cells per mm.^{2}

RT–PCR

Total cellular RNA was extracted from mouse cremaster tissue using an acid guanidinium–phenol–chloroform meth-

od (TRIzol 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 500 ng of cremaster 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 P-selectin, E-selectin and β-actin were as follows: P-selectin (f) 5′-ACG AGC TGG ACG GAC CCG-3′ ; P-selectin (r) 5′-GGC TGG CAC TCA AAT TTA CAG-3′; E-selectin (f) 5′-GGT AGT TGC ACT TTC TGC GG-3′; E-selectin (r) 5′-CCT TCT GTG GCA GCA TGT TC-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′.

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).

Flow cytometry

Endothelial cells were sub-cultured in 12-well plates (2×10^5 cells per well) for 48 h and subsequently incubated with 100 ng ml^{-1} of TNF-α (R & D Systems Europe), MIP-1α and MCP-1 for 3 h at 37°C. Endothelial cells were isolated by trypsination (0.1%) and washed (150 g) for 5 min. Then endothelial cell samples were stained with a FITC-labelled rat anti-mouse P-selectin (RB40.34, 1 μg per 10^6 cells) or a FITC-labelled isotype-matched control antibody (R3-34, 1 μg per 10^6 cells) from Pharmingen. After antibody incubation for 20 min at room temperature, the cell pellet was re-suspended with 0.5 ml PBS and put on ice until analysis, which was performed within 30 min. The intensity of P-selectin expression is given as the mean fluorescence intensity of endothelial cells incubated with the anti-P-selectin antibody divided by the mean fluorescence intensity of cells incubated with the control antibody.

Statistical analysis

Statistical evaluations were performed using Kruskal–Wallis one-way ANOVA on Ranks (Dunnetts method) for unpaired samples. The results are presented as mean values$\pm$s.e.mean. Unless stated otherwise, n represents number of animals.

Results

Neutrophil response to CC chemokines

We observed that administration of MIP-1α and MCP-1 (0–300 ng) dose-dependently increased leukocyte rolling, adhesion and extravascular accumulation (Figure 1a–c, $n=5$). Indeed, histologic analysis disclosed that more than 85% of the leukocytes responding to MIP-1α and MCP-1 comprised neutrophils. At 300 ng of MIP-1α and MCP-1, rolling leukocyte flux was increased by more than 2 fold (Figure 1a, $n=5$). Moreover, this challenge with 300 ng of MIP-1α and MCP-1 enhanced the number of firmly adherent and extravascular neutrophils by more than 6 fold (Figure 1b+c, $n=5$), suggesting that CC chemokines are capable of triggering clear-cut neutrophil recruitment *in vivo*. However, the CXC chemokine MIP-2 was found to be markedly more effective in stimulating infiltration of neutrophils. For example, 30 ng of MIP-2 caused similar neutrophil responses as 300 ng of MIP-1α and MCP-1 (Figure 1a–c).

CC chemokine-induced neutrophil recruitment is mediated by P-selectin

In order to determine the role of endothelial selectins in the neutrophil response to CC chemokine stimulation, we pretreated animals i.v. with monoclonal antibodies directed against mouse P-selectin (RB40.34, 40 μg per mouse) and E-selectin (10E9.6, 40 μg per mouse). Pretreatment with the anti-P-selectin antibody significantly reduced leukocyte rolling in MIP-1α and MCP-1 treated venules by 97% (from 43 ± 11 to 1.5 ± 0.3 cells min^{-1}) and 95% (from 31 ± 6 to 1.6 ± 0.5 cells min^{-1}), respectively (Figure 2a, $P<0.05$ *vs* anti-E, $n=5$). On the other hand, administration of the anti-E-selectin antibody had no effect on leukocyte rolling in CC chemokine treated venules (Figure 2a, $P>0.05$ *vs* control, $n=4-5$). Importantly, administration of the anti-P- and anti-E-selectin antibodies did not change systemic neutrophil count (Table 1). Interestingly, we observed, in parallel to the reduction in rolling, that inhibition of P-selectin function abolished the number of firmly adherent and extravascular neutrophils in response to MIP-1α and MCP-1 challenge (Figure 2b,c, $P<0.05$ *vs* anti-E, $n=5$), suggesting that P-selectin-dependent rolling is a prerequisite in CC chemokine-induced neutrophil adhesion and extravasation. As shown in Table 2, we observed that haemodynamic parameters were similar in all experimental groups.

P- and E-selectin expression

P-selectin expression on cultured endothelial cells was analysed by use of flow cytometry in order to examine if CC chemokines have the capacity to directly activate endothelial cells. Baseline expression of P-selectin was low on non-treated endothelial cells. Challenge with 100 ng ml^{-1} of TNF-α (positive control) for 3 h markedly increased the expression of P-selectin on endothelial cells (Figure 3). However, challenge with 10–1000 ng ml^{-1} of MIP-1α and MCP-1 had no effect on surface expression of P-selectin in endothelial cells (Figure 3). By use of RT–PCR, we observed no expression of E-selectin mRNA in the cremaster

muscle in control mice (Figure 4). Challenge with TNF-α (positive control), MIP-1α and MCP-1 markedly increased gene expression of E-selectin (Figure 4). As expected, P-selectin mRNA was detected at baseline, nonetheless, treatment with TNF-α, MIP-1α and MCP-1 further enhanced the levels of P-selectin mRNA in the cremaster muscle (Figure 4).

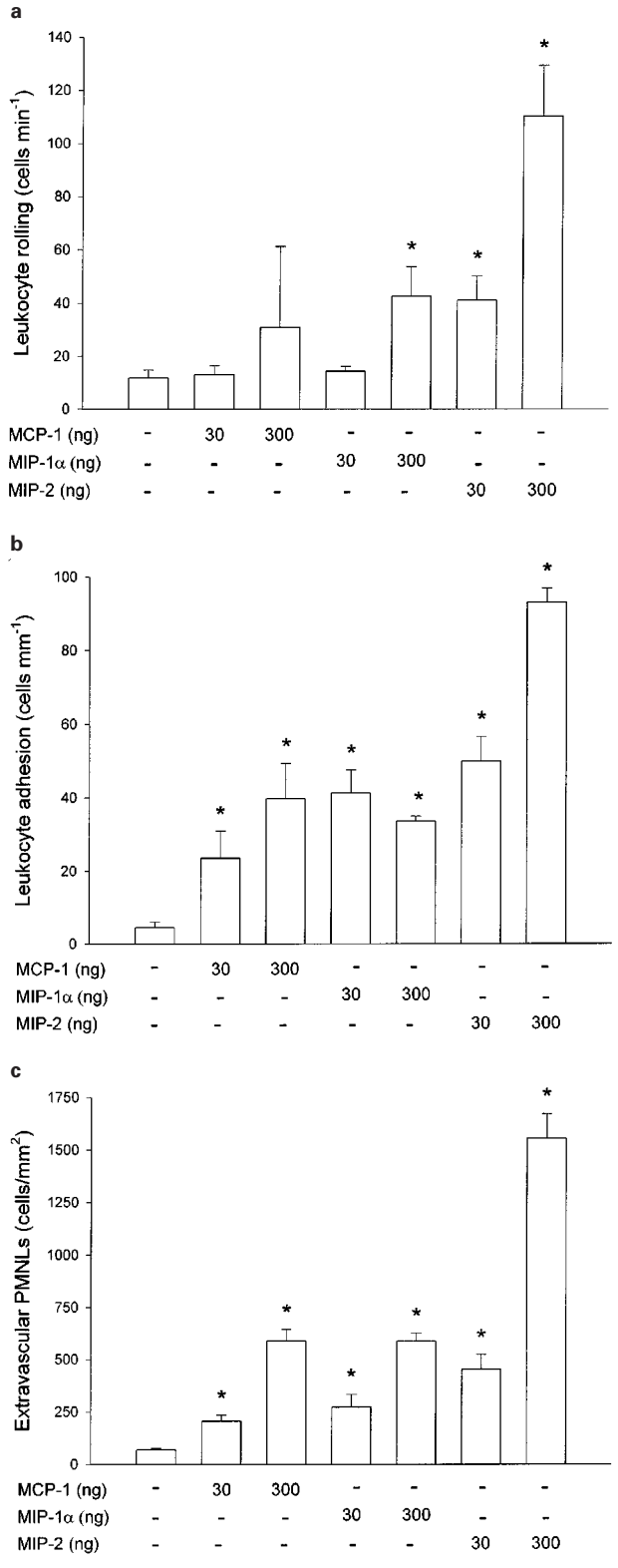

Figure 1 CC chemokines induce neutrophil infiltration. Dose-dependent (0–300 ng per animal) effect of intrascrotal challenge with MCP-1, MIP-1α and MIP-2 on leukocyte (a) rolling (b) firm adhesion and (c) extravascular recruitment of neutrophils in the mouse cremaster muscle. Data represents mean ± s.e.mean and $n=5$. *$P<0.05$ *vs* PBS.

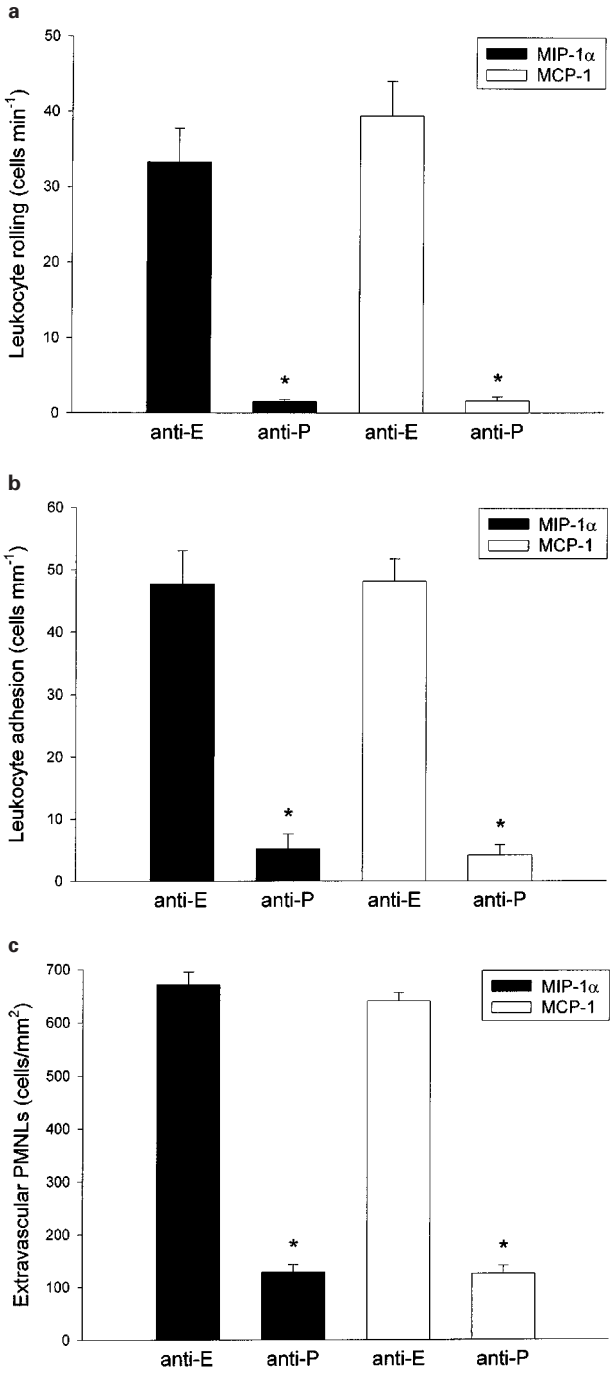

Figure 2 Role of selectins in neutrophil accumulation. Effect of a monoclonal antibody directed against P-selectin (anti-P, 40 μg per mouse) or E-selectin (anti-E, 40 μg per mouse on (a) rolling (b) firm adhesion and (c) extravascular recruitment of neutrophils in the mouse cremaster muscle induced by intrascrotal challenge with 300 ng of MIP-1α and MCP-1 for 3 h. In separate experiments, mice were treated intrascrotally with PBS alone. Data represents mean ± s.e.mean and $n=5$. *$P<0.05$ *vs* anti-E.

Table 1 Systemic leukocyte counts

	PMNL	*MNL*	*Total*
Control ($n=5$)	1.0 ± 0.1	3.2 ± 0.3	4.2 ± 0.4
MIP-1α ($n=5$)	1.2 ± 0.4	3.3 ± 0.4	4.3 ± 0.5
Anti-E + MIP-1α ($n=5$)	1.2 ± 0.1	3.9 ± 0.3	5.2 ± 0.4
Anti-P + MIP-1α ($n=5$)	1.0 ± 0.1	3.6 ± 0.1	4.7 ± 0.2
MCP-1 ($n=5$)	0.8 ± 0.1	3.5 ± 0.3	4.4 ± 0.3
Anti-E + MCP-1 ($n=5$)	1.2 ± 0.1	4.3 ± 0.3	5.5 ± 0.3
Anti-P + MCP-1 ($n=5$)	1.3 ± 0.1	3.8 ± 0.2	5.1 ± 0.2

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 3 h with PBS, MIP-1α (300 ng) and MCP-1 (300 ng) after pretreatment with an anti-P-selectin antibody (Anti-P, 40 μg, i.v.) or an anti-E-selectin antibody (Anti-E, 40 μg, i.v.). Negative controls (Control) received PBS alone. Data are mean ± s.e.mean and represent 10^6 cells ml^{-1} and n represents the number of animals.

Table 2 Haemodynamic parameters in venules of the cremaster muscle

	Diameter (μm)	*Red blood cell velocity* (mm s^{-1})	*Wall shear rate* (s^{-1})
Control ($n=5$)	31.8 ± 1.0	1.6 ± 0.1	258 ± 11
MIP-1α ($n=5$)	33.2 ± 1.1	1.6 ± 0.1	239 ± 16
Anti-E + MIP-1α ($n=5$)	32.7 ± 0.8	1.5 ± 0.2	227 ± 25
Anti-P + MIP-1α ($n=5$)	32.4 ± 0.2	1.4 ± 0.2	232 ± 29
MCP-1 ($n=5$)	34.5 ± 1.0	1.7 ± 0.3	247 ± 37
Anti-E + MCP-1 ($n=5$)	34.5 ± 0.9	1.6 ± 0.2	243 ± 36
Anti-P + MCP-1 ($n=5$)	33.8 ± 1.3	1.4 ± 0.1	257 ± 33

Mice were challenged for 3 h with PBS, MIP-1α (300 ng) and MCP-1 (300 ng) after pretreatment with an anti-P-selectin antibody (Anti-P, 40 μg, i.v.) or an anti-E-selectin antibody (Anti-E, 40 μg, i.v.). Negative controls (Control) received PBS alone. Blood flow velocities were measured off-line by frame-to-frame analysis of the videotaped images. Data are mean ± s.e.mean and n represents the number of animals.

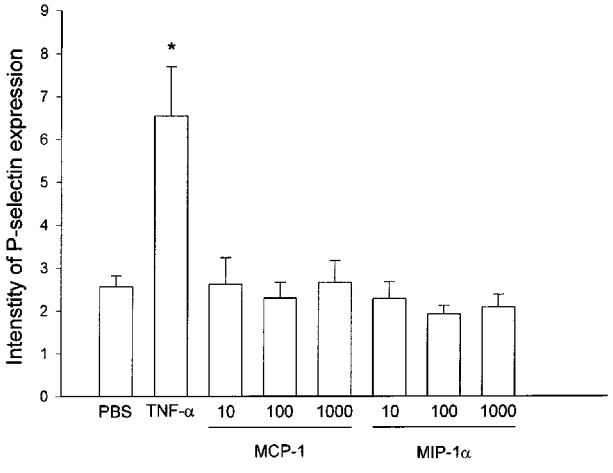

Figure 3 Intensity of P-selectin expression in endothelial cells. Percentage of P-selectin positive endothelial cells after stimulation with PBS, TNF-α (100 ng ml^{-1}), MCP-1 (10–1000 ng ml^{-1}) and MIP-1α (10–1000 ng ml^{-1}) for 3 h. Flow cytometry was performed as described in Methods. Data represents mean ± s.e.mean and $n=4$–5. $^*P<0.05$ *vs* PBS.

Role of mast cells in CC chemokine-induced neutrophil recruitment

We used mutant mice to define the role of tissue mast cells in MIP-1α-provoked neutrophil extravasation. Interestingly, it was found that the neutrophil responses to MIP-1α were markedly reduced in mast cell-deficient mice, i.e. the number of rolling, adherent and transmigrated neutrophils were significantly decreased 52, 84 and 58%, respectively (Figure 5a–c, $P<0.05$ *vs* WT, $n=4$–5). Considering that histamine has been shown to induce leukocyte rolling (Asako *et al*., 1994; Yamaki *et al*., 1998b), it was rational to define the role of histamine in CC chemokine-induced neutrophil responses. However, pretreatment with the histamine-1-receptor antagonist diphenhydramine (10 mg kg^{-1}), which effectively blocks leukocyte rolling (Asako *et al*., 1994; Yamaki *et al*., 1998b), had no effect on MIP-1α-provoked neutrophil responses, i.e. the number of rolling, adherent and extravascular neutrophils was 26 ± 10 cells min^{-1}, 33 ± 12 cells mm^{-1} and 568 ± 87 cells mm^{-2}, respectively ($P>0.05$ *vs* PBS + MIP-1α, $n=6$). Furthermore, in separate experiments, we observed that MIP-2-induced neutrophil responses were also significantly reduced in mast cell-deficient mice (Table 3). There was no difference in microvascular haemodynamics and systemic leukocyte counts between mast cell-deficient mice and wild-types (data not shown).

Discussion

A common dichotomy in chemokine biology holds that CXC chemokines typically regulate neutrophil trafficking and that CC chemokines predominantly coordinate tissue movement of mononuclear leukocytes. Our present study challenges that concept and provides evidence demonstrating that CC chemokines, MIP-1α and MCP-1, have the capacity to provoke neutrophil recruitment *in vivo*. Moreover, our findings suggest that P-selectin, but not E-selectin, supports CC chemokine-induced rolling and that this P-selectin-dependent rolling is a precondition for firm adhesion and extravascular accumulation of neutrophils in response to MIP-1α and MCP-1 stimulation. In contrast to TNF-α, it was found that MIP-1α and MCP-1 could not upregulate P-selectin expression on the surface of endothelial cells *in vitro*. Thus, the P-selectin-dependent neutrophil recruitment induced by CC chemokines *in vivo* is not a direct effect on endothelium. Instead, our results show that CC chemokine-induced neutrophil recruitment is mediated *via* tissue mast cells. These findings demonstrate that CC chemokines are important inducers of tissue neutrophila and help explain the complex networks of chemokines regulating neutrophil trafficking *in vivo*.

The present study documents that MIP-1α and MCP-1 possess the ability to induce all steps in the extravasation process, including rolling, stationary adhesion as well as tissue recruitment of neutrophils *in vivo*. In contrast, most studies on chemotaxis *in vitro* have shown that CC chemokines do not have the capacity to provoke neutrophil migration (Bonecchi *et al*., 1999; McColl *et al*., 1993; Cheng *et al*., 2001). However, these artificial *in vitro* systems comprise only neutrophils with or without endothelial cells and may, thus, neglect indirect effects mediated by other cells

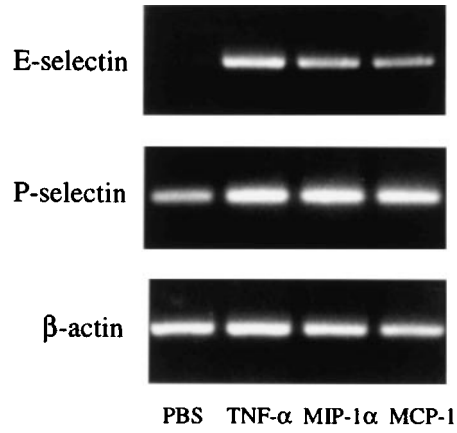

Figure 4 Expression of P- and E-selectin mRNA in the cremaster muscle. β-actin serves as an housekeeping gene. The cremaster muscle was stimulated with PBS, TNF-α (100 ng), MCP-1 (300 ng) and MIP-1α (300 ng) for 3 h. The results presented are from one experiment, which is representative of three others performed.

in multicellular tissues *in vivo*. Indeed, more recent studies have reported that antibodies directed against MIP-1α and MCP-1 reduce neutrophil extravasation *in vivo* in complex models of inflammation, such as septic peritonitis (Matsukawa *et al*., 1999), bacterial meningitis (Diab *et al*., 1999), pulmonary infection (Huffnagle *et al*., 1995) and IgG immune complex-induced tissue injury (Bless *et al*., 2000). Yet, in these studies the mechanisms behind the reduced tissue infiltration of neutrophils in animals passively immunized against CC chemokines were not clarified. A possible mechanism behind the ability of antibodies directed against CC chemokines to interfere with tissue trafficking of neutrophils is that MIP-1α and MCP-1 may contribute indirectly to neutrophil recruitment by provoking macrophage activation and subsequent release of neutrophil-chemotactic substances. Yet, in this context, it is also important to underline the fact that direct effects of CC chemokines on neutrophil accumulation may prevail in complex inflammatory reactions considering that activation (IFN-γ and GM-CSF) of neutrophils causes upregulation of CCR1 (Bonecchi *et al*., 1999; Cheng *et al*., 2001). Importantly, the results in our present study document that MIP-1α and MCP-1 provoke a significant influx of neutrophils *in vivo*. It is noticeable that the potency of CC chemokines to promote neutrophil recruitment was substantially lower than that of a CXC chemokine (i.e. MIP-2). Nonetheless, our present findings are in line with a previous study showing that cutaneous injection of human subjects with MIP-1α induces clear-cut recruitment of neutrophils (Lee *et al*., 2000).

Adhesion molecules support interactions between neutrophils and the microvascular endothelium (Butcher, 1991). The selectin family of adhesion molecules (L-, E- and P-selectin) are particularly important in mediating leukocyte rolling along the endothelium (Carlos & Harlan, 1994). While L-selectin is mainly important for lymphocyte homing in lymphoid tissue, the endothelial selectins (E- and P-selectin) have been implicated in supporting neutrophil rolling in postcapillary venules of non-lymphoid tissues (Butcher, 1991; Carlos & Harlan, 1994). We have previously shown that

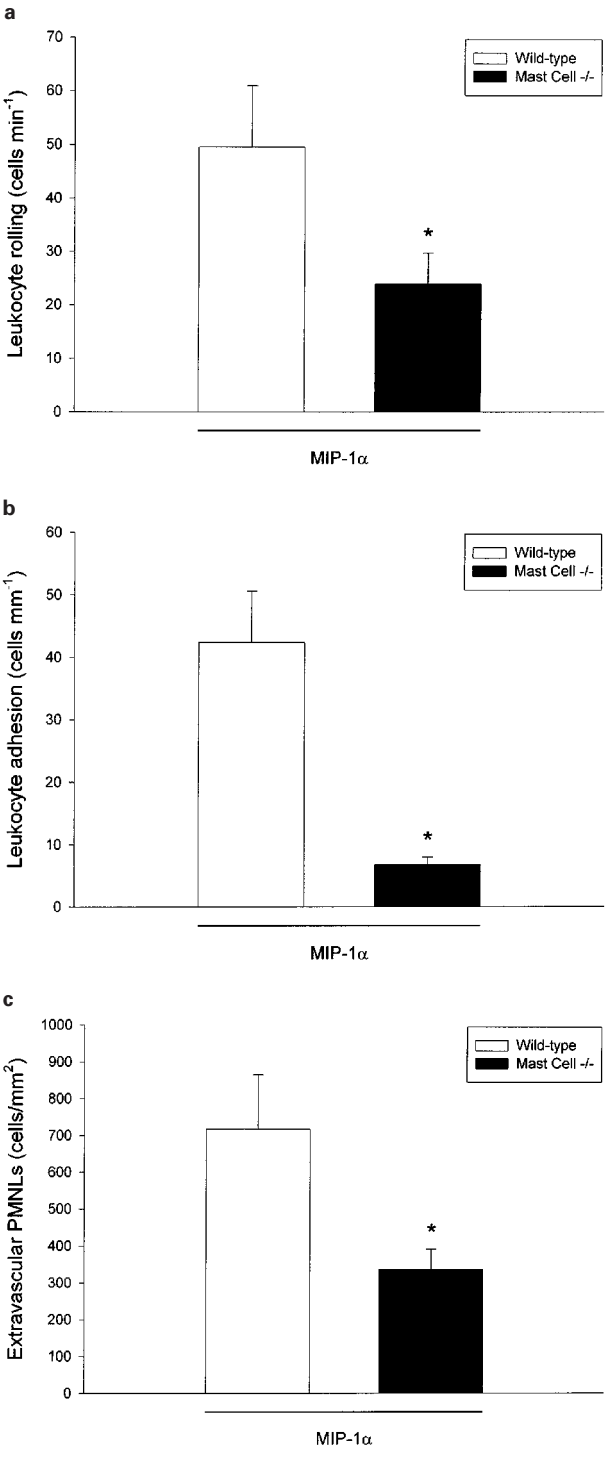

Figure 5 Role of mast cells in CC chemokine-induced neutrophil infiltration. MIP-1α-induced (a) rolling (b) firm adhesion and (c) extravascular recruitment of neutrophils in mast cell-deficient ($-/-$) and wild-type mice. Animals were challenged with 300 ng of MIP-1α intrascrotally for 3 h. Data represents mean$\pm$s.e.mean and $n=4-5$. $^*P<0.05$ *vs* wild-type.

CXC chemokines provoke P-selectin-dependent neutrophil-endothelium interactions *in vivo* (Zhang *et al*., 2001), however, this is the first study showing that CC chemokines has the capacity to increase leukocyte rolling along the

Table 3 MIP-2-induced leukocyte recruitment in mast cell-deficient mice

	Rolling (cells min^{-1})	*Firm adhesion* (cells mm^{-1})	*Extravascular PMNLs* (cells mm^{-2})
Control ($n=4$)	13.1 ± 3.5	5.3 ± 1.7	69.8 ± 9.4
MIP-2 in WT mice ($n=4$)	110.9 ± 19.2*	93.3 ± 3.9*	1560.3 ± 118.3*
MIP-2 in MC-deficient mice ($n=4$)	15.3 ± 2.9#	16.1 ± 6.0#	440.5 ± 38.2#

Wild-type (WT) and mast cell (MC)-deficient mice were challenged intrascrotally with MIP-2 (300 ng) for 3 h. Negative controls (Control) received PBS alone. The number of rolling and adherent leukocytes were determined by use of intravital microscopy of postcapillary venules in the cremaster muscle. Tissue recruitment of neutrophils (PMNLs) was determined in the extravascular space of the cremaster muscle after giemsa staining. Data are mean $\pm$ s.e.mean and represent and n represents the number of animals. *$P<0.05$ *vs* Control and #$P<0.05$ *vs* MIP-2 in WT mice.

endothelium. Herein, we observed that inhibition of P-selectin function abolished CC chemokine-induced rolling, suggesting that P-selectin plays a critical role in neutrophil recruitment provoked by MIP-1α and MCP-1. In contrast, inhibition of E-selectin function had no effect on neutrophil-endothelium interactions (rolling and adhesion) in response to stimulation with CC chemokines. The observation that E-selectin is not involved in the rolling adhesive process is in line with previous studies showing that blocking E-selectin has no effect on TNF-α-induced leukocyte rolling (Wan *et al*., 2002) and that leukocyte rolling is intact in E-selectin-deficient mice (Milstone *et al*., 1998). The role of E-selectin in the extravasation process of neutrophils remains elusive but may be involved in events downstream of rolling, such as activation and adhesion of rolling leukocytes (Milstone *et al*., 1998; Ley *et al*., 1998; Simon *et al*., 2000). Yet, the predominant role of P-selectin in mediating the rolling adhesive interaction observed in the present study has previously also been documented in other systems, such as mast cell-dependent acute inflammation (Thorlacius *et al*., 1994; Hickey *et al*., 1999), ischaemia-reperfusion injury (Kanwar *et al*., 1998; Sawaya *et al*., 1999; Singbartl & Ley, 2000) and TNF-α- and CXC chemokine-induced neutrophil recruitment (Mansson *et al*., 2000; Zhang *et al*., 2001; Robinson *et al*., 1999). Moreover, we observed that inhibition of P-selectin-dependent rolling completely inhibited firm attachment and tissue influx of neutrophils in response to MIP-1α and MCP-1 challenge. These findings indicate that P-selectin-mediated rolling is a precondition for the subsequent adhesion and transmigration of neutrophils in response to CC chemokine challenge *in vivo*. Considered together with other studies in the literature, it may be suggested that rolling supported by P-selectin on the microvascular endothelium constitutes a fundamental and common pathway for cytokine- and chemokine-provoked neutrophil infiltration.

A key feature in the extravasation process of inflammatory cells is activation of endothelial cells. Activated endothelium expresses P-selectin, which has been documented to be of paramount importance in supporting neutrophil rolling and recruitment (Mansson *et al*., 2000; Ley *et al*., 1995; Zhang *et al*., 2001; Robinson *et al*., 1999; this study). As previously described by Weller *et al*. (1992), we observed that TNF-α was a potent inducer of P-selectin expression on the surface of isolated endothelial cells *in vitro*. In contrast, challenge with MIP-1α and MCP-1 had no effect on the expression of P-selectin on endothelial cells, indicating that CC chemokines do not have the capacity to directly activate endothelial cells *in vitro*. On the other hand, our findings showing that MIP-1α and MCP-1 trigger influx of neutrophils into the extravascular compartment, which was completely abolished by injection of an anti-P-selectin antibody, suggest that CC chemokines indirectly activate the vascular endothelial cells *in vivo*. Moreover, our data on gene expression in the cremaster muscle demonstrates that MIP-1α and MCP-1 increase P- and E-selectin mRNA levels, indicating that the endothelial cells are indeed activated in response to CC chemokine challenge *in vivo*. In this context, it is interesting to note that we found, herein, that MIP-1α-induced neutrophil recruitment was greatly reduced in mice lacking mast cells, suggesting that CC chemokine-mediated endothelial cell activation and neutrophil responses are mediated *via* mast cell activation. This notion extends on previous studies showing that MIP-1α and MCP-1 have the capacity to activate mast cells (Alam *et al*., 1994). Moreover, our findings are also in line with recent studies documenting that mast cells express CC chemokine receptors, such as CCR1, CCR2, CCR3 and CCR5 (Oliveira & Lukacs, 2001). Indeed, activated mast cells release a number of substances, including histamine, leukotrienes and TNF-α with the ability to increase P-selectin expression on endothelial cells and in turn promote neutrophil rolling and extravascular recruitment (Ley *et al*., 1995; Yamaki *et al*., 1998a; Ley, 1994; Kanwar *et al*., 1995). Our present data suggest that histamine is not a critical mediator of CC chemokine-induced neutrophil infiltration. In a recent study by Matsukawa *et al*. (1999) it was reported that MCP-1 indirectly provokes peritoneal recruitment of neutrophils *via* production of leukotriene B_4, although the cellular origin of this leukotriene remains to be determined. In this context, it is interesting to note that Mercer-Jones *et al*. (1999) reported that mast cells may play a role as an intermediary cell in MIP-2-provoked neutrophil recruitment in the peritoneum. Indeed, we found here in the cremaster muscle that MIP-2-induced extravasation of neutrophils was reduced by more than 71%. Considered collectively, it may be forwarded that mast cells may constitute a common feature in both CC and CXC chemokine-induced infiltration of neutrophil *in vivo*.

Taken together, this study demonstrates that CC chemokines, MIP-1α and MCP-1, have the ability to provoke all steps in the extravasation process of neutrophils (rolling-adhesion-transmigration). We found that P-selectin plays a dominant role in CC chemokine-induced neutrophil recruitment by supporting initial rolling adhesive interaction. Moreover, this P-selectin-mediated rolling is a precondition for the subsequent firm adhesion and transmigration of neutrophils provoked by MIP-1α and MCP-1. However,

MIP-1α and MCP-1 were not capable of directly activating endothelial cells (i.e. increase P-selectin expression). Instead, CC chemokine-induced endothelial cell activation and neutrophil recruitment appears to be regulated by tissue mast cells. Collectively, our novel data suggest that CC chemokines may also be important targets in order to control pathological recruitment of neutrophils in multicellular tissues *in vivo*.

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