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European Journal of Pharmacology 427 (2001) 277–283

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Redundant function of macrophage inflammatory protein-2 and KC in tumor necrosis factor-α-induced extravasation of neutrophils in vivo

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Received 28 May 2001; received in revised form 16 July 2001; accepted 20 July 2001

Abstract

Tumor necrosis factor-α (TNF-α) stimulates the expression CXC chemokines, i.e. macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), and neutrophil extravasation. However, the individual role of MIP-2 and KC in the recruitment process of neutrophils in vivo remains elusive. By use of intravital microscopy in the mouse cremaster muscle, we analyzed the effect of specific inhibition of CXC chemokines, alone and together, on TNF-α-induced leukocyte rolling, firm adhesion and recruitment. After stimulation with TNF-α, the mRNA levels of both MIP-2 and KC were increased. Notably, separate administration of antibodies directed against MIP-2 and KC had no effect on TNF-α-induced neutrophil extravasation. In contrast, combined injection of anti-MIP-2 and anti-KC antibodies markedly inhibited extravascular migration of neutrophils. Moreover, MIP-2 and KC dose-dependently increased neutrophil recruitment, however, no synergistic effect of combined stimulation with MIP-2 and KC on the neutrophil response was found. Taken together, these data suggest that MIP-2 and KC are functionally redundant in TNF-α-induced neutrophil accumulation and that neutralization of both MIP-2 and KC may be necessary in order to reduce accumulation of neutrophils in cytokine-activated tissues.

Keywords: Chemokine; Inflammation; Intravital microscopy; Leukocyte; Microcirculation

1. Introduction

Infiltration of leukocytes is important in order to eliminate bacterial invasion but may also cause tissue damage. Leukocyte recruitment is a multistep process comprised of initial rolling, which slows down the velocity of the leukocytes followed by firm adhesion to the microvascular endothelium and transendothelial migration (Butcher, 1991). Numerous studies have shown that leukocyte rolling is mainly supported by the selectin family of adhesion molecules, whereas firm adhesion and transmigration are regulated by β_2-integrins (Carlos and Harlan, 1994). Tumor necrosis factor α (TNF-α) is a potent inducer of neutrophil infiltration and inhibition of TNF-α has been shown to be effective in the treatment of Mb Crohn and rheumatic diseases (Bell and Kamm, 2000; Forre et al., 2000). However, TNF-α per se is a weak chemoattractant and the chemotactic effect of TNF-α on tissue trafficking of leukocytes appears to be dependent on induction and secretion of chemokines (Liu et al., 2000; McColl and Clark-Lewis, 1999; Smart and Casale, 1994). The chemokines are classified into two major subfamilies, CXC and CC, on the basis of structural properties (Zlotnik and Yoshie, 2000). The murine CXC chemokines, comprising macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), are functionally characterized by their ability to specifically activate and recruit neutrophils (Bacon and Oppenheim, 1998; Zlotnik and Yoshie, 2000). CXC chemokines have been implicated in the pathogenesis of several important clinical conditions, such as endotoxemia-induced lung injury (Schmal et al., 1996). Notably, it has been demonstrated that TNF-α regulates the expression of MIP-2 and KC in the skin and lung (Czermak et al., 1999; Tessier et al., 1997). However, the relative contribution of MIP-2 and KC in tissue recruitment of leukocytes in response to TNF-α challenge remains elusive and it is not known whether MIP-2 and KC may interact in a synergistic way with respect to neutrophil accumulation.

The objective of this study was to examine the expression and role of MIP-2 and KC in TNF-α-induced leukocyte infiltration. Moreover, we wanted to study whether

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01235-3

CXC chemokines may functionally interact and synergistically increase leukocyte responses. For this purpose, we used intravital microscopy in the mouse cremaster muscle.

2. Materials and methods

2.1. Animals and intravital microscopy

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. 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. 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 observations of the cremaster muscle microcirculation were undertaken after a 15-min equilibration time. Intrascrotal injection of TNF-α, MIP-2 and KC (R&D Systems Europe, Abingdon, Oxon, UK) at indicated doses, diluted in 0.15 ml phosphate-buffered saline (PBS) was performed 2 h prior to microscopic observation. In order to delineate the role of MIP-2 and KC in TNF-α-induced leukocyte recruitment, monoclonal antibodies directed against MIP-2 (20 μg per mouse, clone 40605.111, R&D Systems Europe) and KC (20 μg per mouse, clone 48415.111, R&D Systems Europe), alone or in combination, were given locally immediately prior to intrascrotal administration of TNF-α. In separate experiments, the effectiveness of the anti-MIP-2 and anti-KC antibodies were tested against 2-h stimulation with recombinant 500 ng of MIP-2 and 500 ng of KC. Observations of the cremaster microcirculation were made using an Olympus microscope (BX50WI, Olympus Optical, Hamburg Germany) equipped with water immersion lenses (×40/NA 0.75 and ×63/NA 0.90). The microscopic image was televised (Sony Triniton) using a charge-coupled device videocamera (FK 6990 Cohu, Pieper, 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 26–35 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. 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 per mm. 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, USA). 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 and Lipowsky, 1987).

2.2. 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., 1998). Leukocyte emigration was quantified by counting the number of extravascular polymorphonuclear and mononuclear leukocytes per high power field observed along a randomly selected venule in each preparation and expressed as the number of cells per mm^2.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from mouse cremaster tissue using an acid guanidinium-phenol-chloroform method (Trizol Reagent; GIBCO-BRL Life Technologies, Grand Island, NY) and treated with RNase-free DNase (DNase 1; 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). Each reaction contained 500 ng of cremaster total RNA as 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 1 cycle of cDNA synthesis at 50 °C for 30 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C and extension at 72 °C for 1 min, 1 cycle of final extension at 72 °C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primers sequences of MIP-2, KC and β-actin were as follows: MIP-2 (f) 5′-GCT TCC TCG GGC ACT CCA GAC-3′, MIP-2(r) 5′-TTA GCC TTG CCT TTG TTC AGT AT-3′; KC (f) 5′-GCC AAT GAG CTG CGC TGT CAA TGC-3′, KC(r) 5′-CTT GGG GAC ACC TTT TAG CAT CTT-3′; β-actin

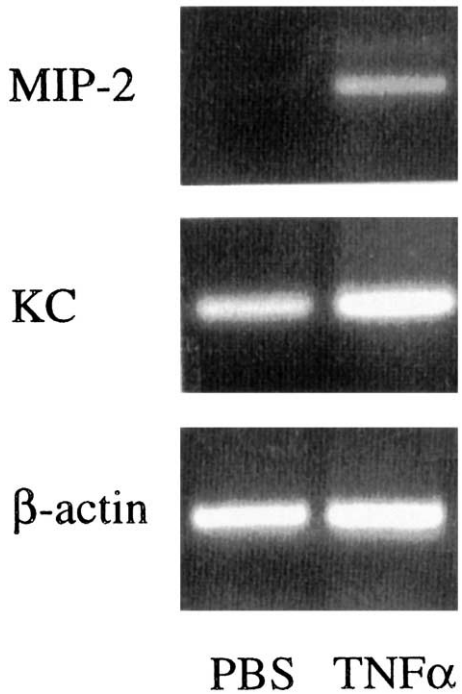

Fig. 1. Expression of MIP-2 and KC mRNA in the mouse cremaster muscle in response to administration of PBS and TNF-α (0.1 μg) for 2 h. B-actin serves as an housekeeping gene. The results presented are from one experiment, which is representative of three others performed.

(f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′.

2.4. Statistical analysis

Statistical evaluations were performed using Mann–Whitney rank sum test for unpaired samples. The results are presented as mean values ± S.E.M. and *n* represents the number of animals.

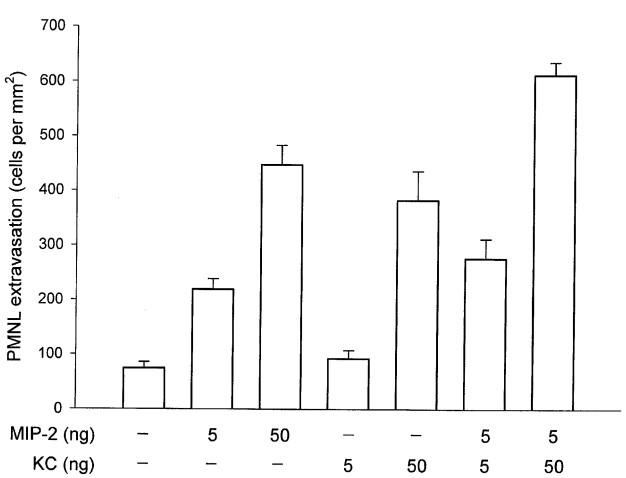

Fig. 2. Effect of intrascrotal challenge with 5 and 50 ng of MIP-2 and KC on neutrophil extravasation. MIP-2 and KC were administered alone or in combination as indicated and the number of extravascular neutrophils were measured after 2 h in the cremaster muscle. Data represents mean ± S.E.M. and $n = 5$.

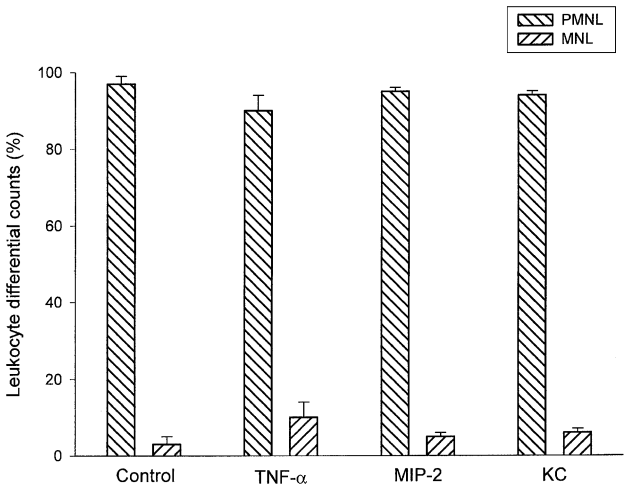

Fig. 3. Extravascular leukocyte differential counts. Animals were treated intrascrotally for 2 h with PBS (Control), TNF-α (0.1 μg), MIP-2 (50 ng) and KC (50 ng). The percentage of polymorphonuclear leukocytes (PMNL) and mononuclear leukocytes (MNL) were determined in the extravascular space of Giemsa stained cremaster muscles. Data are mean ± S.E.M. and $n = 5–7$.

3. Results

3.1. Expression of CXC chemokines

The recognition of the important role of CXC chemokines in directing tissue trafficking of neutrophils and promoting pathological inflammation has provided new

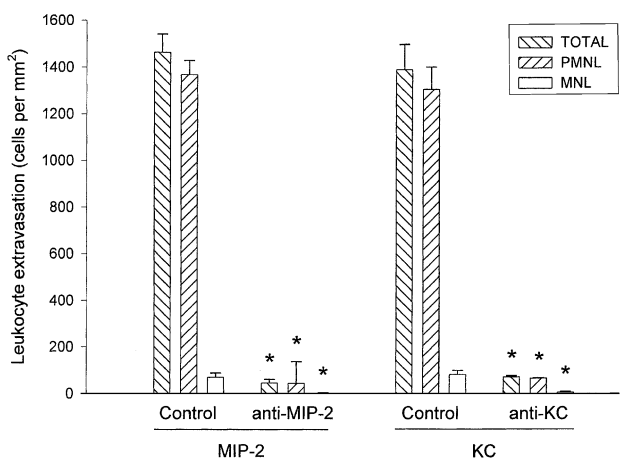

Fig. 4. Effect of monoclonal antibodies directed against MIP-2 (anti-MIP-2, 20 μg per mouse) and KC (anti-KC, 20 μg per mouse). The antibodies were administered locally immediately prior to 2 h of intrascrotal challenge with high doses of MIP-2 (500 ng) and KC (500 ng). Extravascular leukocytes (Total) were subtyped into polymorphonuclear (PMNL) or mononuclear (MNL) leukocytes. Data represent mean ± S.E.M. $^{*}P < 0.05$ vs. Control and $n = 5$.

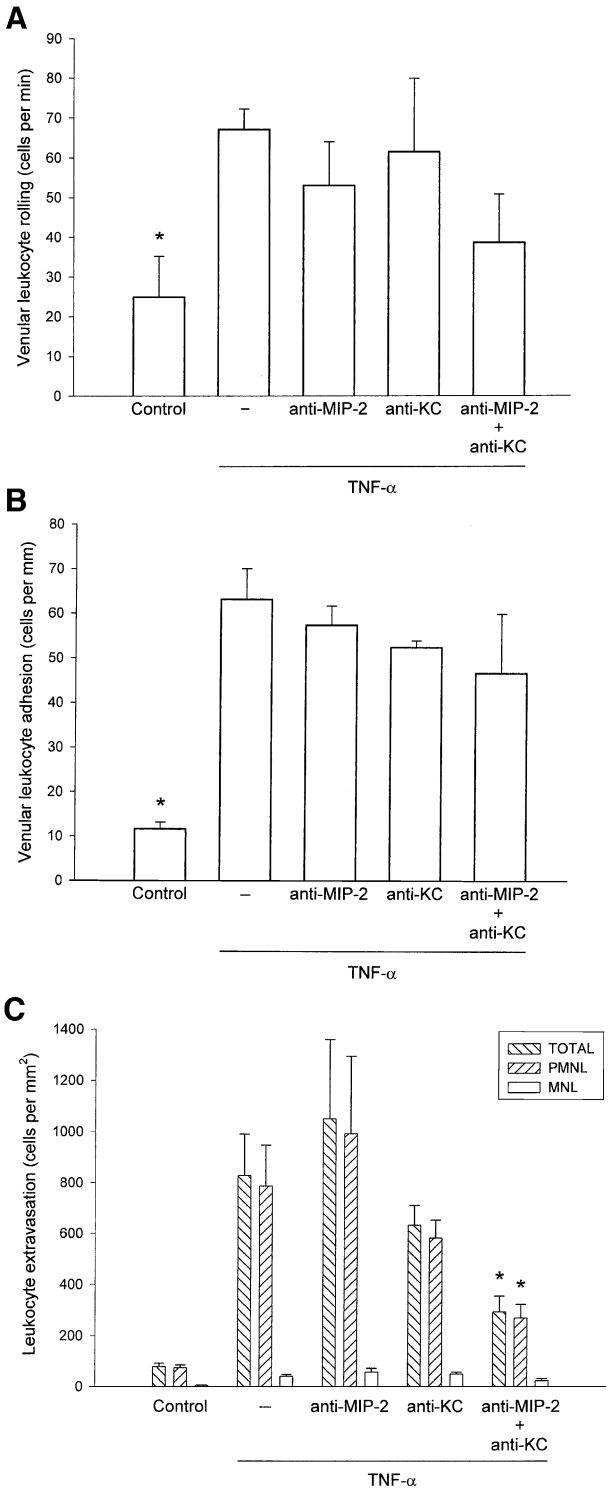

Fig. 5. Effect of monoclonal antibodies directed against MIP-2 (anti-MIP-2, 20 μg per mouse) and KC (anti-KC, 20 μg per mouse) on TNF-α-induced leukocyte (A) rolling (B) firm adhesion and (C) extravascular recruitment in the mouse cremaster muscle. The antibodies were administered locally immediately prior to intrascrotal challenge with 0.1 μg of TNF-α for 2 h. Extravascular leukocytes (Total) were subtyped into polymorphonuclear (PMNL) or mononuclear (MNL) leukocytes. Data represents mean ± S.E.M. $^{*}P < 0.05$ vs. PBS + TNF-α and $n = 5–7$.

potential targets for therapeutic intervention (Bacon and Oppenheim, 1998; Schmal et al., 1996; Zlotnik and Yoshie, 2000). We examined the mRNA expression of MIP-2 and KC in the striated muscle in the present study. Total RNA was isolated from the cremaster muscle, reverse transcribed into cDNA and PCR amplificated with specific primer for MIP-2 and KC. The results showed that, in control mice, only KC mRNA could be detected whereas MIP-2 mRNA was not expressed in the cremaster muscle tissue (Fig. 1). Importantly, after TNF-α challenge (0.1 μg for 2 h) the mRNA expression of both MIP-2 and KC were increased (Fig. 1), suggesting that both MIP-2 and KC are expressed in the cremaster muscle in vivo in response to TNF-α challenge.

3.2. Role of CXC chemokines in TNF-α-induced leukocyte recruitment

In order to study a possible synergistic interaction between CXC chemokines, we initially identified threshold doses of MIP-2 and KC with respect to induction of leukocyte extravasation (Fig. 2, $n = 5$). It was found that 5 ng of MIP-2 or KC provoked a subtle increase in the number of extravascular leukocytes, whereas 50 ng of MIP-2 or KC caused a clear-cut leukocyte response in the mouse cremaster muscle tissue (Fig. 2, $n = 5$). However, combined administration of 5 ng of MIP-2 and 5 ng of KC did not result in a synergistic effect on neutrophil infiltration, but rather an additive response (Fig. 2, $n = 5$). Moreover, it was found that injection of 5 ng of MIP-2 together with 50 ng of KC caused an increase in neutrophil extravasation reflecting the sum of the individual effects of these chemokines (Fig. 2, $n = 5$), indicating that MIP-2 and KC do not act in a synergistic manner with respect to tissue infiltration of neutrophils. Differential analysis revealed that the leukocyte infiltrate in response to TNF-α and CXC chemokine challenge comprised more the 90% polymorphonuclear leukocytes, while less than 10% were

Table 1
Systemic leukocyte differential counts

	PMNLs	MNLs	Total
Control	0.8 ± 0.1^{a}	1.8 ± 0.3	2.6 ± 0.4^{a}
TNF-α	1.4 ± 0.2	2.6 ± 0.3	4.0 ± 0.4
Anti MIP-2 + TNF-α	0.9 ± 0.2	2.3 ± 0.6	4.2 ± 0.4
Anti KC + TNF-α	2.3 ± 0.3^{a}	3.2 ± 0.3	5.5 ± 0.4^{a}
Anti MIP-2 and anti KC + TNF-α	1.9 ± 0.3	3.9 ± 0.7	5.8 ± 1.1

Animals were treated intrascrotally with PBS (Control) and tumor necrosis factor-α (TNF-α) for 2 h. In separate experiments, monoclonal antibodies against MIP-2 (anti-MIP-2) and or KC (anti-KC) were administered alone or together locally immediately before TNF-α challenge. Blood samples were collected from the tail and analyzed in a hemocytometer. The cells were defined as polymorphonuclear leukocytes (PMNLs) and mononuclear leukocytes (MNLs). Data are mean ± S.E.M. and represent 10^{6} cells ml^{-1}. $^{a}P < 0.05$ vs. TNF-α alone and $n = 5–7$.

Table 2
Hemodynamics

	Venular diameter (μm)	Red blood cell velocity (mm s^{-1})	Wall shear rate (s^{-1})
Control	29 ± 0.8	1.5 ± 0.2	260 ± 45
TNF-α	29 ± 0.8	1.6 ± 0.2	273 ± 28
Anti-MIP-2 + TNF-α	32 ± 1.1	1.9 ± 0.1	288 ± 79
Anti-KC + TNF-α	34 ± 1.0	1.8 ± 0.1	265 ± 29
Anti-MIP-2 and anti-KC + TNF-α	31 ± 1.4	1.8 ± 0.1	295 ± 50

Animals were treated intrascrotally with PBS (Control) and TNF-α (0.1 μg) for 2 h. In separate experiments, monoclonal antibodies against MIP-2 (anti-MIP-2, 20 μg per mouse) and or KC (anti-KC, 20 μg per mouse) were locally administered, alone or together, immediately before TNF-α challenge. Data are mean ± S.E.M. and $n = 3–7$.

mononuclear leukocytes (Fig. 3), suggesting that neutrophils constituted the majority of leukocytes in this study.

Next, we tested the effectiveness of monoclonal antibodies directed against MIP-2 and KC in order to identify an optimal antibody dose, which could abolish the respective chemokine function in vivo. As demonstrated in Fig. 4, we observed that 20 μg of anti-MIP-2 and anti-KC antibodies inhibited MIP-2-and KC-provoked neutrophil infiltration by 97% and 95%, respectively. By use of these effective doses of anti-MIP-2 and anti-KC antibodies, we wanted to examine the role of CXC chemokines in TNF-α-induced leukocyte recruitment. Intrascrotal challenge with TNF-α (0.1 μg, 2 h) markedly increased leukocyte rolling, firm adhesion and extravascular recruitment (Fig. 5a–c, $P < 0.05$ vs. control). Notably, local administration of anti-MIP-2 and/or anti-KC antibodies had no effect on TNF-α-induced leukocyte rolling and firm adhesion in the postcapillary venules of cremaster muscle (Fig. 5a–b, $n = 5$). Treatment with the anti-MIP-2 or anti-KC antibody alone did not decrease the number of extravascular neutrophils in response to TNF-α challenge (Fig. 5c, $P > 0.05$ vs. TNF-α alone, $n = 5–7$). In contrast, local co-administration of anti-MIP-2 and anti-KC antibodies significantly reduced TNF-α-induced neutrophil extravasation by 65% (Fig. 5c, $P < 0.05$ vs. TNF-α alone, $n = 5–7$). Together, these data suggest that MIP-2 and KC are redundant with respect to chemotactic function on neutrophils in response to TNF-α. In addition, it is important to note that administration of anti-MIP-2 and/or anti-KC antibodies did not change systemic leukocyte counts (Table 1) or hemodynamic parameters (Table 2).

4. Discussion

This study provides evidence showing that CXC chemokines may be redundant in TNF-α-induced leukocyte recruitment. Thus, we found that functional interference with MIP-2 or KC alone could not reduce the neutrophil response to TNF-α. However, immunoneutralization of both MIP-2 and KC markedly reduced tissue accumulation of neutrophils provoked by TNF-α. Moreover, it was observed that MIP-2 and KC exerted an additive and not a synergistic effect on neutrophil recruitment. Taken together, our novel data suggest that MIP-2 and KC are functionally redundant and that combined inhibition of CXC chemokines may be necessary to reduce TNF-α-induced neutrophil infiltration.

TNF-α is an important regulator of numerous molecules, such adhesion molecules and chemokines, which collectively coordinate the inflammatory response (Liu et al., 2000; Smart and Casale, 1994; Tessier et al., 1997; Thorlacius et al., 1997b). Herein, we found that stimulation with TNF-α increased the mRNA levels of both MIP-2 and KC in the cremaster muscle, indicating that these CXC chemokines are concomitantly present in the extravascular tissue. This notion is in line with recent studies demonstrating that TNF-α is a potent regulator of MIP-2 and KC expression in the skin and lung (Tessier et al., 1997; Czermak et al., 1999). Noteworthy, it was observed that KC, but not MIP-2, was expressed in resting tissue. The functional significance of this baseline expression of KC mRNA is not known, although it is interesting to note that a similar baseline gene expression of KC was recently found in the skin (Biedermann et al., 2000). It may be speculated that such a baseline expression of KC mRNA may rapidly be translated into a functional protein and thus constitute an efficient defense system in response to bacterial invasion.

Functional interaction between mediators is of great importance in complex inflammatory reactions considering the multitude of substances released. For example, it has been shown that cytokines (i.e. TNF-α and interleukin-1β) synergistically increase leukocyte rolling (Thorlacius et al., 1997b) and that histamine potentiates chemoattractant-induced leukocyte adhesion (Thorlacius et al., 1995, 1997a). Based on our present findings showing that both MIP-2 and KC are concomitantly expressed in response to TNF-α challenge, it is feasible to hypothesize that CXC chemokines may potentiate leukocyte tissue accumulation. However, we found that coadministration of MIP-2 and KC did not potentiate the neutrophil response. In fact, the level of neutrophil infiltration reflected the sum of the individual effects of MIP-2 and KC. Thus, based on these data, it may be concluded that MIP-2 and KC do not act in a synergistic manner with respect to tissue infiltration of neutrophils. This lack of potentiating interaction may be attributable to the fact that MIP-2 and KC stimulate neutrophil activation through the same receptor, i.e. CXC repector-2, which is the high-affinity receptor for both CXC chemokines (Cacalano et al., 1994; Jones et al., 1997). This notion is also in line with our findings demonstrating that MIP-2 and KC have an additive effect on neutrophil accumulation in the extravascular space.

Considering that MIP-2 and KC are present after challenge with TNF-α (this study; Czermak et al., 1999; Tessier et al., 1997), it is imperative to learn more about the individual role these CXC chemokines, not only for a deeper pathophysiologic understanding, but such knowledge is also critical in the development of novel anti-inflammatory strategies directed against diseases characterized by cytokine-induced tissue damage. However, the published literature on the relative role of MIP-2 and KC in neutrophil infiltration is complex and partly contradictory. For example, some groups have reported that inhibition of MIP-2 reduces neutrophil accumulation in the lung (Greenberger et al., 1996; Shanley et al., 1997), whereas others have shown that blocking MIP-2 has no effect on pulmonary recruitment of neutrophils (Moore et al., 2000). Furthermore, Tessier et al. (1997) have demonstrated that immunoneutralization of either MIP-2 or KC alone significantly decreased TNF-α-induced accumulation of neutrophils in the skin. In the present study, we found that monoclonal antibodies directed against MIP-2 and KC had no effect on TNF-α-induced neutrophil recruitment when administered alone. In contrast, coinjection of anti-MIP-2 and anti-KC substantially attenuated the number of extravasated neutrophils in response to TNF-α challenge, suggesting that MIP-2 and KC may be functionally redundant in TNF-α activated tissues. However, we observed no effect of immunoneutralization of MIP-2 and KC on intravascular leukocyte rolling and adhesion provoked by TNF-α. This lack of effect on intravascular leukocyte–endothelium interactions may be attributable to the physical barrier provided by the vascular wall against the diffusion of antibodies to the luminal surface of the microvascular endothelium. Alternatively, the leukocyte–endothelium interactions in response to TNF-α may also be mediated by others substances, such as platelet-activating factor (Kuijpers et al., 1992).

In conclusion, our novel data demonstrate that both MIP-2 and KC are expressed in response to TNF-α challenge in the striated muscle in vivo. Although expressed together, this study shows that MIP-2 and KC do not functionally interact and potentiate leukocyte responses. Moreover, we provide evidence suggesting that MIP-2 and KC are redundant and that inhibition of both MIP-2 and KC may be necessary in order to reduce neutrophil infiltration into tissues activated by TNF-α.

Acknowledgements

This study was supported by Swedish Medical Research Council (K98-27I-11610-03, K2000-04P-13411-01A), Cancerfonden (4265-B99-01XAB), Allmäna sjukhusets i Malmö stiftelse för bekämpande av cancer, MAS fonder, Franke and Margareta Bergqvists stiftelse för främjande av cancerforskning, Malmö University Hospital and Lund University.

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