The chemokine receptor CXCR3 mediates rapid and shear-resistant adhesion-induction of effector T lymphocytes by the chemokines IP10 and Mig

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Integrin-mediated adhesion to the vascular endothelium is an essential step in leukocyte diapedesis. We show that the chemokines 10-kDa inflammatory protein (IP10) and monokine induced by IFN (Mig) induce rapid and transient adhesion of human IL-2-stimulated T lymphocytes (IL-2 T cells) to immobilized integrin ligands through their receptor CXCR3, which is selectively expressed on activated T cells. Induction of adhesion by IP10 and Mig was already observed at subnanomolar concentrations and was maximal at 5–10 nM, resulting in three- to sixfold increase in adhesion of IL-2 T cells over background. No effect was seen with resting naive/memory T cells which lack CXCR3 and migration responses to IP10 and Mig. Both chemokines are produced in human umbilical vein endothelial cells (HUVEC) upon stimulation with IFN-γ and TNF-α. These chemokines induce IL-2 T cell adhesion also when captured on the surface of endothelial cells. Under conditions of flow, IL-2 T cells roll and rapidly adhere to IP10/Mig-expressing HUVEC, and anti-CXCR3 mAb treatment reduces arrest and firm adhesion. This is the first study that shows chemokine-induced adhesion in activated memory/effector T cells which represent the fraction of T cells that are selectively mobilized in inflammation. The critical role of IFN-γ as inducer of IP10/Mig production in HUVEC indicates that these chemokines are essential mediators of effector T cell recruitment to IFN-γ-dependent pathologies.

Key words: Chemokine / Adhesion / Integrin / Endothelium / Inflammation

1 Introduction

Leukocyte diapedesis is an essential event in the recruitment of circulating leukocytes to sites of inflammation and involves distinct steps, including selectin-mediated rolling on the endothelium, integrin-mediated adhesion and transendothelial migration [1–4]. For arrest of rolling and firm adhesion, integrins on leukocytes need to be activated, an event which was shown to depend on signaling through G protein-coupled receptors [5–7]. Ligands for these G protein-coupled receptors are thought to be induced in endothelial cells at sites of inflammation and may include chemokines.

Chemokines are rapidly produced in blood and tissue cells upon induction by pro-inflammatory cytokines and other stimuli and are recognized as principal mediators of leukocyte recruitment [8–11]. Currently, this large family of cytokines consists of three dozen individual proteins of 67 to 103 amino acids and is divided into two major subfamilies, CXC and CC chemokines, based on the arrangement of the first two of four conserved cystein residues. The primary target cells for chemokines are leukocytes. The chemokine receptors are expressed in thousands of copies per cell and upon ligand binding, these cells respond by chemotaxis, enzyme and mediator release and various effector functions. All known chemokine receptors are members of the large family of seven-transmembrane domain receptors which require G proteins for signal transduction but show differences in their cellular expression [8–11]. Thus, the cellular infiltrate is defined largely by the composition of locally produced chemokines as well as the diversity of circulating leukocytes that express the relevant receptors.
In contrast to monocytes and granulocytes which predominate in the early (acute) phase in inflammation, participation of lymphocytes does not occur immediately, depends on antigen recognition and is prominently associated with delayed-type hypersensitivity, anti-viral and autoimmune responses. Responsiveness of T lymphocytes to chemokines depends on cell activation [8, 9]. The large majority of freshly isolated T lymphocytes do not respond to CC chemokines but culturing in the presence of IL-2 was found to result in expansion of activated CD45RO⁺ T lymphocytes of either CD4⁺ or CD8⁺ phenotype which uniformly expressed chemokine receptors and readily migrated to chemokines [12–14]. T cell antigen receptor activation, by means of anti-CD3 ± anti-CD28 or PHA treatment, led to rapid and complete inhibition of T cell migration, indicating that CC chemokines are selective for those T lymphocytes which are clonally expanded after antigen recognition in secondary lymphoid organs [12].

The lymphocyte-activating properties are best described for the CC chemokines RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β and the monocyte chemotactic proteins MCP-1 to MCP-4 which bind to CCR1, CCR2, CCR3 and CCR5 [8–11]. MCP-1 and MIP-1β are highly selective for CCR2 and CCR5, respectively, whereas the other CC chemokines interact with more than one chemokine receptor. Expression of these receptors is not restricted to T lymphocytes, and the functions of the chemokines they bind have been studied in detail in monocytes and granulocytes [8–11, 15]. In a search for chemokine receptors with selective expression in activated effector/memory T cells, we have identified CXCR3, the receptor for the CXC chemokines inflammatory protein (IP)10 and monokine induced by IFN (Mig) [16]. CXCR3 is expressed at high levels in IL-2-stimulated but not resting T lymphocytes and, in contrast to all other chemokine receptors, was found to be absent in any other type of leukocyte. Thus, CXCR3 and its ligands are thought to play an important role in inflammatory conditions with prominent T lymphocyte participation.

In this report, we have examined the effect of IP10 and Mig on adhesion of T lymphocytes to immobilized integrin ligands and cultured human umbilical vein endothelial cells (HUVEC). Importantly, we have performed all studies with IL-2-stimulated memory T lymphocytes (IL-2 T cells) which uniformly expressed CXCR3 and strongly responded to IP10 and Mig [16]. Both IP10 and Mig induced rapid and transient T lymphocyte adhesion via CXCR3. Under flow conditions with IFN-γ/TNF-α-stimulated HUVEC monolayers which produced IP10 and Mig, the majority of rolling IL-2 T cells rapidly adhered and this effect was drastically reduced by anti-CXCR3 mAb. Our findings demonstrate that IP10 and Mig are potent inducers of adhesion of activated effector/memory T lymphocytes, indicating that these chemokines play an essential role in the recruitment of T lymphocytes to sites of inflammation and disease.

2 Results

2.1 IP10 and Mig induced rapid adhesion of IL-2 T cells to integrin ligands

All adhesion experiments were performed with IL-2-stimulated, cultured T lymphocytes prepared from PBL (IL-2 T cells) which express large numbers of CXCR3 and were shown to be highly responsive to IP10 and Mig [16]. To study chemokine-mediated T lymphocyte adhesion, ⁵¹Cr-labeled IL-2 T cells were added to 96-well plates containing chemokines as well as purified, immobilized intercellular adhesion molecule (ICAM)-1 or vascular cell adhesion molecule (VCAM)-1, and cell adhesion was evaluated as described in Section 4.5. Fig. 1 illustrates that 5 nM IP10 and Mig induced rapid and firm adhesion of IL-2 T cells to integrin ligands ICAM-1 and VCAM-1. Maximal chemokine effects (two- to sixfold over background binding) were seen 3 min after addition of IL-2 T cells to chemokines and longer incubations raised the chemokine-independent binding to similar levels as with IP10 and Mig. The transient IP10- and Mig-induced IL-2
T cell adhesion was followed by chemokine-independent maximal binding (20–30 % of input cells bound) at 10 min which declined to a steady-state level of adhesion (10–17 % of input cells bound) upon prolonged incubation (10–60 min). In subsequent studies the incubation time was set at 3 min. Fig. 2 shows that IP10 induced T cell adhesion to ICAM-1 and VCAM-1 at subnanomolar concentrations. Maximal effects (in this experiment approx. 10 % of input cells bound) were observed between 1 nM and 10 nM IP10 which correlates with the range of concentrations shown to induce intracellular Ca\textsuperscript{2+} mobilization and chemotaxis [16]. Similar results were obtained with Mig (not shown), and the concentration of chemokines used in subsequent experiments was set at 5 nM.

IP10 and Mig were very effective in IL-2 T cells but not short-term PHA-activated PBL (PHA T cells) which are low in CXCR3 and do not migrate to IP10 or Mig (Fig. 3). Strongest effects were routinely seen on ICAM-1 (> five-fold over background binding) but binding to VCAM-1 and fibronectin was still considerable (3.3- and 2.5-fold over background binding, respectively), whereas binding of IL-2 T cells in the absence of chemokines was 1.9–2.3 % of input cells. IP10 (4-77), an N-terminal truncation variant which lacks chemokine activity and receptor binding ([17] and M. Loetscher, unpublished) as well as IL-8 (not shown), a chemokine with no activity in T lymphocytes, did not induce T cell adhesion, indicating that enhanced adhesion depended on functional integrity of IP10 and was not due to nonspecific interactions with cell surface proteoglycans [18]. In contrast to IL-2 T cells, freshly isolated or short-term PHA-stimulated PBL are extremely low in CXCR3 and do not respond to IP10 and Mig ([16] and M. Loetscher, unpublished). In agreement, IP10 and Mig did not induce enhanced adhesion of PHA T cells to purified integrin ligands (Fig. 3).

2.2 IP10 and Mig-induced T cell adhesion was mediated by CXCR3

To demonstrate the involvement of CXCR3 in mediating IL-2 T cell adhesion, the effect of anti-chemokine receptor mAb was examined (Fig. 4A). The anti-CXCR3 mAb used was shown to block T cell chemotaxis to IP10 and Mig (S. Qin et al., submitted). Likewise, IL-8 binding and responses in transfected Jurkat cells expressing the type 2 IL-8 receptor CXCR2 was shown to be prevented by the anti-CXCR2 mAb [19], and was used here as an isotype control. Pretreatment of T cells with anti-CXCR3 reduced IP10- and Mig-induced adhesion to ICAM-1 by > 90 % (after deduction of 4.6 % background binding). By contrast, the effect with the anti-CXCR2 mAb was marginal, demonstrating that binding of IP10 and Mig to CXCR3 was required for induction of T cell adhesion. An alternative approach to analyze receptor usage is pretreatment with saturating concentrations of chemokines which renders the cells unresponsive to subse-
Figure 4. IL-2 T cell adhesion is mediated by the IP10/Mig receptor CXCR3. (A) IL-2 T cells were treated with anti-CXCR2 (CXCR2) or anti-CXCR3 (CXCR3) mAb at 100 μg/ml or with buffer alone (none) for 1 h at 0 °C before use. (B) IL-2 T cells were preincubated with 100 nM Mig, IP10 or RANTES, or buffer alone (none) for 40 min at 37 °C and washed with ice-cold RPMI-Hepes buffer before use. Rapid induction (3 min) of adhesion by 5 nM Mig (hatched bars), IP10 (crosshatched bars) or RANTES (closed bars), or by buffer alone (open bars) to immobilized ICAM-1 was evaluated.

Data are expressed as in Fig. 1, and are representative of three independent experiments.

Figure 5. Expression of transcripts for IP10, Mig and other chemokines in HUVEC.

To postulate that IP10 and Mig are important regulators of transendothelial migration, it was necessary to show that these chemokines were produced by endothelial cells. Previously, some leukocytes and tissue cells were shown to produce IP10 and Mig upon induction with LPS or IFN-γ [8, 9, 21–25]. No information was available on expression of IP10 and Mig in endothelial cells. Fig. 5 demonstrates that transcripts for both IP10 and Mig were expressed in cultured HUVEC after co-stimulation with 10 ng/ml TNF-α and 1000 U/ml IFN-γ, whereas TNF-α or IFN-γ alone did not induce detectable levels of mRNA. This is in contrast to IL-8 and MCP-1 which were readily expressed in TNF-α-treated HUVEC, and IFN-γ alone or in combination with TNF-α did not show any further effect. In addition, the kinetics of mRNA expression differed greatly. Northern blot signals for IP10 and Mig were not detected before 4 h of stimulation and were maximal at 8–16 h whereas abundant mRNA for IL-8 and MCP-1 was seen as early as 1 h and reached highest levels at 4 h. We have noticed a variation in the extent of IP10 and Mig expression in HUVEC cultures from different donors but Northern blot signals for IP10 and Mig were consistently lower than for IL-8 and MCP-1. Transcript expression fully correlated with protein secretion (Fig. 6). Stimulation of HUVEC with the com-
Figure 6. Secretion of IP10, Mig and other chemokine proteins by stimulated HUVEC. Supernatants of HUVEC cultures stimulated with TNF-α (open bars), IFN-γ (hatched bars) or combination of TNF-α and IFN-γ (closed bars) were analyzed for chemokine proteins and corresponded to HUVEC cultures studied in Fig. 5. Concentrations (nM) for Mig, IP10, IL-8 and MCP-1 were measured by Sandwich ELISA as described in Sect. 4.4. Data are expressed as mean chemokine concentrations (± SD) of triplicate wells. Eight independent experiments were performed for Mig and IP10 protein determination, and three independent experiments for IL-8 and MCP-1.

2.4 IP10 and Mig captured on HUVEC induced adhesion of T cells

The production of IP10 and Mig by TNF-α/IFN-γ-stimulated HUVEC is not only evidenced by their accumulation in the cell culture supernatants. Stimulated HUVEC retain on their cell surface IP10 and Mig, as assessed by immunostaining (not shown), which was possibly due to binding to cell surface proteoglycans [18]. IP10 and Mig were also captured by unstimulated HUVEC when added exogeneously, and Fig. 7 illustrates that the cell-associated chemokines retained their function. 4.7 % and 4.8 % of input IL-2 T cells adhered to IP10- and Mig-treated HUVEC, respectively, as compared to 2.3 % of cells that adhered to untreated HUVEC. As expected, no effect was seen with the inactive IP10 variant IP10(4-77). By contrast, RANTES or MCP-1 were not functional in this assay, suggesting that these chemokines were not captured/presented by the HUVEC monolayers.

2.5 CXCR3-mediated rapid adhesion of IL-2 T cells under conditions of flow

Under physiological conditions, immunocompetent T cells need to escape from the blood circulation at sites of inflammation by rapid adherence to the endothelium which is followed by transmigration. To study the involvement of CXCR3 in IL-2 T cell adhesion to HUVEC under flow conditions, experiments were performed in a
Table 1. CXCR3-mediated IL-2 T cell adhesion under flow conditions

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>HUVECa) Pretreatmentb)</th>
<th>Adherencec)</th>
<th>Transmigrationd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IFN-γ/TNF-α</td>
<td>control</td>
<td>225 (100 %)</td>
</tr>
<tr>
<td></td>
<td>anti-CXCR3</td>
<td>108 (48 %)</td>
<td>66 (61 %)</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ/TNF-α</td>
<td>control</td>
<td>155 (100 %)</td>
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<td></td>
<td>anti-CXCR3</td>
<td>107 (69 %)</td>
<td>42 (39 %)</td>
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<tr>
<td>3</td>
<td>IFN-γ/TNF-α</td>
<td>control</td>
<td>187 (100 %)</td>
</tr>
<tr>
<td></td>
<td>anti-CXCR3</td>
<td>116 (63 %)</td>
<td>52 (45 %)</td>
</tr>
<tr>
<td>4</td>
<td>–/–</td>
<td>control</td>
<td>0</td>
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<td>anti-CXCR3</td>
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a) HUVEC monolayers after treatment with IFN-γ and TNF-α or without.
b) Before use in flow chamber, IL-2 T cells were treated with anti-CXCR2 (control) or anti-CXCR3 mAb.
c) Total numbers of adherent IL-2 T cells counted during 5 min of infusion at 1 dyne/cm² in an optical field of 0.80 mm²; percent (%) values indicate fractions of bound anti-CXCR3-treated IL-2 T cells as compared to controls (100 %).
d) Total number of transmigrated IL-2 T cells counted as above; percent (%) values indicate fractions of adherent anti-CXCR3-treated and control IL-2 T cells that transmigrated.

flow chamber and analyzed by video-imaging [27]. For expression of chemokines, HUVEC monolayers were stimulated with TNF-α and IFN-γ, washed and inserted into the flow chamber. After pretreatment with either anti-CXCR3 or anti-CXCR2 mAb (control), IL-2 T cells were injected at a steady flow rate of 1 dyne/cm² and interactions with HUVEC and transmigration were evaluated during a time interval of 5 min in an optical field of 0.80 mm². Table 1 summarizes the results from three independent experiments, performed with independently prepared IL-2 T cell cultures and stimulated HUVEC monolayers. Pretreatment of IL-2 T cells with the control mAb resulted in the firm adhesion of 155–225 cells/field during 5 min observations whereas blocking of CXCR3 with the specific mAb reduced the adhesion by 40 ± 8 % (n=3). Detachment of adherent cells was not observed and all cells eventually crossed the HUVEC monolayers after an adhesion time of 2.2 ± 0.5 min (n=6). Interestingly, transmigration across HUVEC monolayers was not inhibited by treatment with anti-CXCR3 mAb when compared with the CXCR2 mAb control. Adhesion to unstimulated HUVEC monolayers under flow was not observed and the few IL-2 T cells which showed tethering motions were rapidly cleared.

3 Discussion

Adhesion of circulating leukocytes to vascular endothelium and invasion of inflamed tissue is a complex process which involves a large variety of adhesion molecules and, as evidenced in recent studies, chemokines and their G protein-coupled receptors [1, 2, 9]. Most adhesion molecules are shared by different types of leukocytes and their ligands are regulated by inflammatory cytokines. Yet, the large variety of inflammatory conditions are known to differ greatly in the composition of their cellular infiltrates. Chemokines are the largest family of cytokines which are produced locally during inflammation and have all types of leukocytes as targets [8, 9, 15]. In contrast to expression of adhesion molecules, chemokine receptor expression varies among the different types of leukocytes, and the chemokines they bind are not uniformly present in inflammatory conditions. Therefore, chemokines may determine large parts of the selectivity in leukocyte recruitment. Since signaling through G protein-coupled receptors was shown to be required for transmigration [6, 7], endothelial cell-derived chemokines may provide selectivity at an early stage in leukocyte recruitment.

In the present study we have examined the effect of the two CXC chemokines IP10 and Mig on adhesion of T lymphocytes to integrin ligands and cultured HUVEC monolayers. All experiments were performed with IL-2-stimulated T lymphocytes generated from PBL which are known to express high levels of the IP10/Mig receptors, CXCR3, and were shown to be highly responsive to IP10 and Mig [16]. In this respect, our work differs from all previous studies which dealt with resting T lymphocytes from peripheral blood or related leukemia cell lines with undefined chemokine receptor phenotypes [28–33]. Of note, freshly prepared or short-term PHA-activated PBL are poor targets for chemokine action, and chemokine receptor expression is restricted to activated effector/memory T lymphocytes which are a minor subset in PBL [12, 13, 16, 34]. As an exception, the CXC chemokine SDF-1 (stromal cell-derived factor-1) acts on resting as well as activated T lymphocytes but its synthesis is not up-regulated in inflammation. The principal function of SDF-1 is therefore thought to be unrelated to leukocyte recruitment in inflammation [8, 9].
Strong adhesion responses (three- to sixfold over background binding) were observed at 1–10 nM of IP10 and Mig which correlates well with chemokine-induced Ca2+ mobilization in CXCR3-transfected cell lines as well as in IL-2 T cells [16]. Moreover, as in the Ca2+ response which is typically transient, maximal adhesion was obtained 3 min after addition of IP10 or Mig and declined afterwards to a “steady-state” level of IP10/Mig-independent T lymphocyte adhesion. The kinetics observed may be an overestimation, due to methodological constraints, of the “true” induction time (seconds, rather than minutes) which needs to be short to be effective on rolling/tethering leukocytes under shear stress.

Treatment with blocking mAb to CXCR3 or cellular desensitization with excess of IP10 or Mig demonstrated that the transient adhesion effect seen with the two chemokines was mediated by the specific receptor CXCR3. Firm adhesion of a large portion (10–17%) of cells during prolonged incubation (> 30 min) may be attributed to the general state of activation of cultured T lymphocytes, as noted previously [35–37]. It should be kept in mind that efficient transendothelial migration of circulating leukocytes at sites of inflammation requires rapid induction of adhesion to the endothelium (see below) and, therefore, delayed-type adhesion of leukocytes is unlikely to contribute to this process [38].

Other chemokines do also promote leukocyte adhesion. IL-8, which is not a chemoattractant for IL-2 T cells [9, 12, 17], was shown to induce rapid integrin-dependent adhesion of polymorphonuclear phagocytes, possibly by induction of rapid fusion of granules containing the integrins with the plasma membrane [39, 40, 41]. In monocytes, transient and differential adhesion induction was observed with multiple CC chemokines [42]. Similarly, RANTES, MIP-1α, MIP-1β and MCP-1 were reported to induce adhesion of resting T lymphocytes or cultured T leukemia cell lines to immobilized ICAM-1, VCAM-1 and extracellular matrix proteins [28, 29, 32, 33, 42, 43]. Generally, adhesion was examined 15–60 min after addition of chemokines and rapid and transient effects, as shown here, were not studied. As an exception, MCP-1 was reported to induce maximal adhesion of T lymphocytes to fibronectin between 4 min to 7 min of stimulation [33]. However, no increased adhesion to endothelium-associated adhesion molecules (ICAM-1 and VCAM-1) was observed which led the authors to conclude that MCP-1 is not involved in triggering firm adhesion of T lymphocytes to the vascular wall.

Similar to the state of pre-activation in cultured T lymphocytes, chemokines themselves may influence delayed-type adhesion under static conditions. Chemokines have been reported to induce T lymphocyte activation that is unrelated to chemokine responses which are generally short in duration (enzyme and mediator release, production of oxygen radicals, cytoskeletal rearrangement). For example, micromolar concentrations of RANTES were shown to cause T lymphocyte proliferation, focal adhesion and cytokine production, which was probably due to chemokine receptor-independent signaling events [44, 45]. Another study reports co-stimulation with nanomolar concentrations of MCP-1, MIP-1α, MIP-1β and RANTES in T lymphocyte proliferation and IL-2 production [43]. Binding to proteoglycans, as shown in detail for IP10 [18], may induce cell activation and could explain the long-term responses seen in T lymphocytes [43–45]. In summary, it is reasonable to postulate that delayed-type adhesion of resting T lymphocytes, which are extremely low in CXCR3 and other chemokine receptors, was the outcome of chemokine receptor-independent cell activation.

To propose the involvement of IP10 and Mig in diapedesis of circulating T lymphocytes, it was essential to demonstrate that endothelial cells are capable of producing and, to avoid dilution by blood flow, capturing these chemokines. Both IP10 and Mig were produced and secreted by cultured HUVEC after induction with TNF-α and IFN-γ. Addition of these cytokines alone or sequentially did not result in IP10 or Mig expression. By contrast, IFN-γ alone was sufficient to induce these chemokines in monocytes/macrophages and related cell lines [21–25]. The gene for IP10 contains binding motifs for several transcription factors, including interferon stimulus responsive elements and NF-κB binding sequences [46]. Possibly, culturing of monocytes/macrophages led to up-regulation of NF-κB protein and thereby substituted for exogenous TNF-α. The strict requirement for IFN-γ sets IP10 and Mig apart from most other chemokines whose expression is not affected or even inhibited by IFN-γ [8, 9, 15]. As an example, we have shown that IL-8 and MCP-1 were highly expressed in HUVEC upon stimulation with TNF-α whereas IFN-γ alone or in combination with TNF-α had no further effect. Also, both IP10 and Mig are highly basic proteins with isoelectric points of 10.8 and 11.1, respectively, and were shown to interact strongly with cell surface proteoglycans [18]. Here, we demonstrate that cultured HUVEC captured both IP10 and Mig and presented these chemokines in a functional manner to IL-2 T cells.

Further evidence for an important role of CXCR3 and its ligands in the transendothelial migration of T lymphocytes stems from adhesion experiments under flow conditions. IL-2 T cells tethered under flow on nonactivated, cultured HUVEC monolayers but did not arrest. Using TNF-α/IFN-γ-stimulated HUVEC monolayers, however, rolling T lymphocytes firmly attached and traversed the...
endothelial cell monolayers. Rapid adhesion was prevented to a large degree (40 ± 8 %, n=3) upon pretreatment of IL-2 T cells with anti-CXCR3 blocking mAb, demonstrating that signaling through CXCR3 was a critical step in the induction of tight adhesion. The blocking effect with anti-CXCR3 mAb was not complete, suggesting that other HUVEC-derived ligands may have acted in a CXCR3-independent fashion on rolling T lymphocytes.

In contrast to previous reports [28–33], we have correlated induction of integrin-dependent T lymphocyte adhesion with responsiveness to IP10 and Mig (chemo-tactic migration, Ca²⁺ mobilization), and expression of CXCR3 [16]. Here we show that the IP10 and Mig-induced T lymphocyte adhesion is a rapid and transient event which is in full agreement with Bordetella pertussis toxin-sensitive chemokine responses in leukocytes [8, 9, 15]. The selective expression of CXCR3 in activated effector/memory T lymphocytes and the requirement of IFN-γ to produce its ligands IP10 and Mig merit special attention. To our knowledge, this is the first chemokine/chemokine receptor system that can be postulated to be important in diapedesis and recruitment of immunocompetent T lymphocytes to sites of inflammation and disease, and the first report to show T cell adhesion inhibition under conditions of flow with a single chemokine receptor antagonist.

4 Materials and methods

4.1 Antibodies and reagents

Rabbit anti-IP10, anti-Mig and anti IL-8 Ab were generated in outbred animals by i.m. injections with synthetic chemokines in PBS plus CFA for immunization, and in PBS plus IFA for booster injections. Ab were purified by protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) chromatography. For biotinylation, purified Ab in 0.1 M NaHCO₃, pH 8.5 were treated with succinimidyl-6-(biotin-amido-)hexanoate (Pierce, Rockford, IL) and subsequently dialyzed against PBS. The generation of anti-MCP-1 Ab has been described [47].

mAb LS77 – 1C6.2 (anti CXCR3, IgG1) was obtained by immunizing mice with a synthetic peptide corresponding to the N-terminal 37 residues of CXCR3. mAb 1C6 totally blocks the binding and chemotactic responses of T cells to IP10. A full description of this mAb is described elsewhere (S. Qin et al., submitted). The mAb LS37 – 6C6.4 (anti CXCR2, IgG1) was described in detail elsewhere [34].

VCAM-mCox and ICAM-mCox are fusion proteins consisting of seven extracellular domains of human VCAM-1 or five extracellular domains of human ICAM-1, respectively, with the murine immunoglobulin kappa light chain constant (mCox) region at the C terminus. Using a rat anti-mCox affinity column, these proteins were purified from culture supernatants of SF9 cells infected with recombinant baculovirus. Rat anti-mCox mAb was purified from conditioned culture medium from the rat hybridoma cell line 187.1 (ATCC no. HB 58) using Gammabind G-Sepharose (Pharmacia) according to the manufacturer’s instructions. The rat anti-mCox was linked to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions, and equilibrated with Tris-buffered saline, pH 7.0. The bound mCox fusion proteins were eluted with 0.1 M glycine, pH 2.2, and immediately neutralized with 2.0 M Tris, pH 8.0.

4.2 Cell preparations and culture

Cultures of HUVEC monolayers were generated from umbilical cords, obtained from the Placenta Transfusion Laboratory of the Women’s Hospital, University of Bern, after informed consent of the mothers. Endothelial cells were recovered from umbilical cords by collagenase treatment and supplemented with M-199 culture medium (Gibco BRL, Paisley, GB) containing 20 % FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 25 μg/ml heparin (Fluka, Buchs, Switzerland) and 0.6 % endothelial cell growth supplement isolated from calf brain as described [48]. HUVEC were grown in gelatin-coated culture dishes and used after not more than five passages. Human PBL were isolated from donor blood buffy coats by separation on Ficoll-Paque followed by two steps of plastic adherence depletion of monocytes. To obtain IL-2 T cells, PBL were cultured in complete RPMI 1640 (10 % FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 × non-essential amino acids and 50 μM 2-ME, all from Gibco BRL), supplemented with recombinant human IL-2 at 100 U/ml. Cell density was maintained at 1–2.5 × 10⁶ cells/ml, and cells were used between 10 and 26 days of culture. The phenotype of PBL cultured with IL-2 has been described in detail [12]. THP-1 cells were obtained from Dr. A. Tobler (Department of Hematology, Insel-Hospital, University of Bern, Switzerland).

4.3 Northern blot analysis

Total RNA from cultured HUVEC was extracted, separated by agarose gel electrophoresis and blotted onto Nylon membranes as described [12]. Hybridization probes were generated by PCR with total cDNA from IFN-γ stimulated THP-1 monocytic cells as template and the following amplification primers: For synthesis of IP10 DNA, primers 5’-TTTCTGAAGCGTACCTCGTACCTG and 5’-TATCGTCAAGCTTGAAGATCTC were used, resulting in a fragment of 231 bp, corresponding to positions 142 to 372 in the published sequence (Gene Bank Accession no. M17752, 21]. Primers for Mig DNA synthesis were 5’-TTTCTGCAAAGGAACCTCCATTACAC and 5’-TATCGTCAAGCTTGAAGATCTC, and amplified DNA of 415 bp corresponded to positions 21 to 435 in the published
sequence (Gene Bank Accession no. X72755, [25]). For the generation of the MCP-1 probe, the primers used for PCR were: 5'-CTGTTCCGGCGAAGCTGCAGTCTCCGCT and 5'-CTGGTTCCGGCGAAGCTGCAGTCTCCGCT, resulting in a DNA fragment of 352 bp corresponding to positions 46 to 375 of the MCP-1 gene (Gene Bank Accession no. M24545). Preparation of IL-8 DNA has been described [49]. Both MCP-1 and IL-8, probes were amplified from of cDNA isolated from LPS-stimulated monocytes. PCR amplifications were performed for 30 cycles (95 °C for 15 s, 48 °C for 30 s, 72 °C for 45 s) on a Master Cycler 5330 (Eppendorf-Netheler-Hinz, Hamburg, Germany) as described [16]. After subcloning and sequencing, purified insert DNA were labeled with [α-32P] dATP (Amersham, Amersham, GB) (> 1 × 10^9 dpm/μg DNA) and used in Northern blot hybridization experiments [12]. Radioactive signals were analyzed on a Phosphorlmager (Molecular Dynamics, Sunnyvale, CA).

4.4 ELISA
Sandwich ELISA for quantification of chemokines was performed according to Engvall et al. [50] with modifications. Briefly, for IP10, Mig and MCP-1, Maxi-Sorp™ ELISA plates (Nunc, Roskilde, Denmark) were coated with anti-chemokine Ab in 0.1 M carbonate buffer, pH 9.6, overnight at 4 °C. After washing twice with Tris-buffered saline containing 0.05% Tween-20 (TBS-Tween), ICAM-1 and VCAM-1 were incubated at 2.5 mg/ml in carbonate buffer, pH 9.6, overnight at 4 °C washed twice with TBS-Tween, TBS. Wells were blocked with a 2% BSA solution, washed, and test samples (HUVEC culture supernatants) and purified chemokines (standards), diluted in TBS-Tween plus 2% FCS, were added and incubated overnight at 4 °C. After extensive washings (TBS-Tween, TBS), biotinylated anti-chemokine Ab were added, incubated for 3 h at 37 °C, and wells were washed as above. Following incubation with ExtrAvidin®-coupled alkaline phosphatase (Sigma, St. Louis, MO) for 30 min at room temperature and washing, phosphatase activity on p-nitrophenyl phosphate (Sigma) (1 mg/ml in diethanolamine-HCl, pH 9.8) was allowed to occur for 1 h at 37 °C. The reaction was stopped with 5 M NaOH, and absorbance at 405 nm was measured in an ELISA reader (Molecular Dynamics). For IL-8, the anti-IL-8 mAb WS-4 [51] was used as primary Ab, and rabbit anti-IL-8 Ab were used as secondary Ab which were detected with anti-rabbit IgG coupled to alkaline phosphatase (Bio-Rad, Richmond, CA). The ELISA allowed the determination of chemokine concentrations in the range of 10–150 pM.

4.5 Static adhesion assays
Adhesion of T lymphocytes to immobilized human recombinant adhesion molecules (ICAM-1, VCAM-1) was performed as described recently [52]. Briefly, 96-well Maxi-Sorp™ ELISA plates were coated with rat anti-murine IgG c-domain mAb YFC51.1 at 2 μg/ml in carbonate buffer, pH 9.6, overnight at 4 °C. Alternatively, ELISA plates were coated with 5 μg/ml fibronectin (Sigma). After blocking with 2% BSA in TBS, ICAM-1 and VCAM-1 were incubated at 2.5 μg/ml and 0.75 μg/ml, respectively in TBS-Tween supplemented with 2% FCS for 1 h at 37 °C. After washing, 50 μl RPMI 1640 medium, supplemented with 0.5% BSA and 25 mM Hepes, pH 7.4 (RPMI-Hepes) were added to the wells. Chemokines were diluted in RPMI-Hepes and added in threefold final concentrations to the wells, and the plates were kept on an ice-water mixture (0°C) till use. After labeling of T lymphocytes with 50 μCi Na51CrO4 (Amersham) [37], 100-μl cell aliquots (2.5 × 10^5 cells) in ice-cold RPMI-Hepes were added to the plates which were kept for an additional 30 min at 0 °C. Wells were sealed with acetic acid foil and, to start induction of adhesion, plates were immediately transferred to a 37 °C water bath and kept floating for the time indicated. After removing of unbound cells by washing twice with prewarmed (37 °C) PBS supplemented with 1 mM Ca2+, 1 mM Mg2+ and 0.5% BSA by careful immersion and flipping of the plates [52], adherent cells were lysed with 100 μl 1% Triton-X-100 and radioactivity was determined in a MR 480 automatic gamma counter (Kontron, Zürich, Switzerland). Background adhesion to BSA only was always below 0.1% of input cells. For adhesion to HUVEC monolayers, chemokines at 100 nM were added to confluent HUVEC monolayers in 96-well plates for 1 h at 0 °C, and washed with 100 μl RPMI-Hepes per well with subsequent flipping [52]. This was performed for a total of three times to remove unbound chemokines. Wells were then filled with 100 μl RPMI-Hepes/well kept at 0 °C till use. Binding of T lymphocytes to these HUVEC monolayers was performed as described above.

4.6 Laminar flow adhesion assay
Adhesion assays under conditions of flow were performed as described by Alon et al. [27]. Confluent HUVEC monolayers cultured in 6-well plates were stimulated with 10 ng/ml TNF-α and 1000 IU IFN-γ for 20 h, washed with HBSS (Gibco BRL), supplemented with 10 mM Hepes, 0.2% human serum albumin, 1 mM Mg2+ and 2 mM Ca2+ (binding buffer) and used as the lower wall in a parallel wall flow chamber [6]. The flow chamber was mounted onto the stage of an inverted phase-contrast microscope and T lymphocytes (10^5/assay), after treatment with 100 μg/ml of anti-CXCR3 or anti-CXCR2 (control) mAb for 1 h at 0 °C, were diluted tenfold with prewarmed (37 °C) binding buffer and perfused through the parallel wall flow chamber. The flow rate was set at 1 dyne/cm² and adhesive events were recorded on videotape. Firm adhesion and transmigration was determined by counting the number of cells that adhered and/or transmigrated the HUVEC monolayers during 5 min of flow in a given optical field (0.80 mm²).

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5 References


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